Br. J. Pharmac. (1984), **81**, 277–282

Antagonism of tetrodotoxin- and procaine-induced axonal blockade by adenine nucleotides in the frog sciatic nerve

J.A. Ribeiro & A.M. Sebastião

Laboratory of Pharmacology, Department of Cell Biology, Gulbenkian Institute of Science, 2781 – Oeiras, Portugal

1 The effects of adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), and adenosine on compound action potentials were investigated in de-sheathed frog-sciatic nerve preparations.

2 ATP and ADP but not adenosine antagonized the inhibitory action of tetrodotoxin (TTX) on nerve conduction. AMP had little or no antagonistic effect on TTX-induced axonal block. ATP was more effective than ADP. The effects of the nucleotides were related to the degree of the TTX-induced inhibition and were more evident where the blockade was more intense.

3 ATP and ADP but not adenosine antagonized the procaine-induced axonal blockade which, in some experiments, was completely reversed by these nucleotides. ATP and ADP were of similar potency.

4 The axonal blockade induced by pentobarbitone was not antagonized by ATP, ADP, AMP or adenosine.

5 The possibility that ATP stimulates a TTX-sensitive sodium channel is discussed.

Introduction

Adenosine triphosphate (ATP) and related nucleotides are released from axons during depolarization (Abood *et al.*, 1962; Kuperman *et al.*, 1964b). When exogenously applied to myelinated (Okamoto *et al.*, 1964; Ribeiro & Dominguez, 1978) or unmyelinated (see Stone, 1981) axons as well as to neuromuscular junctions (Bishop *et al.*, 1963; Ribeiro *et al.*, 1979), these nucleotides appear to be devoid of effect. However, if the neuromuscular junctions have been previously inhibited either pre- or postsynaptically, ATP and/or adenosine decrease neuromuscular transmission (e.g. Ribeiro, 1982). It therefore seemed of interest to investigate the effects of purines on axonal conduction of progressively inhibited preparations.

A preliminary account of some of this work has been presented to the Pharmacological Society (Ribeiro & Sebastião, 1983).

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 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. For the dissection, the technique used 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. The bathing solution contained (mM): NaCl 117, KCl 2.5, NaH_2PO_4 1, Na_2HPO_4 1, $MgCl_2$ 1.2 and $CaCl_2$ 1.8. The pH of the bathing solution was 7.0 and readjusted with NaOH or HCl to this value where necessary.

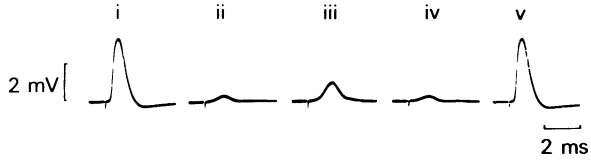

Figure 1 Effect of ATP on the amplitude and duration of a compound action potential recorded from a frog sciatic nerve: (i) before applying tetrodotoxin (TTX), (ii) effect after 20 min in TTX (60 nM), (iii) effect after 10 min in TTX (60 nM) + ATP (5 mM); (iv) 10 min after returning to an ATP-free solution containing 60 nM TTX; (v) illustrates the amplitude and duration of the compound action potential recorded 30 min after returning to the control bathing solution. Each trace consists of six consecutive superimposed action potentials.

Drugs

Drugs used were: procaine hydrochloride (B.D.H.); tetrodotoxin (Sankyo); pentobarbitone sodium (May & Baker); adenosine, adenosine 5′-monophosphate, adenosine 5′-diphosphate (sodium salt); adenosine 5′-triphosphate (sodium salt), (Sigma); ethylenediamine-tetraacetic acid (EDTA) (Sigma).

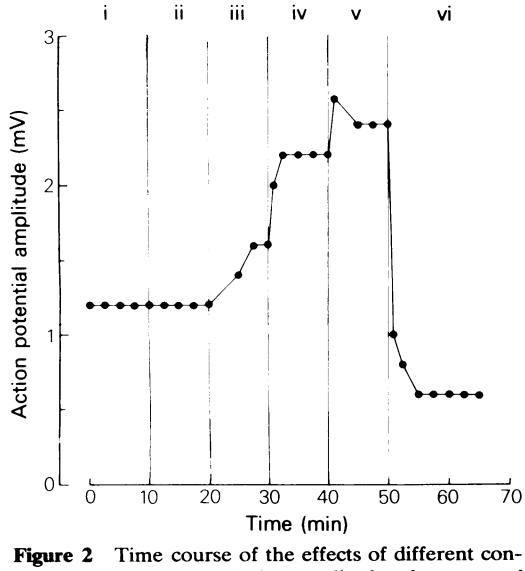

Figure 2 Time course of the effects of different concentrations of ATP on the amplitude of compound action potentials: (i) and (vi) tetrodotoxin (TTX, 100 nM), (ii) TTX (100 nM) + ATP (0.1 mM); (iii) TTX (100 nM) + ATP (1 mM); (iv) TTX (100 nM) + ATP (5 mM), (v) TTX (100 nM) + ATP (10 mM). Compound action potential amplitude in the bathing solution before applying TTX was 8.0 mV and its value 30 min after (vi) was 11.2 mV.

Statistics

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

Results

Tetrodotoxin

The effect of adenosine triphosphate Figure 1 illustrates the effect of ATP (5mM) on the amplitude of a compound action potential recorded from a frog sciatic nerve in the presence of TTX (60 nM). As can be seen, ATP antagonized the inhibition induced by TTX on action potential amplitude. The effect was concentration-dependent as seen in the experiment illustrated in Figure 2, which shows the time course of the effect of ATP (0.1–10 mM) on action potential amplitude. The full effect of ATP (5 mM) was usually seen in the first 5–10 min that followed its application to the nerve, and was easily washed out. Following ATP, TTX (100 nM) decreased the action potential amplitude in a similar way to that observed before using ATP (Figure 2). This means that the increase in action potential amplitude observed during application of ATP in the presence of TTX was caused by ATP itself and did not occur as a consequence of spontaneous recovery. Similar experiments were carried out on 15 different nerves and the results are summarized in Figures 3 and 4.

The effect of ATP on action potential amplitude depended upon the degree of axonal blockade induced by TTX. For a given blockade, ATP caused a concentration-dependent increase in action potential amplitude, but where the blockade was more pronounced ATP produced greater effects (see Figure 3). In order to detect the ATP-induced increase in action potential amplitude, this has to be reduced by TTX to less than 60% of its initial value in the bathing solution. The maximal effect of ATP (which usually represented more than a threefold increase of the action potential amplitude in TTX) was seen when the level of the toxin-induced blockade was below 10% of the value of the action potential amplitude in the bathing solution; however, ATP had no antagonistic effect on the TTX-induced axonal blockade when this was complete. There was considerable variation in the concentration of TTX that caused the same degree of nerve conduction inhibition in different nerves. However, when the same nerve was exposed to different concentrations of TTX, the effects were always concentration-dependent. The range of TTX concentrations needed to induce axonal conduction blockade, which was

sensitive to the action of ATP, was within 15 to 130 nM.

No tachyphylaxis to the action of ATP was observed. Thus, no diminution was seen in its effect on action potential amplitude when the preparation was bathed in TTX (60 nM) plus ATP (5 mM) for more than 5 h.

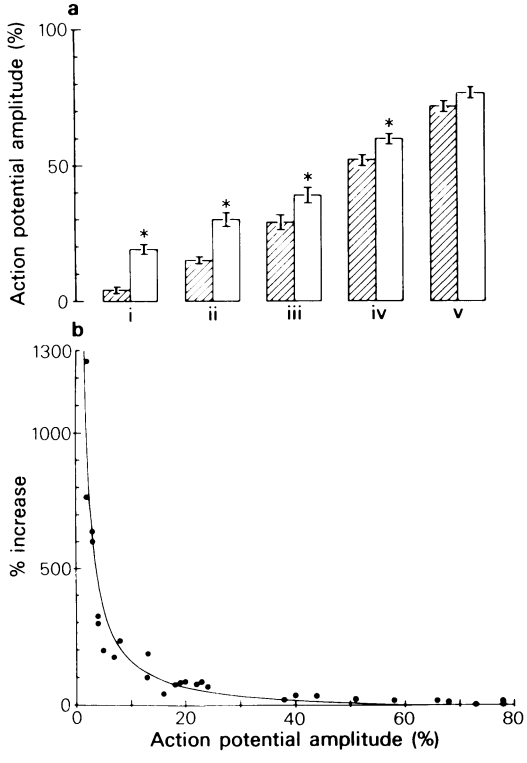

Figure 3 Influence of tetrodotoxin (TTX) induced axonal blockade on the effect of ATP (5 mM) on the amplitude of compound action potentials. (a) Illustrates how different degrees of blockade affect the action of ATP, hatched columns, TTX alone; open columns, TTX plus ATP: (i) 2–10% of the control action potential amplitude in the bathing solution, average concentration (nM) of TTX needed to cause the blockade ± s.e. mean, 68 ± 4.4, n = 10; (ii) 10–20%, 64 ± 10.8, n = 8; (iii) 20–40%, 49 ± 6.8, n = 6; (iv) 40–60%, 45 ± 6.7, n = 6, (v) 60–80%, 32 ± 6.4, n = 8. The amplitude of the action potential in control bathing medium is taken as 100%. The vertical bars represent s.e. mean. *P < 0.05. Mean action potential amplitude in the bathing solution ± s.e. mean, 7.0 ± 0.7 mV. (b) Computed plot of the effect of ATP on the compound action potential amplitude when it was previously blocked by TTX. The abscissae are the amplitudes in presence of TTX as a percentage of the action potential amplitude in the bathing solution. The ordinates are percentage increase in the amplitude of the compound action potential in the presence of TTX. The different 29 observations were taken from 7 experiments. $y = (1915.78/x) - 31.58$.

Comparison of the effects of ATP, ADP, AMP and adenosine A comparison of the effects of these substances (0.1–10 mM) was made in six different experiments and a summary of the results obtained with ATP, ADP and AMP is shown in Figure 4. ATP and ADP produced a concentration-dependent effect, ATP was more potent than ADP, at least by a factor of two whilst AMP had little or no effect on the TTX-induced axonal blockade (Figure 4). Adenosine (0.1–7.5 mM) did not reverse the inhibition induced by TTX on nerve conduction.

Sodium chloride In order to know whether the sodium in the ATP molecule (which was a disodium salt) was responsible for the increase in the action potential amplitude caused by ATP in the presence of TTX, an experiment was designed in which NaCl (10 mM) was applied to a nerve previously blocked by TTX (70 nM). NaCl did not modify the depressant action of the toxin on nerve conduction, although the preparation did respond in the usual way to a concentration of ATP (5 mM) containing the same amount of sodium, i.e. ATP applied in the presence of TTX (70 nM) caused an increase in the action potential amplitude of about 2.5 fold.

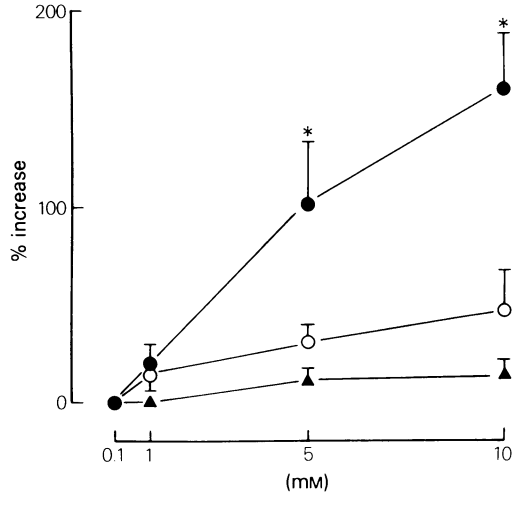

Figure 4 Comparison of the effects of ATP (●), ADP (○) and AMP (▲) on tetrodotoxin (TTX) induced axonal blockade. The ordinates are percentage increases in the amplitude of compound action potentials recorded in the presence of TTX 60–130 nM (average action potential amplitude in the presence of TTX was 12.0 ± 2.3% of the action potential amplitude in the bathing solution). The vertical bars represent s.e. mean and are shown when they exceed the symbols: *$P < 0.05$. Average amplitude in the bathing solution 9.0 ± 0.7 mV. Each point is the average of 4 to 6 experiments.

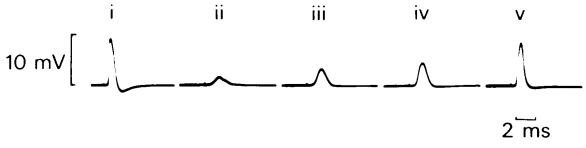

Figure 5 Effect of ADP on the procaine-induced axonal blockade in the frog-sciatic nerve. The amplitude and duration of the compound action potential recorded: (i) in the bathing solution before applying procaine; (ii) effect after 30 min in procaine (5 mM); (iii) after 15 min in ADP (2.5 mM) + procaine (5 mM); (iv) after 15 min in ADP (5 mM) + procaine (5 mM); (v) 20 min after returning to a drug-free bathing solution. Each trace consists of six superimposed action potentials.

EDTA It has been suggested that calcium ions can pass through the TTX-sensitive sodium channels (Baker *et al.*, 1971). In order to explore whether ATP increased the calcium permeability of the TTX-blocked sodium channels, the nucleotide (5–10 mM) was used in two experiments in which EDTA (5 mM) was added to a calcium-free bathing solution. In these conditions, ATP applied in the presence of TTX caused an increase in action-potential amplitude similar to that observed when normal calcium is present in the bathing solution.

The effects of purines on the axonal blockade induced by procaine and pentobarbitone

Procaine ATP and ADP (2.5–7.5 mM) antagonized the inhibitory effect of procaine on action-potential amplitude (Figures 5 and 6). These effects were concentration-dependent and their magnitude was related to the concentration of procaine (Figure 7). For example, ATP (7.5 mM) caused complete recovery of the amplitude of an action potential which had

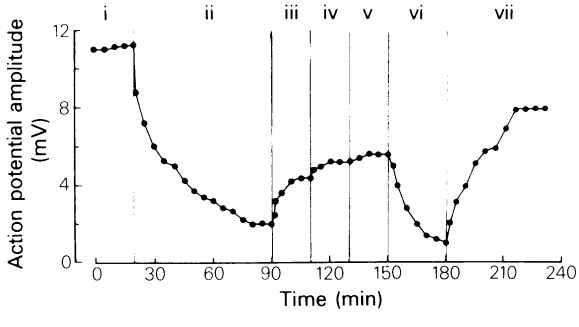

Figure 6 Time course of the effects of different concentrations of ATP on the amplitude of a compound action potential: (i) and (vii) bathing solution; (ii) and (vi) procaine (5 mM); (iii) procaine (5 mM) + ATP (2.5 mM); (iv) procaine (5 mM) + ATP (5 mM); (v) procaine (5 mM) + ATP (7.5 mM).

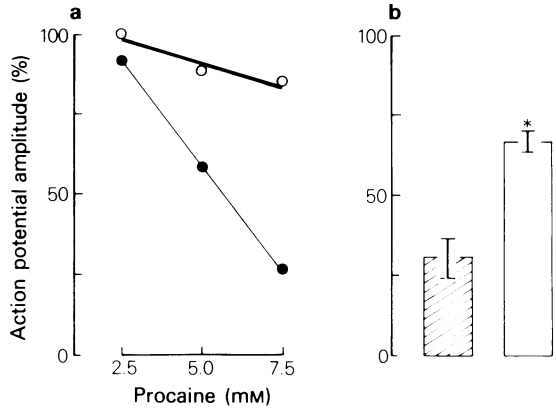

Figure 7 Effect of ATP on compound action potential amplitude in the presence of procaine. (a) Concentration-dependent effects of procaine recorded in the absence (●) and in the presence of ATP (5 mM) (○). 100% = compound action potential amplitude in the bathing solution = 1.2 mV. (b) Results pooled from 10 experiments in which ATP (5 mM) was applied to preparations previously blocked with procaine (5 mM): procaine alone, hatched column; procaine plus ATP 5 mM, open column. 100% = compound action potential amplitude in the bathing solution = 3.7 ± 1.3 mV. $^*P < 0.05$.

been reduced by procaine (5 mM) to about 50% of its control amplitude. For each concentration of ATP, the full effect was usually seen within 10 min after starting its application and the response to both procaine and ATP was completely reversed with a 20 to 40 min wash of control bathing solution (i.e. pre- and post-control measurements did not differ significantly). When their actions were compared on the same nerve, ADP produced its effect more slowly than ATP. For instance, in one experiment, ADP took approx. 15 min to produce its maximal effect, whereas ATP, at the same concentration, took approx. 7 min.

No tachyphylaxis to the action of ATP was observed, since no reduction of its activity was detected when the nerve was kept for more than 5 h in ATP (5 mM) applied in the presence of procaine (5 mM).

In two experiments in which adenosine (5 mM) was used, this substance did not antagonize the procaine (5 mM) – induced axonal blockade.

As was the case when NaCl was used in the presence of TTX, no effect on action potential amplitude was detected when NaCl (10 mM) was applied to axons in the presence of procaine (5 mM).

Pentobarbitone In three experiments ATP (5 mM) and adenosine (7.5 mM) were applied in the presence of pentobarbitone. An axonal conduction blockade of the same magnitude as that observed with TTX or

procaine was induced by pentobarbitone (2–4 mM). However neither ATP nor adenosine modified its depressant effect on action potential amplitude.

Discussion

The present results show that ATP and ADP can antagonize both TTX- and procaine-induced inhibition of action potential amplitude. In the presence of TTX, ATP was more effective than ADP; AMP had little or no effect. In the presence of procaine ATP and ADP had similar potency.

Adenosine did not antagonize the axonal blockade produced by TTX or procaine, suggesting that the energy-rich phosphate bonds of the nucleotides may be responsible for their antagonistic effects. Indeed, the intensity of the antagonism of TTX blockade correlates with the number of phosphate bonds.

The results obtained with ATP and ADP in the presence of procaine confirm the findings of others (Kuperman *et al.*, 1964a; Kraynack *et al.*, 1980), who observed that ATP and ADP antagonize procaine-induced conduction blockade. In the present investigation AMP has not been tested but according to Kuperman *et al.* (1964a) its potency is similar to that of ATP or ADP.

No resting potential was measured in the present work and hence we do not know whether ATP depolarized or hyperpolarized the nerve membrane in the presence of TTX or procaine, and how this could influence its effect on action potential amplitude. However, Akasu *et al.* (1983) have shown that ATP depolarizes the postsynaptic membrane in sympathetic ganglia and during depolarization the amplitude of the fast excitatory postsynaptic potentials decreases. Thus, the increase in action potential amplitude caused by ATP does not appear to be related to its ability to depolarize nerve membranes.

ATP reversed almost completely the procaine-induced axonal blockade but not that induced by TTX, which was only partially antagonized. These differences might be related to the way these anaesthetics affect nerve conduction; TTX is a specific blocker of the external opening of the voltage-sensitive sodium channels of the axonal membrane (Narahashi *et al.*, 1964), whereas procaine is thought to act mainly by displacing membrane-bound calcium, influencing the sodium-ion transport in an indirect manner (Blaustein & Goldman, 1966).

Kuperman *et al.* (1964a) suggested that the effect of ATP on procaine-induced axonal blockade depends on calcium because it is smaller when calcium is removed from the bathing solution by the addition of EDTA. However, this chelating agent did not modify the action of ATP against TTX-induced axonal blockade (present work), suggesting that the TTX-sensitive calcium influx (Baker *et al.*, 1971) does not contribute to the increase caused by ATP in action potential amplitude, observed in the presence of TTX.

The sodium of the ATP molecule does not appear to be responsible for the increase in action potential amplitude caused by ATP in the presence of TTX or procaine, since increasing sodium concentration in the bathing solution did not modify the depressant action of these anaesthetics on nerve conduction. This also precludes the remote possibility of ATP antagonizing the effects of these drugs through an increase in the external sodium concentration via stimulation of Na^+, K^+–ATPase; furthermore, ATP hardly penetrates cell membranes (e.g. Glynn, 1968).

In isolated intestinal epithelial cells, ATP enhances the unidirectional influx of sodium (Kimmich & Randles, 1982). If this applies to nerve cells, through voltage-dependent sodium channels, the possibility of ATP increasing membrane-sodium permeability might explain its antagonistic effect on TTX-induced axonal blockade. Whether this antagonism implies an increase in the conductance of unit sodium channels or the total number of available sodium channels as was proposed for the ion channels operated by the acetylcholine receptor to explain the increase in acetylcholine sensitivity caused by ATP at the end-plate membrane (Akasu *et al.*, 1981) or at the subsynaptic membrane in the sympathetic ganglia (Akasu *et al.*, 1983), cannot be answered by the present investigation.

A.M.S. is in receipt of a Gulbenkian Foundation Research Studentship.

References

ABOOD, L.G., KOKETSU, K. & MIYAMOTO, S. (1962). Outflux of various phosphates during membrane depolarization of excitable tissues. *Am. J. Physiol.*, **202**, 469–474.

AKASU, T., HIRAI, K. & KOKETSU, K. (1981). Increase of acetylcholine-receptor sensitivity by adenosine triphosphate: a novel action of ATP on ACh-sensitivity. *Br. J. Pharmac.*, **74**, 505–507.

AKASU, T., HIRAI, K. & KOKETSU, K. (1983). Modulatory actions of ATP on nicotinic transmission in bullfrog sympathetic ganglia. In *Physiology and Pharmacology of Adenosine Derivatives*. ed. Daly, J., Kuroda, Y., Phillis,

J.W., Shimizu, H. & Ui, M. pp. 165–171. New York: Raven Press.

BAKER, P.F., HODGKIN, A.L. & RIDGWAY, E.B. (1971). Depolarization and calcium entry in squid giant axons. *J. Physiol.*, **218,** 709–755.

BISHOP, H., FRAZER, A.C., ROBINSON, G.B. & SCHNEIDER, R. (1963). The nature of the antiperistaltic factor from wheat gluten. *Br. J. Pharmac. Chemother.*, **21,** 238–243.

BLAUSTEIN, M.P. & GOLDMAN, D.E. (1966). Competitive action of calcium and procaine on lobster axon. *J. gen. Physiol.*, **49,** 1043–1063.

GLYNN, I.M. (1968). Membrane adenosine triphosphatase and cation transport. *Br. med. Bull.*, **24,** 165–169.

KIMMICH, G.A. & RANDLES, J. (1982). An ATP- and Ca^{2+}–regulated Na^{+} channel in isolated intestinal epithelial cells. *Am. J. Physiol.*, **243,** C116–C123.

KRAYNACK, B.J., GINTAUTAS, J. & RACZ, G.B. (1980). Antagonism of procaine conduction block by adenosine 5′-triphosphate *in vitro*. *Proc. West. Pharmac. Soc.*, **23,** 429–432.

KUPERMAN, A.S., OKAMOTO, M., BEYER, A.M. & VOLPERT, W.A. (1964a). Procaine action: antagonism by adenosine triphosphate and other nucleotides. *Science, N.Y.*, **144,** 1222–1223.

KUPERMAN, A.S., VOLPERT, W.A. & OKAMOTO, M. (1964a). Release of adenine nucleotide from nerve axons. *Nature, Lond.*, **204,** 1000–1001.

NARAHASHI, T., MOORE, J.W. & SCOTT, W.R. (1964). Tetrodotoxin blockage of sodium conductance increase in lobster giant axons. *J. gen. Physiol.*, **47,** 965–974.

OKAMOTO, M., ASKARI, A. & KUPERMAN, A.S. (1964). The stabilizing actions of adenosine triphosphate and related nucleotides on calcium – deficient nerve. *J. Pharmac. exp. Ther.*, **144,** 229–235.

RIBEIRO, J.A. (1982). The decrease of neuromuscular transmission by adenosine depends on previous neuromuscular depression. *Archs int. Pharmacodyn. Ther.*, **255,** 59–67.

RIBEIRO, J.A. & DOMINGUEZ, M.L. (1978). Mechanisms of depression of neuromuscular transmission by ATP and adenosine. *J. Physiol., Paris*, **74,** 491–496.

RIBEIRO, J.A., DOMINGUEZ, M.L. & GONÇALVES, M.J. (1979). Purine effects at the neuromuscular junction and their modification by theophylline, imidazole and verapamil. *Archs int. Pharmacodyn. Ther.*, **238,** 206–219.

RIBEIRO, J.A. & SEBASTIÃO, A.M. (1983). The effect of adenosine triphosphate on tetrodotoxin axonal blockade. *Br. J. Pharmac.*, **79,** 364P.

STAFF OF THE DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF EDINBURGH (1968). *Pharmacological Experiments on Isolated Preparations.* p. 116. Edinburgh: Livingstone Ltd.

STONE, T.W. (1981). Physiological roles for adenosine and adenosine 5′-triphosphate in the nervous system. *Neuroscience*, **6,** 523–555.

(Received May 27, 1983.
Revised July 28, 1983.)