British Journal of Pharmacology (1996) 119, 253-260

Preferential activation of excitatory adenosine receptors at rat hippocampal and neuromuscular synapses by adenosine formed from released adenine nucleotides

[1]*Rodrigo A. Cunha, †Paulo Correia-de-Sá, *Ana M. Sebastião & *,†J. Alexandre Ribeiro

*Laboratory of Pharmacology, Gulbenkian Institute of Science, 2781 Oeiras codex, Portugal and †Laboratory of Pharmacology, ICBAS, University of Oporto, 4000 Oporto, Portugal.

1 In the present work, we investigated the action of adenosine originating from extracellular catabolism of adenine nucleotides, in two preparations where synaptic transmission is modulated by both inhibitory A_1 and excitatory A_{2a}-adenosine receptors, the rat hippocampal Schaffer fibres/CA1 pyramid synapses and the rat innervated hemidiaphragm.

2 Endogenous adenosine tonically inhibited synaptic transmission, since 0.5-2 uml^{-1} of adenosine deaminase increased both the population spike amplitude (30±4%) and field excitatory post-synaptic potential (f.e.p.s.p.) slope (27±4%) recorded from hippocampal slices and the evoked [^{3}H]-acetylcholine ([^{3}H]-ACh) release from the motor nerve terminals (25±2%).

3 α,β-Methylene adenosine diphosphate (AOPCP) in concentrations (100-200 μM) that almost completely inhibited the formation of adenosine from the extracellular catabolism of AMP, decreased population spike amplitude by 39±5% and f.e.p.s.p. slope by 32±3% in hippocampal slices and [^{3}H]-ACh release from motor nerve terminals by 27±3%.

4 Addition of exogenous 5'-nucleotidase (5 uml^{-1}) prevented the inhibitory effect of AOPCP on population spike amplitude and f.e.p.s.p. slope by 43-57%, whereas the P_2 antagonist, suramin (100 μM), did not modify the effect of AOPCP.

5 In both preparations, the effect of AOPCP resulted from prevention of adenosine formation since it was no longer evident when accumulation of extracellular adenosine was hindered by adenosine deaminase (0.5-2 uml^{-1}). The inhibitory effect of AOPCP was still evident when A_1 receptors were blocked by 1,3-dipropyl-8-cyclopentylxanthine (2.5-5 nM), but was abolished by the A_2 antagonist, 3,7-dimethyl-1-propargylxanthine (10 μM).

6 These results suggest that adenosine originating from catabolism of released adenine nucleotides preferentially activates excitatory A_2 receptors in hippocampal CA1 pyramid synapses and in phrenic motor nerve endings.

Keywords: Adenosine; ecto-nucleotidases; adenosine receptors; hippocampus; neuromuscular junction; acetylcholine release

Introduction

In the hippocampus and at neuromuscular junctions, extracellular adenosine can originate from the release of adenosine as such (Cunha & Sebastião, 1993; Lloyd *et al.*, 1993), in parallel with the formation of adenosine from released adenosine triphosphate (ATP) (Terrian *et al.*, 1989; Smith, 1991; Silinsky & Redman, 1995). The formation of extracellular adenosine from released ATP is carried out by an ecto-nucleotidase pathway which sequentially catabolises ATP into AMP and then into adenosine through the action of an ecto-5'-nucleotidase (Richardson *et al.*, 1987; Cunha & Sebastião, 1991), which is feed-forwardly inhibited by ATP and/or ADP (Naito & Lowenstein, 1985; Richardson *et al.*, 1987; Cunha & Sebastião, 1991).

The rat hippocampal Schaffer fibres/CA1 pyramid synapses (Cunha *et al.*, 1994a) and the rat innervated hemidiaphragm (Correia-de-Sá *et al.*, 1991), are two preparations where synaptic transmission is known to be modulated by both inhibitory adenosine A_1 receptors and excitatory adenosine A_{2a} receptors. We recently showed that in these preparations the action of extracellular endogenous adenosine results from a balance between tonic A_1/A_{2a} receptor activation (Cunha *et al.*, 1994b; Correia-de-Sá & Ribeiro, 1996). Thus, in view of the regulatory potential of the ecto-nucleotidase pathway for the accumulation of extracellular adenosine, we decided to investigate the role of adenosine formed from the ecto-nucleotidase pathway in relation to the differential activation of both inhibitory adenosine A_1 and excitatory A_{2a} receptors in these two preparations.

Preliminary accounts of some of the results have already been published (Correia-de-Sá *et al.*, 1994a; Cunha *et al.*, 1994d; Ribeiro *et al.*, 1994).

Methods

Electrophysiological recording of neuronal excitability in rat hippocampal slices

Male Wistar rats (140 g) were decapitated under halothane anaesthesia and the brain rapidly removed into ice-cold artificial cerebro-spinal fluid (CSF) of the following composition (mM): NaCl 115, KCl 3, KH_2PO_4 1.2, $MgSO_4$ 2, $CaCl_2$ 1.2, $NaHCO_3$ 25, glucose 10, pH 7.4, gassed with a 95% O_2 and 5% CO_2 mixture. One hippocampus was dissected free and slices (400 μm thick) were cut perpendicularly to the long axis of the hippocampus and allowed to recover for at least 1 h in gassed CSF, at room temperature. One slice was then transferred to a 1 ml recording chamber for submerged slices and continuously superfused with gassed CSF solution, kept at 30.5°C, at a flow rate of 3 ml/min. Drugs were added to this superfusion solution.

[1] Author for correspondence.

Electrophysiological recordings of field excitatory post-synaptic potentials (f.e.p.s.p.s.) and/or population spikes was as previously described (Sebastião *et al.*, 1990; Cunha *et al.*, 1994a). Monopolar stimulation (rectangular pulses of 0.1 ms applied once every 10 s) was delivered through an electrode placed on the Schaffer collateral/commissural fibres, in the *stratum radiatum* near the CA3/CA1 border. Orthodromically-evoked f.e.p.s.ps were recorded through an extracellular microelectrode (4 M NaCl, 2–4 MΩ resistance) placed in the *stratum radiatum* of the CA1 area, whereas simultaneous or alternative recording of orthodromically-evoked population spikes were through a similar extracellular microelectrode placed in the *stratum pyramidale* of the CA1 area. The intensity of the stimulus (120–600 μA) was adjusted to evoke either the largest f.e.p.s.p. without population spike contamination or a population spike with an amplitude near 50% of the population spike amplitude obtained with supramaximal stimulation. The individual responses were displayed on a Tektronix (2430A) digitalizing oscilloscope and the averages of 8 or 16 consecutive responses were digitally recorded on a Tekmate computer. f.e.p.s.p. responses were quantified either as the amplitude or as the initial slope of the averaged f.e.p.s.p.s whereas population spike responses were quantified as the amplitude of the averaged population spikes. In each preparation, perfusion of the slice with the tested drugs was begun only after a stable response was recorded.

When testing the ability of any drug (AOPCP, ADA, DPCPX, DMPX, 5'-nucleotidase, NBTI, EHNA or suramin) to modify the effect of purines (AMP, ADP, ATP, adenosine or AOPCP), the effect of the purine was tested first in the absence of other drugs; the purine was then washed out, and the drug was perfused until its full effect could be observed and for at least 20 min before perfusing the purine in the presence of the drug. The two substances were then washed out and the effect of the purine in the absence of other drugs was usually tested again.

[^{3}H]-acetylcholine release from rat innervated hemidiaphragm

The experiments were carried out on rat phrenic nerve-hemidiaphragm preparations (8 mm width) from Wistar rats of either sex of about 200 g in weight. The procedures used for labelling the preparations and measuring evoked [^{3}H]-acetylcholine ([^{3}H]-ACh) released were as previously described (Correia-de-Sá *et al.*, 1991) with minor modifications. Briefly, the preparations were superfused (3 ml min^{-1}) in 3 ml organ baths at 37°C with Tyrode solution continuously gassed with 95% O_2 and 5% CO_2, containing (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 11.2 and choline 0.001. After a 30 min equilibration period, the perfusion was stopped and the nerve endings were labelled during 40 min with 1 μM [^{3}H]-choline (specific activity 2.5 μCi $nmol^{-1}$) under electrical stimulation at 1 Hz with supramaximal rectangular pulses of 15 V and 40 μs duration. After the end of the labelling period, the preparations were again superfused (15 ml min^{-1}) and the nerve stimulation stopped. From this time onwards, hemicholinium-3 (10 μM) was present to prevent uptake of choline. After a 60 min period of washout, the perfusion was stopped, and 3 ml bath samples were collected every 3 min by emptying and refilling again the organ bath with the solution in use. Aliquots (1 ml) of the incubation medium were added to 6 ml of Packard Insta Gel II scintillator. Radioactivity was measured in a Beckman LS 3801 scintillation spectrophotometer.

The phrenic nerve was supramaximally stimulated with rectangular pulses of 40 μs duration and with a frequency of 5 Hz. The voltage amplitude was usually fixed at a value three times that which caused a maximal contraction, and did not exceed 15 V. Two stimulation periods were used: at 12 min (S_1) and at 39 min (S_2) after the end of washout (zero time). The number of pulses delivered in each train was 750. Note that electrical stimulation of the phrenic nerve increases the release of [^{3}H]-ACh, which is abolished in the absence of calcium ions in the bathing solution or by 0.3 μM tetrodotoxin, while the output of [^{3}H]-choline remains unchanged (Wessler & Kilbinger, 1986; Correia-de-Sá & Ribeiro, 1994). Therefore, evoked [^{3}H]-ACh release was calculated by subtracting the basal tritium outflow from the total tritrium outflow during the stimulation period (cf. Correia-de-Sá *et al.*, 1991). Averaged evoked [^{3}H]-ACh release was $30.3 \pm 1.7 \times 10^3$ d.p.m.g^{-1} of wet weight of preparation ($n = 19$). Tested purines were added 15 min before S_2 and were present up to the end of the experiments. Their effects were expressed by the ratios S_2/S_1, i.e. the ratio between the evoked [^{3}H]-ACh release during the second stimulation period (in the presence of the tested purines) and the evoked [^{3}H]-ACh release during the first stimulation period (without the tested purines). In control conditions, i.e. without addition of drugs, the S_2/S_1 value was 0.81 ± 0.03 ($n = 8$). When testing the ability of any drug (AOPCP, ADA, DPCPX, DMPX, NBTI or EHNA) to modify the effect of AMP or of AOPCP, the preparations were pretreated with these drugs 15 min before each stimulation period and were washed out immediately after each stimulation period, i.e. they were present in both S_1 and S_2. When the same drug was present in both S_1 and S_2, the S_2/S_1 ratios were not appreciably different from those obtained in control conditions, i.e. without addition of drugs.

Drugs and solutions

Adenosine, adenosine 5'-triphosphate disodium salt (ATP), adenosine 5'-monophosphate sodium salt (AMP), adenosine 5'-O-(α,β-methylene)-diphosphonate sodium salt (α,β-methylene ADP, AOPCP), 5'-nucleotidase (E.C. 3.1.3.5), S-(4-nitrobenzyl)-6-thioinosine (NBTI), choline chloride and hemicholinium-3 were from Sigma (Dorset, U.K.). Adenosine deaminase (ADA, type VI, 1803 u/ml, EC 3.5.4.4) was also purchased from Sigma (Dorset, U.K.), in a suspension in 50% (v/v) glycerol in potassium phosphate (pH 6.0) and dilutions of this suspension were used, together with appropriate corrections of glycerol and KH_2PO_4 content of all control solutions. 1,3-Dipropyl-8-cyclopentylxanthine (DPCPX), 3,7-dimethyl-1-propargylxanthine (DMPX) and suramin were from RBI (Natick, MA, U.S.A.). Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) was purchased from Burroughs Wellcome Co (Research Triangle Park, NC, U.S.A.) and [methyl-^{3}H]-choline chloride (ethanol solution, 80 Ci $mmol^{-1}$) was obtained from Amersham (Buckinghamshire, U.K.). All other reagents were of the highest purity available. DPCPX was made up into a 5 mM stock solution in 99% dimethylsulphoxide (DMSO) 1% NaOH (1 M) (v/v). NBTI and DMPX were made up into a 10 mM DMSO solution. ENHA was made up as a 5 mM solution in ethanol. All stock solutions were stored as frozen aliquots at −20°C. Aqueous dilutions of these stock solutions were made daily and appropriate solvent controls were done. The pH of the superfusion solution was not changed by the addition of the drugs in the maximum concentrations applied to the preparations.

Statistics

The data are expressed as mean ± s.e.mean from n observations. The significance of the means differences was calculated by Student's t test. $P < 0.05$ was considered to represent significant differences.

Results

Rat hippocampal slices – exogenously added purines

The amplitude of population spikes recorded in CA1 pyramids of rat hippocampal slices was inhibited by addition of either adenosine (2–12.6 μM; $EC_{50} = 6.3$ μM, $n = 4$), AMP (3–30 μM;

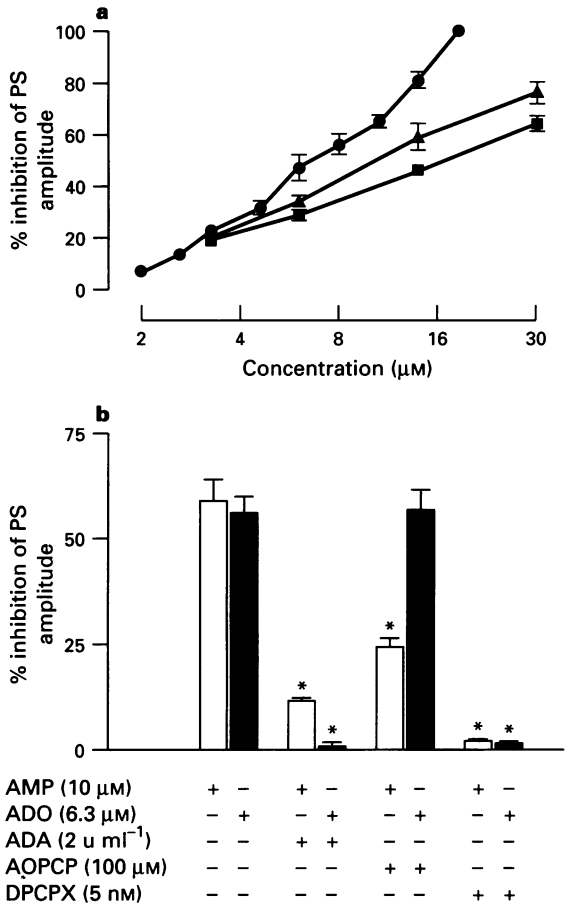

Figure 1 (a) Inhibition of the amplitude of population spikes (PS), recorded extracellularly in hippocampal CA1 pyramids, by exogenously added adenosine (●), AMP (▲) or ATP (■), and (b) modification of the inhibitory effect of AMP (10 μM, open columns) and adenosine (ADO, 6.3 μM, solid columns) by adenosine deaminase (ADA, the enzyme that converts adenosine into its inactive metabolite inosine), by the inhibitor of ecto-5'-nucleotidase, α,β-methylene ADP (AOPCP), or by the A_1 antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX). In (a), the ordinates represent the percentage of inhibition of population spike amplitude produced by adenosine, AMP or ATP in relation to the amplitude of the population spike in control conditions (i.e. in the absence of any added drug to the perfusion solution). In (b), the ordinates represent the percentage inhibition of population spike amplitude caused by adenosine or AMP in the presence of the drugs indicated below each column. 0% corresponds to the population spike amplitude in control conditions (i.e. without any added drug or upon addition of ADA, AOPCP or DPCPX) and 100% corresponds to blockade of population spikes. In each experiment, ADA, AOPCP or DPCPX were applied to the preparations 20–25 min before testing the effect of adenosine or AMP in their presence. The effect of adenosine or AMP in the absence and in the presence of ADA, AOPCP or DPCPX was always compared in the same experiment. $*P<0.05$ (Student's paired t test) when comparing with the effect of AMP or adenosine alone. The results are mean±s.e.mean of 2 to 8 experiments in (a) and of 3 to 4 experiments in (b). In (a) the s.e.means are shown when they exceed the symbols in size.

$EC_{50}=11.6$ μM) or ATP (3–30 μM; $EC_{50}=14.6$ μM) (Figure 1a). As shown in Figure 1b, the inhibitory effect of AMP (10 μM) was attenuated (80±2%, $n=3$) by ADA (2 u ml^{-1}), the enzyme that converts adenosine into its inactive metabolite, inosine (98±2% inhibition of the effect of 6.3 μM adenosine, $n=3$). The inhibitory effect of ATP (10 μM) was also attenuated (75±5%, $n=3$) by ADA (2 u ml^{-1}). AOPCP (100 μM), which inhibits the catabolism of AMP (10 μM) by 86% (Cunha *et al.*, 1992), attenuated the inhibitory effect of AMP (10 μM) by 58±6% ($n=4$) as well as the inhibitory effect of ATP (10 μM) by 51±8% ($n=3$). This ability of AOPCP to attenuate the inhibitory effect of ATP and AMP might not be attributed to the inhibitory effect of AOPCP itself, since AOPCP (100 μM) did not modify the inhibitory effect of adenosine (6.3 μM, $n=3$). Taken together, these observations suggest that ATP and AMP have to be catabolised into adenosine to inhibit neuronal excitability. Activation of A_1 receptors is probably responsible for the inhibitory effects of exogenously applied ATP, AMP and adenosine since DPCPX (5 nM), an A_1 antagonist in the hippocampus (see Sebastião *et al.*, 1990), prevented the inhibitory effects of AMP (96±1%, $n=3$), ATP (92±3%, $n=3$) and adenosine (97±1%, $n=4$) on the amplitude of population spikes in CA1 pyramids.

Rat hippocampal slices–endogenously released purines

To investigate the role of endogenous extracellular adenosine originating from released adenine nucleotides, we compared the effect of ADA, which removes all the endogenous extracellular adenosine, with the effect of AOPCP, which only prevents the formation of adenosine from the extracellular catabolism of adenine nucleotides. ADA (0.5–5 u mol^{-1}) increased population spike amplitude, and the maximal increase (30±4%, $n=11$), fully prevented by EHNA (25 μM), an inhibitor of ADA (Henderson *et al.*, 1977), was observed with 2 u ml^{-1} ADA (Table 1). A similar facilitatory effect was found with the A_1 adenosine receptor antagonist, DPCPX (5 nM, 22±5% increase, $n=5$), indicating that endogenous extracellular adenosine is doing an overall tonic inhibition of neuronal excitability, in agreement with previous reports (Mitchell *et al.*, 1993; Dunwiddie & Diao, 1994). As previously observed (Sebastião & Ribeiro, 1992; Cunha *et al.*, 1994b), DMPX (10 μM) caused a 14±2% facilitation of neuronal excitability ($n=4$), which could be attributed to its low A_2/A_1 selectivity (4–10 times, see Ukena *et al.*, 1986) and the preponderance of A_1 over A_2 receptors in the hippocampus (Cunha *et al.*, 1994a).

Surprisingly, the prevention of the formation of extracellular adenosine from endogenously released adenine nucleotides by AOPCP produced an inhibitory effect (Figure 2). Partial inhibition of ecto-5'-nucleotidase with 50 μM AOPCP (57% inhibition of 10 μM AMP catabolism, see Cunha *et al.*, 1992) produced a 16±1% ($n=3$) inhibition of population spike amplitude, while a maximal inhibition of ecto-5'-nucleotidase with 100 μM AOPCP (86% inhibition of 10 μM AMP catabolism, see Cunha *et al.*, 1992) produced a 39±5% inhibition of the amplitude of population spike ($n=17$) and a 32±3% inhibition of the f.e.p.s.p. slope (n = 10) (Table 1). The inhibitory effect of AOPCP could be attributed to: (1) the formation of adenosine from adenosine disphosphate (ADP), since the batches of AOPCP used are consistently contaminated with up to 2.7% ADP (1.0–2.7% as assessed by h.p.l.c., data not shown); (2) a direct effect of AOPCP on purinoceptors (see Wiklund *et al.*, 1985; Bailey & Hourani, 1992); (3) the prevention of the formation of adenosine from released adenine nucleotides, which would preferentially activate excitatory adenosine receptors.

To evaluate the hypothesis of ADP contamination, we tested the effect of ADP (2.5 μM) on neuronal excitability. ADP (2.5 μM) inhibited population spike amplitude by 14±3% ($n=4$), but in the presence of AOPCP (100 μM), ADP (2.5 μM) inhibited the amplitude of population spikes by only 6±2% ($n=3$) and, thus, cannot account for the inhibitory effect of AOPCP. To exclude further the contribution of ADP contamination to the effect of AOPCP, we tested the effect of AOPCP on the amplitude of population spike in the presence of DPCPX, at a concentration (5 nM) that fully antagonizes maximally effective concentrations of 2-chloroadenosine on population spike amplitude (Sebastião *et al.*, 1990). In these conditions, AOPCP (100 μM) still inhibited the amplitude of the population spike by 23±5% ($n=5$) (Figure 3a), indicating that the formation of adenosine from contaminant ADP was not the main cause of the inhibitory effect of AOPCP. AOPCP

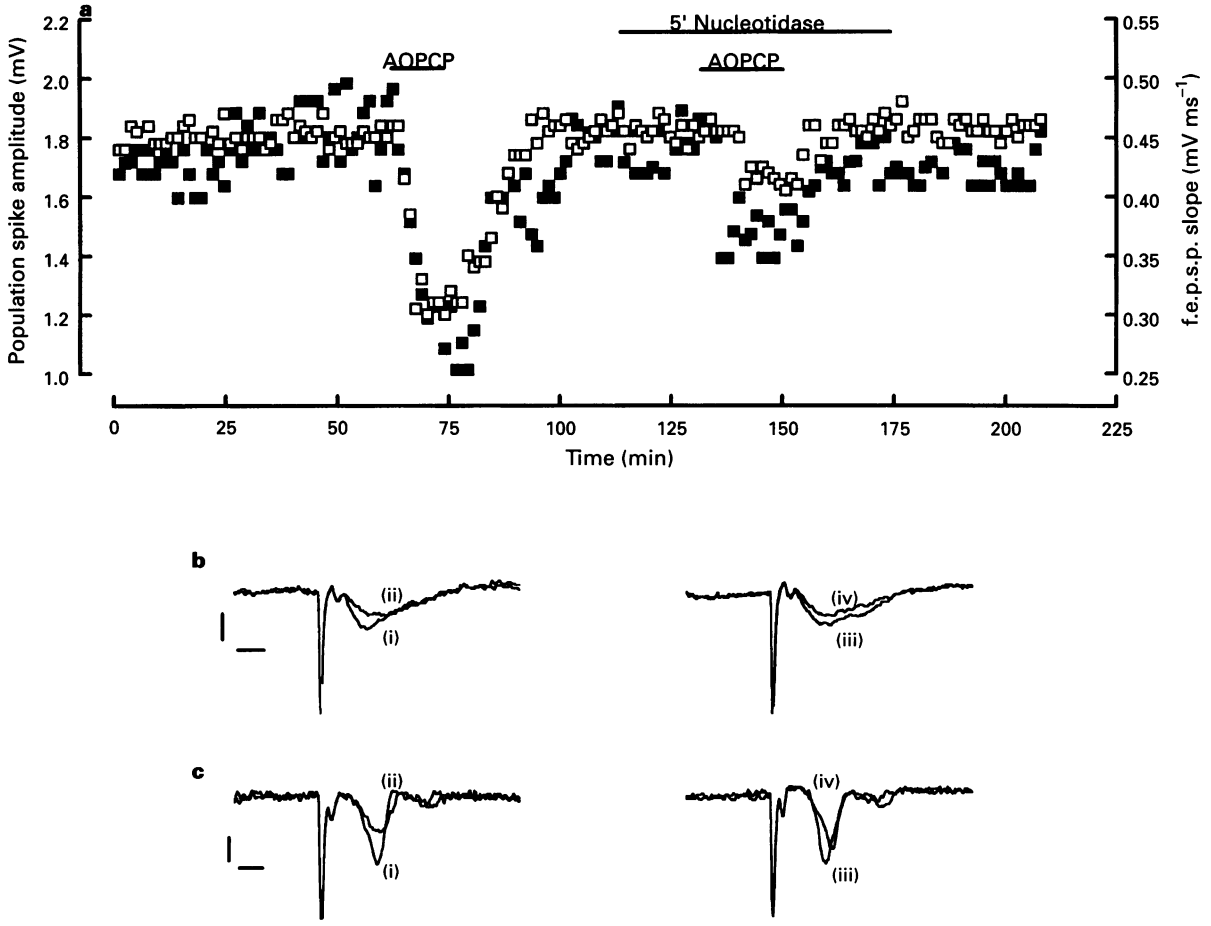

Figure 2 Effect of the inhibitor of ecto-5'-nucleotidase, α,β-methylene ADP (AOPCP) on population spike amplitude (□) and field excitatory postsynaptic potential (f.e.p.s.p.) slope (■), and ability of exogenously added 5'-nucleotidase to attenuate these inhibitory effects. In panel (a) is shown the time course of the amplitude of averages of 8 consecutive population spikes and the slope of averages of 8 consecutive f.e.p.s.p's recorded simultaneously from the CA1 area of the hippocampus. The hippocampal slice was perfused with AOPCP (100 μM) either in the absence or in the presence of 5'-nucleotidase (5 u ml^{-1}), as indicated by the bars above the figure. In panels (b) and (c) are shown recordings of f.e.p.s.p's (b) and population spikes (c), corresponding to 60 min ((i) control), 80 min ((ii) AOPCP), 130 min ((iii) 5'-nucleotidase) and 150 min ((iv) 5'-nucleotidase + AOPCP) in (a). Each recording consists of a stimulus artefact, followed by the presynaptic volley and either f.e.p.s.p. (b) or the population spike (c), and correspond to the average of 8 consecutive responses. The calibration bars in (b) correspond to 500 μV and 5 ms and in (c) correspond to 500 μV and 10 ms.

is also probably not affecting the removal of endogenous adenosine since the simultaneous blockade of the nucleoside transport, with 10 μM NBTI, and of adenosine deamination, with 25 μM ENHA (which by themselves produced a 10–13% inhibition of population spike amplitude and f.e.p.s.p. slope), did not alter the effect of AOPCP on synaptic transmission and neuronal excitability ($n=2$). A direct action of AOPCP on P_2-purinoceptors (hypothesis 2) also seems unlikely, since the antagonist of P_2-purinoceptors, suramin (100 μM) (which by itself produced a 20–25% inhibition of f.e.p.s.p. slope), did not modify the effect of 100 μM AOPCP ($n=2$). The observation that ADA (2 u ml^{-1}) markedly inhibited the effect of 100 μM AOPCP on population spike amplitude (84±5%, $n=3$) (Figure 3a) also argues against a direct effect of AOPCP on purinoceptors. This attenuation of the effect of AOPCP by ADA is compatible with hypothesis 3. Thus, when extracellular adenosine accumulation was prevented with ADA, the physiological consequence of the prevention of adenosine formation from released adenine nucleotides, is no longer evident. The observation that the effect of AOPCP (100 μM) on f.e.p.s.p. slope and population spike amplitude was attenuated (43–57%, $n=2$) by exogenously added 5'-nucleotidase (5 u ml^{-1}) (Figure 2) further suggests that AOPCP depresses synaptic transmission and neuronal excitability by inhibiting ecto-5'-nucleotidase. To investigate whether the inhibitory effect of AOPCP could result from inhibition of adenosine formation and, therefore, prevention of the activation of the excitatory adenosine receptors, we used the A_2 antagonist, DMPX. The concentration of DMPX used (10 μM) almost completely antagonized the excitatory effect of the A_{2a} selective agonist, CGS 21680 (10–30 nM), in CA1 pyramid synapses (Sebastião & Ribeiro, 1992). As shown in Figure 3a, DMPX (10 μM) prevented by 94±2% ($n=3$) the inhibitory effect of AOPCP (100 μM).

Rat innervated hemidiaphragm – endogenously released purines

As shown in Table 1, the modulation of electrically-evoked [^{3}H]-acetylcholine ([^{3}H]-ACh) release by endogenous extracellular adenosine at the rat innervated hemidiaphragm was similar to that observed in the CA1 pyramid synapses. ADA, at a concentration of 0.5 u ml^{-1}, maximally enhanced neurotransmitter release (Table 1), indicating that endogenous extracellular adenosine is producing an overall tonic inhibition of evoked [^{3}H]-ACh release. In contrast, prevention by AOPCP of adenosine formation from the extracellular catabolism of released adenine nucleotides, significantly reduced the release of [^{3}H]-ACh (Table 1). Partial inhibition of ecto-5'-nucleotidase with 50 μM AOPCP (57% inhibition of 10 μM AMP

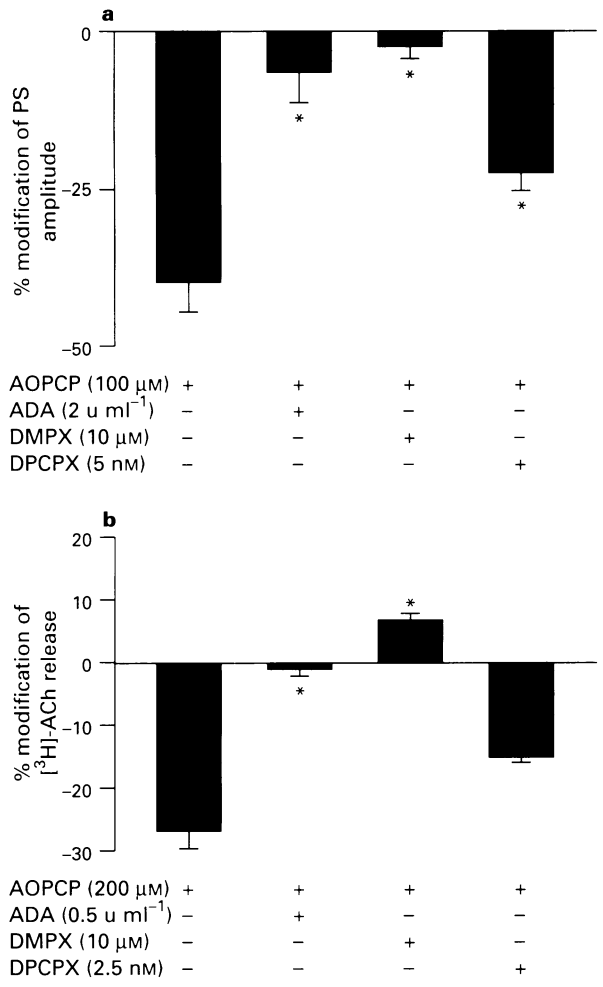

Figure 3 Attenuation of the inhibitory effect of α,β-methylene ADP (AOPCP) on hippocampal excitability (a) and on [^{3}H]-acetylcholine ([^{3}H]-ACh) release from motor nerve terminals (b) caused by the enzyme that converts adenosine into its inactive metabolite inosine, adenosine deaminase (ADA, 2 u ml^{-1}), by the A_2 antagonist, 3,7-dimethyl-1-propargylxanthine (DMPX), or by the A_1 antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX). In (a), the ordinates represent percentage modification of the population spike (PS) amplitude caused by AOPCP (100 μM) alone (left column) and by AOPCP (100 μM) in the presence of the drug indicated below each column. In each experiment, drugs were applied to the preparations 20–25 min before testing the effect of AOPCP in their presence. The effect of AOPCP in the absence and in the presence of the tested drugs was always compared in the same experiment. In (b), the ordinates represent percentage modification of the S_2/S_1 ratio caused by AOPCP (200 μM) alone (left column) and AOPCP (200 μM) in the presence of the drug indicated below each column. AOPCP was added 15 min before S_2, and ADA, DMPX or DPCPX were added 15 min before and were immediately washed out after each stimulation period (S_1 and S_2); the S_2/S_1 ratios obtained in these conditions, i.e. with ADA, DMPX or DPCPX present in both S_1 and S_2, were not statistically different from the ratio obtained in control conditions. *$P<0.05$ when compared with the inhibitory effect of AOPCP alone. The results are mean ± s.e.mean of 3 experiments in (a) and of 2 to 4 experiments in (b).

Table 1 Comparison of the effect of removal of all endogenous extracellular adenosine by use of adenosine deaminase (ADA), and removal of only the extracellular adenosine originating from the catabolism of released adenine nucleotides by α,β-methylene ADP (AOPCP), on hippocampal excitability and on [^{3}H]-acetylcholine ([^{3}H]-ACh) release from motor nerve terminals

	% change of population spike amplitude in hippocampal slices	*% change of f.e.p.s.p. slope in hippocampal slices*	*% change of [^{3}H]-ACh release from motor nerve terminals*
ADA	30 ± 4%*	27 ± 4%*	25 ± 2%*
AOPCP	−39 ± 5%*	−32 ± 3%*	−27 ± 3%*

In hippocampal slices, the effect of ADA (2 u ml^{-1}) on population spike amplitude and on field excitatory post-synaptic potential (f.e.p.s.p.) slope was derived from 11 and 15 experiments respectively, and that of AOPCP (100 μM) from 17 and 10 experiments respectively. In innervated hemidiaphragm, the effect of ADA (0.5 u ml^{-1}) was derived from 5 experiments, and that of AOPCP (200 μM) from 4 experiments. Note that positive values indicate excitatory effects and negative values indicate inhibitory effects. The results are mean ± s.e.mean. *$P<0.05$ as compared with 0%.

catabolism, see Cunha & Sebastião, 1991) produced a 21 ± 3% ($n=4$) inhibition of [^{3}H]-ACh release, while a complete blockade of ecto-5′-nucleotidase with 200 μM AOPCP (100% inhibition of 10 μM AMP catabolism, see Cunha & Sebastião, 1991) produced a 27 ± 3% ($n=4$) inhibition of [^{3}H]-ACh release.

As in hippocampal slices, pharmacological modification of the inhibitory effect of AOPCP on [^{3}H]-ACh release suggests that the effect of AOPCP was mostly due to a reduction of the excitatory tonus produced by endogenous extracellular adenosine originating from the catabolism of released adenine nucleotides. Thus, as shown in Figure 3b, AOPCP (200 μM) still inhibited by 15 ± 1% ($n=2$) [^{3}H]-ACh release in the presence of the A_1 antagonist, DPCPX (2.5 nM); in the presence of ADA (0.5 u ml^{-1}), AOPCP (200 μM) produced virtually no effect (−1 ± 1% of control, $n=4$), and the A_2 antagonist, DMPX (10 μM) transformed the inhibitory effect of AOPCP (200 μM) into a small facilitatory action (7 ± 1%, $n=3$, $P<0.05$). The possible interference of AOPCP with adenosine inactivation mechanisms was excluded since the simultaneous presence of NBTI (30 μM) and ENHA (50 μM) (which by themselves enhanced transmitter release by about 60%, see Correia-de-Sá & Ribeiro, 1996) did not significantly change the inhibitory effect of AOPCP ($n=5$).

Rat innervated hemidiaphragm – exogenously added purines

In contrast to what was observed in hippocampal slices where both AMP and adenosine consistently inhibited synaptic transmission, exogenously added AMP and adenosine produced different patterns of modulation of [^{3}H]-ACh release in the rat innervated hemidiaphragm. As shown in Figure 4a, exogenously added AMP (1–500 μM) enhanced the evoked [^{3}H]-ACh release from motor nerve endings. Maximum enhancement of [^{3}H]-ACh release was observed with 30 μM and 100 μM of AMP; 500 μM AMP caused a smaller effect than 100 μM, but still enhanced [^{3}H]-ACh release. This facilitatory effect of AMP contrasts with the biphasic effect obtained with exogenously added adenosine (10–500 μM): adenosine (30 μM) reduced evoked [^{3}H]-ACh release by 32 ± 3% ($n=4$) through activation of A_1 receptors, while at high concentrations (100–500 μM), adenosine consistently increased evoked [^{3}H]-ACh release through activation of A_{2a} receptors (Correia-de-Sá & Ribeiro, 1996).

As shown in Figure 4b, the facilitatory effect of 100 μM AMP (31 ± 5% of control, $n=4$) was prevented by ADA (0.5 u ml^{-1}). AOPCP (200 μM), which blocks ecto-5′-nucleotidase at neuromuscular junctions (Cunha & Sebastião, 1991; Smith, 1991), prevented the excitatory effect of 100 μM AMP (Figure 4b). These observations suggest that AMP has to be catabolised into adenosine to enhance ACh release. The

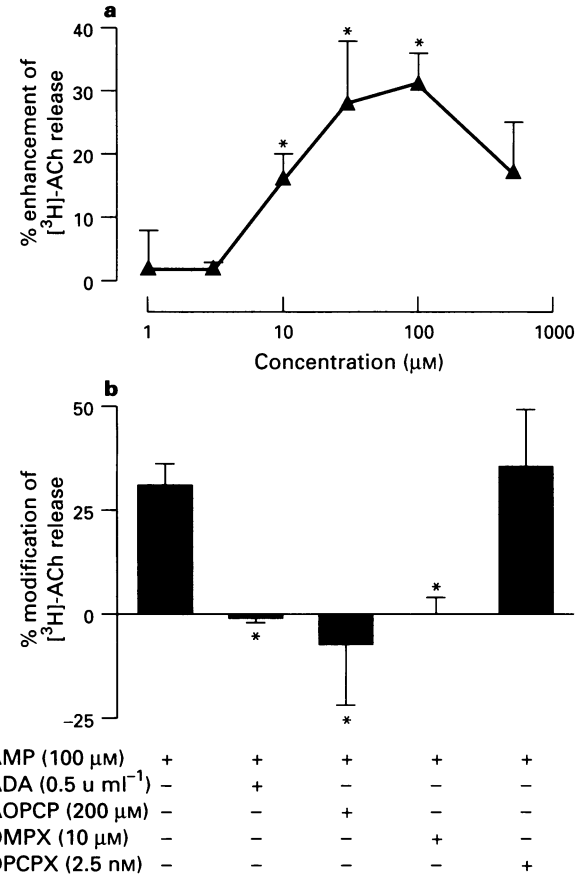

Figure 4 (a) Facilitation of the evoked (5 Hz, 750 pulses, 40 μs duration) release of [^{3}H]-acetylcholine ([^{3}H]-ACh) from phrenic nerve endings by exogenously added AMP, and (b) modification of the facilitatory effect of AMP by adenosine deaminase (ADA, the enzyme that converts adenosine into its inactive metabolite inosine), by the inhibitor of ecto-5′-nucleotidase, α,β-methylene ADP (AOPCP), by the A_2 antagonist, 3,7-dimethyl-1-propargylxanthine (DMPX), or by the A_1 antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX). In (a), the ordinates represent the percentage increase of the S_2/S_1 ratio produced by AMP added 15 min before S_2. The average S_2/S_1 ratio in control conditions (i.e. absence of AMP) was 0.81 ± 0.03 ($n=8$). In (b), the ordinates are percentage changes in the S_2/S_1 ratio caused by AMP (100 μM) alone (left column) and by AMP (100 μM) in the presence of the drug indicated below each column. AMP was added 15 min before S_2 and ADA, AOPCP, DMPX or DPCPX were added 15 min before and were immediately washed out after each stimulation period (S_1 and S_2); the S_2/S_1 ratio obtained in these conditions, i.e. with ADA (0.5 u ml^{-1}), AOPCP (200 μM), DMPX (10 μM) or DPCPX (2.5 nM) present in both S_1 and S_2, were not statistically different from the ratio obtained in control conditions. $^*P<0.05$ when compared with zero percentage in (a) or when compared with the facilitatory effect of AMP alone in (b). The results are mean ± s.e.mean of 2 to 4 experiments in (a) and of 3 to 5 experiments in (b).

AMP-induced facilitation of evoked [^{3}H]-ACh release is probably mediated by an A_2 receptor, since it was prevented by the A_2 antagonist, DMPX (Figure 4b), when this compound was applied in a concentration (10 μM) which completely blocked the facilitatory effect of the selective adenosine A_{2a} receptor agonist, CGS 21680 (3 nM), on evoked [^{3}H]-ACh release from the phrenic motor nerve terminals (Correia-de-Sá & Ribeiro, 1994). In contrast, the A_1 antagonist, DPCPX (2.5 nM), failed to modify the facilitatory effect of AMP (100 μM) on evoked neurotransmitter release ($n=3$) (Figure 4b). Both antagonists, in the concentrations described here, and when applied in both stimulation periods, had no effect on the S_2/S_1 ratio.

Discussion

The present results show that the inhibitor of ecto-5′-nucleotidase, α,β-methylene adenosine diphosphate (AOPCP) (Naito & Lowenstein, 1985), inhibited synaptic transmission in both rat hippocampal CA1 pyramid synapses and at the rat innervated hemidiaphragm. The prevention of the inhibitory effect of AOPCP by adenosine deaminase (ADA) indicates that the effect of AOPCP is mediated by changes in the levels of endogenous extracellular adenosine. This effect of AOPCP is probably due to its ability to inhibit ecto-5′-nucleotidase since exogenously added 5′-nucleotidase attenuated the inhibitory effect of AOPCP. The finding that the effect of AOPCP was abolished by the adenosine A_2 antagonist, DMPX, suggests that AOPCP inhibits synaptic transmission by precluding the activation of excitatory adenosine receptors. These observations suggest that the effect of AOPCP is due to its ability to block the formation of adenosine from catabolism of released adenine nucleotides, and that this catabolism is tightly coupled to activation of the excitatory adenosine receptors. Thus, the present results constitute the first demonstration that excitatory adenosine receptors can be activated by endogenous adenosine in CNS glutamatergic synapses. The excitatory adenosine receptors that enhance synaptic transmission in both the Schaffer fibres/CA1 pyramid synapses (Cunha *et al.*, 1994a) and at the innervated hemidiaphragm (Correia-de-Sá *et al.*, 1991) are of the A_{2a} subtype. It thus seems likely that the excitatory receptors activated by adenosine formed from the extracellular catabolism of released adenine nucleotides are of the A_{2a} subtype.

That a direct action of AOPCP on P_2-purinoceptors could explain its inhibitory effects seems unlikely, since the effect of AOPCP was not prevented by the P_2-purinoceptor antagonist, suramin, and was prevented by ADA and by DMPX. This could suggest that AOPCP was catabolized into adenosine, which would then activate the inhibitory adenosine receptors. However, formation of adenosine from catabolism of AOPCP was not detected (data not shown; detection limit for adenosine used was 20 nM; see Cunha & Sebastião, 1993), and it was not expected to occur due to the resistance of the methylene bond between the phosphorus α and β of this compound (Cascalheira & Sebastião, 1992). Moreover, the effect of AOPCP was not prevented by the A_1 antagonist, DPCPX in concentrations (2.5–5 nM) which block the inhibitory effect of maximally effective concentrations of the 2-chloroadenosine in both preparations (Sebastião *et al.*, 1990). An effect of AOPCP on the pathways of endogenous extracellular adenosine removal can also be excluded since the adenosine transport inhibitor, NBTI, and the adenosine deaminase inhibitor, EHNA, failed to modify the effect of AOPCP. These observations, together with the inability of AOPCP to affect the action of adenosine also rule out the possibility that the effect of AOPCP might be due to modification of A_1 receptor function, and also exclude that the ADP contamination of AOPCP could account for the effect of AOPCP. A direct action of AOPCP on A_2 receptors can be excluded due to the prevention of the effect of AOPCP by ADA; moreover, AOPCP does not affect binding of the A_{2a} agonist, [^{3}H]-CGS21680 (Pirotton & Boeynaems, 1993). Finally, the observation that DMPX prevented the effect of AOPCP while DPCPX only attenuated it, suggests that, in spite of the poor A_2/A_1 selectivity of DMPX (Ukena *et al.*, 1986), this xanthine is acting by impairment of A_2 receptor activation. A possible reason for the ability of DPCPX to attenuate the effect of AOPCP could be the existence of A_1/A_2 receptor interaction (see Cunha *et al.*, 1994a; Correia-de-Sá & Ribeiro, 1996), so that tonic A_1 receptor activation might be needed to enhance A_2 activation. This would also account for the absence of facilitatory action of exogenously added adenosine in the presence of DPCPX.

In both preparations, removal of all the endogenous extracellular adenosine with ADA had an opposite effect to that of removing only the adenosine that originates from the catabolism of released adenine nucleotides. In CNS preparations,

global stimulation of the preparations leads to a predominant release of adenosine as such with little contribution of catabolism of released adenine nucleotides to adenosine formation (Pedata *et al.*, 1990; Lloyd *et al.*, 1993); but if focal stimulation of these preparations is used, or if isolated nerve endings are stimulated, both the release of adenosine and of adenine nucleotides occurs (Wieraszko *et al.*, 1989; White & MacDonald, 1990), and catabolism of released adenine nucleotides constitute a major source of endogenous extracellular adenosine (Richardson *et al.*, 1987; White & MacDonald, 1990; Cunha *et al.*, 1994c). The feed-forward inhibition of ecto-5'-nucleotidase by ATP and/or ADP tightly regulates the concentration of adenosine formed from released adenine nucleotides, according to the amounts of ATP released (Meghji *et al.*, 1992; James & Richardson, 1993): if the amount of ATP released is small, adenosine will be formed in a 'linear' manner, but if the amount of ATP released is great, extracellular AMP will accumulate until the levels of ATP and ADP fall below the values required to inhibit ecto-5'-nucleotidase; thereafter, reduction of ecto-5'-nucleotidase inhibition will force a burst-like formation of adenosine, which will reach a transient high concentration in the synaptic cleft (James & Richardson, 1993), sufficient to activate excitatory A_2 receptors. Away from active zones, adenosine is probably mainly released as such (see Mitchell *et al.*, 1993) and, therefore, formed in a more diffuse manner, which reaches concentrations sufficient only to activate inhibitory A_1 receptors. The use of ADA would remove the preponderant inhibitory homeostatic tonus (Dunwiddie & Diao, 1994), whereas AOPCP would relieve mainly the tonic excitatory synaptic control of neurotransmitter release.

At the rat neuromuscular junction, low concentrations of adenosine (10–100 μM) inhibit [^{3}H]-acetylcholine release by activating A_1 receptors, while high concentrations (>100 μM) enhance [^{3}H]-acetylcholine release by activating A_{2a} receptors (Correia-de-Sá & Ribeiro, 1996). Release of ATP and feed-forward inhibition of ecto-5'-nucleotidase, which lead to a burst-like formation of massive amounts of adenosine, may be a strategy to form high enough local concentrations of adenosine to activate A_{2a} receptors. Thus, one would expect that A_{2a} receptor activation might play an important role in facilitatory mechanisms of synaptic transmission derived from increasing frequencies of stimulation, such as tetanic facilitation. According to this idea, we have recently reported that increasing the stimulation pulse duration (to 1 ms) or during high frequency (>10 Hz) stimulation of the rat motor nerve terminals, the tonic adenosine A_{2a} receptor-mediated facilitatory effect on neurotransmitter release predominates (Correia-de-Sá *et al.*, 1994b) over the tonic A_1 inhibition. Making a parallel with the neuromuscular junction, one can speculate on the possible role of activation of excitatory A_2 receptors by adenosine generated from the catabolism of released adenine nucleotides in synaptic plasticity phenomena in hippocampal CA1 pyramid synapses. At these synapses, long-term potentiation (LTP) stimulation paradigms produce a massive release of adenine nucleotides (Wieraszko *et al.*, 1989), and LTP is modulated by excitatory A_{2a} receptor activation (de Mendonça & Ribeiro, 1994).

Another interesting point in the present work was the observation that, in hippocampal slices, exogenously added and endogenously released adenine nucleotides produced opposite effects as modulators of neuronal excitability. This is probably due to the need of exogenously added adenine nucleotides to cross several layers of cells in the slice before reaching the group of synapses whose activity is being recorded. The more common localization of ecto-5'-nucleotidase is in glial cells (Zimmermann, 1992), although ecto-5'-nucleotidase is also located on hippocampal nerve terminals (Cunha *et al.*, 1992; Zimmermann *et al.*, 1993). Thus, exogenously added adenine nucleotides could be efficiently converted into adenosine, not reaching the nerve endings as adenine nucleotides. Adenosine would activate the most abundant (Cunha *et al.*, 1994a), synaptically and mainly non-synaptically-located (Tetzlaff *et al.*, 1987; Swanson *et al.*, 1995) adenosine receptors, the inhibitory A_1 receptors in the hippocampus. In contrast, endogenously released adenine nucleotides will probably be released near the synaptically located ecto-5'-nucleotidase, which, as described in this paper, appears to be closely associated with excitatory A_2 receptors. At the neuromuscular junction, no inconsistency was observed between the effect of exogenously added and endogenously released adenine nucleotides, probably as a consequence of both the lower ratio of extra-synaptic/synaptic ecto-5'-nucleotidase, and the thinner preparation.

It is worthwhile noting that at the frog motor nerve endings (Ribeiro & Sebastião, 1987; Redman & Silinsky, 1994) as well as at the mouse phrenic nerve endings (Silinsky & Redman, 1995) stimulated at moderate frequencies (0.5–1 Hz), AOPCP *increases* acetylcholine release, whereas at rat phrenic nerve endings stimulated at higher frequencies (5 Hz) we observed that AOPCP *decreased* acetylcholine release. Since release of ATP from motor nerve endings is greater the greater the frequency of stimulation (Cunha & Sebastião, 1993) and bolus release of ATP, with the consequent feed-forward inhibition of ecto-5'-nucleotidase, will force a burst-like formation of adenosine (James & Richardson, 1993), it is possible that at moderate frequencies of motor nerve stimulation the amounts of adenosine formed from released adenine nucleotides are not high enough to activate excitatory receptors. However, the frequency of neuronal stimulation might not be the only variable that influenced the effect of AOPCP, since at the CA1 pyramid synapses, we could observe an inhibitory effect of AOPCP, even at low frequency (0.1 Hz) of stimulation. Also, in rat cholinergic nerve terminals of the hippocampus (Cunha *et al.*, 1994c) or striatum (Richardson *et al.*, 1987), the activity of ecto-nucleotidases is linked to activation of inhibitory adenosine A_1 receptors. Before it is possible to predict the importance of the differential activity of the ecto-nucleotidase pathway *vis à vis* differential activation of A_1 or A_2 subtypes of adenosine receptors, it is necessary to know the location and relative densities, of ecto-5'-nucleotidase, in relation to A_1 and of A_2 receptors in a synapse, as well as the relative affinity and efficacy of adenosine for the inhibitory A_1 and excitatory A_2 receptors.

We gratefully acknowledge the skilful technical assistance of Dr M.A. Timóteo in performing [^{3}H]-acetylcholine release experiments. The work was supported by Gulbenkian Foundation, JNICT and EU. R.A.C. is supported by PRAXIS XXI.

References

BAILEY, S.J. & HOURANI, S.M.O. (1992). Effects of purines on the longitudinal muscle of the rat colon. *Br. J. Pharmacol.*, **105**, 885–892.

CASCALHEIRA, J.F. & SEBASTIÃO, A.M. (1992). Adenine nucleotide analogues, including γ-phosphate-substituted analogues, are metabolised extracellularly in innervated frog sartorius muscle. *Eur. J. Pharmacol.*, **222**, 49–59.

CORREIA-DE-SÁ, P. & RIBEIRO, J.A. (1994). Tonic adenosine A_{2A} receptor activation modulates nicotinic autoreceptor function at the rat neuromuscular junction. *Eur. J. Pharmacol.*, **271**, 349–355.

CORREIA-DE-SÁ, P. & RIBEIRO, J.A. (1996). Adenosine uptake and deamination regulate tonic A_{2a}-receptor facilitation of evoked [^{3}H]-ACh release from the motor nerve terminals. *Neuroscience*, **73**, 85–92.

CORREIA-DE-SÁ, P., SEBASTIÃO, A.M. & RIBEIRO, J.A. (1991). Inhibitory and excitatory effects of adenosine receptor agonists on evoked transmitter release from phrenic nerve endings of the rat. *Br. J. Pharmacol.*, **103**, 1614–1620.

CORREIA-DE-SÁ, P., TIMÓTEO, M.A. & RIBEIRO, J.A. (1994a). Role of the ecto-nucleotidase pathway on adenosine neuromodulation at the rat neuromuscular junction: preferential activation of A_{2a}-facilitatory receptors. *Can. J. Physiol. Pharmacol.*, **72**,(Suppl. 1), 426.

CORREIA-DE-SÁ, P., TIMÓTEO, M.A. & RIBEIRO, J.A. (1994b). Tonic adenosine A_1-inhibition and tonic A_{2A}-facilitation of acetylcholine release from the rat motor nerve endings. *Drug. Dev. Res.*, **31**, 258.

CUNHA, R.A., JOHANSSON, B., VAN DER PLOEG, I., SEBASTIÃO, A.M., RIBEIRO, J.A. & FREDHOLM, B.B. (1994a). Evidence for functionally important adenosine A_{2a} receptor in the rat hippocampus. *Brain Res.*, **649**, 208–216.

CUNHA, R.A., MILUSHEVA, E., VIZI, E.S., RIBEIRO, J.A. & SEBASTIÃO, A.M. (1994b). Excitatory and inhibitory effects of A_1 and A_{2A} adenosine receptor activation on the electrically evoked [^{3}H]acetylcholine release from different areas of the rat hippocampus. *J. Neurochem.*, **63**, 207–214.

CUNHA, R.A., RIBEIRO, J.A. & SEBASTIÃO, A.M. (1994c). Purinergic modulation of the evoked release of [^{3}H]acetylcholine from the hippocampus and cerebral cortex of the rat: role of the ectonucleotidases. *Eur. J. Neurosci.*, **6**, 33–42.

CUNHA, R.A. & SEBASTIÃO, A.M. (1991). Extracellular metabolism of adenine nucleotides and adenosine in the innervated skeletal muscle of the frog. *Eur. J. Pharmacol.*, **197**, 83–92.

CUNHA, R.A. & SEBASTIÃO, A.M. (1993). Adenosine and adenine nucleotides are independently released from both the nerve terminals and the muscle fibres upon electrical stimulation of the innervated skeletal muscle of the frog. *Pflügers Arch. Eur. J. Physiol.*, **424**, 503–510.

CUNHA, R.A., SEBASTIÃO, A.M. & RIBEIRO, J.A. (1992). Ecto-5'-nucleotidase is associated with cholinergic nerve terminals in the hippocampus but not in the cerebral cortex of the rat. *J. Neurochem.*, **59**, 657–666.

CUNHA, R.A., SEBASTIÃO, A.M. & RIBEIRO, J.A. (1994d). Opposite effects of exogenous and endogenous adenine nucleotides as modulators of neuronal excitability in the rat hippocampus. *Can. J. Physiol. Pharmacol.*, **72**,(Suppl. 1), 502.

DE MENDONÇA, A. & RIBEIRO, J.A. (1994). Endogenous adenosine modulates long-term potentiation in the hippocampus. *Neuroscience*, **62**, 385–390.

DUNWIDDIE, T.V. & DIAO, L. (1994). Extracellular adenosine concentrations in hippocampal brain slices and the tonic inhibitory modulation of evoked excitatory responses. *J. Pharmacol. Exp. Ther.*, **268**, 537–545.

HENDERSON, J.F., BROX, L., ZOMBOR, G. & HUNTING, D. (1977). Specificity of adenosine deaminase inhibitors. *Biochem. Pharmacol.*, **26**, 1967–1972.

JAMES, S. & RICHARDSON, P.J. (1993). Production of adenosine from extracellular ATP at striatal cholinergic synapse. *J. Neurochem.*, **60**, 219–227.

LLOYD, H.G.E., LINDSTRÖM, K. & FREDHOLM, B.B. (1993). Intracellular formation and release of adenosine from rat hippocampal slices evoked by electrical stimulation or energy depletion. *Neurochem. Int.* **23**, 173–185.

MEGHJI, P., PEARSON, J.D. & SLAKEY, L.L. (1992). Regulation of extracellular adenosine production by ectonucleotidases of adult rat ventricular myocytes. *Am. J. Physiol.*, **263**, H40–H47.

MITCHELL, J.B., LUPICA, C.R. & DUNWIDDIE, T.V. (1993). Activity-dependent release of endogenous adenosine modulates synaptic responses in the rat hippocampus. *J. Neurosci.*, **13**, 3439–3447.

NAITO, Y. & LOWENSTEIN, J.M. (1985). 5'-Nucleotidase from rat heart membranes. Inhibition by adenine nucleotides and related compounds. *Biochem. J.*, **226**, 645–651.

PEDATA, F., PAZZAGLI, M., TILLI, S. & PEPEU, G. (1990). Regional differences in the electrically stimulated release of endogenous and radioactive adenosine and purine derivatives from rat brain slices. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **342**, 447–453.

PIROTTON, S. & BOEYNAEMS, J.M. (1993). Evidence that ATP, ADP and AMP are not ligands of the striatal adenosine A_{2A} receptors. *Eur. J. Pharmacol.*, **241**, 55–61.

REDMAN, R.S. & SILINSKY, E.M. (1994). ATP released together with acetylcholine as the mediator of neuromuscular depression at frog motor nerve endings. *J. Physiol.*, **477**, 117–127.

RIBEIRO, J.A., CORREIA-DE-SÁ, P., CUNHA, R.A. & SEBASTIÃO, A.M. (1994). Adenosine originated from released adenine nucleotides catabolism preferentially activates excitatory A_2 receptors in both the rat neuromuscular junction and hippocampal slices. *Drug Dev. Res.*, **31**, 313, (Abs. 1322).

RIBEIRO, J.A. SEBASTIÃO, A.M. (1987). On the role, inactivation and origin of endogenous adenosine at the frog neuromuscular junction. *J. Physiol.*, **384**, 571–585.

RICHARDSON, P.J., BROWN, S.J., BAILYES, E.M. & LUZIO, J.P. (1987). Ectoenzymes control adenosine modulation of immunoisolated cholinergic synapses. *Nature*, **327**, 232–234.

SEBASTIÃO, A.M. & RIBEIRO, J.A. (1992). Evidence for the presence of excitatory A2 adenosine receptors in the rat hippocampus. *Neurosci. Lett.*, **138**, 41–44.

SEBASTIÃO, A.M., STONE, T.W. & RIBEIRO, J.A. (1990). The inhibitory adenosine receptor at the neuromuscular junction and hippocampus of the rat: antagonism by 1,3,8-substituted xanthines. *Br. J. Pharmacol.*, **101**, 453–459.

SILINSKY, E.M. & REDMAN, R.S. (1995). Adenosine derived from neurally released ATP is the physiologic cause of skeletal neuromuscular depression. In *Adenosine and Adenine Nucleotides: from Molecular Biology to Integrative Physiology*, ed. Belardinelli, L. & Pelleg, A., pp. 113–120. Boston: Kluwer Academic Publishers.

SMITH, D.O. (1991). Sources of adenosine released during neuromuscular transmission in the rat. *J. Physiol.*, **432**, 343–354.

SWANSON, T.H., DRAZBA, J.A. & RIVKEES, S.A. (1995). Adenosine A1 receptors are located predominantly on axons in the rat hippocampal formation. *J. Comp. Neurol.*, **363**, 517–531.

TERRIAN, D.M., HERNANDEZ, P.G., REA, M.A. & PETERS, R.I. (1989). ATP release, adenosine formation, and modulation of dynorphin and glutamic acid release by adenosine analogues in rat hippocampal mossy fiber synaptosomes. *J. Neurochem.*, **53**, 1390–1399.

TETZLAFF, W., SCHUBERT, P. & KREUTZBERG, G.W. (1986). Synaptic and extrasynaptic localization of adenosine binding sites in the rat hippocampus. *Neuroscience*, **21**, 869–875.

UKENA, D., SHAMIM, M.T., PADGETT, W. & DALY, J.W. (1986). Analogues of caffeine: antagonists with selectivity for A_2 adenosine receptors. *Life Sci.*, **39**, 743–750.

WESSLER, I. & KILBINGER, H. (1986). Release of [^{3}H]-acetylcholine from a modified rat phrenic nerve-hemidiaphragm preparation. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **334**, 357–364.

WHITE, T.D. & MACDONALD, W.F. (1990). Neural release of ATP and adenosine. *Ann. N.Y. Acad. Sci.*, **603**, 287–299.

WIERASZKO, A., GOLDSMITH, G. & SEYFRIED, T.N. (1989). Stimulation-dependent release of adenosine triphosphate from hippocampal slices. *Brain Res.*, **485**, 244–250.

WIKLUND, N.P., GUSTAFSSON, L.E. & LUNDIN, J. (1985). Pre- and postjunctional modulation of cholinergic neuroeffector transmission by adenine nucleotides. Experiments with agonist and antagonist. *Acta Physiol. Scand.*, **125**, 681–691.

ZIMMERMANN, H. (1992). 5'-nucleotidase: molecular structure and functional aspects. *Biochem. J.*, **285**, 345–365.

ZIMMERMANN, H., VOGEL, M. & LAUBE, U. (1993). Hippocampal localization of 5'-nucleotidase as revealed by immunocytochemistry. *Neuroscience*, **55**, 105–112.

(Received May 10, 1996
Revised June 9, 1996
Accepted June 10, 1996)