ELSEVIER

European Journal of Pharmacology 332 (1997) 35–42

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Diadenosine polyphosphates inhibit adenosine kinase activity but decrease levels of endogenous adenosine in rat brain.

Suzanne M. Delaney [a], G. Michael Blackburn [b], Jonathan D. Geiger [a,*]

[a] *Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Manitoba, Canada R3E 0W3*

[b] *Department of Chemistry, Krebs Institute, Sheffield University, Sheffield, UK*

Received 10 April 1997; revised 15 April 1997; accepted 15 April 1997

Abstract

Findings in peripheral tissues that diadenosine polyphosphates (Ap_nAs) activate 5′-nucleotidase activity and inhibit adenosine kinase activity in vitro led us to test the hypothesis that Ap_nAs and analogues thereof, through such actions on purine enzymes, increase brain levels of endogenous adenosine in vivo. Accordingly, we tested Ap_nAs for their effects on the in vitro activities of adenosine kinase, adenosine deaminase, AMP deaminase and 5′-nucleotidase and, following unilateral microinjections in rat striatum, on in vivo levels of endogenous adenosine. Adenosine kinase activity was not affected significantly by 5′,5‴-P^1,P^2-diadenosine pyrophosphate (Ap_2A) or by 5′,5‴-P^1,P^3-diadenosine triphosphate (Ap_3A), but was inhibited by 5′,5‴-P^1,P^4-diadenosine tetraphosphate (Ap_4A), 5′,5‴-P^1,P^5-diadenosine pentaphosphate (Ap_5A) and 5′,5‴-P^1,P^6-diadenosine hexaphosphate (Ap_6A); apparent IC_{50} values were 5.0, 3.3 and 500 μM, respectively. Inhibition of adenosine kinase activity by Ap_4A and the four metabolically stable analogues of Ap_4A tested was uncompetitive. Following unilateral intrastriatal injections, adenosine levels, relative to uninjected contralateral striatum, were decreased significantly ($P < 0.05$) by 48% with Ap_4A and by 37% with $AppCH_2ppA$, a metabolically stable analogue of Ap_4A. Striatal levels of adenosine were not affected significantly by Ap_5A or Ap_6A. Cytosolic, but not particulate 5′-nucleotidase activity was inhibited and AMP deaminase activity was increased by some Ap_nAs. Although adenosine kinase inhibitors increase levels of endogenous adenosine and we showed here that Ap_nAs were potent inhibitors of this enzyme, these particular actions of Ap_nAs were not consistent with their effects on levels of endogenous adenosine. © 1997 Elsevier Science B.V.

Keywords: Brain; Adenosine; Diadenosine polyphosphate; Microwave; Adenine nucleotides; (Rat)

1. Introduction

The majority of the CNS actions of adenosine appear to be mediated by cell-surface adenosine receptors (Palmer and Stiles, 1995). Production and release of endogenous adenosine available to act on these receptors has been shown to increase in response to physiological stimuli (Jonzon and Fredholm, 1985; MacDonald and White, 1985) and various pathological events including ischaemia, hypoxia and seizure activity (Van Wylen et al., 1986; Zhang et al., 1993). Endogenous adenosine as well as adenosine analogues have been found to be neuroprotective (Von Lubitz et al., 1995) and levels of endogenous adenosine are controlled by a combination of processes including its transport and release across cell membranes as well as its enzyme-mediated production by 5′-nucleotidase and its removal by either adenosine kinase or adenosine deaminase. Activators and inhibitors of these pathways can act as regulators of endogenous adenosine levels (REAL agents) and REAL agents, through their ability to increase the levels and actions of endogenous adenosine, are being developed as potentially beneficial therapeutic agents (Geiger et al., in press). Specifically, inhibitors of adenosine kinase and adenosine deaminase increase levels of endogenous adenosine as well as potentiate adenosine-mediated effects in the CNS (Mitchell et al., 1993; Zhang et al., 1993; Lloyd and Fredholm, 1995; Golembiowska et al., 1996).

Diadenosine polyphosphates are a group of adenosine-based compounds that may serve as REAL agents. Diadenosine polyphosphates contain two adenosine moieties linked by 2–6 phosphate groups (Ap_nAs, $n = 2–6$).

* Corresponding author. Tel.: (1-204) 789-3542; Fax: (1-204) 783-6915; e-mail: geiger@cc.umanitoba.ca

PII S0014-2999(97)01057-1

Amongst other actions (McClennan, 1992), Ap_4A and Ap_5A have been shown to be potent inhibitors of adenosine kinase from bovine adrenal medulla (Rotllan and Miras-Portugal, 1985), Ap_4A has been found to stimulate 5′-nucleotidase in pig lung and human lymphoid cells (Johnson and Fridland, 1989; Itoh and Yamada, 1990) and Ap_4A reportedly stimulated rat skeletal muscle AMP deaminase (Fernandez et al., 1984). This latter enzyme is highly regulated and tightly controls the availability of the substrate AMP for 5′-nucleotidase (Van den Berghe et al., 1992). Thus, Ap_nAs could increase adenosine levels by multiple actions including adenosine kinase inhibition whereby the removal of adenosine by this enzyme is decreased or 5′-nucleotidase stimulation whereby formation of adenosine is promoted.

We tested the hypotheses that diadenosine polyphosphates act as inhibitors of rat brain adenosine kinase activity in vitro and that Ap_nAs with such inhibitory effects could function as REAL agents to increase adenosine levels in vivo. The in vivo experiments were performed using our model of unilateral intra-striatal injections into rats later killed by high energy focused microwave irradiation. This technique can be used to obtain accurate and precise measurements of striatal levels of endogenous adenosine (Delaney and Geiger, 1995, 1996a). In addition, the striatum may be a relevant site of action for Ap_nAs since their release from this brain region has been demonstrated in vivo (Pintor et al., 1993b, 1995). Here, we showed that although Ap_nAs potently inhibited adenosine kinase activity in vitro, the levels of endogenous adenosine decreased in vivo and thus Ap_nAs may not serve as REAL agents to increase the levels and actions of endogenous adenosine.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats were obtained from the University of Manitoba Central Animal Care facility. All procedures followed Canadian Council on Animal Care guidelines and were approved by the Animal Care Committee at the University of Manitoba. Rats used for intra-striatal injections weighed 170–190 g and rats used for enzyme studies weighed 200–220 g.

2.2. Intra-striatal injections

Animals were anaesthetized with 74 mg/kg sodium pentobarbital (i.p.), placed in a stereotaxic frame and unilateral intra-striatal injections were performed as previously described (Delaney and Geiger, 1995) using the coordinates (in mm) AP 9.0, ML 3.0 and DV 4.5 (Paxinos and Watson, 1986). Drugs were dissolved in 50 mM Tris–HCl (pH 7.4), administered (0.5 μl) using a 30 gauge needle over a 2 min period and the needle was left in place for 1 min post-injection to allow diffusion of drug away from the injection site. Rats were killed 15 min post-injection by high energy focused-microwave irradiation (Cober Instruments) at a power level of 10 kW for 1.25 s as previously described (Delaney and Geiger, 1996a). Brains were removed, ipsilateral and contralateral striata were dissected and striata were analyzed separately for tissue adenosine content using a fluorescence-HPLC method (Delaney and Geiger, 1995, 1996a). Protein determinations for this and all subsequent assays used the method of Lowry et al. (1951) and BSA as standard. Tissue adenosine content was expressed as pmol per mg protein.

2.3. Enyzme activity assays

All enzyme assays were performed on brains (minus cerebellum and brainstem) from rats killed by decapitation. Adenosine kinase (EC 2.7.1.20) activity was assayed by measuring the production of ^{3}H-labeled nucleotides from [^{3}H]adenosine as described previously and was expressed as nmol formed/5 min per mg protein (Gu et al., 1991). 5′-Nucleotidase (EC 3.1.3.5) activity was assayed by measuring the production of inorganic phosphate using an adaptation of the method of Heymann et al. (1984) and was expressed as nmol inorganic phosphate formed/15 min/mg protein. Brains were homogenized in 25 volumes (w/v) of 10 mM Tris-acetate buffer (pH 7.3) containing 0.32 M sucrose using a Teflon-glass homogenizer. Homogenates were centrifuged at $1000 \times g$ for 10 min at 4°C and supernatants were centrifuged first at $9000 \times g$ for 20 min at 4°C and again at $100\,000 \times g$ for 1 h at 4°C before assaying for endo-5′-nucleotidase activity. Post-$100\,000 \times g$ pellets were gently resuspended in 100 mM Tris–HCl buffer (pH 7.0) to give 1.5–3.0 mg protein/ml, homogenized using a Teflon-glass homogenizer and used to determine ecto-5′-nucleotidase activity. The incubation mix for both ecto- and endo-5′-nucleotidase assays contained, in a total volume of 120 μl, 100 mM Tris–HCl buffer (pH 7.0), tissue, 1 mM of AMP or IMP and 10 mM $MgSO_4$. Reaction blanks contained buffer instead of AMP or IMP and in our assays non-specific phosphatases did not contribute to phosphate production. Incubations were for 15 min at 37°C and enzyme activity was terminated by addition of 20 μl of 25% trichloroacetic acid followed by centrifugation at $16\,000 \times g$ for 2 min. Supernatants (100 μl) were removed, added to 900 μl distilled water and following addition of colour reagent (1 ml), inorganic phosphate was measured spectrophotometrically. Adenosine deaminase (EC 3.5.4.4) activity was assayed using a spectrophotometric method to detect formation of ammonia as described previously and activity was expressed as nmol NH_3 formed/h per mg protein (Padua et al., 1990). AMP deaminase (EC 3.5.4.6) activity was assayed as previously described using essentially the same spectrophotometric method as for adenosine deaminase except

that AMP was used at a final concentration of 10 mM and samples were incubated at 37°C for 15 min (Padua et al., 1990). Results for AMP deaminase were expressed as nmol NH_3 formed/15 min per mg protein.

2.4. HPLC of diadenosine polyphosphates

In order to test for the metabolism of Ap_nAs during in vitro enzyme assays, sample aliquots of 100 μl were added to 200 μl of tri-*n*-octylamine/Freon (45:155) and vortexed for 5 s. Following centrifugation for 2 min at $16\,000 \times g$, Ap_nA levels in 70 μl of the top aqueous layer were measured by reverse phase HPLC using a mobile phase of 0.1 M KH_2PO_4 containing 1% methanol (pH 6.0); UV detection was at 254 nm.

2.5. Chemicals

Adenosine, AMP, IMP, P^1–P^2 diadenosine diphosphate (sodium salt), P^1–P^3 diadenosine triphosphate (ammonium salt), P^1–P^4 diadenosine tetraphosphate (ammonium salt), P^1–P^5 diadenosine pentaphosphate (sodium salt) and P^1–P^6 diadenosine hexaphosphate (ammonium salt) were purchased from Sigma (St. Louis, MO, USA) and chloracetaldehyde was purchased from Fluka (Ronkonkoma, New York, NY, USA). All other chemicals were of analytical grade and were obtained from standard laboratory sources. [2,8,5-^{3}H]Adenosine was obtained from New England Nuclear (Montreal, Québec, Canada). Erthyro-9-(2-hydroxy-3-nonyl)adenine HCl was generously supplied by Dr. S. Grossman (Burroughs Wellcome, Research Triangle Park, NC, USA). The following analogues of diadenosine tetraphosphate were synthesized in the laboratory of Dr. G.M. Blackburn (McClennan et al., 1989): diadenosine 5′,5‴-(P^2, P^3-methylene)-P^1,P^4-tetraphosphate ($AppCH_2ppA$), diadenosine 5′,5‴-(P^2, P^3-dichloromethylene)-P^1,P^4-tetraphosphate ($AppCCl_2ppA$), diadenosine 5′,5‴-(P^2, P^3-monofluoromethylene)-P^1,P^4-tetraphosphate (AppCHFppA) and diadenosine 5′,5‴-(P^2, P^3-monochloromethylene)-P^1,P^4-tetraphosphate (AppCHClppA).

2.6. Data analysis

All data were expressed as mean ± S.E.M. Statistical analyses for the effect of Ap_nAs on adenosine kinase activity were conducted using an ANOVA followed by Tukey–Kramer multiple comparisons test. Differences were considered to be significant for $P < 0.05$. Analyses of Ap_4A and $AppCH_2ppA$ inhibition of adenosine deaminase activity were performed using the computer programme ENZYME (Lutz et al., 1986). Statistical analyses for the effect of diadenosine polyphosphates on endogenous levels of adenosine were performed using a paired Student's *t*-test. Differences were considered to be significant for $P < 0.05$. Regression analyses for the effects of Ap_5A and Ap_6A on AMP deaminase activity were performed using GraphPad Prism. The goodness of fit (r^2) was obtained and was considered to be significantly different from zero if $P < 0.05$.

3. Results

The effects of 5 Ap_nAs each at 100 μM on adenosine kinase activity are shown in Table 1. Adenosine kinase activity was decreased significantly by Ap_4A ($P < 0.01$) and Ap_5A ($P < 0.01$). Ap_6A decreased adenosine kinase activity by 40%. When varying concentrations of Ap_nAs were tested, we found that Ap_4A and Ap_5A were more potent and efficacious than Ap_6A; the apparent IC_{50} values were 5.0 μM for Ap_4A, 3.3 μM for Ap_5A and 500 μM for Ap_6A (Fig. 1A). Metabolically stable Ap_4A analogues with substituted carbon atoms inserted between the second and third phosphate in the polyphosphate bridge inhibited adenosine kinase with similar efficacies, however, $AppCCl_2ppA$, was approximately 5-times less potent than the other analogues. The IC_{50} values were 1.9 μM for both $AppCH_2ppA$ and AppCHFppA, 2.1 μM for AppCHClppA and 10.7 μM for $AppCCl_2ppA$ (Fig. 1C). The K_m value for adenosine kinase was 0.5 μM and the V_{max} value was 14.5 nmol/5 min per mg protein. Inhibition of adenosine kinase activity by Ap_4A (Fig. 1B) and $AppCH_2ppA$ (Fig. 1D) was uncompetitive; K_i values were 4.0 and 0.2 μM, respectively.

Inhibitors of adenosine kinase have been shown to increase levels of endogenous adenosine (see Section 1). Therefore, we went on to measure levels of endogenous adenosine after intra-striatal injections of those Ap_nAs that inhibited adenosine kinase activity. Levels of endogenous adenosine in vehicle-injected striata (50 mM Tris–HCl, pH 7.4) of 143 ± 46 were not significantly different from levels in uninjected striata of 147 ± 28 pmol/mg protein; expressed as percentage of the contralateral uninjected striatum, adenosine levels were $108 \pm 25\%$ ($n = 4$). Intrastriatal injection of 10 nmol Ap_4A decreased significantly (paired *t*-test; $P < 0.05$) levels of endogenous adenosine to $52 \pm 13\%$ of those in the uninjected contralateral striata (Fig. 2). The metabolically stable Ap_4A analogue, $AppCH_2ppA$, at a dose of 5 nmol, decreased significantly (paired *t*-test; $P < 0.05$) levels of endogenous adenosine to $63 \pm 13\%$ of contralateral striata, a level not significantly different from that of 10 nmol Ap_4A. Ap_5A and Ap_6A, each at 10 nmol, did not significantly affect levels of endogenous adenosine.

The effects of Ap_4A, Ap_5A and Ap_6A on levels of endogenous adenosine in vivo appeared to be inconsistent with their inhibition of adenosine kinase activity. Therefore, we went on to investigate the effects of Ap_nAs on other enzymes involved in adenosine metabolism. 5′-Nucleotidase is a family of cytosolic and membrane-bound enzymes that dephosphorylate AMP to adenosine and IMP to inosine. Using IMP as substrate, 5′-nucleotidase activity

Table 1
Effects of 100 μM Ap_nA ($n = 2–6$) on adenosine kinase activity

Ap_nA	Adenosine kinase activity (nmol/mg protein per 5 min)
—	9.7 ± 1.1 (13)
Ap_2A	12.2 ± 2.9 (3)
Ap_3A	10.6 ± 1.7 (4)
Ap_4A	0.5 ± 0.2 * (3)
Ap_5A	0.5 ± 0.1 * (3)
Ap_6A	5.9 ± 1.4 (4)

Values represent mean ± S.E.M. Number of determinations in parentheses.
* $P < 0.01$ versus control activity.

was 316 ± 33 nmol/15 min per mg protein ($n = 4$) for brain membranes and 158 ± 29 nmol/15 min per mg protein ($n = 4$) for brain cytosol. 5′-Nucleotidase activity using AMP as substrate was 584 ± 59 nmol/15 min per mg protein ($n = 4$) for brain membranes and 269 ± 38 nmol/15 min per mg protein ($n = 4$) for brain cytosol. Brain membrane 5′-nucleotidase activity with either IMP or AMP was not affected significantly by Ap_nAs. In contrast, in the presence of 100 μM Ap_4A, cytosolic dephosphorylation of AMP was significantly ($P < 0.05$) inhibited by 36% and dephosphorylation of IMP was significantly ($P < 0.05$) inhibited by 56%. Other Ap_nAs did not affect cytosolic AMP or IMP 5′-nucleotidase activity. Concentration-response curves generated for Ap_4A at concentrations ranging from 1 to 1000 μM showed that cytosolic AMP 5′-nucleotidase activity was inhibited maximally by 250 μM Ap_4A to 39 ± 9% of control values (Fig. 3). The metabolically stable Ap_4A analogues $AppCH_2ppA$ and $AppCCl_2ppA$, tested over a 10-fold range of concentrations, did not affect cytosolic 5′-nucleotidase activity suggesting that the effect of Ap_4A was being mediated through products formed from its breakdown.

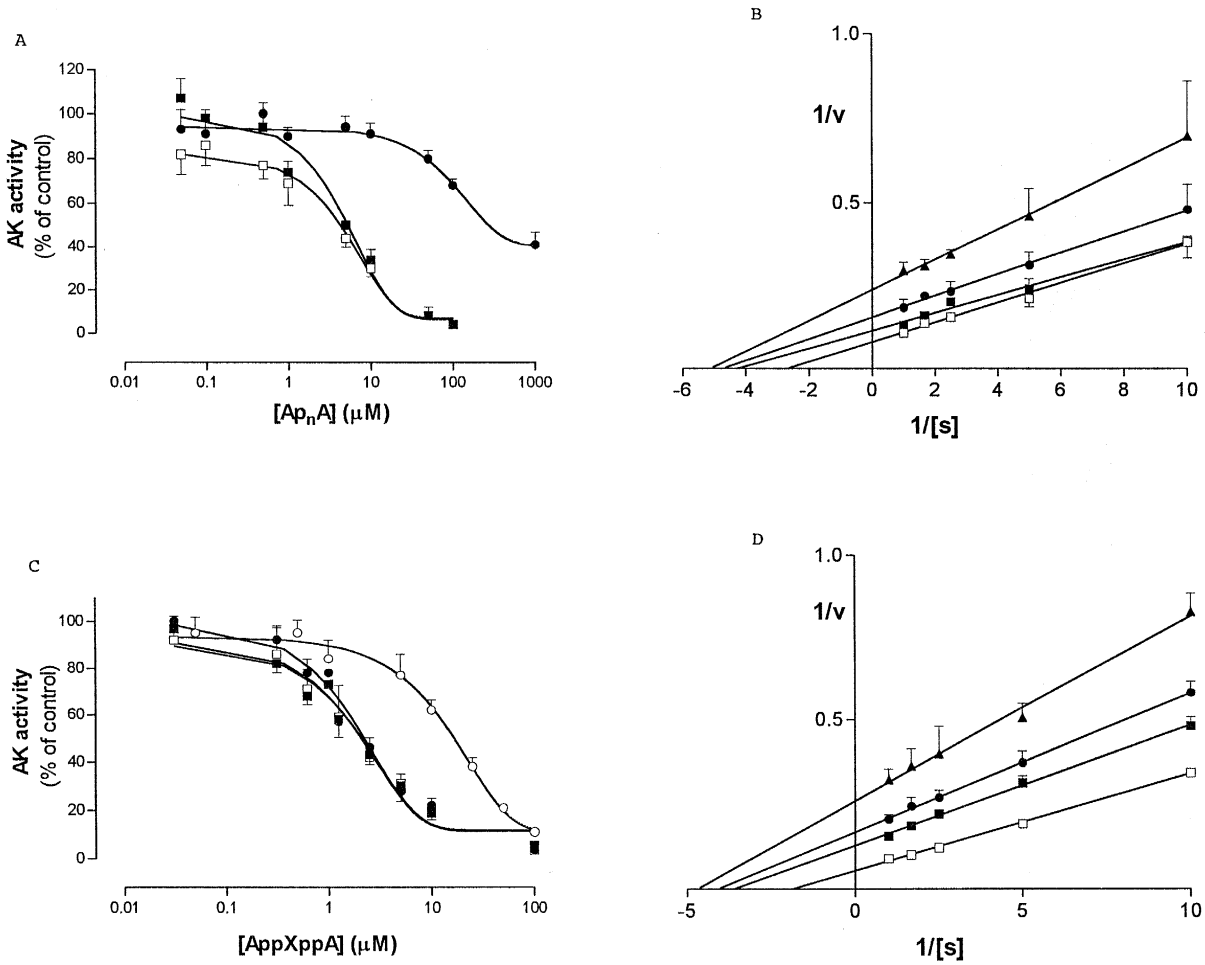

Fig. 1. (A) Effect of increasing concentrations of Ap_4A (closed square), Ap_5A (open square) and Ap_6A (closed circle) on adenosine kinase activity expressed as % of control enzyme activity. (B) Double reciprocal plot of adenosine kinase activity using [^{3}H]adenosine as substrate in the absence (open square) and presence of 2.5 (closed square), 5 (closed circle) and 10 (closed triangle) μM Ap_4A. (C) Effects of increasing concentrations of AppXppA on adenosine kinase activity were expressed as % of control enzyme activity in the absence of Ap_nA where X represents CH_2(closed square), CHCl (closed circle), CHF (open square) and CCl_2 (open circle). (D) Double reciprocal plot of adenosine kinase activity using [^{3}H]adenosine as substrate in the absence (open square) and presence of 0.25 (closed square), 0.5 (closed circle) and 1 (closed triangle) μM $AppCH_2ppA$. Values represent mean ± S.E.M. from at least 3 experiments each performed in triplicate.

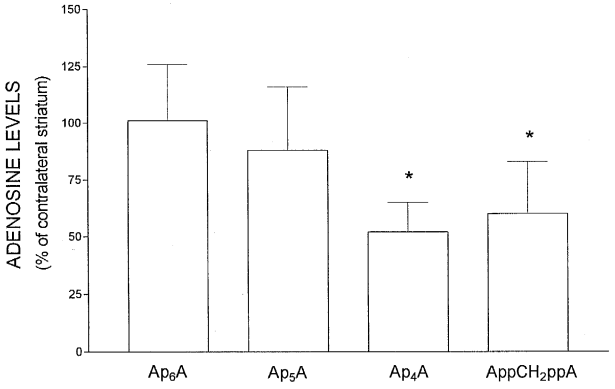

Fig. 2. Levels of endogenous adenosine in striata of rats receiving unilateral injections of 10 nmol Ap_6A ($n=8$), Ap_5A ($n=9$), Ap_4A ($n=6$), or 5 nmol $AppCH_2ppA$ ($n=3$). Levels in injected striata were expressed as a percentage of those in the contralateral uninjected striata. * $P<0.05$ (paired t-test) injected striatum versus uninjected contralateral striatum.

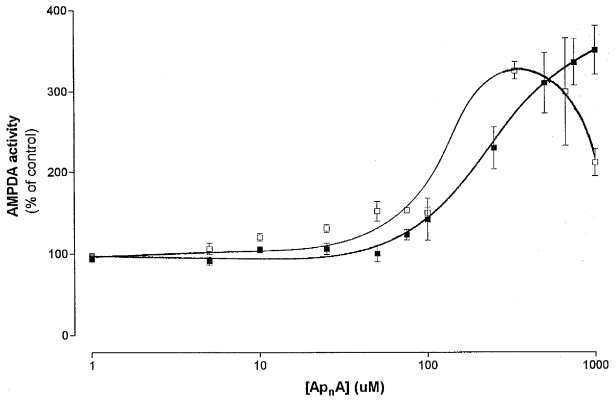

Fig. 4. Effects of increasing concentrations of Ap_5A (closed square) and Ap_6A (open square) on AMP deaminase activity. Values expressed as % of control enzyme activity in the absence of Ap_nA represent mean ± S.E.M. from 4 experiments.

Indeed, inclusion of 250 μM ADP or ATP in the assay system inhibited 5′-nucleotidase activity by 78 and 94%, respectively (data not shown).

AMP deaminase activity tightly regulates levels of AMP available for possible metabolism by 5′-nucleotidase. Preliminary studies showed that AMP deaminase activity levels of 493 ± 27 nmol/15 min per mg protein ($n=8$) were significantly increased 120% by 100 μM Ap_5A ($P<0.05$) and 147% by 100 μM Ap_6A ($P<0.01$). Concentration–response data indicated that AMP deaminase activity was increased to 352 ± 30% by 1 mM Ap_5A, the highest dose tested and that AMP deaminase activity was maximally increased to 327 ± 11% by Ap_6A at 333 μM (Fig. 4). At concentrations above 333 μM, the effects of Ap_6A decreased such that at 1 mM Ap_6A, AMP deaminase activity was 212 ± 17% of control levels. ATP is both an activator of AMP deaminase and a metabolite of Ap_6A breakdown. Under our assay conditions, approximately 21% of the 100 μM Ap_6A added was metabolized and, thus, the maximal amount of ATP that could be produced would be about 40 μM. Nevertheless, even 100 μM ATP was without effect. Previously, Fernandez et al. (1984) plotted $1/v$ against $1/[Ap_4A]$ to obtain the activation constant for the effects of Ap_4A on AMP deaminase activity. We transformed our data and plotted $1/v$ against $1/[Ap_5A]$ and $1/[Ap_6A]$ to obtain activation constants of 1.2 μM and 70 μM for Ap_5A ($r^2=0.97$) and 2.3 μM for Ap_6A ($r^2=0.91$; $P<0.05$) on AMP deaminase activity (data not shown).

Studies on adenosine deaminase activity, another enzyme responsible for adenosine breakdown, showed that control levels of 96 ± 8 nmol/15 min per mg protein ($n=11$) were not affected significantly by Ap_nAs.

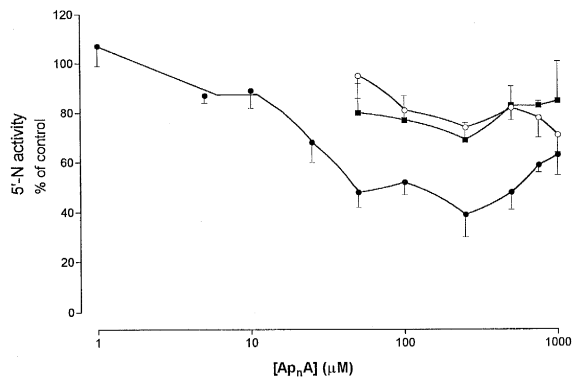

Fig. 3. Effects of increasing concentrations of Ap_4A (closed circle), $AppCH_2ppA$ (open circle) and $AppCCl_2ppA$ (closed square) on cytosolic AMP 5′-nucleotidase activity. Values expressed as % of control activity in the absence of Ap_nA represent mean ± S.E.M. from at least 3 experiments each performed in duplicate.

4. Discussion

Ap_nAs are endogenously produced and are known to have both intra- and extracellular effects (Hoyle, 1990; Remy, 1992; Pintor and Miras-Portugal, 1993, 1995) including regulation of enzymes involved in purine metabolism (Sillero and Cameselle, 1992). Ap_nAs, at least in some peripheral tissues, have been found to inhibit adenosine kinase activity (Rotllan and Miras-Portugal, 1985) and stimulate 5′-nucleotidase activity (Johnson and Fridland, 1989; Itoh and Yamada, 1990). Accordingly, we hypothesized that, Ap_nAs might act as regulators of endogenous adenosine levels (REAL agents) and increase adenosine levels via their actions on key purine metabolizing enzymes such as adenosine kinase and 5′-nucleotidase. This is an issue of some biological and therapeutic importance because adenosine is thought to act as an endogenous neuroprotectant, the levels of which would be expected to increase if, for example, the enzymes adenosine kinase and adenosine deaminase that are responsible for its removal are inhibited, and/or if the enzyme 5′-nucleotidase responsible for its formation was stimulated.

Ap_4A and Ap_5A have previously been found to inhibit adenosine kinase from bovine adrenal medulla and here, for the first time, we showed that Ap_4A, Ap_5A and Ap_6A, but not Ap_2A and Ap_3A inhibited adenosine kinase activity in rat brain. Adenosine kinase, an enzyme that catalyzes the phosphorylation of adenosine to 5′-AMP, is important in regulating levels of endogenous levels of adenosine. Inhibition of adenosine kinase has previously been found to potentiate the levels and actions of adenosine in a variety of CNS tissues and preparations (Lloyd and Fredholm, 1995; Golembiowska et al., 1996). However, even though the levels of adenosine kinase activity, the Michealis–Menton kinetics and inhibition profile (uncompetitive) measured here were similar to those reported previously (Yamada et al., 1980; Rotllan and Miras-Portugal, 1985; Gu et al., 1991), the levels of endogenous adenosine in vivo were not increased by intra-striatal injections of Ap_nAs. To the contrary, we found that Ap_4A significantly decreased levels of endogenous adenosine in rat striatum in vivo. Ap_nAs are known to be metabolized both by intra- and extra-cellular enzymes (Rodriguez-Pascual et al., 1992; Ramos and Rotllan, 1995) and to eliminate the possibility that the effects of Ap_4A on adenosine kinase activity and on levels of endogenous adenosine were mediated by products of Ap_4A breakdown, we tested a series of metabolically stable Ap_4A analogues (McClennan et al., 1989). These analogues (AppXppA where X = CH_2, CCl_2, CHCl or CHF) were also potent inhibitors of adenosine kinase and, like Ap_4A, AppCH_2ppA significantly decreased levels of endogenous adenosine. Thus, Ap_nAs as such appear to be effecting changes to adenosine kinase activity and levels of endogenous adenosine.

The observed decrease in levels of endogenous adenosine caused by Ap_nAs might be explained by Ap_nA-induced decreases in 5′-nucleotidase activity. It has been found previously that 5′-nucleotidase activity in human lymphoid cells and pig lung was stimulated by Ap_nAs irrespective of whether the substrate for 5′-nucleotidase was AMP or IMP (Johnson and Fridland, 1989; Itoh and Yamada, 1990). However we found that particulate 5′-nucleotidase activity was unaffected while cytosolic dephosphorylation of both AMP and IMP was inhibited to a similar extent by Ap_4A. Although this finding may, at first, appear to help explain the observed decreases in levels of endogenous adenosine, the observed inhibition of cytosolic AMP 5′-nucleotidase by Ap_4A was not duplicated by AppCH_2ppA and AppCCl_2ppA which suggested to us that at least part of the effects on 5′-nucleotidase activity were due to Ap_4A metabolites. Indeed, metabolism of, for example Ap_4A, would result in the formation of AMP and ATP (Rodriguez-Pascual et al., 1992; Ramos and Rotllan, 1995) and ATP has been found to affect cytosolic 5′-nucleotidase activity (Orford et al., 1991) in a concentration-related pattern similar to that observed here for Ap_4A. Therefore, the observed results with the metabolically stable Ap_4A analogues appear to eliminate Ap_4A inhibition of 5′-nucleotidase as the reason for the observed decreased levels of endogenous adenosine.

We found that AMP deaminase activity was activated by Ap_5A and Ap_6A in a manner similar to that described for rat skeletal muscle (Fernandez et al., 1984). Because skeletal muscle contains almost exclusively isozyme A while brain contains isozymes B and C (Van den Berghe et al., 1992), it appears that activation of AMP deaminase by Ap_nAs is not isoform dependent. Increased AMP deaminase activity would be expected to decrease the amount of AMP available for adenosine formation by 5′-nucleotidase. However, because Ap_5A and Ap_6A, but not Ap_4A increased AMP deaminase activity, these findings do not help explain the observed Ap_4A-induced decreases in levels of endogenous adenosine.

Adenosine levels are also regulated by transport processes and under experimental conditions similar to those used in the present study, we have shown that dilazep, an inhibitor of adenosine uptake, can increase adenosine levels (Delaney and Geiger, 1996b). Thus, decreased adenosine levels may result from stimulation of adenosine transport. Nucleoside uptake is sensitive to regulation by purinergic P_{2Y} receptors (Sen et al., 1993) and Ap_4A, acting through P_2 purinergic receptors has been shown to regulate nitrobenzythioinosine-sensitive equilibrative nucleoside transporters (Sen et al., 1993; Delicado et al., 1994). It is presently unclear which subtype of purinoceptors might be involved because Ap_nAs interact with P_{2X}, P_{2Y}, P_{2U} (MacKenzie et al., 1988; Hoyle et al., 1989; Pintor et al., 1991; Lazarowski et al., 1995) and, the putative P_{2D} and P_4 receptors (Pintor et al., 1993a; Pintor and Miras-Portugal, 1995). However, even though P_{2Y} and P_{2X} purinoceptor subtypes have been located in the striatum (Kidd et al., 1995; Zhang et al., 1995), the rank order of Ap_nA potencies for decreasing adenosine levels ($Ap_4A > Ap_5A > Ap_6A$) observed here most closely resemble Ap_nA competition for [^{35}S]adenosine 5′-*O*-(2-thiodiphosphate) binding to putative P_{2D} receptors in rat brain synaptic terminals (Pintor et al., 1993a) and Ap_nA stimulation of [^{3}H]inositol phosphate formation in cultured astrocytoma cells expressing the cloned human P_{2U}-purinoceptor (Lazarowski et al., 1995). Thus, the decrease in adenosine levels may have been due to Ap_nA actions on adenosine transporters possibly mediated through P_2 purinoceptors. Interestingly, it has been reported previously that injections of amphetamine in rat striatum were found to increase levels of adenosine as well as levels of Ap_4A and Ap_5A (Pintor et al., 1995), but from our work here, it appears clear that the amphetamine and not the resultant increases in Ap_4A and Ap_5A caused the increases in adenosine.

Acknowledgements

This work was supported by a grant from the Medical Research Council of Canada (MRC). S.M.D. and J.D.G.

were supported by MRC Studentship and Scientist awards, respectively.

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