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Enhancement of tetrodotoxin-induced axonal blockade by adenosine, adenosine analogues, dibutyryl cyclic AMP and methylxanthines in the frog sciatic nerve

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1 The effects of adenosine, adenosine analogues (N^6-cyclohexyladenosine (CHA), L-N^6-phenylisopropyladenosine (L-PIA), D-N^6-phenylisopropyladenosine (D-PIA), N^6-methyladenosine and 2-chloroadenosine), adenine, inosine, hypoxanthine, cyclic AMP and its analogue the dibutyryl cyclic AMP (db cyclic AMP), and methylxanthines (theophylline, caffeine and isobutylmethylxanthine (Ibmx) on compound action potentials were investigated in de-sheathed sciatic nerve preparations of the frog.

2 Adenosine and its analogues enhanced, in a concentration-dependent manner, the inhibitory action of tetrodotoxin (TTX) on nerve conduction. The order of potencies was: CHA > D-PIA > L-PIA > N^6-methyladenosine > 2-chloroadenosine > adenosine.

3 The adenosine metabolites, inosine and hypoxanthine, were inactive on TTX-induced axonal blockade. Adenine enhanced the inhibitory action of TTX on nerve conduction, but was less effective than adenosine.

4 db Cyclic AMP, but not cyclic AMP, mimicked the inhibitory effect of adenosine on nerve conduction.

5 Methylxanthines did not antagonize the effect of adenosine on TTX-induced axonal block and in high concentrations also mimicked the effect of adenosine on nerve conduction.

6 The possibility of adenosine acting on TTX-induced axonal block through an adenosine receptor positively coupled to adenylate cyclase is discussed.

Introduction

Adenosine is released from nerve fibres upon electrical stimulation (Maire *et al.*, 1982). When applied exogenously, this substance does not affect either axonal (Okamoto *et al.*, 1964; Ribeiro & Dominguez, 1978) or nerve terminal (Silinsky, 1984) action potentials. In a recent study, Ribeiro & Sebastião (1984) demonstrated that adenosine triphosphate (ATP) and adenosine diphosphate (ADP) antagonize the inhibitory action of tetrodotoxin (TTX) on nerve conduction; adenosine monophosphate (AMP) had little or no effect, and adenosine did not reverse the inhibition induced by TTX on action potential amplitude. It has been shown that adenosine promotes accumulation of cyclic AMP in peripheral axons (Roch & Salamin, 1976) and that a cyclic AMP-dependent protein kinase can selectively phosphorylate the TTX-sensitive subunit of the sodium channel (Costa *et al.*, 1982). The present work was undertaken to investigate further the effect of adenosine on nerve conduction of preparations partially blocked by TTX.

Methods

The experiments were carried out at room temperature (22–25°C) on the partially de-sheathed frog sciatic nerve trunk taken from autumn frogs (*Rana ridibunda*). The preparations were mounted in a Perspex chamber in which a Perspex block was fitted with electrodes for stimulating the nerve trunk and for recording the action potentials. The preparations were arranged so that the bathing solution or the solutions containing the drugs could be applied as

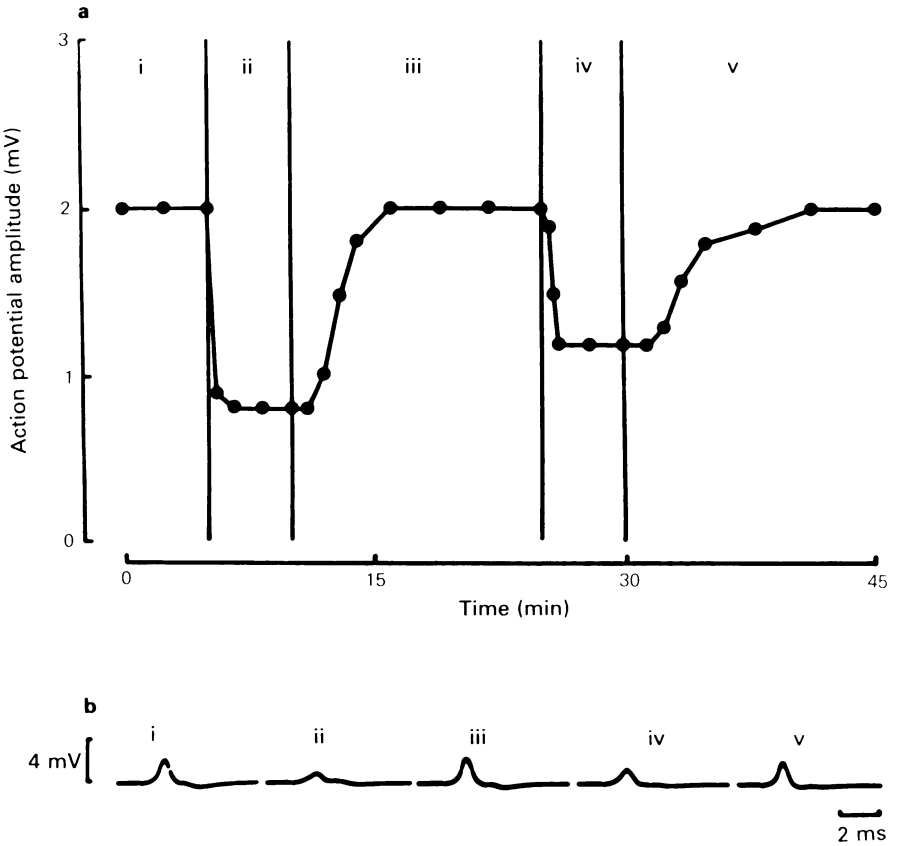

Figure 1 Effect of adenosine on the amplitude and duration of a compound action potential recorded from a frog-sciatic nerve. (a) Time course of the effect of adenosine: (i), (iii) and (v) tetrodotoxin (TTX, 80 nM); (ii) TTX (80 nM) + adenosine (5 mM); (iv) TTX (80 nM) + adenosine (2 mM). In (b) are shown compound action potentials corresponding to (i) 4, (ii) 9, (iii)19, (iv) 29 and (v) 44 minutes for time in (a) (each trace consists of six consecutive superimposed action potentials). Action potential amplitude in the bathing solution before applying TTX was 12.0 mV and its value after returning to the bathing solution was 8.8 mV.

pulses of 500 μl to the de-sheathed part of the trunk. The tissue as a whole was kept moist because the bottom of the chamber contained the bathing solution and the top was tightly sealed with paraffin wax to prevent evaporation. The technique used for the dissection was that described by the Staff of the Department of Pharmacology of the University of Edinburgh (1968). The nerve was stimulated supramaximally with rectangular pulses of 0.01 ms duration applied once every 5 s. Throughout the experiments compound action potentials were recorded in the conventional way and photographed.

Solutions and drugs

The bathing solution (pH = 7.0) contained (mM): NaCl 117, KCl 2.5, NaH_2PO_4 1, Na_2HPO_4 1, $MgCl_2$ 1.2 and $CaCl_2$ 1.8. Drugs used were tetrodotoxin (Sankyo); adenosine, 2-chloroadenosine, N^6-methyladenosine, adenine, inosine, hypoxanthine, adenosine 3′,5′-monophosphate (cyclic AMP), dibutyryl cyclic adenosine 3′,5′-monophosphate (db cyclic AMP), caffeine, theophylline (Sigma); N^6-cyclohexyladenosine (CHA), L-N^6-phenylisopropyladenosine (L-PIA), D-N^6-phenylisopropyladenosine (D-PIA), 1,3-isobutylmethylxanthine (Ibmx) (R.B.I.). L-PIA and D-PIA were made up into a 50 mM stock solution in dimethylsulphoxide (DMSO) containing 120 mM NaCl and dilutions of this were used. The pH of the solutions was readjusted to 7.0 with NaOH or HCl where necessary.

Statistics

The significance of the differences between means was calculated using Student's t test. P values of 0.05 or less were considered to represent significant differences.

Results

The effect of adenosine

Figure 1 illustrates the effect of adenosine (2–5 mM) on the amplitude and duration of a compound action potential recorded from a frog sciatic nerve in the presence of TTX (80 nM). As can be seen, adenosine enhanced in a concentration-dependent manner the inhibitory effect of TTX on action potential amplitude (see also Figure 2). The full effect of adenosine was usually seen in the first 5 min that followed its application to the nerve and was easily washed out in 5–15 min, i.e., following adenosine, TTX decreased the action potential amplitude in a similar way to that observed before using adenosine (Figure 1).

In order to detect the adenosine-induced enhancement of the inhibitory action of TTX on action potential amplitude, the latter has to be reduced by this toxin to less than 50% of its initial value in the bathing solution. In preparations in which the amplitude of the action potential recorded in the presence

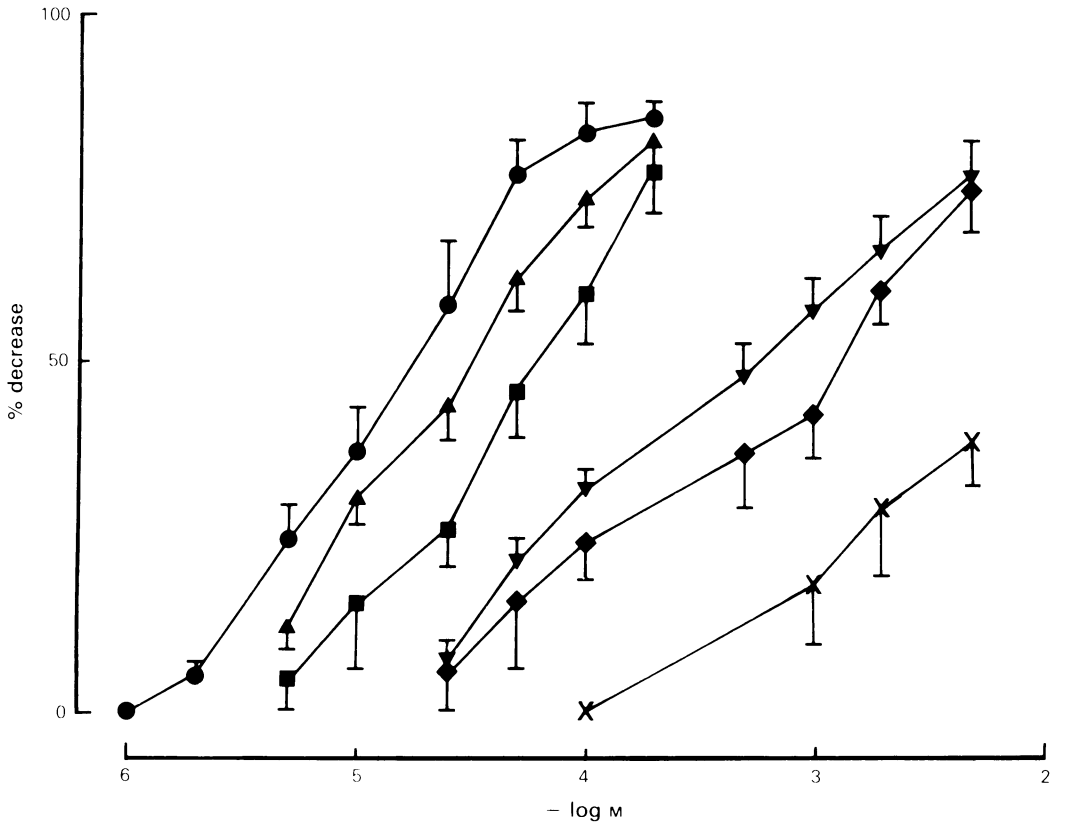

Figure 2 Concentration-response curves for the effect of N^6-cyclohexyladenosine (CHA) (●), D-N^6-phenylisopropyladenosine (D-PIA) (▲), L-N^6-phenylisopropyladenosine (L-PIA) (■), N^6-methyladenosine (▼), 2-chloroadenosine (◆) and adenosine (×) on tetrodotoxin (TTX)-induced axonal blockade. The ordinates are percentage decreases in the amplitude of the compound action potentials recorded in the presence of TTX 30–130 nM (average action potential amplitude in the presence of TTX was 19 ± 1.3% of the action potential amplitude in the bathing solution). 0% is the action potential amplitude in the presence of TTX, and 100% represents a complete inhibition of action potential amplitude. The vertical bars represent s.e.mean and are shown when they exceed the symbols. Average action potential amplitude in the bathing solution 5.7 ± 0.5 mV. Each point is the average of 4 to 6 experiments.

of TTX was above 50% of its value in the bathing solution, adenosine (1–5 mM) had little or no effect on the TTX-induced axonal blockade and in TTX-free solutions adenosine was devoid of effect on action potential amplitude. In the experiment illustrated in Figure 1, TTX (80 nM) decreased the action potential amplitude to 17% of its initial value in the bathing solution. Similar TTX-induced inhibitions were used in the remaining experiments, with an average action potential amplitude recorded in the presence of TTX (20–130 nM) of 21.6 ± 1.3% of its initial value in the bathing solution.

Adenosine analogues and metabolites In order to know whether the effect of adenosine on TTX-induced axonal blockade was mediated through a specific adenosine receptor, the effects of the stable adenosine analogues, 2-chloroadenosine, N^6-methyladenosine, L-N^6-phenylisopropyladenosine (L-PIA), D-N^6-phenylisopropyladenosine (D-PIA) and N^6-cyclohexyladenosine (CHA) on nerve conduction were investigated. As occurred with adenosine, all these compounds (1 μM–5 mM) were inactive when applied to axons in the absence of TTX. However, when the action potential amplitude had been decreased by this toxin, the adenosine analogues enhanced in a concentration-dependent manner the inhibitory effect of TTX on nerve conduction (Figure 2). A complete inhibition (100% decrease in the action potential amplitude recorded in the presence of TTX) was never achieved for CHA, which behaved as the most potent of the adenosine analogues tested (see Figure 2). The effects of L-PIA and D-PIA could not be attributed to its solvent, DMSO, since the maximum concentration of DMSO (0.4% v/v) present in the PIA solutions applied to the nerves, was inactive on TTX-induced axonal block. The full effect of each substance was always seen in the first 5 min after its application. The effects of N^6-methyladenosine and 2-chloroadenosine were usually washed out in 10–15 min and those of CHA, L-PIA and D-PIA in 20–40 min after the preparations returned to the control TTX solution.

Table 1 shows the values for the concentration of each substance that produced half maximal effect (ED_{50}) calculated from the data of Figure 2. These data were obtained in the presence of TTX (30–130 nM) which decreased the action potential amplitude to 19 ± 1.3% of its initial amplitude in the bathing solution. The averages of the TTX-induced blockades used to test each substance did not differ statistically ($P > 0.05$). It is evident from Table 2 that the rank order of potencies for adenosine and its derivatives was CHA > D-PIA > L-PIA > N^6-methyladenosine > 2-chloroadenosine > adenosine.

Since electrical activity in nerve fibres causes the release of adenine, inosine and hypoxanthine (Maire *et al.*, 1982), the effects of these substances on the axonal blockade induced by TTX (30–40 nM) were investigated in three experiments. Neither inosine (5 mM) nor hypoxanthine (5 mM) changed the inhibition induced by TTX on nerve conduction. Adenine, 1 mM, had little or no effect on TTX-induced axonal blockade, and 2 and 5 mM, caused a decrease in the amplitude of the action potentials recorded in the presence of TTX of 11 ± 5.9% and 35 ± 6.2% respectively; only the effect of adenine 5 mM was statistically different from the control ($P < 0.05$).

The effect of dibutyryl cyclic AMP

Figure 3A illustrates the time course of the effect of dibutyryl cyclic AMP (db cyclic AMP) (1 mM) on the amplitude of a compound action potential recorded in the presence of TTX (40 nM). The effect of adenosine (1 mM) was also studied in order to compare the effects of both substances in the same preparation. As can be seen db cyclic AMP reversibly enhanced the inhibitory effect of TTX on action potential amplitude and its effect was similar to that observed with adenosine. The effects of another concentration (5 mM) of adenosine and db cyclic AMP on TTX (80 nM)-induced inhibition of action poten-

Table 1 ED_{50} values and the relative potencies obtained from the data shown in Figure 2

	CHA	*D-PIA*	*L-PIA*	*N^6-Me-Ad*	*2-Cl-Ad*	*Adenosine*
ED_{50} (μM)	12	22	41	313	550	6412
Relative potency	534	291	156	20	12	1

CHA: N^6-cyclohexyladenosine; D-PIA: D-N^6-phenylisopropyladenosine; L-PIA L-N^6-phenylisopropyladenosine; N^6-Me-Ad: N^6-methyladenosine; 2-Cl-Ad; 2-chloroadenosine. Relative potency: ED_{50} of adenosine/ED_{50} of each agonist. ED_{50} values were determined on the regression lines (method of least squares, correlation coefficients ranged from 0.990 to 0.998) of the linear part of the dose-response curves shown in Figure 2, and represent the concentration of each substance that produced 50% of the maximal effect of CHA.

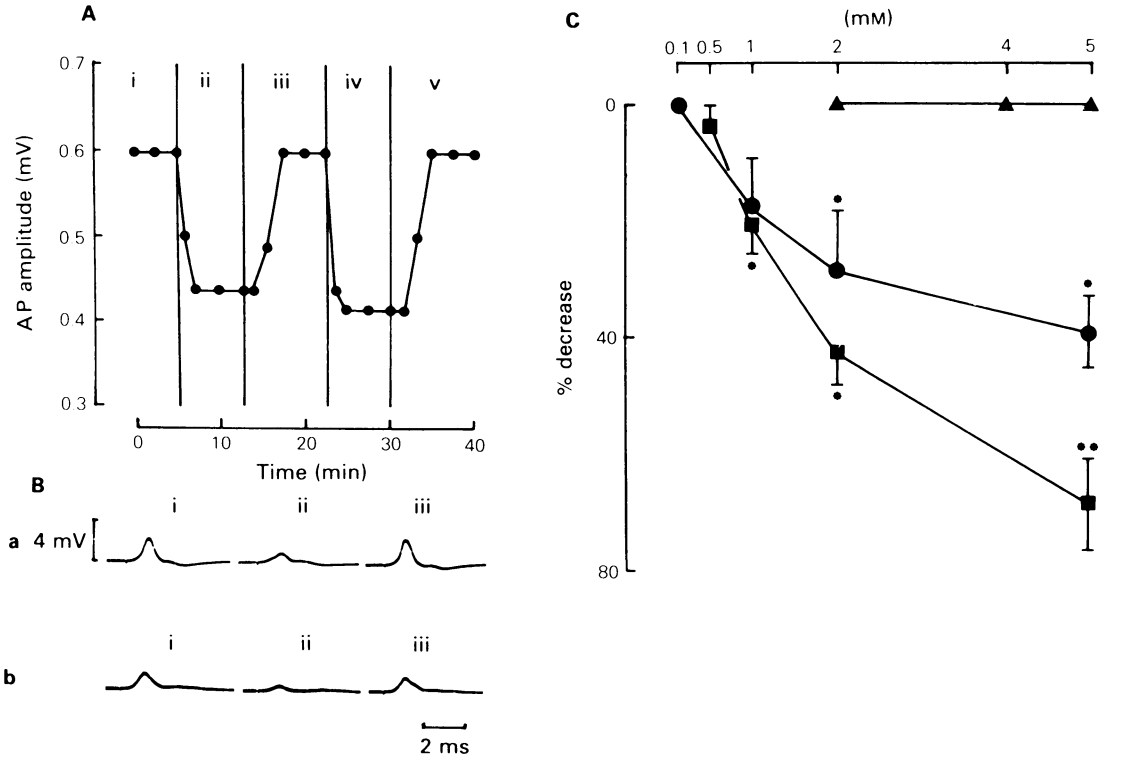

Figure 3 Effects of adenosine and dibutyryl cyclic AMP (db cyclic AMP) on the tetrodotoxin (TTX)-induced axonal blockade in frog-sciatic nerve preparations. (A) Time course of the effects of adenosine and db cyclic AMP on the amplitude of compound action potentials: (i), (iii) and (v) TTX (40 nM); (ii) TTX (40 nM) + adenosine (1 mM); (iv) TTX (40 nM) + db cyclic AMP (1 mM). Compound action potential amplitude in the bathing solution before applying TTX was 11.0 mV and its value 15 min after returning to the bathing solution was 10.6 mV. (B) Compound action potentials recorded from two other experiments: (a) effect of adenosine. (i) and (iii) TTX (80 nM); (ii) TTX (80 nM) + adenosine (5 mM); Compound action potential amplitude in the bathing solution = 12 mV. (b) effect of db cyclic AMP. (i) and (iii) TTX (80 nM); (ii) TTX (80 nM) + db cyclic AMP (5 mM). Compound action potential amplitude in the bathing solution = 5.5 mV. Each trace consists of six consecutive superimposed action potentials. (C) Comparison of the effects of adenosine (●), db cyclic AMP (■) and cyclic AMP (▲). The ordinates are percentage decreases in the amplitude of compound action potentials recorded in the presence of TTX 20–130 nM (averaged action potential amplitude: 23 ± 2% of the action potential amplitude in the bathing solution). The vertical bars represent ± s.e.mean and they are shown when they exceed the symbols. $*P<0.05$ as compared to control (action potential amplitude in TTX); $**P<0.05$ as compared to adenosine. Averaged amplitude in the bathing solution 7.9 ± 0.6 mV. Each point is the average of 3 to 7 experiments.

tial amplitude, recorded from a different experiment, are compared in Figure 3B; similar qualitative effects were observed, though db cyclic AMP was more effective than adenosine. This greater effectiveness was confirmed statistically and is shown in Figure 3C. This figure compares concentration-response curves for db cyclic AMP (0.5–5 mM) and adenosine (0.1–5 mM) as well as illustrating the absence of effect of cyclic AMP (2–5 mM) on the TTX-induced axonal blockade. As with adenosine, db cyclic AMP caused a concentration-dependent enhancement of the inhibitory action of TTX on nerve conduction. This cyclic nucleotide was also devoid of effect on action potential amplitude in TTX-free solutions (cf. Horn & McAfee, 1977).

The effects of methylxanthines

In order to know whether methylxanthines serve as antagonists of the effect of adenosine on nerve conduction, theophylline, caffeine and isobutylmethylxanthine (Ibmx), were tested in preparations

inhibited by TTX. This inhibition was of the same order of magnitude as that used usually to test adenosine in the absence of methylxanthines. In these conditions the methylxanthines (0.01–0.05 mM) neither modified the inhibitory effect of TTX on nerve conduction, nor antagonized the effect of adenosine (1–5 mM). In higher concentrations (0.1–1 mM) these xanthines enhanced in a concentration-dependent manner the inhibitory effect of TTX on nerve conduction (Figure 4 and Table 2) and hence, in these cases antagonism of the adenosine responses could not be tested. As happens with adenosine, these substances were devoid of effect on nerve action potential amplitude, when applied in the absence of TTX (Horn & McAfee, 1977). 5′-N-ethylcarboxamide adenosine (NECA; a gift from Byk Gulden, Konstanz, F.R.G.), 100 μM–5 mM, enhanced TTX-induced axonal blockade (ED_{50} = 459 μM, n = 3). In the rank order of potencies for adenosine and its derivatives NECA was between N^6-Me-Ad and 2-Cl-Ad (see Table1).

Discussion

The present results show that adenosine and its stable analogues (CHA, D-PIA, L-PIA, N^6-methyl adenosine and 2-chloroadenosine) enhanced the TTX induced inhibition of action potential amplitude and that this effect was mimicked by db cyclic AMP but not by cyclic AMP. The absence of effect of cyclic AMP could be attributed to the difficulty with which it crosses cell membranes (Henion *et al.*, 1967). The methylxanthines (theophylline, caffeine and Ibmx), in concentrations which were ineffective on TTX-induced axonal blockade did not prevent the inhibitory effect of adenosine, and in higher concentrations (> 0.1 mM) enhanced the inhibitory action of TTX on nerve conduction. Inosine and hypoxanthine were devoid of effect on the TTX-induced axonal block. Adenine enhanced the inhibitory action of TTX on action potential amplitude but was less effective than adenosine, i.e., adenosine caused a statistically significant effect in concentrations > 2 mM (Figure 3C), whereas, adenine produced a statistically significant effect only at 5 mM.

The effect of adenosine contrasts with the antagonistic effect of ATP on TTX-induced axonal blockade (Ribeiro & Sebastião, 1984). This might indicate the presence in axons of two different populations of purinoceptors (Burnstock, 1978), P_2 more sensitive to ATP which mediates its effect against the TTX-induced axonal blockade, and P_1 more sensitive to adenosine responsible for the enhancement of the TTX-induced inhibition on nerve conduction. The effects of the adenosine analogues, which are resistant to uptake and metabolism, observed in the present work are consistent with the concept that this P_1 adenosine receptor is externally located on the axons and therefore it is an R-type adenosine receptor. The R-type adenosine receptors can be classified into R_a/A_2 and R_i/A_1 types (Londos & Wolff, 1977; van Calker *et al.*, 1979). The R_a/A_2 adenosine receptor operates through activation of adenylate cyclase and consequent increase in intracellular cyclic AMP levels. The present results showing that both adenosine and db cyclic AMP enhance the TTX-induced axonal blockade, taken together with the observation that adenosine stimulates accumulation of cyclic AMP in peripheral axons (Roch & Salamin,

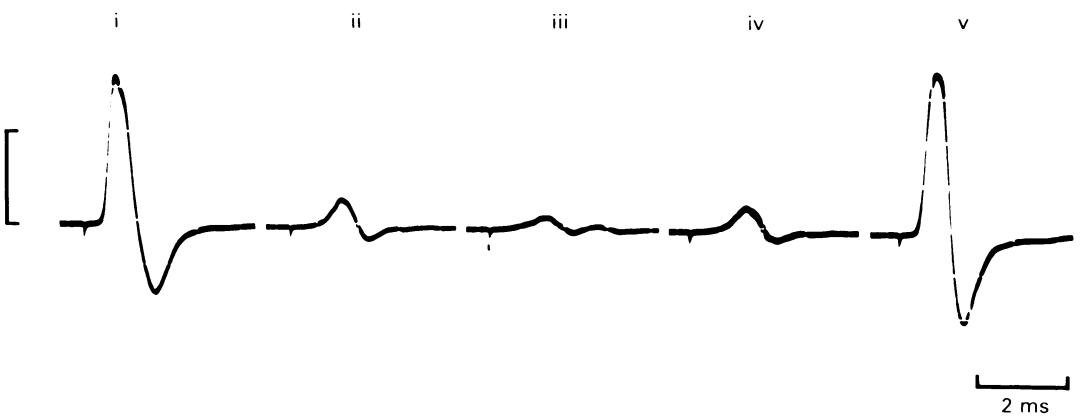

Figure 4 Effect of theophylline on the amplitude and duration of a compound action potential recorded from a frog-sciatic nerve: (i) before applying tetrodotoxin (TTX), (ii) effect after 30 min in TTX (100 nM), (iii) effect after 10 min in TTX (100 nM) + theophylline (1 mM), (iv) 20 min after returning to a theophylline-free solution containing 100 nM TTX, (v) illustrates the amplitude and duration of the compound action potential recorded 15 min after returning to the control bathing solution. The vertical calibration bar in (i) and (v) is 4 mV, and in (ii), (iii) and (iv) is 2 mV. Each trace consists of six consecutive superimposed action potentials.

Table 2 Effects of methylxanthines on the amplitude of action potentials inhibited by tetrodotoxin (TTX)

	% decrease in the action potential amplitude caused by different concentrations (mM)		
	0.1	0.5	1.0
Theophylline	11.7±8.0 ($n=3$)	33.2±1.8* ($n=3$)	65.0±2.0* ($n=3$)
Caffeine	25.0 ($n=1$)	51.6±5.6* ($n=5$)	75.0±1.0* ($n=2$)
Ibmx	10.8±6.4 ($n=4$)	39.7±2.3* ($n=4$)	53.2±5.9* ($n=3$)

Ibmx: isobutylmethylxanthine.
Values are presented as means ± s.e.mean.
Averaged action potential amplitude in the presence of TTX (30–130 nM): 27.7 ±3.1% of the action potential in the bathing solution. Averaged action potential in the bathing solution: 6.4±0.85 mV. *$P<0.05$.

1976), strongly suggests that the effect of adenosine on nerve conduction is operated via increase in cyclic AMP levels, and hence, through an R_a/A_2 kind of receptor. Furthermore, the presently observed absence of stereoselectivity for the isomers of PIA (cf. Smellie *et al.*, 1979; Collis, 1983), together with the present findings showing that the concentrations of the adenosine analogues required to produce half maximal effects on the TTX-induced axonal block were in the micromolar range (cf. Daly, 1983), support the view that the receptor for adenosine on the axons is an A_2-adenosine receptor.

The present observation that methylxanthines do not antagonize the action of adenosine on the TTX-induced axonal block could argue the existence of an A_2-adenosine receptor on axons. However, some A_2-adenosine receptors are relatively insensitive to xanthines (Daly, 1983; Shimizu, 1983). It is interesting to note that the methylxanthines in high concentrations caused an inhibitory effect on nerve conduction similar to that obtained with adenosine or db cyclic AMP. This might result from their ability to inhibit phosphodiesterases (see Wu *et al.*, 1982) and by this mechanism to increase cyclic AMP levels. In fact, it has been shown that theophylline increases cyclic AMP in desheathed frog sciatic nerves (Horn & McAfee, 1977).

Besides a pharmacological characterization of adenosine receptors through use of methylxanthines as antagonists, recent proposals of their classification on the basis of the relative potency of the adenosine agonists have been advanced (see Daly, 1983). The order of potencies for the adenosine agonists, observed in the present work was as follows: CHA > D-PIA > L-PIA > N^6-methyladenosine > 2-chloroadenosine > adenosine. This order does not fit the potency series usually proposed for the A_2-receptor agonists (see Daly, 1983). However, it now appears that the A_2 adenosine receptor is a mixture of subclasses each possessing variable affinities towards adenosine analogues as well as methylxanthines (Daly, 1983; Shimizu, 1983).

In summary, two out of the four criteria proposed for a suspected adenosine receptor positively coupled to adenylate cyclase (Daly, 1983) are now satisfied for axons: (1) adenosine promotes accumulation of cyclic AMP in peripheral axons (Roch & Salamin, 1976); (2) the effect of adenosine is mimicked by db cyclic AMP.

It has been shown that a cyclic AMP-dependent protein kinase can selectively phosphorylate the α-subunit of the sodium channel (Costa *et al.*, 1982) and that cyclic AMP-mediated phosphorylation can modulate the activity of ion channels (see Siegelbaum & Tsien, 1983). The possibility of adenosine decreasing the entry of sodium by promoting phosphorylation of the voltage-dependent sodium channels, via cyclic AMP, might explain its enhancing effect on TTX-induced axonal blockade. Whether the effect of adenosine now described implies a decrease in the number of functional sodium channels, the probability that an individual sodium channel is open or the current through a single sodium channel when it is open needs further investigation.

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