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Adenosine monophosphate as a mediator of ATP effects at P1 purinoceptors

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1 When perfused with a medium containing no added magnesium and 4-aminopyridine (4AP) (50 μM) hippocampal slices generated epileptiform bursts of an interictal nature. We have shown in a previous study that adenosine 5′-triphosphate (ATP) depressed epileptiform activity and that this effect was blocked by the adenosine A_1 receptor antagonist cyclopentyltheophylline but was not affected by adenosine deaminase. This implied that ATP might act indirectly at P1 receptors or at a xanthine-sensitive P2 receptor. The aim of the present study was to investigate further the action of ATP on epileptiform activity.

2 ATP can be metabolized by ecto-nucleotidases to adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP) and adenosine, respectively. Each of these metabolites can activate receptors in its own right: P2 receptors for ADP and P1 receptors for AMP and adenosine.

3 We now show that both AMP and ATP (50 μM) significantly decrease epileptiform discharge rate in a rapid and reversible manner. 5′Adenylic acid deaminase (AMP deaminase, AMPase) (0.2 u ml^{-1}), when perfused alone did not significantly alter the discharge rate over the 10 min superfusion period used for drug application. When perfused concurrently with AMP (50 μM), AMP deaminase prevented the depressant effect of AMP on discharge rate.

4 AMP deaminase, at a concentration of 0.2 u ml^{-1} which annulled the effect of AMP (50 μM), prevented the inhibitory activity of ATP (50 μM). A higher concentration of ATP (200 μM) depressed the frequency of spontaneous bursts to approximately 30% control and this response was also prevented by AMP deaminase.

5 Superfusion of the slices with 5′-nucleotidase also prevented the inhibitory activity of ATP on epileptiform discharges.

6 The results suggest that AMP mediates the inhibitory effects of ATP on epileptiform activity, a conclusion which can explain the earlier finding that cyclopentyltheophylline but not adenosine deaminase inhibited the effect of ATP. A corollary to this is that, when examining the pharmacology of ATP, care must be taken to inactivate AMP with AMP deaminase, as well as adenosine with adenosine deaminase, before a direct action of ATP on P1 receptors can be postulated. Failure to do so may have led to erroneous conclusions in some previous studies of nucleotide activity on nucleotside receptors.

Keywords: AMP; ATP; epileptiform activity; ectonucleotidases; purines; adenosine

Introduction

Adenosine 5′-triphosphate (ATP) is increasingly regarded as an important extracellular neuromodulator in the CNS as well as peripherally. The application of ATP can induce inward current and depolarization in some populations of central neurones such as those in the cerebellum (Ikeuchi & Nishizaki, 1996), nucleus tractus solitarius (Ueno *et al*., 1992), hypothalamus (Chen *et al*., 1994) and locus coeruleus (Shen & North, 1993; Frohlich *et al*., 1996), while in the hippocampus inward currents, including those for calcium are produced in response to ATP application (Inoue *et al*., 1992; 1995; Balachandran & Bennett, 1996; Dave & Mogul, 1996). ATP has been proposed as a fast excitatory neurotransmitter in the medial habenula and locus coeruleus, responsible for producing evoked and miniature excitatory postsynaptic potentials (e.p.s.p.) (Edwards *et al*., 1992; Nieber *et al*., 1997). In the light of these results, there is a growing interest in the nature of the receptors for ATP in the CNS.

Receptors for the purine nucleotides and nucleosides were classified by Burnstock (1978) into P2 sites, sensitive primarily to ATP and ADP, and P1 sites responding to adenosine and AMP. However, several recent studies have claimed that these categories are no longer valid, as evidence has been obtained for nucleotides producing responses which are blocked by xanthines and are therefore presumed to be mediated by P1 receptors (von Kugelgen *et al*., 1992; Cunha *et al*., 1994; Barajas-Lopez *et al*., 1995; King *et al*., 1996). A similar problem was encountered in our earlier study of the effects of ATP on epileptiform discharges induced in hippocampal slices (Ross *et al*., 1998a). It was found that ATP could depress epileptiform bursting in a manner which was resistant to adenosine deaminase but was antagonized by 8-cyclopentyl-1,3-dimethylxanthine (CPT) (Ross *et al*., 1998a), a result which led us to the conclusion that ATP might act indirectly at P1 receptors or at a xanthine-sensitive P2 receptor.

However, ATP is susceptible to metabolism by a variety of enzymes, which can produce adenosine 5′-monophosphate (AMP) as well as adenosine (Zimmerman, 1996). It was, therefore, of interest to investigate further the modulation of epileptiform activity by ATP in the CA3 region of the hippocampus taking into account the possible actions of these metabolites.

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Methods

Male Wistar rats (180–250 g) were anaesthetized with urethane (1.3 g kg^{-1}, i.p.) before being killed by cervical dislocation. Transverse hippocampal slices (450 μm) were prepared with a McIlwain tissue chopper. The slices were kept within an interface chamber containing artificial cerebrospinal fluid (aCSF) gassed with 95% O_2–5% CO_2 for at least one hour before use. The composition of the aCSF was as follows (mM): NaCl 115, $NaHCO_3$ 25, KCl 2, KH_2PO_4 2.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2 and glucose 10; saturated with 95% O_2–5% CO_2. After incubation individual slices were transferred to a 1 ml submersion chamber which was continually perfused with aCSF or modified aCSF at a rate of 3.5–4 ml min^{-1}. The temperature of the chamber was maintained at approximately 34°C.

A bipolar stimulation electrode was placed in the hippocampal CA3 region to allow orthodromic stimulation of the mossy fibres. The response was recorded via a glass capillary electrode in the pyramidal cell layer of the CA3 region. Stimulation (0.2 Hz) was applied briefly to check the viability of the slice and the correct positioning of the recording electrode, after which stimulation was halted and the perfusing medium changed from normal aCSF to magnesium-free aCSF containing 4-aminopyridine (4AP) at 50 μM. After approximately 5–20 min spontaneous bursts of population spikes occurred which were continuously recorded on a Gould storage oscilloscope and a Grass pen recorder and subsequently plotted as frequency against time. Drugs were perfused for a minimum of 10 min. The control frequency (bursts per minute) was calculated as the mean of the 3 observations immediately preceding start of drug perfusion. The effect of added agents was measured both as the mean of the final 3 observations made during the 10 min period of perfusion and as the maximum amount of inhibition produced during the period of application. Results are expressed as a percentage of the control rate $\pm$ s.e.mean for n slices. Statistical analysis of control against test rate was carried out by use of a paired Student's t test. Multiple comparisons were made by Student's t-test (paired or unpaired) or analysis of variance (ANOVA) followed by a Student-Newman-Keuls *post-hoc* test. $P<0.05$ was taken to indicate significance.

Drugs

All drugs except AMP deaminase were dissolved in distilled water to form a stock solution before being diluted further in normal or modified aCSF. AMP deaminase was dissolved directly in modified aCSF, then filtered to remove a resulting residue. This process did not alter the activity of the enzyme.

5′-Adenylic acid deaminase (AMPase; E.C. 3.5.4.6), 5′-nucleotidase (E.C.3.1.3.5), ATP, and 4-aminopyridine were purchased from Sigma Chemical Company. AMP was purchased from British Drug Houses.

Results

Epileptiform bursts of an interictal nature were generated when hippocampal slices were perfused with medium containing no added magnesium and 4-aminopyridine (4AP at 50 μM). The resulting activity occurred at a frequency between 0.08–0.6 Hz, with a duration of the individual bursts ranging from 150–400 ms. The frequency and burst duration remained consistent during wash periods in individual slices.

AMP deaminase (E.C. 3.5.4.6)

AMP deaminase (0.2 u ml^{-1}) tended to decrease the rate of epileptiform activity to a small extent, but over the 10 min perfusion period this did not reach significance (Figure 1). AMP at 50 μM depressed the rate of epileptiform activity by approximately 60% (Figure 2a,b). This effect was rapid in onset and had a slight tendency to drift back towards control during the perfusion time (Figure 2a). AMP deaminase metabolizes AMP to inosine 5′-monophosphate (IMP) which was inactive (data not shown). The depression of activity caused by AMP (50 μM) was totally inhibited when AMP deaminase (0.2 u ml^{-1}) was co-perfused with AMP (Figure 2).

ATP also reduced the frequency of spontaneous activity in this model when tested at 50 μM or 200 μM (Figure 3). ATP is metabolized through a number of stages by ecto-ATPases, one of the resulting metabolites being AMP. In order to neutralize any AMP produced during the perfusion of ATP, AMP deaminase was used at the concentration which inhibited the effect of AMP at 50 μM. The responses to ATP at both 50 and 200 μM were reduced by the enzyme. There was a tendency for the rate of bursting to decline progressively throughout the application of the enzyme but, by the end of the ATP application, AMP deaminase had still reduced the effect sufficiently that burst frequency was not significantly below control levels (Figure 3c). Indeed, the small degree of inhibition produced by ATP plus AMP deaminase after 10 min of perfusion was similar to that produced by the enzyme alone (see Figure 1) and may therefore be attributed to an action of the enzyme itself. The maximum amount of inhibition produced by ATP was reduced by AMP deaminase from 96 $\pm$ 3.45% to 20 $\pm$ 11.98%.

Adenosine deaminase

In order to estimate the extent to which the effects of AMP were mediated by adenosine formed from it by nucleotidase activity, the combination of AMP and adenosine deaminase was examined. As seen in Figure 4, a concentration of adenosine deaminase of 0.2 u ml^{-1}, which we have shown is sufficient to abolish responses to adenosine at 50 μM (Ross *et al.*, 1998a) was only able to reduce partially the effect of AMP.

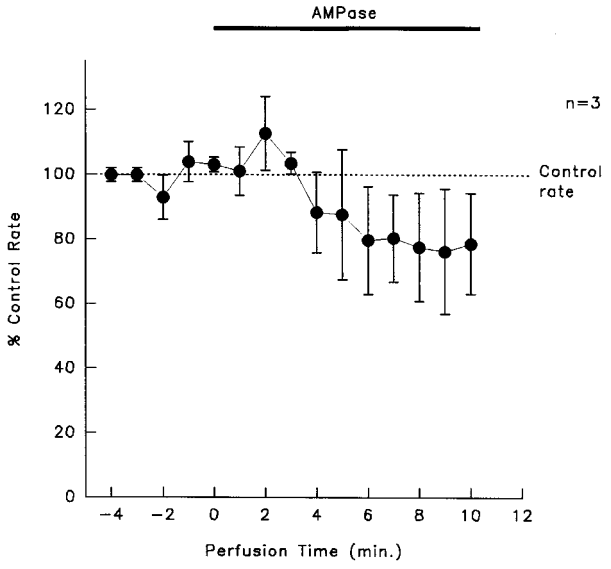

Figure 1 The effect of AMP deaminase on the rate of epileptiform activity.

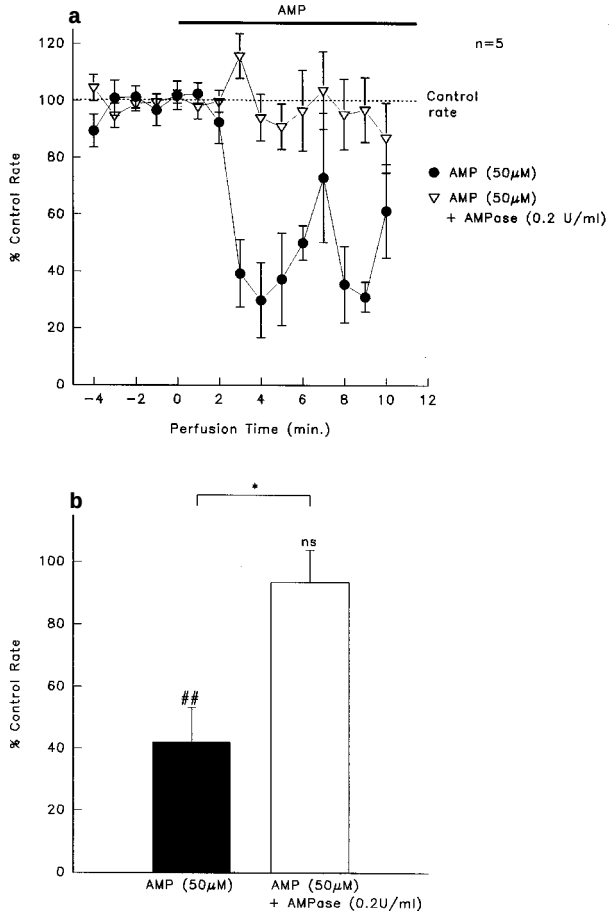

Figure 2 The time course of the decrease in discharge rate by AMP and the inhibition by AMP deaminase is shown in (a). The mean effect at the end of a 10 min perfusion is summarized in (b). AMP significantly altered the rate from control ($\#\#P<0.01$). AMP deaminase significantly reduced the effect of AMP ($*P<0.05$).

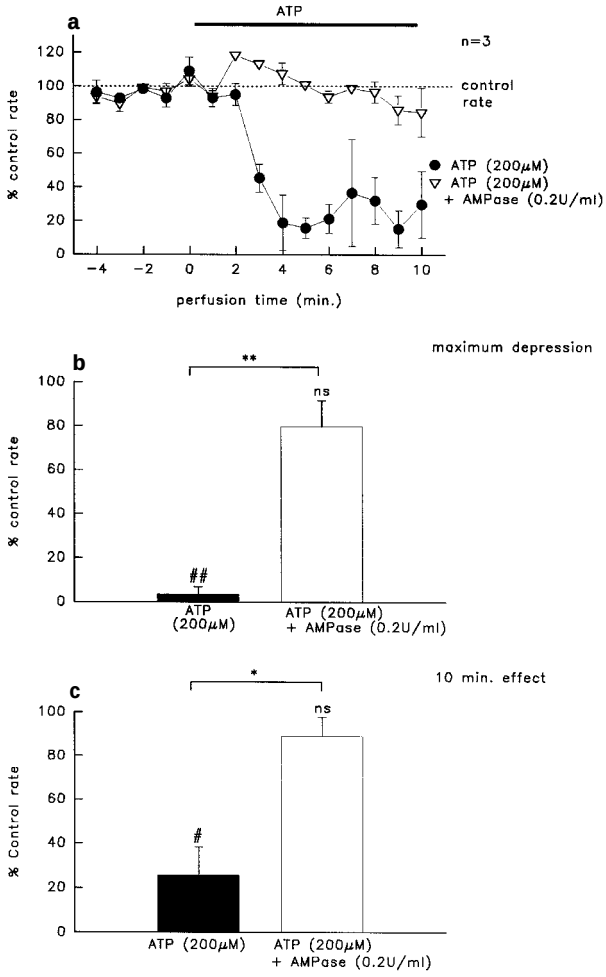

Figure 3 (a) The time course of the effect of ATP (200 μM) and ATP + AMP deaminase on the rate of activity. The maximum inhibition produced by ATP is summarized in (b) and the net effect at the end of 10 min superfusion is shown in (c). Only ATP alone significantly changed the rate from control. AMP deaminase significantly inhibited the effect of ATP. The maximum effect results are calculated as the mean $\pm$ s.e.mean of the maximum effect in individual slices, not necessarily at the same time point. Hence the maximum and terminal values differ slightly between the time course graph and the histograms. $\#P<0.05$, $\#\#P<0.01$ relative to control levels; $*P<0.05$, $**P<0.01$ for difference between the columns.

This effect was much more obvious towards the end of a 10 min perfusion period, when the depression of control burst rate was reduced from approximately 40% to less than 20%, than earlier in perfusion when the maximum inhibition of rate was reduced from around 65% to near 40%. The reduction by adenosine deaminase was significant at both times, but the depressant effect of AMP remained significant at both times. The concentration of adenosine deaminase used in this experiment was likely to have been near maximal, since in a further three experiments the concentration was increased tenfold to 2 u ml^{-1}, but the same profile of change was observed, with a roughly 50% reduction of the maximal AMP response.

5′-Nucleotidase (E.C. 3.1.3.5)

In addition to its metabolic deamination by AMP deaminase, AMP can be hydrolyzed to adenosine by the action of 5′-nucleotidase (E.C.3.1.3.5). The effects of AMP and ATP were therefore examined during superfusion of the brain slices with this enzyme at a concentration of 0.2 u ml^{-1}. Adenosine deaminase was routinely included in these experiments to remove the adenosine formed from the nucleotides. Figure 5 illustrates that the inhibitory effect of AMP was completely prevented by this combination of enzymes.

In the presence of 5′-nucleotidase and adenosine deaminase, ATP (50 μM) showed an initial depression which did not occur with AMP, although the inhibitory effect of ATP was reduced to a level which was not significantly different from control values (Figure 6). By the end of the 10 min superfusion period the discharge rate had returned to control rates (Figure 6).

Discussion

Epileptiform bursts

In vitro models of epileptiform activity have been used extensively to study factors involved in the generation maintenance and regulation of epileptic episodes. Hippocampal slices generate epileptiform activity of an interictal nature when perfused with a medium containing zero magnesium or 4-aminopyridine (4AP) (Voskuyl & Albus, 1985; Mody *et al.*, 1987; Schniederman & MacDonald, 1987; Swartwelder *et al.*, 1988; Lewis *et al.*, 1989; Avoli *et al.*, 1996). In this study it was found that the combination of zero magnesium and 4AP produced spontaneous bursts of activity more consistently

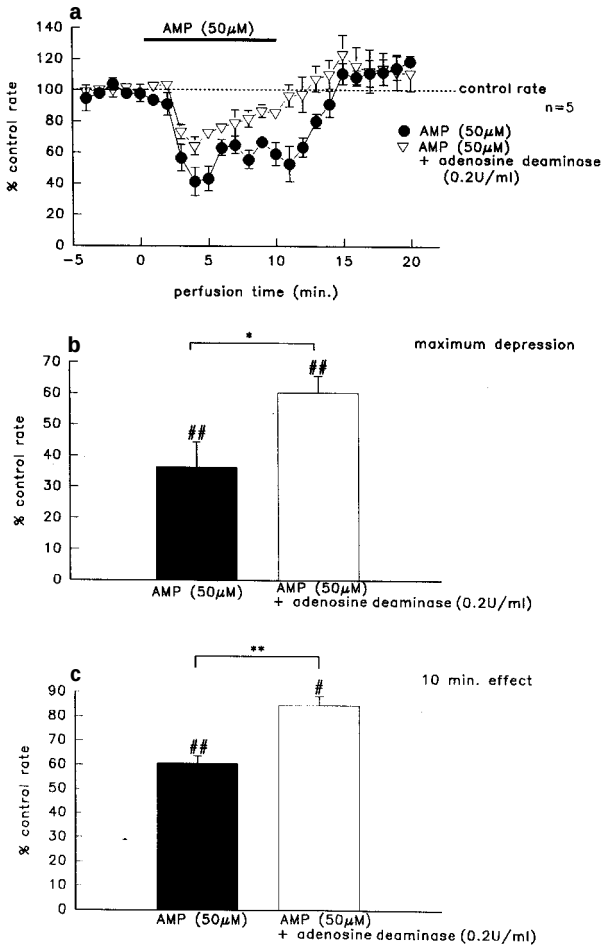

Figure 4 (a) The time course of inhibition by adenosine deaminase of the depression of activity produced by AMP (50 μM). (b) and (c) Indicate the maximum and 10 min effect, respecitvely, of AMP both alone and in the presence of adenosine deaminase. The maximum effect results are calculated as the mean $\pm$ s.e.mean of the maximum effect in individual slices, not necessarily at the same time point. Hence the maximum and terminal values differ slightly between the time course graph and the histograms. $\#P<0.05$; $\#\#P<0.01$ relative to control levels; $*P<0.05$, $**P<0.01$ for difference between the columns.

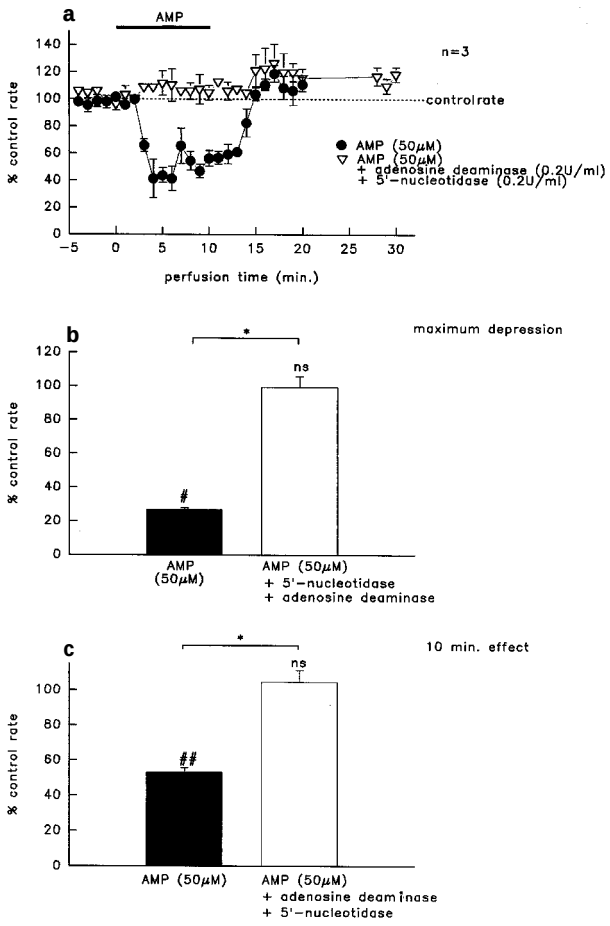

Figure 5 The time course of the effect of AMP alone and with 5′-nucleotidase and adenosine deaminase is shown in (a). The maximum extent of inhibition and the 10 min effect are analysed in (b) and (c), respectively. The maximum effect results are calculated as the mean $\pm$ s.e.mean of the maximum effect in individual slices, not necessarily at the same time point. Hence the maximum and terminal values differ slightly between the time course graph and the histograms. $\#P<0.05$, $\#\#P<0.01$ relative to control levels; $*P<0.05$, for difference between the columns.

than either individual component. The perfusion of slices with a 0 Mg/4AP combination medium results in paroxysmal depolarizing shifts when recorded intracellularly in hippocampal slices (Ross *et al.*, 1998b) and in neocortical neurones (Siniscalchi *et al.*, 1997). Paroxysmal depolarizing shifts are the intracellular correlate for abnormal wave activity known as interictal spikes seen in electroencephalogram recordings in the periods between motor seizures (Ayala *et al.*, 1970). Therefore, the spontaneous discharges in this study are considered to represent a form of interictal epileptiform activity.

ATP effects

ATP is now regarded as playing almost as important a role extracellularly as it does intracellularly, with a large number of studies devoted to understanding how ATP is involved physiologically (Hoyle & Burnstock, 1991). We have previously shown that ATP can depress epileptiform activity in an *in vitro* model in a manner not characteristic of the involvement of classical P2 purinoceptors. An excitatory effect of ATP was mimicked by α, β-methylene ATP and blocked by suramin and pyridoxal-phosphate-6-azophenyl-2′-4′-disulphonic acid (PPADS) and was attributed to P2X receptors (Ross *et al.*, 1998a). In contrast, the depressant effect of ATP was prevented by P1 (adenosine) receptor antagonist, 8-cyclopentyl-1,3-dimethylxanthine, but not by adenosine deaminase. This led us to conclude that either ATP was acting on a P1 receptor or that P2 inhibitory receptors could be blocked by xanthines.

One of the major problems with investigating the effect of ATP is that metabolism occurs. The metabolism of ATP is carried out primarily by a group of enzymes known as the ectoATPases or ectonucleotidases, which were first described by Engelhardt (1957; see Gordon 1986; Stone & Simmonds, 1991). These enzymes, which have their active site facing the extracellular medium (Nagy *et al.*, 1983; Grondal & Zimmerman, 1986), differ from their intracellular counterparts (Nagy *et al.*, 1986; Zimmerman, 1996). The metabolism of ATP by ectoenzymes has been demonstrated in numerous brain regions including vestibular neurones (Cummins & Hyden, 1962), hippocampal neurones (Cunha *et al.*, 1992; 1994) and synaptosomes from a number of brain regions including the

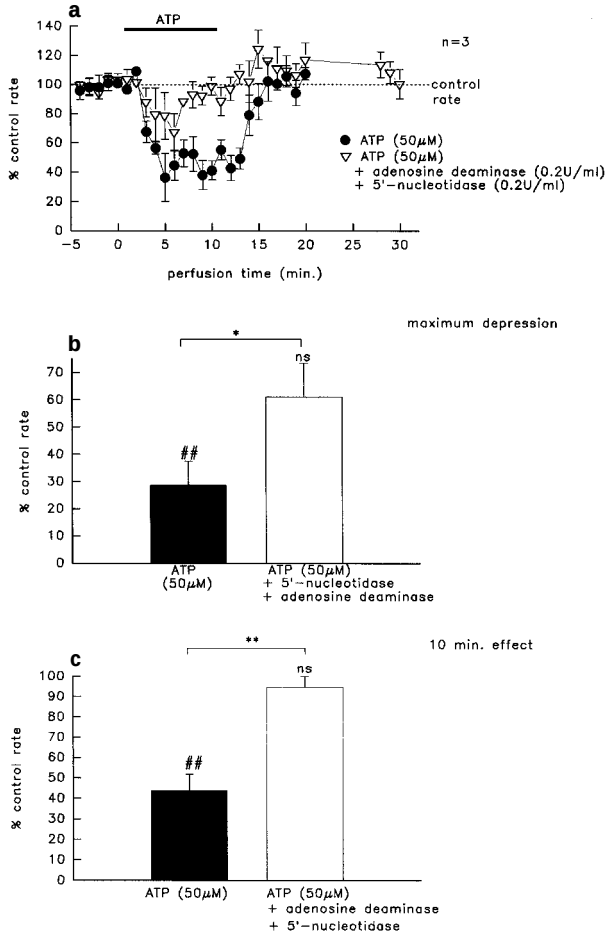

Figure 6 (a) The inhibition by 5′-nucleotidase and adenosine deaminase of the depression of activity produced by ATP (50 μM). (b) and (c) Represent the maximum and 10 min effect, respectively, of ATP both alone and in the presence of 5′-nucleotidase and adenosine deaminase. The maximum effect results are calculated as the mean$\pm$s.e.mean of the maximum effect in individual slices, not necessarily at the same time point. Hence the maximum and terminal values differ slightly between the time course graph and the histograms. $\#\#P<0.01$ relative to control levels; $*P<0.05$, $**P<0.01$ for difference between the columns.

cortex (Lin & Way, 1982) and the hippocampus (Nagy *et al*., 1986). Thus, these enzymes represent a method for terminating the action of ATP released endogenously (Richardson & Brown, 1987; Terrian *et al*., 1989; Kennedy *et al*., 1996). EctoATPases are calcium- and magnesium-dependent, although only one cation has to be present for the enzymes to be active (Grondal & Zimmerman, 1986; Nagy *et al*., 1986). Therefore, the enzymes should continue to be active in the present work despite the omission of magnesium from the medium. The formation of AMP from ADP or ATP results from the action of either ectoATPase, ectoADPase or adenylate kinase which is also thought to be an ecto-enzyme (Nagy *et al*., 1989). The final enzyme involved in ATP catabolism is 5′-nucleotidase which is also located on the external aspect of membranes (Lee *et al*., 1986) and dephosphorylates AMP to form adenosine.

In some instances it has been shown that the majority of ATP is metabolized in a biological system within 30 s (Green *et al*., 1995), although an equally convincing body of work has failed to find substantial metabolism (Welford & Anderson, 1988; Matsuoka *et al*., 1995). The major metabolites are, in order of their formation, ADP, AMP and adenosine and each of these has agonist activity at one of the purine receptors: ATP and ADP act primarily at P2 receptors, while adenosine and AMP act primarily at P1 sites (Burnstock, 1978). Hence, when attributing an effect to ATP, the possible involvement of all these metabolites should be considered.

The attribution of an effect to the direct action of ATP is usually substantiated by the use of non-hydrolyzable ATP analogues which are P2 receptor agonists, for example α,β-methyleneATP or ATPγS, or the inhibition by known selective P2 receptor antagonists. However, a number of recent studies have reported effects of ATP which are inhibited by P1 receptor antagonists (von Kugelgen *et al*., 1992; Cunha *et al*., 1994; Barajas-Lopez *et al*., 1995; King *et al*., 1996) but not P2 antagonists, leading to the concept that nucleotides can activate P1 receptors. Our earlier results were entirely consistent with this, since ATP depressed epileptiform activity in a manner insensitive to adenosine deaminase but inhibited by the A_1 antagonist cyclopentyltheophylline (Ross *et al*., 1998a). The role of metabolites in these studies was ruled out mainly by using adenosine deaminase to degrade any adenosine formed, and α,β-methyleneADP, a 5′-nucleotidase inhibitor, to halt the production of adenosine from AMP. However, the involvement of AMP itself was not considered by us or previous groups (Von Kügelgen *et al*., 1992; Kurz *et al*., 1993; Cunha *et al*., 1994; Barajas-Lopez *et al*., 1995; King *et al*., 1996).

In the present study, AMP exerted a depressant effect in our model of epileptiform activity which is in agreement with the inhibitory effects of AMP on hippocampal population spikes (Dunwiddie & Hoffer, 1980; Dicori & Henry, 1984; Salter & Henry, 1985). ATP also depressed spontaneous activity in a similar manner. However, AMP deaminase inhibited the action of ATP (50 or 200 μM) so that ATP no longer produced a significant depression of discharge rate. The percentage inhibition of activity rate noted in the later stages of perfusion with AMP deaminase and ATP was similar to that seen with the enzyme perfused alone and thus could be due to an intrinsic action of the enzyme.

It should be emphasized that the involvement of AMP demonstrated here does not necessarily apply to all instances where ATP responses are blocked by P1 receptor antagonists, since some groups failed to block ATP responses with AMP deaminase, thus substantiating their suggestion that ATP was able to activate P1 receptors in some tissues (Griese *et al*., 1991; Bo *et al*., 1993; Côte *et al*., 1993).

In summary, the depression of epileptiform activity by ATP is annulled by the enzymes AMP deaminase and 5′-nucleotidase, suggesting that AMP is the mediator of this ATP effect. The inhibition of the ATP response by cyclopentyltheophylline found previously suggests that A_1 receptors are involved. This study, therefore, suggests that caution should be made when using ATP in the absence of nucleotidase inhibitors and that all possible metabolites, including AMP, need to be considered and not just adenosine.

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