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ON THE ROLE, INACTIVATION AND ORIGIN OF ENDOGENOUS ADENOSINE AT THE FROG NEUROMUSCULAR JUNCTION

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SUMMARY

1. The effects of adenosine deaminase, inosine, alkylxanthines (8-phenyltheophylline (8-PT), theophylline and isobutylmethylxanthine (IBMX)), dipyridamole, α,β-methylene ADP (AOPCP) and ATP analogues (α,β-methylene ATP and β,γ-methylene ATP) on evoked end-plate potentials (e.p.p.s) were investigated in innervated sartorius muscles of the frog, in which twitches had been prevented with tubocurarine. The effects of 8-PT and IBMX on the amplitude and quantal content of e.p.p.s were also investigated in innervated sartorius muscles of the frog, in which twitches had been prevented with high-magnesium solutions.

2. Adenosine deaminase reversibly increased the amplitude of e.p.p.s and prevented the reduction caused by exogenously applied adenosine on e.p.p. amplitude. The increase caused by adenosine deaminase was equivalent to the decrease caused by $12 \pm 5{\cdot}8$ μM-adenosine on e.p.p. amplitude.

3. Inosine, the product of adenosine deamination, was virtually devoid of effect on e.p.p.s.

4. The adenosine receptor antagonists at the frog neuromuscular junction, 8-PT and theophylline, increased in a concentration-dependent manner the amplitude of e.p.p.s in the presence of tubocurarine. 8-PT increased the amplitude and quantal content of e.p.p.s in the presence of high magnesium. IBMX, which does not behave as an adenosine receptor antagonist at the frog neuromuscular junction, decreased the amplitude of e.p.p.s in the presence of tubocurarine or high-magnesium solutions.

5. Dipyridamole, an adenosine uptake blocker, decreased the amplitude of e.p.p.s, and in a concentration that did not affect neuromuscular transmission potentiated the depressing effect of adenosine, but not that of 2-chloroadenosine, on the amplitude of e.p.p.s.

6. AOPCP, an inhibitor of 5′-nucleotidase, increased the amplitude of e.p.p.s and markedly attenuated the depressing effect of ATP, but not that of adenosine, on e.p.p. amplitude.

7. The ATP analogue, α,β-methylene ATP, which is not a substrate for 5′-nucleotidase, was virtually devoid of effect on e.p.p.s. β,γ-Methylene ATP, which can be a substrate for 5′-nucleotidase, mimicked the depressing effect of ATP on e.p.p. amplitude, an effect which was also reduced by AOPCP.

8. It is concluded that in conditions in which the initial quantal content is assumed to be normal (1) endogenous adenosine depresses neuromuscular transmission, (2) at

the neuromuscular junction adenosine is inactivated through a dipyridamole-sensitive uptake process, and (3) released adenine nucleotides might contribute to the pool of endogenous adenosine which modulates neuromuscular transmission.

INTRODUCTION

Exogenously applied adenosine decreases transmitter release at the neuromuscular junction (Ginsborg & Hirst, 1972; Ribeiro & Walker, 1975) by activating a xanthine-sensitive adenosine receptor (Ribeiro & Sebastião, 1985). Adenosine deaminase enhances transmission at the presynaptically inhibited neuromuscular junction, an effect which has been interpreted as endogenous adenosine exerting an inhibitory 'tone' over neuromuscular transmission (Sebastião & Ribeiro, 1985).

The experiments described below provide evidence for the role of endogenous adenosine as a modulator of neuromuscular transmission in preparations paralysed post-synaptically with tubocurarine, and also provide evidence that adenosine is inactivated through an adenosine uptake mechanism at the neuromuscular junction.

On the other hand, it is known that ATP is released from active motor nerve endings (Silinsky, 1975) and when exogenously applied decreases transmitter output from motor nerve terminals with similar potency as adenosine (Ribeiro & Walker, 1975). Since extracellular ATP can be converted into adenosine through the action of ecto-ATPases followed by that of ecto-5′-nucleotidase (Keller & Zimmermann, 1983; Nagy, Shuster & Rosenberg, 1983; Kreutzberg, Heymann & Reddington, 1986), it was considered of interest to know whether released ATP contributes to the pool of endogenous adenosine which modulates transmission at the neuromuscular junction.

A brief account of some of the results has already appeared (Ribeiro & Sebastião, 1986).

METHODS

The experiments were carried out at room temperature (22–25 °C) on innervated sartorius muscles of the frog (*Rana ridibunda*). The preparations were mounted in a Perspex chamber of 5 ml capacity through which the solutions flowed continuously at a rate of 5 ml min^{-1} via a roller pump. The bath volume was kept constant by suction. Solutions were changed by transferring the inlet tube of the pump from one flask to another. This involved a minimum of disturbance to the preparation and allowed prolonged recording from the same fibre with many solution changes. However, because of the slow rate of flow it was not possible to estimate rates of onset of the effects of the substances. The change-over times in the Figures of this paper indicate the times at which the inlet tube of the pump was transferred to a new solution.

Evoked end-plate potentials (e.p.p.s) were recorded in the conventional way (Fatt & Katz, 1951) with intracellular electrodes filled with 3 M-KCl and 10–20 MΩ resistance. The bath electrode was an Ag–AgCl pellet. The nerve was stimulated supramaximally with rectangular pulses of 10 μs duration applied once every 2 s. Evoked responses of sixty-four consecutive stimuli were averaged, after amplification, with a Datalab DL-4000 computer. The output of the computer was coupled to a pen recorder. The usual procedure was to continue to record averages in the same solution until a stable value was obtained, i.e. until two successive averages differed by less than 2%. The nerve was stimulated at a constant rate throughout the experiments and at least 30 min to 1 h before the recording was begun. The time from the beginning to the peak of each individual e.p.p. was less than 1 ms.

The normal bathing solution (pH 7·0) contained (mM): NaCl, 117; KCl, 2·5; NaH_2PO_4, 1;

Na_2HPO_4, 1; $MgCl_2$, 1·2; $CaCl_2$, 1·8. Usually the twitches of the muscle in response to nerve stimulation were prevented by addition of tubocurarine (0·8–1·2 μM) to the bath; occasionally, the twitches were prevented by increasing the concentration of magnesium ($MgCl_2$, 10–11 mM) in the bathing solution.

Statistical analysis

The significance of differences between means was calculated using the Student's *t* test. *P* values of 0·05 or less were considered to represent statistically significant differences.

Drugs

The following drugs used were: adenosine deaminase type VI, adenosine, 2-chloroadenosine, ATP, α,β-methylene ADP, α,β-methylene ATP, β,γ-methylene ATP, 5′-nucleotidase, theophylline, inosine (Sigma); 8-phenyltheophylline, 1,3-isobutylmethylxanthine (R.B.I.); dipyridamole (Boehringer Ingelheim). 8-phenyltheophylline and dipyridamole were made up in 10 mM stock solutions in 80% methanol (v/v) containing 0·2 M-NaOH and dilutions of these solutions were used. Adenosine deaminase (1500 i.u. ml^{-1}) was purchased in a suspension in 50% glycerol (v/v) with 10 mM-potassium phosphate (pH 6·0) and dilutions of this suspension were used. The pH of the bathing solution did not change by the addition of the drugs in the maximum concentrations applied to the preparations.

RESULTS

The effect of adenosine deaminase

Fig. 1 illustrates the effect of adenosine deaminase (2·5 i.u. ml^{-1}), an enzyme which deaminates adenosine into inosine (e.g. Arch & Newsholme, 1978), on the average amplitude of e.p.p.s recorded from a frog sartorius muscle fibre in the presence of tubocurarine (1 μM). As can be seen, the enzyme reversibly increased the average amplitude of e.p.p.s without modifying their decay phase or the membrane resting potential of the muscle fibre. Similar effects were observed in another nine experiments, the mean increase in e.p.p.s amplitude caused by adenosine deaminase (2·5 i.u. ml^{-1}) being $23 \pm 2·7$% for the ten experiments. Used in the same concentration (2·5 i.u. ml^{-1}) this enzyme completely abolished the depressing effect of adenosine (50 μM) on e.p.p. amplitude.

The full effect of adenosine deaminase (2·5 i.u. ml^{-1}) on e.p.p. amplitude was usually seen in the first 20 min after starting its perfusion and disappeared within 30 min after returning the preparations to the control bathing solution (Fig. 1*B*). The increase in e.p.p. amplitude caused by adenosine deaminase could not be attributed to its suspension medium (50% glycerol with 10 mM-potassium phosphate, pH 6·0) since all the adenosine deaminase-free solutions contained equivalent amounts (0·17% v/v) of adenosine deaminase suspension medium.

Inosine

In order to investigate whether the enhancing action of adenosine deaminase on neuromuscular transmission could be a consequence of its ability to increase the levels of inosine, the effect of this adenosine metabolite on e.p.p.s was investigated in three experiments performed on frog sartorius muscles paralysed with tubocurarine (0·9–1 μM). In these experiments the amplitude of e.p.p.s recorded in the control bathing solutions ranged from 2·0 to 11·5 mV and inosine (100 μM) had virtually no effect on e.p.p.s, though the preparations did respond in the usual way to adenosine (2·5–10 μM), which decreased the average amplitude of e.p.p.s.

Comparison of the enhancing action of adenosine deaminase and the depressing effect of adenosine

Adenosine deaminase is a protein which probably does not cross cell membranes from outside to inside. Its specificity for adenosine and the ability of erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), an inhibitor of the enzymatic properties of adenosine deaminase (Agarwal, Spector & Parks, 1977), to prevent the action of this

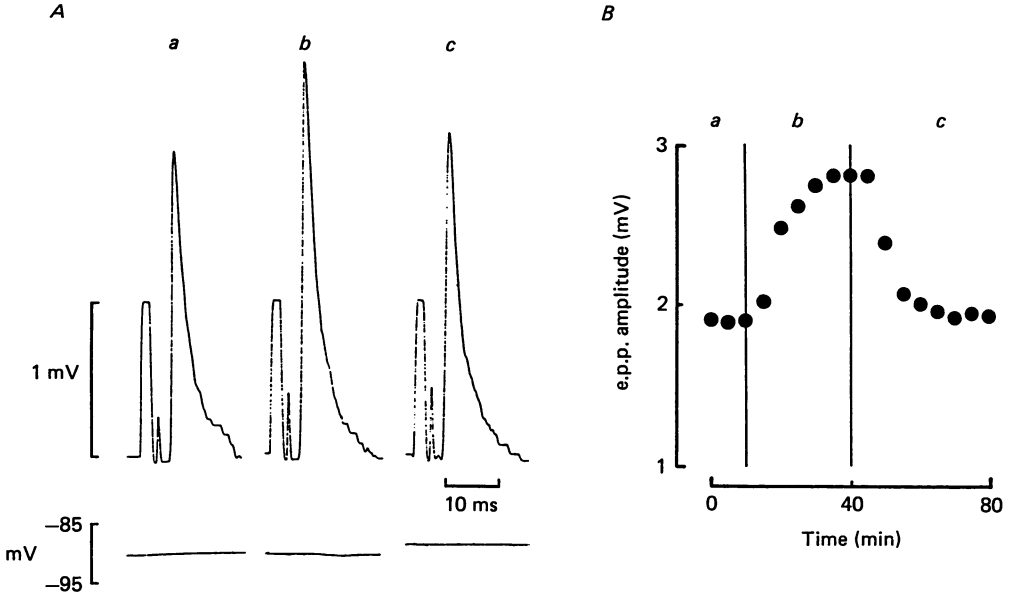

Fig. 1. Effect of adenosine deaminase on the averaged amplitude of evoked end-plate potentials (e.p.p.s) recorded from a frog sartorius muscle fibre. Solutions contained tubocurarine (1 μM) which prevented muscle action potentials and twitches in response to nerve stimulation. *A*, upper part: pen recorder traces of averaged e.p.p.s recorded: in the control bathing solution, before applying adenosine deaminase (*a*); 15 min after starting perfusion of adenosine deaminase (2·5 i.u. ml^{-1}) (*b*); 30 min after returning to the control bathing solution (*c*). Each evoked response is the computed average of sixty-four successive e.p.p.s and is preceded by a calibration pulse of 1 mV amplitude and 2 ms duration. Lower part: membrane resting potential. *B*, time course of the effect of adenosine deaminase in another experiment. The ordinate shows the amplitudes of the computed averages of sixty-four successive e.p.p.s and the abscissa the times the averaging began. *a* and *c*, control bathing solution; *b*, adenosine deaminase (2·5 i.u. ml^{-1}). Membrane resting potential: −96 mV.

enzyme on neuromuscular transmission (Sebastião & Ribeiro, 1985), taken together with the present observation that inosine has no effect on e.p.p.s recorded from frog sartorius muscles, suggest that the enhancing effect of adenosine deaminase on e.p.p. amplitude results from its ability to inactivate endogenous adenosine located extracellularly at the neuromuscular junction. In order to infer the order of magnitude of the endogenous concentrations of adenosine in the synaptic cleft (see Schubert, Lee, Reddington & Kreutzberg, 1983), the enhancing action of adenosine deaminase on e.p.p. amplitude was compared with the depressing effect of different

concentrations of adenosine exogenously applied to the same fibre. Results from five experiments are summarized in Table 1. As can be seen, there was considerable variation between the effects of the same concentration of exogenous adenosine in different fibres. This conforms with the results obtained by others in the rat diaphragm (cf. Ginsborg & Hirst, 1972). The enhancing action of adenosine deaminase in different fibres also varied. The increase caused by adenosine deaminase on e.p.p. amplitude was equivalent to the decrease in e.p.p. amplitude caused by $12 \pm 5{\cdot}8$ μM exogenously applied adenosine, a value determined from the linear part of the log concentration–response curves for adenosine.

TABLE 1. Comparison of the depressing effect of adenosine and the enhancing effect of adenosine deaminase on the amplitude of evoked end-plate potentials (e.p.p.s)

Fibre	[Adenosine] (μM)	Percentage decrease in e.p.p. amplitude caused by adenosine	Percentage increase in e.p.p. amplitude caused by adenosine deaminase (2·5 i.u. ml^{-1})
1	1	13	
	2·5	22	45
2	10	10	
	25	23	
	50	35	23
3	1	11	
	2·5	29	
	5	40	16
4	2·5	11	
	5	29	25
5	1	18	
	2·5	30	
	5	34	17

Averaged amplitude of e.p.p.s in the control bathing solution: $4{\cdot}5 \pm 0{\cdot}8$ mV. Averaged resting membrane potential: -95 ± 3 mV. Solutions contained tubocurarine (0·8–1·1 μM).

Alkylxanthines

The adenosine receptor at the frog neuromuscular junction is competitively antagonized by 8-phenyltheophylline (8-PT) and theophylline, but not by isobutylmethylxanthine (IBMX) (Ribeiro & Sebastião, 1985). In order to know whether endogenous activation of this receptor affects neuromuscular transmission, it was considered of interest to investigate the effects of 8-PT, theophylline and IBMX on e.p.p.s.

Fig. 2. illustrates the results obtained in a typical experiment in which 8-PT (5 μM) was applied to a frog sartorius muscle paralysed with tubocurarine (1·1 μM). This xanthine reversibly increased the average amplitude of the e.p.p.s without modifying their decay phase or the membrane resting potential of the muscle fibre. Similar results were obtained when 8-PT was applied to magnesium (10–11 mM)-paralysed muscles, the mean increase in e.p.p. amplitude caused by 5 and 10 μM-8-PT being $33 \pm 7{\cdot}5\%$ ($n = 3$, $P < 0{\cdot}05$) and $48 \pm 0{\cdot}6\%$ ($n = 3$, $P < 0{\cdot}05$), respectively. The quantal content of the e.p.p.s, determined by the variance method (Hubbard, Llinás &

Quastel, 1969) in a magnesium (11 mM)-paralysed fibre, was augmented 43 % by 8-PT (10 μM) without significant modification of the quantal size, which suggests that the enhancing effect of 8-PT on neuromuscular transmission was mainly presynaptic, increasing the evoked release of the transmitter.

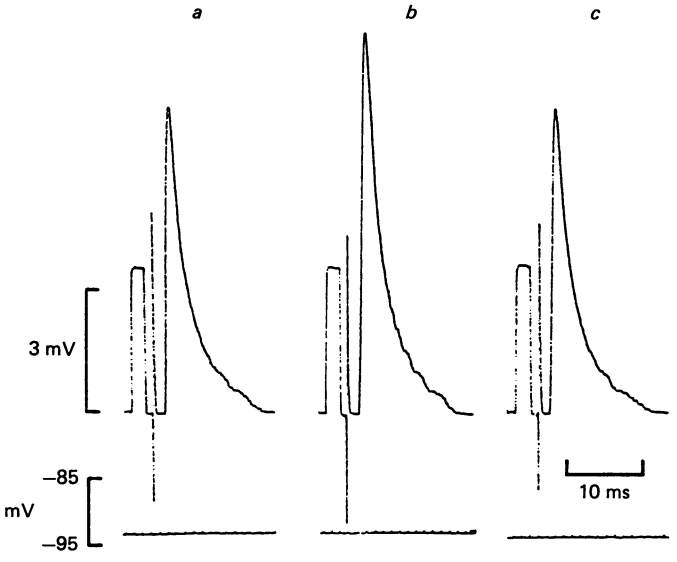

Fig. 2. Effect of 8-phenyltheophylline (8-PT) on the averaged amplitude of evoked end-plate potentials (e.p.p.s). Solutions contained tubocurarine (1·1 μM). Upper part: pen-recorder traces of averaged e.p.p.s recorded: in the control bathing solution before applying 8-PT (*a*); 15 min after starting perfusion of 8-PT (5 μM) (*b*); 30 min after returning to the control bathing solution (*c*). Calibration pulse: 3·5 mV amplitude and 2 ms duration. Lower part: membrane resting potential. Details as in legend to Fig. 1.

The full effect of 8-PT on e.p.p. amplitude was usually observed within 5–15 min after starting its perfusion and disappeared within 10–30 min after returning the preparations to the control bathing solutions. The effect of 8-PT on neuromuscular transmission cannot be attributed to its solvent (80 % methanol with 0·2 M-NaOH) since the maximum concentration (0·1 % v/v) of solvent present in the 8-PT solutions did not affect e.p.p. amplitude.

Theophylline also increased e.p.p. amplitude, though being less potent than 8-PT; thus, increases of 37 ± 3 % ($n = 2$) and 47 ± 1 % ($n = 2$) in e.p.p. amplitude were obtained with 100 and 200 μM-theophylline, respectively.

In contrast with the effects of 8-PT and theophylline, IBMX (0·1–1 mM) decreased the amplitude, increased the duration and prolonged the decay phase of e.p.p.s recorded from muscle fibres paralysed either with magnesium (10–11 mM, four experiments) or with tubocurarine (0·8–1·2 μM, two experiments). These effects of IBMX were reversible and concentration dependent as can be concluded from the results illustrated in Fig. 3. In a magnesium (11 mM)-paralysed muscle fibre, it was observed that IBMX (0·5 mM) decreased the quantal content (determined by the variance method), the quantal size and the average amplitude of e.p.p.s by 18, 14

and 36%, respectively, suggesting that this xanthine may have both pre- and post-synaptic inhibitory actions on neuromuscular transmission. When used in lower concentrations (10–50 μM) IBMX was virtually devoid of effect on neuromuscular transmission.

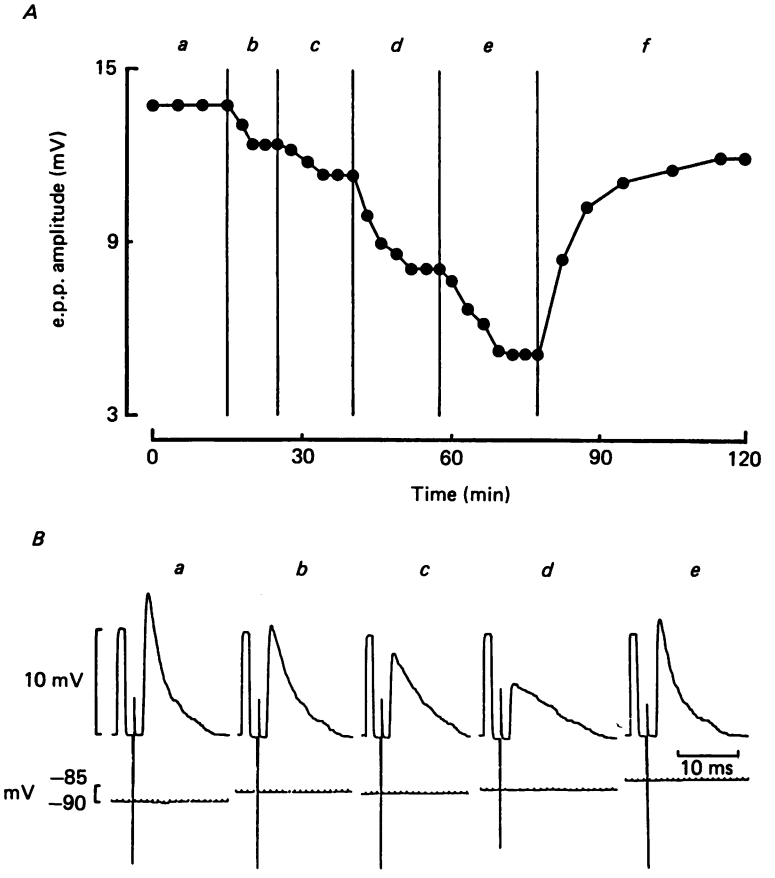

Fig. 3. Effect of 1,3-isobutylmethylxanthine (IBMX) on evoked end-plate potentials (e.p.p.s). Solutions contained 10 mM-magnesium which prevented muscle action potentials and twitches in response to nerve stimulation. *A*, time course of the effects of different concentrations of IBMX. The ordinate shows the amplitudes of the computed averages of sixty-four successive e.p.p.s and the abscissa the times the averaging began. *a* and *f*, control bathing solution; *b*, IBMX (0·1 mM); *c*, IBMX (0·2 mM); *d*, IBMX (0·5 mM); *e*, IBMX (1 mM). *B*, upper part: pen recorder traces of averaged e.p.p.s corresponding to: *a*, 14; *b*, 39; *c*, 55; *d*, 75; *e*, 120 min for time in *A*. Each evoked response is preceded by a calibration pulse of 10 mV amplitude and 2 ms duration. Lower part: membrane resting potential.

Dipyridamole

The observation that the adenosine deaminase inhibitor, EHNA, does not modify the amplitude of e.p.p.s, though preventing the enhancing action of exogenously applied adenosine deaminase on neuromuscular transmission (Sebastião & Ribeiro, 1985), suggests that at the neuromuscular junction deamination of adenosine is not

the main mechanism involved in the inactivation of the nucleoside. We decided therefore to investigate the effect of an adenosine uptake blocker, dipyridamole (e.g. Wu & Phillis, 1984), on neuromuscular transmission and on the effect of exogenously applied adenosine.

Fig. 4*A* illustrates the results obtained in a typical experiment in which dipyridamole (1 μM) was applied to a muscle in the presence of tubocurarine (0·9 μM). It is evident that in this concentration dipyridamole decreased the average amplitude of

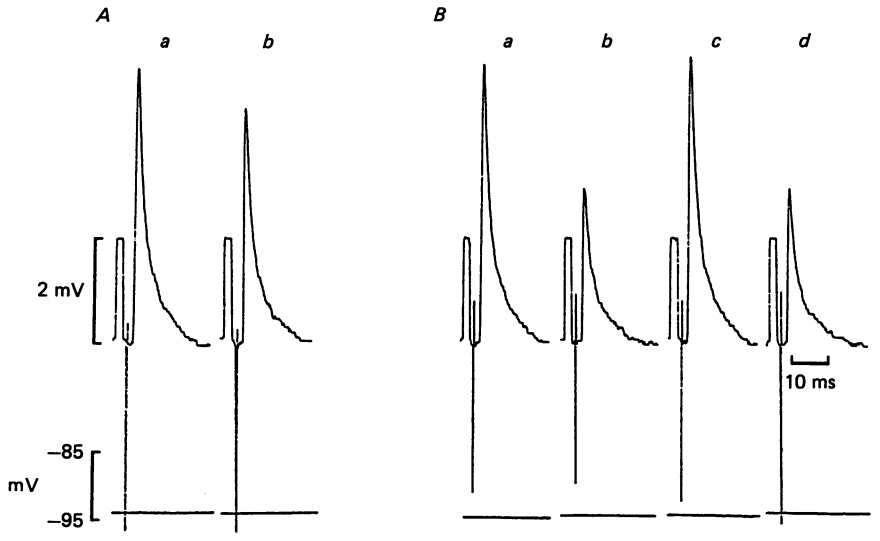

Fig. 4. Effect of dipyridamole on the averaged amplitude of evoked end-plate potentials (e.p.p.s). Solutions contained tubocurarine (0·9 μM). Upper part: pen recorder traces of averaged e.p.p.s. Calibration pulse: 2 mV amplitude and 2 ms duration. Lower part: membrane resting potential. *A*, effect of dipyridamole in the absence of adenosine. Responses obtained in the control bathing solution, before applying dipyridamole (*a*); 25 min after starting perfusion of dipyridamole (1 μM) (*b*). *B*, effect of dipyridamole when applied in the presence of a supramaximal concentration of adenosine. *a* and *c*, control bathing solution; *b*, adenosine (100 μM); *d*, dipyridamole (1 μM) + adenosine (100 μM). Recordings in *A* and *B* were obtained from the same end-plate. Details as in legend to Fig. 1.

e.p.p.s, without modifying their decay phase or the resting membrane potential of the muscle fibre. Similar results were obtained in another experiment, the average decrease in e.p.p. amplitude caused by dipyridamole (1 μM) in the two experiments being $22 \pm 2\%$.

The full effect of dipyridamole (1 μM) on e.p.p. amplitude was observed within 20 min after starting its perfusion and its action persisted for more than 60 min after returning the preparation to the control bathing solution without dipyridamole. This difficulty in reversing the action of dipyridamole conforms with the results obtained by others in the guinea-pig longitudinal muscle–myenteric plexus preparation (Dowdle & Maske, 1980).

The depressing action of dipyridamole on neuromuscular transmission was not additive with that of a supramaximal concentration (100 μM) of adenosine (Fig. 4*B*),

which might suggest that the effect of dipyridamole results from its ability to increase the amounts of extracellular endogenous adenosine.

Fig. 5 illustrates the action of a subthreshold concentration (0·1 μM) of dipyridamole (i.e. a concentration that by itself did not affect neuromuscular transmission) on the concentration–response curve for the depressing effect of adenosine on e.p.p. amplitude. In each end-plate the effects of different concentrations of adenosine on the averaged amplitude of e.p.p.s were tested cumulatively, first in the absence and

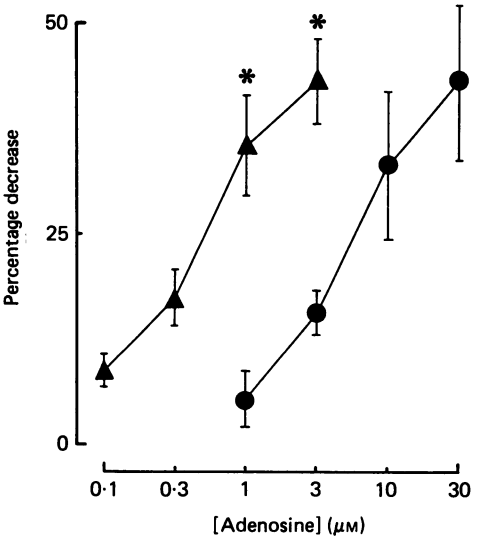

Fig. 5. Action of dipyridamole, in a concentration (0·1 μM) virtually devoid of effect on evoked end-plate potentials (e.p.p.s), on the concentration–response curve for the depressing effect of adenosine on e.p.p.s amplitude. The ordinate shows the percentage decreases in the amplitude of e.p.p.s recorded from tubocurarine (0·9–1·2 μM)-paralysed muscle fibres. ▲, adenosine + dipyridamole; ●, adenosine. 0% is the e.p.p. amplitude in the control bathing solution (3·2 ± 0·7 mV) and 100% represents a complete inhibition of e.p.p.s. In each experiment the effects of adenosine in the absence and in the presence of dipyridamole were compared in the same end-plate. The results are the average of four experiments. Averaged resting membrane potential: −95 ± 0·2 mV. *$P < 0·05$ (paired Student's t test) when compared with the effect of the same concentration of adenosine in the absence of dipyridamole. The vertical bars represent ± S.E. of mean.

then in the presence of dipyridamole (0·1 μM). The pre-incubation time with dipyridamole before testing the effect of adenosine in its presence was approximately 30 min. It is evident from the results summarized in Fig. 5 that dipyridamole (0·1 μM) shifted to the left the concentration–response curve for adenosine, the nucleoside being about fourteen times more potent in decreasing e.p.p. amplitude when applied to the preparations in the presence of dipyridamole.

Tested in the same conditions dypiridamole (0·1 μM) did not modify the concentration–response curve for the depressing effect of 2-chloroadenosine (0·1–3 μM) on e.p.p. amplitude. Also, when the actions of dipyridamole (0·1 μM) on the depressing effects of adenosine and 2-chloroadenosine on e.p.p. amplitude were compared in the same end-plate, it was observed that dipyridamole potentiated the

effect of adenosine but not that of 2-chloroadenosine; thus, when applied to the preparation in the absence and in the presence of dipyridamole, 2-chloroadenosine (0·3 μM) decreased the average amplitude of e.p.p.s by 21 and 22%, respectively, whereas adenosine (3 μM) reduced e.p.p. amplitude by 16% in the absence and 40% in the presence of dipyridamole.

The effects of dipyridamole on neuromuscular transmission cannot be attributed to its solvent (80% methanol, 0·2 M-NaOH) since all dipyridamole-free solutions contained equivalent amounts (0·001–0·01% v/v) of solvent.

5′-Nucleotidase inhibition

To study whether ATP contributes to the pool of endogenous adenosine which modulates transmission at the neuromuscular junction we used α,β-methylene ADP (AOPCP) an inhibitor of 5′-nucleotidase (Burger & Lowenstein, 1970), the enzyme responsible for the final step in the hydrolysis of ATP into adenosine (e.g. Kreutzberg *et al.* 1986).

AOPCP (50 μM) increased the amplitude (11·3 $\pm$ 1·2%, $n = 6$, $P < 0.05$) of e.p.p.s recorded from tubocurarine (1–1·2μM)-paralysed muscle fibres, its full effect being usually observed within 10 min after its application to the preparations. The decay phase of e.p.p.s or the resting membrane potential of the muscle fibres were virtually unaffected by AOPCP (50 μM).

In order to know whether AOPCP could influence the action of exogenously applied ATP, experiments were designed in which the depressing effects of several concentrations of ATP on neuromuscular transmission in the absence and in the presence of AOPCP were compared in the same end-plate. Fig. 6*A* illustrates the results obtained in a typical experiment. As can be seen AOPCP (50 μM) prevented the depressing effect of ATP (1 μM) on e.p.p. amplitude and markedly attenuated the effect of higher (2·5–10 μM) concentrations of this nucleotide. A similar experiment was performed in another preparation and a summary of the results obtained in both experiments is illustrated in Fig. 6*B*. It is evident that the log concentration–response curve for the depressing effect of ATP on e.p.p. amplitude is shifted to the right by AOPCP (50 μM) by a factor of about 14. AOPCP (50 μM) did not affect the depressing action of adenosine (10 μM) on neuromuscular transmission, which caused a similar decrease in e.p.p. amplitude when applied to a tubocurarine (1·1 μM)-paralysed muscle fibre either in the presence or in the absence of AOPCP.

If the observed attenuation of the depressing effect of ATP on neuromuscular transmission by AOPCP is due to its ability to inhibit ecto-5′-nucleotidase, it should be expected that appropriate concentrations of exogenously applied 5′-nucleotidase could reverse that action of AOPCP. This was tested in one experiment in which ATP (10 μM) was applied to a tubocurarine (1·1 μM)-paralysed muscle fibre, either alone, in the presence of AOPCP (50 μM) and in the presence of AOPCP (50 μM) plus 5′-nucleotidase (2·5 i.u. ml^{-1}). 5′-Nucleotidase reversed the attenuation of the depressing effect of ATP by AOPCP, 5′-nucleotidase (2·5 i.u. ml^{-1}) by itself slightly decreased (9 $\pm$ 1·0%, $n = 2$) e.p.p. amplitude.

ATP analogues

The finding that AOPCP reduces the depressing effect of ATP on neuromuscular transmission, suggests that ATP has to be hydrolysed into adenosine by 5′-

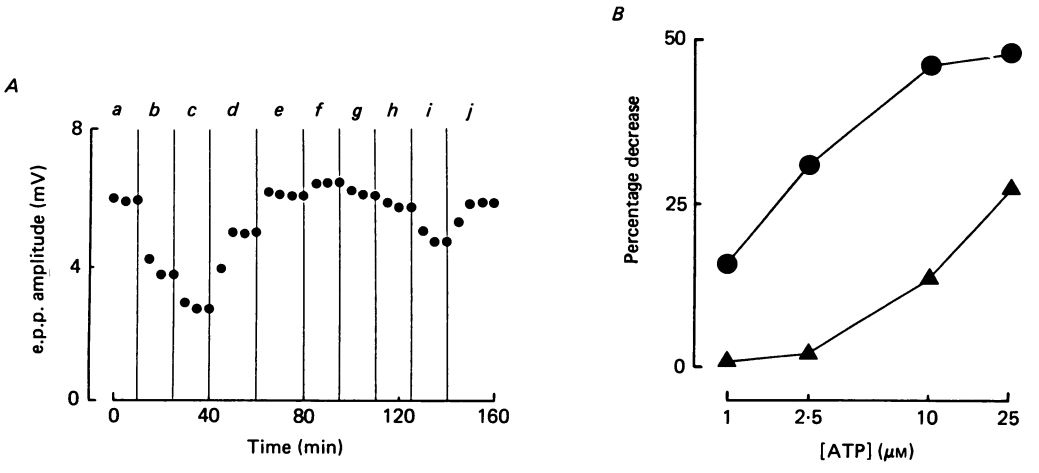

Fig. 6. Effect of α,β-methylene ADP (AOPCP) on the depressing action of ATP on the amplitude of evoked end-plate potentials (e.p.p.s) recorded from muscle fibres paralysed with tubocurarine (1·1–1·2 μM). *A*, time course of the effects of different concentrations of ATP in the absence and in the presence of AOPCP. The ordinate shows the amplitudes of the computed averages of sixty-four successive e.p.p.s and the abscissa the times averaging began. *a*, *e* and *j*, control bathing solution; *b*, ATP (2·5 μM); *c*, ATP (10 μM); *d*, ATP (1 μM); *f*, AOPCP (50 μM); *g*, AOPCP (50 μM) + ATP (1 μM); *h*, AOPCP (50 μM) + ATP (2·5 μM); *i*, AOPCP (50 μM) + ATP (10 μM). Resting membrane potential: −93 mV. *B*, concentration–response curves for the effect of ATP in the absence (●) and in the presence (▲) of AOPCP (50 μM). The ordinate shows the percentage decreases of e.p.p. amplitude caused by ATP. 0% is the amplitude of e.p.p.s in the control bathing solution (5·7 ± 0·2 mV) and 100% represents a complete inhibition of e.p.p.s. The results are the averages of two experiments. In each experiment the effects of ATP in the absence and in the presence of AOPCP were compared in the same end-plate. Averaged resting membrane potential: −92 ± 2 mV.

nucleotidase in order to produce its depressing effect at the neuromuscular junction. To investigate this further, we tested the effects of α,β-methylene ATP and β,γ-methylene ATP on e.p.p.s recorded from tubocurarine (1·1–1·2 μM)-paralysed muscle fibres.

α,β-Methylene ATP (10–50 μM), which is not a substrate for 5′-nucleotidase (Yount, 1975), was virtually devoid of effect on e.p.p.s recorded from ATP (1–25 μM)-sensitive muscle fibres (two experiments). In contrast, β,γ-methylene ATP (1–50 μM), which can be hydrolysed into AMP and adenosine (Yount, 1975), decreased in a concentration-dependent manner the amplitude of e.p.p.s, without modifying their decay phase or the resting membrane potential of the muscle fibres (three experiments). Comparing the effects of β,γ-methylene ATP and ATP on the same end-plate it was observed that ATP was more potent than β,γ-methylene ATP by a factor of about four, i.e. the decrease in e.p.p. amplitude caused by 2·5 μM-ATP was of similar magnitude to that caused by 10 μM-β,γ-methylene ATP (Fig. 7). As occurred with ATP, the depressing effect of β,γ-methylene ATP on neuromuscular transmission was markedly attenuated by AOPCP (50 μM) (Fig. 8). This suggests that the depressing effect of this ATP analogue on neuromuscular transmission results from its hydrolysis to adenosine.

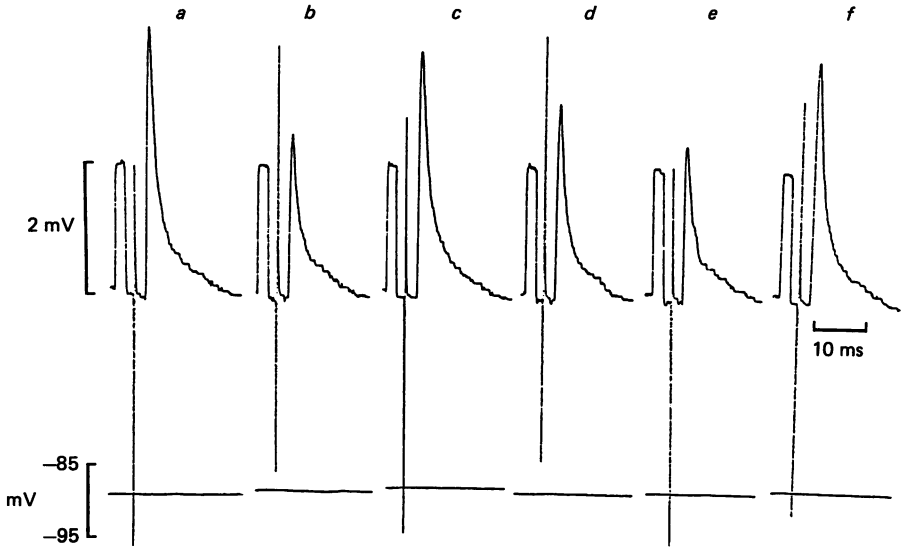

Fig. 7. Comparison of the effects of ATP and β,γ-methylene ATP on the amplitude of evoked end-plate potentials (e.p.p.s). Solutions contained tubocurarine (1·2 μM). Upper part: pen recorder traces of averaged e.p.p.s. Calibration pulse: 2 mV amplitude and 2 ms duration. Lower part: membrane resting potential. Responses recorded in the control bathing solution before applying ATP (*a*); 10 min after starting perfusion of ATP (2·5 μM) (*b*); 10 min after returning to the control bathing solution (*c*); 10 min after starting perfusion of β,γ-methylene ATP (2·5 μM) (*d*); 10 min after starting perfusion of β,γ-methylene ATP (10 μM) (*e*); 15 min after returning to the control bathing solution (*f*). Details as in legend to Fig. 1.

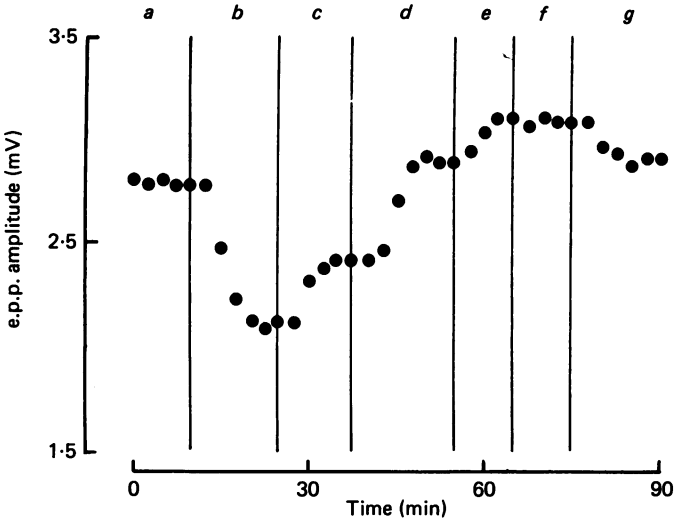

Fig. 8. Time course of the effects of different concentrations of β,γ-methylene ATP in the absence and in the presence of α,β-methylene ADP (AOPCP, 50 μM), on the amplitude of evoked end-plate potentials (e.p.p.s) recorded from a muscle fibre paralysed with tubocurarine (1 μM). The ordinate shows the amplitudes of the computed averages of sixty-four successive e.p.p.s and the abscissa the times averaging began. *a* and *d*, control bathing solution; *b*, β,γ-methylene ATP (2·5 μM); *c*, β,γ-methylene ATP (1 μM); *e*, AOPCP (50 μM); *f*, AOPCP (50 μM)+β,γ-methylene ATP (1 μM); *g*, AOPCP (50 μM)+β,γ-methylene ATP (2·5 μM). Membrane resting potential: −93 mV.

DISCUSSION

The present results showing that adenosine deaminase increased e.p.p. amplitude in tubocurarine-paralysed muscles, reinforce the idea that adenosine is an endogenous modulator of neuromuscular transmission (Sebastião & Ribeiro, 1985) and indicate that this also occurs in conditions where the initial quantal content of e.p.p.s is assumed to be normal. The enhancing action of adenosine deaminase on neuromuscular transmission was equivalent to the depressing action of about 12 μM exogenously applied adenosine, indicating that the levels of endogenous adenosine which modulate neuromuscular transmission are in the low micromolar range. Comparing the enhancing action of adenosine deaminase with the depressing effect of adenosine in the rat (Dunwiddie & Hoffer, 1980) and rabbit (Jackisch, Strittmatter, Kasakov & Hertting, 1984) hippocampus, it is possible to estimate similar amounts of endogenous adenosine (see also Schubert *et al.* 1983).

Also supporting the idea that extracellular endogenous adenosine inhibits transmission at the neuromuscular junction are the present observations that the adenosine receptor antagonists 8-PT and theophylline in micromolar concentrations facilitate neuromuscular transmission. In previous reports the enhancing action of theophylline on neuromuscular transmission was interpreted in terms of its phosphodiesterase inhibitor properties (Goldberg & Singer, 1969; Wilson, 1974; but see Ginsborg & Hirst, 1972). The increase in e.p.p. amplitude caused by micromolar concentrations of 8-PT and theophylline does not appear to be related to their ability to inhibit phosphodiesterases, and is probably related to their capacity to antagonize the adenosine receptor since (1) the concentrations of 8-PT and theophylline which already enhanced neuromuscular transmission are below those usually needed to inhibit phosphodiesterases (cf. Smellie, Davis, Daly & Wells, 1979), (2) 8-PT is less potent than theophylline in inhibiting phosphodiesterases (Smellie *et al.* 1979; Wu, Phillis & Nye, 1982), is more potent than theophylline as an adenosine receptor antagonist at the neuromuscular junction (Ribeiro & Sebastião, 1985) and was more potent than theophylline in facilitating neuromuscular transmission, (3) IBMX, a potent phosphodiesterase inhibitor (Smellie *et al.* 1979) which does not behave as an antagonist of the adenosine receptor at the frog neuromuscular junction (Ribeiro & Sebastião, 1985), did not enhance neuromuscular transmission and at high concentrations even decreased e.p.p. amplitude.

Dipyridamole, a well-known adenosine uptake blocker (e.g. Wu & Phillis, 1984) decreased e.p.p. amplitude, and when used in a concentration that by itself did not affect neuromuscular transmission, markedly potentiated the depressing effect of exogenous adenosine on the amplitude of e.p.p.s. Since the depressing effect of dipyridamole on e.p.p. amplitude was not additive with that of a supramaximal concentration of adenosine and the depressing effect of 2-chloroadenosine, an adenosine analogue with a low affinity for the adenosine uptake system (Jarvis, Martin & Ng, 1985), on e.p.p. amplitude was not affected by dipyridamole, it is likely that the effects of this substance at the neuromuscular junction result from its ability to inhibit the uptake of adenosine. The presently observed effects of dipyridamole on neuromuscular transmission taken together with the finding that the adenosine deaminase inhibitor EHNA (Agarwal *et al.* 1977), in a concentration that blocked the enhancing action of adenosine deaminase on neuromuscular transmission, did not

affect e.p.p. amplitude (Sebastião & Ribeiro, 1985) indicate that at the neuromuscular junction, as it occurs in the central nervous system (Nimit, Skolnick & Daly, 1981; Jackisch *et al.* 1984), adenosine uptake is more important than adenosine deamination in the regulation of extracellular adenosine concentrations.

Another point of interest in the present work is the finding that AOPCP, a 5′-nucleotidase inhibitor (Burger & Lowenstein, 1970), increases e.p.p. amplitude. AOPCP is a nucleotide which presumably does not cross cell membranes and consequently should inhibit ecto-5′-nucleotidase without affecting intracellular 5′-nucleotidase. At least two possibilities can be advanced to explain the enhancing action of AOPCP on neuromuscular transmission: (1) released ATP (Silinsky, 1975), after being hydrolysed into adenosine, tonically depresses neuromuscular transmission, (2) the intact ATP molecule enhances neuromuscular transmission. This second possibility seems unlikely since the stable ATP analogue, α,β-methylene ATP (Yount, 1975) had no effect on neuromuscular transmission. The observations that AOPCP attenuates the depressing effect of ATP, but not that of adenosine, on neuromuscular transmission, and that this action of AOPCP can be surmountable by exogenously applied 5′-nucleotidase, suggest that its action on neuromuscular transmission might result from its ability to prevent hydrolysis of adenine nucleotides into adenosine.

The increase caused by AOPCP in e.p.p. amplitude was about 49 % of the increase caused by adenosine deaminase in the same experimental conditions. Assuming that the effect of AOPCP results from its ability to prevent hydrolysis of released adenine nucleotides into adenosine, one can estimate that endogenous adenine nucleotides can contribute at least 40 to 50 % to the pool of extracellular endogenous adenosine which modulates neuromuscular transmission. This value may be an underestimate if not all 5′-nucleotidase activity was inhibited by AOPCP (cf. Fredholm, Jonzon, Lindgren & Lindström, 1982).

In conclusion, the present results indicate that (1) when the quantal content of e.p.p.s is assumed to be normal extracellular endogenous adenosine tonically depresses neuromuscular transmission, (2) a dipyridamole-sensitive adenosine uptake process is involved in the inactivation of adenosine at the neuromuscular junction, and (3) released adenine nucleotides might contribute to the pool of endogenous adenosine which modulates neuromuscular transmission.

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