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On the adenosine receptor and adenosine inactivation at the rat diaphragm neuromuscular junction

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1 The effects of adenosine and adenosine analogues 2-chloroadenosine (CADO), L-N^6-phenylisopropyladenosine (L-PIA), D-N^6-phenylisopropyladenosine (D-PIA), N^6-cyclohexyladenosine (CHA) and 5'-N-ethylcarboxamide adenosine (NECA) on evoked endplate potentials (e.p.ps) and on twitch tension were investigated in innervated diaphragms of the rat.

2 Adenosine and its analogues decreased, in a concentration-dependent manner, the amplitude of both the e.p.ps and the twitch responses evoked by nerve stimulation. The order of potency in decreasing the twitch tension was CHA, L-PIA, NECA > D-PIA > CADO > adenosine. L-PIA was about 8 times more potent than D-PIA. Neither adenosine nor the adenosine analogues affected the twitch responses of directly stimulated tubocurarine-paralysed muscles.

3 8-Phenyltheophylline (8-PT), theophylline and isobutylmethylxanthine (IBMX), in concentrations virtually devoid of effect on neuromuscular transmission, antagonized the inhibitory effect of 2-chloroadenosine. The order of potency of the alkylxanthines as antagonists of the adenosine receptor at the rat diaphragm neuromuscular junction was 8-PT > IBMX > theophylline. The antagonism by these xanthines was shown to be competitive, the pA_2 value for 8-PT being 7.16. In concentrations slightly higher than those used to test its ability to antagonize the adenosine receptor, IBMX and 8-PT increased the amplitude of e.p.ps without modifying their decay phase or the resting membrane potential of the muscle fibre.

4 The adenosine uptake inhibitor, nitrobenzylthioinosine (NBI) and the adenosine deaminase inhibitor, erythro-9(2-hydroxy-3-nonyl)adenine (EHNA), in concentrations virtually devoid of effect on neuromuscular transmission, potentiated the inhibitory effect of adenosine at the rat diaphragm neuromuscular junction. The potentiation factors were about 2.6 for NBI (5 μM), 2.2 for EHNA (25 μM) and 4.6 for the combination of NBI (5 μM) and EHNA (25 μM).

5 It is concluded that both uptake and deamination contribute to the inactivation of adenosine at the rat diaphragm neuromuscular junction and that in this preparation the inhibitory effect of adenosine on transmission is mediated by a xanthine-sensitive adenosine receptor with an agonist profile which does not fit the criteria for its classification either as an A_1 or A_2-adenosine receptor.

Introduction

The inhibitory effect of adenosine on neurotransmitter release was first detected at the rat diaphragm neuromuscular junction (Ginsborg & Hirst, 1971; 1972). However, no studies have been performed to investigate how adenosine is inactivated or to characterize the adenosine receptor in this preparation. The equipotency of adenosine and ATP at the rat diaphragm neuromuscular junction (Ribeiro & Walker, 1975) suggests the involvement of a P_1 (Burnstock, 1978)/R-type (Londos & Wolf, 1977) purinoceptor. It has recently been reported that at the frog sartorius neuromuscular junction, where adenosine is inactivated by uptake (Ribeiro & Sebastião, 1987), the adenosine receptor that mediates the inhibitory action of this nucleoside on transmission has an agonist profile with N^6-cyclohexyladenosine (CHA), L-N^6-phenylisopropyladenosine (L-PIA) and 5'-N-ethylcarboxamide adenosine (NECA) being almost equipotent and more potent than 2-chloroadenosine (CADO) (Ribeiro & Sebastião, 1985). This agonist profile does not fit the A_1/A_2 criteria for adenosine receptor classification (see Ribeiro & Sebastião, 1986a). In this preparation the inhibitory effect of 2-chloroadenosine on transmission is antagonized by 8-phenyltheophylline (8-PT) and theophylline, but not by isobutylmethylxanthine

(IBMX), which, however, in several preparations is an adenosine receptor antagonist and increases transmitter release (e.g. Jackisch *et al.*, 1984).

The present work was undertaken to characterize the adenosine receptor at the rat diaphragm neuromuscular junction, by use of stable adenosine analogues as agonists and the alkylxanthines, 8-PT, theophylline and IBMX as antagonists. We also studied how adenosine is inactivated by using the adenosine uptake inhibitor, nitrobenzylthioinosine (NBI) and the adenosine deaminase inhibitor, erythro-9(2-hydroxy-3-nonyl)adenine (EHNA).

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

Methods

The experiments were carried out on isolated preparations of the phrenic nerve-diaphragm of the rat. The rats used were Wistar strain, of either sex and of no more than 200 g in weight.

Endplate potentials recordings

Evoked endplate potentials (e.p.ps) were recorded from single fibres of innervated strips of the diaphragm at room temperature (22–25°C). The strips (approx. 8 mm wide) were mounted in a Perspex chamber of 3 ml capacity through which the solutions flowed continuously at a rate of 3 ml min^{-1} via a roller pump, the bath level being kept constant by suction. The solutions were changed by transferring the inlet tube of the pump from one flask to another. This involved a minimum disturbance of 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 determine rates of onset of the effects of the drugs. The changeover times in the Figures of this paper indicate the times at which the inlet tube of the pump was transferred to a new solution.

The e.p.ps were recorded in the conventional way (Fatt & Katz, 1951) with intracellular electrodes filled with KCl (3 M) and of 10–20 MΩ resistance; the bath electrode was a Ag-AgCl pellet. The nerve was stimulated supramaximally with rectangular pulses of 20 μs duration applied once every 2 s. Evoked responses of 64 or 128 consecutive stimuli were averaged after amplification with a Datalab DL-4000 computer. The output of the computer was coupled to a pen recorder. The resting membrane potential of the muscle fibre was continuously monitored and displayed in a second channel of the pen recorder. The usual procedure was to continue to record averages of consecutive e.p.ps in the same solution until a stable value was obtained, i.e., until two successive averages differed by less than 5%.

Muscle contraction recordings

When recording twitch responses the innervated diaphragm strips were set up in a 25 ml bath through which the solutions flowed continuously with the aid of a roller pump. The flow rate was 50 ml min^{-1} during the first 2 min after changing the solutions and 10 ml min^{-1} until the next changeover of solutions. Unless otherwise specified, the experiments were performed at 37°C; occasionally they were performed at room temperature (22–25°C). The nerve was stimulated supramaximally with rectangular pulses of 100 μs duration applied once every 5 s. The tension developed (T) was recorded isometrically at a resting tension of 50 mN with a Sanborn transducer and displayed on a Hewlett-Packard recorder. The maximum rate of rise of tension (dT/dt_{max}) was recorded simultaneously with T. In some experiments the twitch responses evoked by direct stimulation of the muscle fibres were also recorded; supramaximal pulses of 0.8–1.5 ms duration were applied once every 5 s by means of two palador wires. In these experiments tubocurarine was added to the bath in a concentration (5 μM) sufficient to cause complete blockade of neuromuscular transmission.

Solutions and drugs

The normal bathing solution (pH 7.4) contained (mM): NaCl 117, KCl 5, $NaHCO_3$ 25, NaH_2PO_4 1.2, glucose 11, $CaCl_2$ 2.5, $MgCl_2$ 1.2. When recording e.p.ps the Mg^{2+} concentration was adjusted to prevent twitches of the muscles in response to nerve stimulation. In order to reduce the safety margin of neuromuscular transmission (Paton & Waud, 1967) when recording twitch responses the Mg^{2+} concentration was increased (7.5 – 9.5 mM) or submaximal concentrations (0.7–0.8 μM) of tubocurarine were added. The solutions applied to the preparations were continuously gassed with 95% O_2 and 5% CO_2.

Drugs used were: adenosine, 2-chloroadenosine (CADO), S-(*p*-nitrobenzyl)-6-thioinosine (NBI), theophylline, (+)-tubocurarine hydrochloride (Sigma, St Louis, MO, U.S.A.); N^6-cyclohexyladenosine (CHA), 1,3-isobutylmethylxanthine (IBMX), D-N^6-phenylisopropyladenosine (D-PIA), L-N^6-phenylisopropyladenosine (L-PIA), 8-phenyltheophylline (8-PT) (Research Biochemicals Inc., Wayland, MA, U.S.A.). Erythro-9(2-hydroxy-3-nonyl)adenine (EHNA) was a gift from Burroughs Wellcome, Greenville, U.S.A., and 5'-N-ethylcarboxamide adenosine was a gift from Byk Gulden, Konstanz, F.R.G. L-PIA and

D-PIA were made up in 50 mM stock solutions in dimethylsulphoxide (DMSO) containing 120 mM NaCl; 8-PT and NBI were made up in 10 mM stock solutions in 80% methanol/20% molar NaOH (v/v). Dilutions of these stock solutions were used.

Statistics

The values are given as mean ± s.e. mean. The significance of the differences between the means was calculated by Student's *t* test. *P* values of 0.05 or less were considered to represent significant differences.

Results

Adenosine and adenosine analogues

Comparison between the effects of 2-chloroadenosine on endplate potentials and on nerve-evoked twitch responses. Figure 1 illustrates the effect of the adenosine analogue, CADO, on the amplitude of evoked endplate potentials (e.p.ps) recorded from a muscle fibre of an innervated rat diaphragm paralysed with high Mg^{2+} ($MgCl_2$ 19 mM). CADO (1–30 μM) decreased in a concentration-dependent manner the average amplitude of e.p.ps, without modifying their decay phase or the resting membrane potential of the muscle fibre (Figure 1B). The full effect of each concentration of CADO on the amplitude of e.p.ps was usually observed within 10 to 15 min after starting its application to the preparations and was washed out within 30 to 40 min after returning to the control bathing solution (Figure 1A).

The effect of CADO on the nerve-evoked twitch responses of a rat diaphragm preparation is shown in Figure 2. The Mg^{2+} concentration of the solutions was 7.5 mM, which decreased the amplitude of the nerve-evoked twitches to 57% of its value in the normal (Mg^{2+} 1.2 mM) bathing solution. In this condition CADO (1–10 μM) caused a concentration-dependent decrease in the twitch amplitude, the effect being reversed after 35 min washing (Figure 2A). As can be seen in Figure 2B, CADO affected to the same extent both the amplitude and the maximum rate of rise of the nerve-evoked twitches.

Comparing the results shown in Figures 1 and 2, one can conclude that CADO decreased the amplitude of e.p.ps and the twitch amplitude within a similar range of concentrations and in a quantitatively similar way. This might indicate that the inhibitory action of this adenosine analogue on twitch responses evoked by nerve stimulation is due to a reduction in neuromuscular transmission and not to a direct effect on muscle contraction. To investigate this further an experiment was performed with direct stimulation of the diaphragm in the presence of tubocurarine in a concentration (5 μM) that caused complete inhibition of neuromuscular transmission. Under these conditions CADO (50 μM) was virtually devoid of effect on twitch amplitude, though a five times lower concentration (10 μM) of CADO, applied to the same preparation under indirect stimulation in the absence of tubocurarine, had caused its typical inhibitory effect (49% reduction) on twitch amplitude.

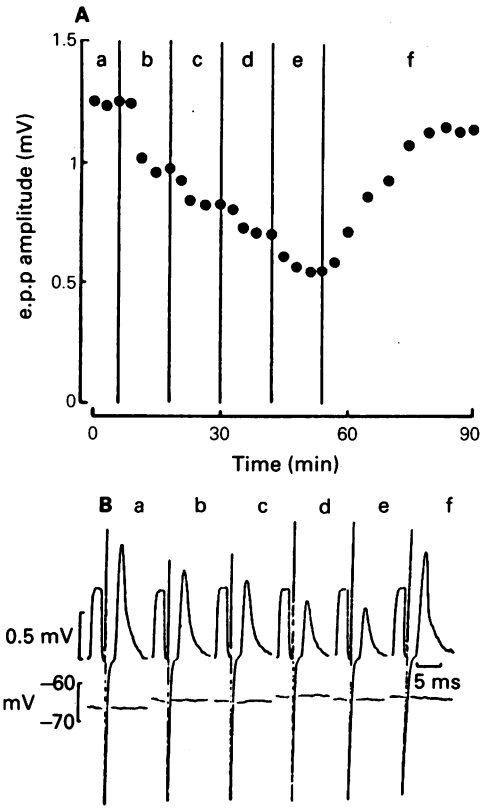

Figure 1 Effect of 2-chloroadenosine (CADO) on the averaged amplitude of evoked endplate potentials (e.p.ps) recorded from a muscle fibre of the rat diaphragm. Solutions contained 19 mM Mg^{2+} which prevented muscle action potentials and twitches in response to nerve stimulation. (A) Time course of the effects of different concentrations of CADO. The ordinates are the computed averages of 128 successive e.p.ps and the abscissae the times averaging began. (a) and (f) control bathing solution; (b) CADO (1 μM); (c) CADO (3 μM); (d) CADO (10 μM); (e) CADO (30 μM). (B) Upper part: pen-recorder traces of averaged e.p.ps corresponding to: (a) 6, (b) 18, (c) 30, (d) 42, (e) 54, (f) 90 min for time in (A). Each response is preceded by a calibration pulse of 750 μV amplitude and 2 ms duration. Lower part: membrane resting potential.

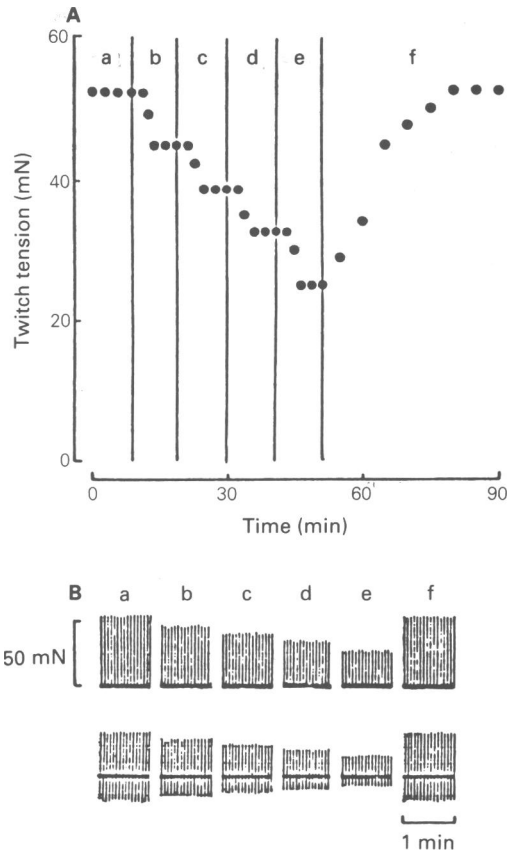

Figure 2 Effect of 2-chloroadenosine (CADO) on the twitch responses evoked by nerve stimulation of a rat diaphragm. (**A**) Time course of the effects of different concentrations of CADO. (a) and (f) control bathing solution; (b) CADO (1 μM); (c) CADO (2.5 μM); (d) CADO (5 μM); (e) CADO (10 μM). (**B**) Pen recorder traces of nerve evoked twitch responses (upper part) and maximum rate of rise of tension (lower part) corresponding to (a) 8, (b) 18, (c) 30, (d) 40, (e) 50, (f) 90 min for time in (**A**). Solutions contained 7.5 mM Mg^{2+} which decreased the nerve evoked twitch responses to 57% of its amplitude in the normal (Mg^{2+} 1.2 mM) bathing solution.

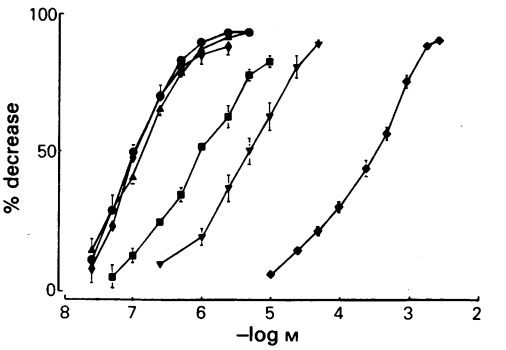

Figure 3 Concentration-response curves for the inhibitory effects of N^6-cyclohexyladenosine (CHA, ●), L-N^6-phenylisopropyladenosine (L-PIA, ♦), 5'-N-ethylcarboxamide adenosine (NECA, ▲), D-N^6-phenylisopropyladenosine (D-PIA, ■), 2-chloroadenosine (CADO, ▼) and adenosine (◆) on twitch responses evoked by nerve stimulation of rat diaphragm muscles. The ordinates are percentage decreases in twitch amplitude recorded in high Mg^{2+} (7.5–9.5 mM) bathing solutions (averaged twitch amplitude in the high Mg^{2+} control bathing solutions was 44 ± 1.6% of the twitch amplitude in the normal (Mg^{2+} 1.2 mM) bathing solution). 0% is the twitch amplitude in the high Mg^{2+} control bathing solutions and 100% represents a complete inhibition of the twitches. The vertical bars represent ± s.e. mean and are shown when they exceed the symbols in size. Averaged twitch amplitude in the normal bathing solution: 74 ± 4.6 mN. Each point is the average of 3 to 7 experiments.

Rank order of potency of the adenosine analogues All the adenosine analogues tested (L- and D-PIA, CHA, NECA and CADO) decreased in a concentration-dependent manner both the amplitude and the maximum rate of rise of tension evoked by indirect stimulation of rat diaphragms. Concentration-response curves for the inhibitory effect of adenosine and its analogues on nerve-evoked twitch amplitude are shown in Figure 3. The effects of L-PIA and D-PIA could not be attributed to its solvent DMSO, since the maximum concentration of DMSO (0.02% v/v) present in the PIA solutions applied to the preparations was devoid of effect on the twitch responses.

Table 1 shows the mean values for the concentration of each adenosine receptor agonist that produced 50% of the maximal effect (EC_{50}) calculated from the data of Figure 3. It is evident that the rank order of potency of adenosine and its derivatives was CHA, L-PIA, NECA > D-PIA > CADO > adenosine.

Since the data shown in Table 1 were obtained in 7.5–9.5 mM Mg^{2+} solutions, which decreased the amplitude of the nerve-evoked twitch responses to about 50% of its value in the normal (Mg^{2+} 1.2 mM) bathing solution, we also studied the potency of CADO in inhibiting the amplitude of nerve-evoked twitches in normal Mg^{2+} (1.2 mM) solutions; four experiments were performed and the amplitude of nerve-evoked twitches was decreased with tubocurarine (0.7–0.8 μM) to 53 ± 0.8% of its value in the normal bathing solution. In these conditions, CADO (1–10 μM) also caused concentration-dependent decreases in twitch amplitude, the EC_{50} value being

Table 1 Relative potencies of the adenosine receptor agonists

	EC_{50} (nM)	*Relative potency*
CHA	97 ± 10 ($n = 13$)	2987
L-PIA	111 ± 21 ($n = 12$)	2610
NECA	115 ± 13 ($n = 12$)	2519
D-PIA	918 ± 74 ($n = 10$)	316
CADO	3885 ± 555 ($n = 12$)	75
Adenosine	289715 ± 34936 ($n = 17$)	1

Relative potency: EC_{50} of adenosine/EC_{50} of each agonist. EC_{50} values were determined on the regression lines (method of least squares, correlation coefficients with $P < 0.05$) of the linear part of the concentration-response curves shown in Figure 3, and represent the concentration of each agonist ± s.e. mean (n = number of observations from 3 to 7 experiments) that produced 50% of the maximal effect; this was taken as 92% decrease in twitch tension which was about the maximal effect obtained for CHA. CHA, N^6-cyclohexyladenosine; L-PIA, L-N^6-phenylisopropyladenosine; NECA, 5'-N-ethylcarboxamide adenosine; D-PIA; D-N^6-phenylisopropyladenosine; CADO, 2-chloroadenosine.

$3.4 \pm 0.7\,\mu$M, which is similar to the EC_{50} value ($3.9 \pm 0.6\,\mu$M, cf. Table 1) of CADO in the preparations bathed with high Mg^{2+} (7.5–9.5 mM) solutions.

Adenosine and its analogues when applied to the innervated diaphragms in the highest concentration used to test their effects on neuromuscular transmission were devoid of effect on the twitch responses evoked by direct stimulation of the muscles in which transmission had been prevented with a supramaximal concentration of tubocurarine (5 μM).

Adenosine receptor antagonists

The ability of alkylxanthines (theophylline, 8-PT and IBMX) to antagonize the inhibitory effect of CADO on twitch responses evoked by indirect stimulation of rat diaphragms was investigated. In this set of experiments the effect of different concentrations of CADO in the absence, in the presence and again in the absence of alkylxanthines was compared sequentially in the same experiment. Alkylxanthines were used in concentrations devoid of effect on twitch responses and were applied to the preparations at least 30 min before applying CADO in their presence. Figure 4 illustrates the action of 8-PT (0.1–0.5 μM), IBMX (1–5 μM) and theophylline (10–50 μM) on the inhibitory effect of CADO (10 μM) on neuromuscular transmission. As can be seen, the three alkylxanthines antagonized in a concentration-dependent manner the inhibitory effect of CADO, the effect of this adenosine analogue being significantly lower ($P < 0.05$) in the presence of these xanthines. The concentrations of 8-PT, IBMX and theophylline that produced 50% antagonism (IC_{50}) of the inhibitory effect of CADO (10 μM) calculated from the data shown in Figure 4B were 0.2 μM, 4.5 μM and 32 μM, respectively.

The antagonism caused by these alkylxanthines was reversed in 10 to 20 min, i.e. after removal of the xanthines from the bath, CADO (10 μM) inhibited the nerve-evoked twitch responses in a similar manner to that observed before applying the xanthines to the preparations.

In the presence of 8-PT (0.1–2.5 μM), the log concentration-response curve for the inhibitory effect of CADO on twitch amplitude was shifted to the right in a near parallel way, the shift being the greater the higher the concentration of 8-PT (Figure 5A). The pA_2 value for 8-PT, calculated by the method of Schild (Arunlakshana & Schild, 1959) was 7.16, the slope of the regression line of the Schild plot (Figure 5B) being 1.14. From the data shown in Figure 5A it appears that the maximal effect of CADO is not modified by 8-PT (0.1–0.5 μM); a full concentration-response curve for CADO in the presence of the highest concentration (2.5 μM) of 8-PT was not performed due to the high concentrations (> 1 mM) of CADO needed to observe its maximal effect in the presence of the antagonist. Kinetic analysis by the double reciprocal plot of the data shown in Figure 5A suggested that the maximal effect of CADO in the presence of 8-PT (0.1–2.5 μM) remains virtually unchanged, whereas its apparent affinity was decreased (Figure 5C).

The antagonism by 8-PT of the inhibitory effect of CADO on neuromuscular transmission could not be attributed to its solvent (80% methanol/20% molar NaOH, v/v) since the maximal concentration (0.025% v/v) of solvent present in the 8-PT solutions applied to the preparations did not antagonize the inhibitory action of CADO on the nerve-evoked twitch responses.

In order to test whether the antagonistic action of IBMX and theophylline against the inhibitory effect of CADO on neuromuscular transmission was surmountable by a supramaximal concentration of CADO, experiments were designed in which the effect of CADO (500 μM) on the amplitude of nerve-evoked twitches was compared in the presence and in the absence of the xanthines. In these experiments neither IBMX (5 μM) nor theophylline (50 μM) affected significantly the inhibitory effect of CADO (500 μM) on neuromuscular transmission. Thus, the adenosine analogue decreased the amplitude of the nerve-evoked twitches by $92 \pm 2.4\%$ in the absence of the xanthines and by $91 \pm 3.4\%$ and $92 \pm 1.7\%$ in

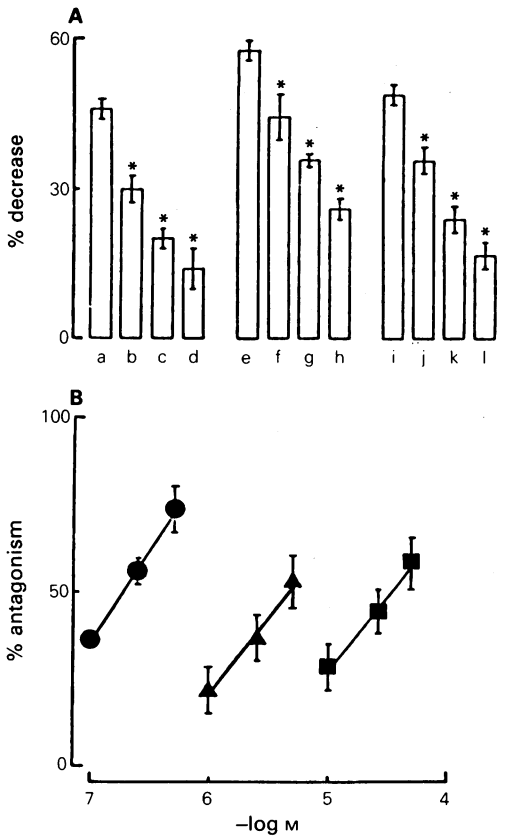

Figure 4 Comparison of the potencies of 8-phenyltheophylline (8-PT), isobutylmethylxanthine (IBMX) and theophylline in antagonizing the inhibitory effect of 2-chloroadenosine (CADO) on twitch responses evoked by nerve stimulation of rat diaphragms. (**A**) The left panel (a–d) illustrates the antagonism by 8-PT, the middle panel (e–h) the antagonism by IBMX and the right panel (i–l) the antagonism by theophylline. The ordinates are percentage decreases in twitch amplitude caused by (a), (e) and (i) CADO (10 μM) in the absence of antagonists; (b), (c) and (d) CADO (10 μM) in the presence of 0.1, 0.25 and 0.5 μM 8-PT respectively; (f), (g) and (h) CADO (10 μM) in the presence of 1, 2.5 and 5 μM IBMX, respectively; (j) (k) (l) CADO (10 μM) in the presence of 10, 25 and 50 μM theophylline, respectively. Each column represents pooled data from 3 to 5 experiments and in each panel (a–d; e–h; i–l) results obtained from the same experiments are compared. Solutions contained 8.0 mM Mg^{2+} which decreased the twitch amplitude to 49 $\pm$ 1.5% of its value (78 $\pm$ 3.6 mN) in the normal (Mg^{2+} 1.2 mM) bathing solution. The vertical bars represent $\pm$s.e. mean. *$P < 0.05$ as compared to the effect of CADO alone in the same experiments. (**B**) Ordinates: percentage antagonism of the inhibitory effect of CADO (10 μM) caused by different concentrations of 8-PT (●), IBMX (▲) and theophylline (■). The percentage antagonism was calculated as $100 \times (E_0 - E_A)/E_0$ where E_0 and E_A represent the effect of CADO (10 μM) in the absence and in the presence of antagonist, respectively. Data were obtained from the same experiments as in (**A**). The vertical bars represent $\pm$s.e. mean. The regression lines were calculated by the method of least squares (correlation coefficients with $P < 0.05$).

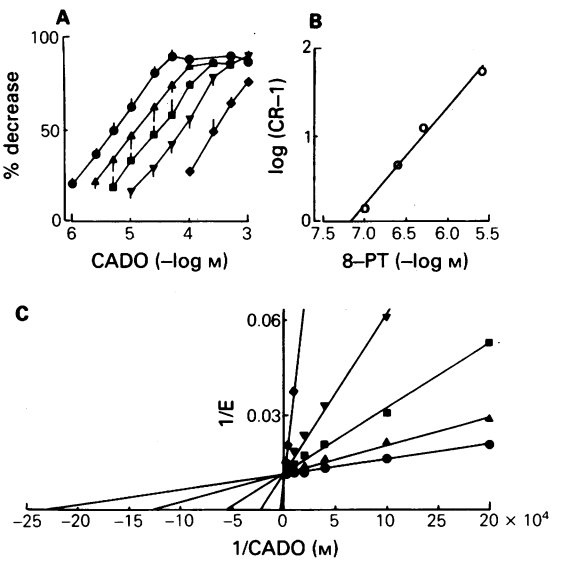

Figure 5 Effect of 8-phenyltheophylline (8-PT) on the concentration-response curve for the inhibitory effect of 2-chloroadenosine (CADO) on neuromuscular transmission. (**A**) Ordinates: percentage decrease in twitch amplitude caused by different concentrations of CADO in the absence (●) and in the presence of 0.1 μM (▲), 0.25 μM (■), 0.5 μM (▼) and 2.5 μM (◆) 8-PT. The vertical bars represent $\pm$s.e. mean. Each point is the average of 4 to 7 experiments. The solutions contained 7.5–9.5 mM Mg^{2+}. Averaged twitch tension in the high-Mg^{2+} control bathing solutions was 45 $\pm$ 2.7% of its value (77 $\pm$ 6.1 mN) in the normal (Mg^{2+} 1.2 mM) bathing solution. (**B**) Schild plot of the data shown in (**A**) where CR represents the concentration-ratios calculated from the log concentration-response curves for CADO at the level of 46% inhibition (EC_{50}) of twitch amplitude. The regression line ($y = 1.14x + 8.17$) was calculated by the method of least squares (correlation coefficient with $P < 0.05$). The pA_2 value for 8-PT ($-x$ intercept) was 7.16. (**C**) Double reciprocal plot of the data shown in (**A**) where C is the concentration of CADO and E is the percentage decrease in twitch tension caused by CADO. The symbols have the same meaning as in (**A**). The regression lines were calculated by the method of least squares (correlation coefficients with $P < 0.05$). The apparent K_D values ($-1/x$ intercept) of CADO in the absence and in the presence of 0.1 μM, 0.25 μM, 0.5 μM and 2.5 μM 8-PT were 4.3 μM, 7.4 μM, 17.5 μM, 41.8 μM and 266 μM, respectively, and the calculated maximal effects (1/y intercept) were 91%, 85%, 86%, 84% and 99%, respectively.

the presence of IBMX (5 μM) or theophylline (50 μM), respectively (four experiments).

Isobutylmethylxanthine at room temperature The finding that IBMX antagonizes the inhibitory effect of CADO on transmission at the rat diaphragm neuromuscular junction at 37°C (present work) but does not antagonize the adenosine receptor at the frog sartorius neuromuscular junction at room temperature (Ribeiro & Sebastião, 1985), prompted an investigation into the antagonistic effect of IBMX on the rat diaphragm at room temperature (22–25°C). IBMX (5 μM) antagonized by 45 $\pm$ 6.0% (n = 5) the inhibitory effect of a submaximal concentration of CADO (25 μM) on the twitch amplitude, the antagonism being reversed by removing the xanthine from the bath. This suggests that temperature has little or no effect on the ability of IBMX to antagonize the adenosine receptor at the rat diaphragm neuromuscular junction.

Effects of isobutylmethylxanthine and 8-phenyltheophylline on endplate potentials Figure 6 illustrates the effect of IBMX (5–500 μM) on the amplitude of e.p.ps recorded from a rat diaphragm paralysed with Mg^{2+} ($MgCl_2$ 21 mM). IBMX increased e.p.ps amplitude, the effects of 25 μM and 500 μM being of similar magnitude and more pronounced than that of 5 μM IBMX. In the range of concentrations used (1–500 μM) this xanthine was virtually devoid of effect on the resting membrane potential of the muscle fibres or on the decay phase and duration of the e.p.ps (six experiments).

As with IBMX, 8-PT (10 μM) also increased the amplitude of e.p.ps recorded from rat diaphragm muscle fibres paralysed with high Mg^{2+} (19–20 mM), the mean increase in e.p.ps amplitude being 32 $\pm$ 7.1% (n = 5). In this concentration 8-PT completely antagonized the inhibitory effect of CADO (10 μM), which when applied to the same endplate in the absence of 8-PT decreased the average amplitude of e.p.ps by 40% (n = 2). In these experiments the solvent of 8-PT was present in similar amounts (0.1% v/v) in all solutions without 8-PT, thus neither the increase in e.p.ps amplitude caused by 8-PT nor its ability to prevent the effect of CADO can be attributed to the solvent. The resting membrane potential of the muscle fibres and the duration or decay phase of the e.p.ps were virtually unaffected by 8-PT (10 μM).

Inactivation of adenosine

The actions of NBI, an adenosine uptake blocker (Paterson *et al.*, 1977) and of EHNA, an adenosine deaminase inhibitor (Agarwal *et al.*, 1977), on the inhibitory effect of adenosine on neuromuscular

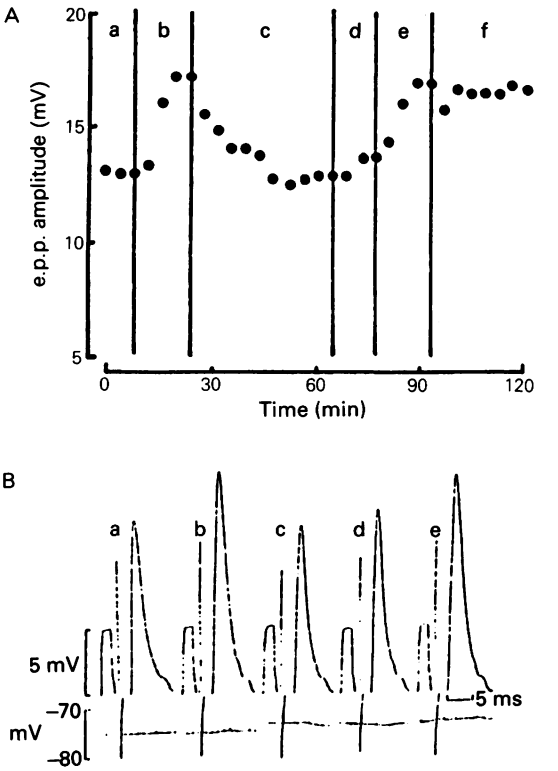

Figure 6 Effect of isobutylmethylxanthine (IBMX) on the averaged amplitude of evoked endplate potentials (e.p.ps) recorded from a rat-diaphragm. Solutions contained 21 mM Mg^{2+} which prevented muscle action potentials and twitches in response to nerve stimulation. (A) Time course of the effects of different concentrations of IBMX. The ordinates are the computed averages of 128 successive e.p.ps and the abscissae the times averaging began. (a), (c) and (f) control bathing solution; (b) IBMX (25 μM); (d) IBMX (5 μM); (e) IBMX (500 μM). (B) Upper part: pen-recorder traces of averaged e.p.ps corresponding to (a) 8, (b) 24, (c) 52, (d) 77 and (e) 90 min for time in (A). Each response is preceded by a calibration pulse of 5 mV amplitude and 2 ms duration. Lower part: membrane resting potential. Note that the effect of IBMX in high concentration (500 μM) was not washed out during 30 min that followed the return to the bathing solution without IBMX.

transmission in the rat diaphragm were investigated. In each experiment different concentrations of adenosine were applied to the preparations in a cumulative manner, first in the absence of NBI or EHNA; adenosine was then washed out and a new concentration-response curve for adenosine, now in the presence of NBI or EHNA was performed. The comparisons between the concentration-response

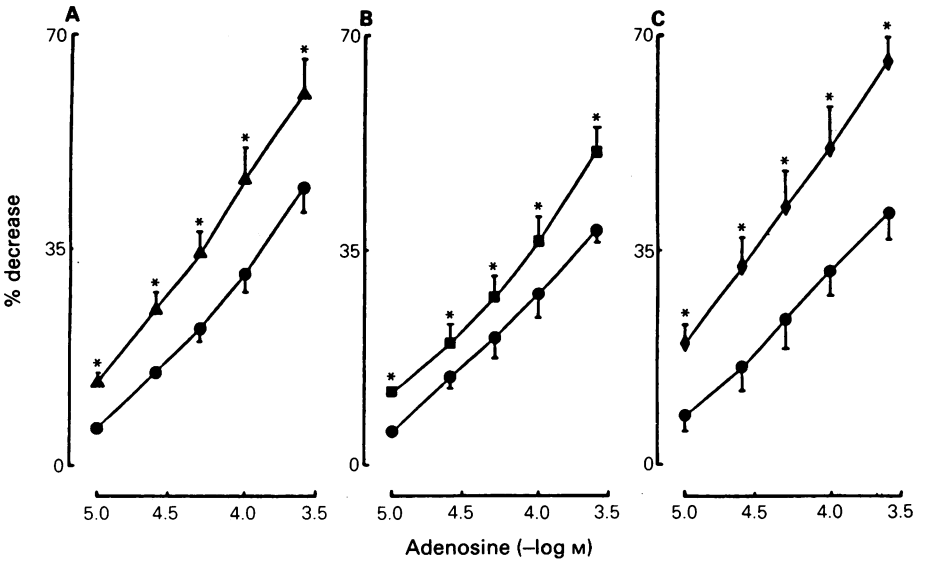

Figure 7 Actions of nitrobenzylthioinosine (NBI) (**A**), erythro-9(2-hydroxy-3-nonyl)adenine (EHNA) (**B**) and NBI + EHNA (**C**) on the concentration-response curves for the inhibitory effect of adenosine on neuromuscular transmission. The ordinates are percentage decreases in nerve-evoked twitch tension caused by adenosine (●), adenosine in the presence of NBI (5 μM) (▲), adenosine in the presence of EHNA (25 μM) (■) and adenosine in the presence of NBI (5 μM) + EHNA (25 μM) (◆). Each panel represents pooled data from 4 to 7 experiments. Solutions contained 8.0–9.0 mM Mg^{2+} which decreased the twitch amplitude to 44 ± 2.2% of its value (57 ± 4.6 mN) in the normal (Mg^{2+} 1.2 mM) bathing solution. 0% is the twitch amplitude in the high Mg^{2+} control bathing solutions and 100% represents a complete inhibition of the twitches. The vertical bars represent ±s.e. mean and are shown when they exceed the symbols in size. *$P < 0.05$ (paired Student's t test as compared to the effect of adenosine alone in the same experiments). The concentrations of adenosine that caused 35% inhibition of the twitch amplitude, determined on the regression lines (method of the least squares, correlation coefficients with $P < 0.05$) of the linear part of the concentration-response curves for adenosine were: (**A**) 127 μM in the absence and 48 μM in the presence of NBI (5 μM); (**B**) 185 μM in the absence and 83 μM in the presence of EHNA (25 μM); (**C**) 139 μM in the absence and 30 μM in the presence of NBI (5 μM) + EHNA (25 μM).

curves for adenosine in the absence or in the presence of NBI or EHNA were made using data from the same experiments because there is considerable variation between the quantitative effects of the same concentration of adenosine in different experiments. NBI or EHNA were used in concentrations virtually devoid of effect on twitch amplitude and the pre-incubation time with these substances before applying adenosine in its presence was about 10 min.

As can be seen in Figure 7A, NBI (5 μM) shifted to the left the log concentration-response curve for the inhibitory effect of adenosine (10–250 μM) on the amplitude of the nerve-evoked twitches. EHNA (25 μM) also shifted to the left the log concentration-response curve for adenosine (10–250 μM) (Figure 7B) though to a smaller extent than NBI. Increasing the concentration of NBI up to 20 μM or EHNA up to 50 μM did not cause a further potentiation of the effect of adenosine. However, when both NBI (5 μM) and EHNA (25 μM) were present, the shift of the log concentration-response curve for adenosine (10–250 μM) (Figure 7C) was greater than that caused by each inhibitor of adenosine inactivation alone. The potentiation factors, determined as the ratio between the concentrations of adenosine that caused 35% inhibition of the twitch tension when applied to the preparations in the absence and in the presence of NBI (5 μM), EHNA (25 μM) and NBI (5 μM) + EHNA (25 μM) were 2.6, 2.2, and 4.6, respectively (see Legend to Figure 7).

The action of NBI on the effect of adenosine could not be attributed to its solvent (80% methanol/20% molar NaOH, v/v), since the concentration of solvent (0.05% v/v) present in the NBI solutions applied to the preparations did not affect the inhibitory action of adenosine on neuromuscular transmission. Neither NBI (5 μM) nor EHNA (25 μM) nor a mixture of the two, potentiated the inhibitory effect of CADO (0.25–25 μM) on the amplitude of the twitches evoked by nerve stimulation (three experiments).

Discussion

The present results show that adenosine and its stable analogues decreased the amplitude of both e.p.ps and the twitch responses evoked by indirect stimulation of rat diaphragm muscle fibres. The rank order of potency for adenosine and its analogues in decreasing the twitch tension was CHA, L-PIA, NECA > D-PIA > CADO > adenosine; L-PIA was about 8 times more potent than D-PIA. The inhibitory effect of these substances on twitch responses cannot be attributed to a direct action on contractile mechanisms since adenosine and its analogues were devoid of effect on twitch amplitude when the diaphragms were directly stimulated in the presence of a supramaximal concentration of tubocurarine. The inhibition of neuromuscular transmission caused by adenosine and its analogues should be attributed to a presynaptic action of these substances, i.e. to a decrease in the evoked release of acetylcholine from the phrenic nerve endings (cf. Ginsborg & Hirst, 1972; Ribeiro & Walker, 1975). The spontaneous release of acetylcholine in the mouse diaphragm is also decreased by adenosine analogues, the rank order of potency being L-PIA > NECA > CADO (Singh *et al.*, 1986).

It is known that Mg^{2+} ions increase the binding of adenosine receptor agonists to brain membranes (Goodman *et al.*, 1982; Yeung *et al.*, 1985). In the present work it was observed that the concentrations of CADO needed to decrease e.p.ps amplitude from preparations in which the twitches were prevented by 19 mM to 21 mM Mg^{2+}, were the same as those needed to inhibit nerve-evoked twitches in preparations where the safety margin of neuromuscular transmission (see Paton & Waud, 1967) was reduced with 7.5 mM to 9.5 mM Mg^{2+}. Also, the EC_{50} value for CADO to inhibit twitch responses in the presence of Mg^{2+} (7.5–9.5 mM) was similar to its EC_{50} value in experiments in which normal (1.2 mM) concentrations of Mg^{2+} were used and the safety margin of neuromuscular transmission (see Paton & Waud, 1967) was reduced with submaximal concentrations (0.7–0.8 μM) of tubocurarine. Thus, it appears that the Mg^{2+} concentrations used in the present work did not markedly affect the inhibitory action of adenosine receptor agonists on neuromuscular transmission. Furthermore, the adenosine receptor agonist profile found in the rat diaphragm neuromuscular junction with CHA, L-PIA NECA as the most potent agonists and more potent than CADO, is similar to the agonist profile described for the adenosine receptors that mediate decreases in the electrically evoked release of the transmitter from most preparations of the peripheral and central nervous systems where lower Mg^{2+} concentrations in the bath were used. This is the case for the frog sartorius neuromuscular junction (Ribeiro & Sebastião, 1985), guinea-pig ileum (Paton & Webster, 1984; Christofi & Cook, 1986), rat anococcygeus (Stone, 1983), rat vas deferens (Paton & Webster, 1984), rabbit portal vein (Brown & Collis, 1983) and rabbit hippocampus (Jackisch *et al.*, 1984; 1985; Feuerstein *et al.*, 1985). As previously discussed (Hughes & Stone, 1983; Stone, 1983; Ribeiro & Sebastião, 1985; Christofi & Cook, 1986; Church *et al.*, 1986; Ribeiro & Sebastião, 1986a; Singh *et al.*, 1986) an order of agonist potency in which CHA, L-PIA and NECA were almost equipotent and more potent than CADO does not fit the A_1/A_2 criteria for adenosine receptor classification. At the A_1-adenosine receptors, CHA and L-PIA are more potent than CADO, which is more potent than NECA, and at the A_2-adenosine receptors NECA is more potent than CADO, which is more potent than CHA and L-PIA (e.g. Daly, 1983).

The effects of NECA are usually more rapid in onset than those of L-PIA and CHA and this has been used as an argument to explain the high potency of NECA as an inhibitor of neurotransmitter release (Collis, 1985). In the present work the effect of NECA was also observed after shorter times than those observed for L-PIA or CHA, but the experimental conditions used allowed us to wait long enough for the observation of the full effect of each concentration of the adenosine receptor agonists, and thus the equipotency of NECA, L-PIA and CHA should not be considered as a consequence of the time course of their effects. The adenosine receptor which has an agonist profile with CHA, L-PIA and NECA as the most potent agonists and more potent than CADO, was recently named A_3 (Ribeiro & Sebastião, 1986a). According to this criterion the adenosine receptor that mediates inhibition of neuromuscular transmission at the rat diaphragm neuromuscular junction might be classified as an A_3-adenosine receptor. However, in the absence of distinct antagonist profiles for the putative extracellular adenosine receptors, the nature of the presynaptic adenosine receptor at the neuromuscular junction cannot be conclusively established.

8-PT, IBMX and theophylline, in concentrations that did not modify the nerve-evoked twitch responses, antagonized the inhibitory effect of CADO on neuromuscular transmission. The order of potency of the antagonists, with 8-PT > IBMX > theophylline, conforms with the results obtained by others in the central nervous system (Smellie *et al.*, 1979; Wu *et al.*, 1982). The findings that 8-PT decreases the apparent affinity of CADO without decreasing its maximal effect, and that the Schild plot for 8-PT was linear with a slope near 1, indicate that the adenosine receptor at the rat diaphragm neuromuscular junction is competitively

antagonized by 8-PT. A detailed analysis of the nature of the antagonism by the less potent antagonists, theophylline and IBMX, was not done in the present work but the observation that the antagonism by these xanthines was surmountable by a supramaximal concentration of CADO suggests that the antagonism by IBMX and theophylline is also of a competitive nature.

In the present work IBMX increased the amplitude of e.p.ps, an effect that might be related to its ability to antagonize endogenous adenosine which tonically inhibits transmission at the rat diaphragm neuromuscular junction. In contrast, at the frog sartorius neuromuscular junction, IBMX does not antagonize the adenosine receptor (Ribeiro & Sebastião, 1985) and in submillimolar concentrations has postsynaptic inhibitory effects on transmission (Ribeiro & Sebastião, 1987), that are probably related to its ability to block the opening of acetylcholine receptors and to increase the rate constant of receptor closure (Silinsky & Vogel, 1987). Whether the apparently different behaviour of IBMX against the adenosine receptors at the rat diaphragm and frog sartorius neuromuscular junctions reflects different sensitivities of both species to IBMX itself, rather than differences in the adenosine receptor cannot be answered in the present investigation. Nevertheless, the hypothesis (Ribeiro & Sebastião, 1985) that the absence of antagonism by IBMX is a general characteristic of the adenosine receptors at the neuromuscular junction can no longer be sustained.

The inhibitory effect of adenosine on transmission at the rat diaphragm neuromuscular junction was potentiated by NBI, an adenosine uptake blocker (Paterson *et al.*, 1977) and by EHNA, an adenosine deaminase inhibitor (Agarwal *et al.*, 1977). The potentiation of the effect of the nucleoside by these adenosine inactivation inhibitors was specific for adenosine, since the inhibitory effect of CADO, an adenosine analogue which is not a substrate for adenosine deaminase (e.g. Daly, 1983) and has low affinity for the adenosine uptake system (Jarvis *et al.*, 1985), was not potentiated by NBI or EHNA. The ability of NBI and EHNA to potentiate the inhibitory effect of adenosine on neuromuscular transmission indicates that at the rat diaphragm neuromuscular junction, adenosine is inactivated by uptake and deamination. This contrasts with the frog sartorius neuromuscular junction (Ribeiro & Sebastião, 1987) and rabbit hippocampus (Jackisch *et al.*, 1984; 1985; Feuerstein *et al.*, 1985) where adenosine uptake appears to be the main if not the only relevant process for inactivation of extracellular adenosine. The finding that in the rat diaphragm neuromuscular junction the potentiation of the inhibitory effect of adenosine caused by NBI was more pronounced than that caused by EHNA when both adenosine inactivation inhibitors were used in supramaximal concentrations, indicates that in this preparation adenosine uptake might be more important than adenosine deamination in the regulation of the extracellular adenosine levels. The potentiation of the effect of adenosine by EHNA and NBI, used together, was not greater than the sum of the potentiation factors caused by each inhibitor of adenosine inactivation used alone. This contrasts with what happens in rat cerebral cortical slices, where the action of an adenosine deaminase inhibitor is more pronounced when the adenosine uptake system is blocked (Nimit *et al.*, 1981), and suggests that at the rat diaphragm neuromuscular junction the adenosine uptake system and adenosine deaminase act in an independent manner to regulate the levels of extracellular adenosine.

One point may be noted in relation to the low potency of exogenous adenosine at the rat diaphragm neuromuscular junction, even in the presence of adenosine inactivation inhibitors. It is known that considerable amounts of ATP are released from the phrenic nerve terminals upon nerve stimulation (Silinsky, 1975), and in cholinergic synapses extracellular ATP can be hydrolysed into adenosine (Zimmermann *et al.*, 1986; Ribeiro & Sebastião, 1987). Evidence that endogenous adenosine might be present at the rat diaphragm neuromuscular junction was provided by the present observations that 8-PT in a concentration (10 μM) that completely antagonized the inhibitory effect of CADO (10 μM) on the amplitude of e.p.ps increases the amplitude of e.p.ps, and that IBMX also increases their amplitude, its maximal effect being obtained with concentrations (25 μM) not expected to cause maximal inhibition of phosphodiesterases (cf. Smellie *et al.*, 1979). Thus, the low potency of exogenously applied adenosine at the rat diaphragm neuromuscular junction might reflect, at least in part, partial occupation of the adenosine receptors with endogenous adenosine.

In conclusion, adenosine inhibits transmission at the rat diaphragm neuromuscular junction by activating a xanthine-sensitive receptor with an agonist profile that does not fit the A_1/A_2-criteria for adenosine receptor classification. Inactivation of extracellular adenosine at the rat diaphragm, in contrast with what occurs in other preparations from the peripheral and central nervous systems (e.g. Jackisch *et al.*, 1984; 1985; Feuerstein *et al.*, 1985; Ribeiro & Sebastião, 1987), might occur through both uptake and deamination.

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