ATP released together with acetylcholine as the mediator of neuromuscular depression at frog motor nerve endings

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1. The hypothesis that ATP released by presynaptic stimulation is hydrolysed to adenosine and mediates prejunctional neuromuscular depression was tested at vertebrate neuromuscular junctions. Electrophysiological recordings of evoked acetylcholine (ACh) release and perineural ionic currents at motor nerve endings were made using the frog cutaneous pectoris nerve–muscle preparation. Either tubocurarine or α-bungarotoxin was used to block muscle contractions.

2. Either α,β-methylene ADP (which inhibits ecto-5′nucleotidases and thus prevents the degradation of ATP to adenosine) or selective adenosine receptor antagonists (8-cyclopentyl alkyl xanthines) prevented the inhibitory effects of *exogenous* ATP on ACh release in response to low-frequency nerve stimulation. These results confirm earlier findings that ATP must be hydrolysed to adenosine to inhibit ACh release.

3. The presence of α,β-methylene ADP completely prevented neuromuscular depression in response to repetitive high-frequency nerve stimulation (0·5–1 Hz). α,β-Methylene ADP had no effect on ACh secretion under conditions where ACh release is well maintained (low-frequency stimulation, 0·05 Hz).

4. Selective adenosine receptor antagonists completely eliminated neuromuscular depression produced by repetitive high-frequency nerve stimulation (1·0 Hz) but had no effect on ACh release at low frequencies of stimulation (0·05 Hz).

5. Exogenous adenosine deaminase (5 i.u. ml^{-1}), which degrades adenosine to its inactive nucleoside inosine, also eliminated neuromuscular depression but had no significant effect on ACh release at frequencies of nerve stimulation too low to produce prejunctional depression.

6. During maximal neuromuscular depression, the effects of exogenous adenosine or 2-chloroadenosine, an adenosine agonist, were occluded.

7. The calcium-sensitive component of perineurial recordings of motor nerve terminal currents did not change during depression or during application of adenosine receptor antagonists and adenosine deaminase, suggesting that neuromuscular depression in this species was not associated with changes in presynaptic Ca^{2+} currents.

8. These results suggest that, under the conditions of these experiments, endogenous ATP, after hydrolysis to adenosine, causes prejunctional neuromuscular depression. This inhibitory effect of endogenous adenosine occurs at a site distal to the locus of Ca^{2+} entry in the frog.

The skeletal neuromuscular junction is designed for the rapid, faithful communication of information between motor nerve and skeletal muscle. The efficiency of this communication process is limited by the neuromuscular depression that ensues with even brief repetitive stimulation (Eccles, Katz & Kuffler, 1941; Liley & North, 1953; Takeuchi, 1958; Betz, 1970; Christensen & Martin, 1970); this depression is a consequence of the reduction in the release of the neurotransmitter acetylcholine (ACh) during continuous nerve stimulation (Otsuka, Endo & Nonamura, 1962). There are at least two important phases of presynaptic depression. At very high release rates, depression is correlated with the depletion of vesicular ACh stores in the nerve ending (Heuser, Reese, Jan, Jan & Evans, 1979). At

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more normal levels of release, presynaptic depression is not associated with measurable depletion of available ACh stores (for reviews, see Hubbard, 1973; Silinsky, 1985).

It has been suggested that the inability to reconcile the simple vesicle hypothesis with quantitative theories of neuromuscular depression at normal levels of ACh release could be due to the co-release of ACh with the presynaptic inhibitory modulator ATP (Silinsky, 1975, p. 161). According to this hypothesis, adenosine derivatives are released by motor nerve impulses and depress the amount of ACh released in response to a subsequent impulse without producing measurable changes in available ACh quanta. Indeed, ATP is released together with ACh in stoichiometric amounts from motor nerve endings (Silinsky, 1975; Schweitzer, 1987; Smith & Lu, 1991; Smith, 1991). Furthermore, exogenous ATP inhibits ACh release (Ribeiro & Walker, 1975) but the effect is likely to be due to the hydrolysis of ATP to its metabolite, adenosine (Ginsborg & Hirst, 1972; Silinsky, 1980, 1984; Ribeiro & Sebastiao, 1987).

Previous studies have revealed only a small role for adenosine in neuromuscular depression, suggesting that the preponderance of neuromuscular depression is unrelated to endogenous adenosine derivatives (Meriney & Grinnell, 1991; see also Ribeiro & Sebastiao, 1987; Bennett, Karunanithi & Lavidis, 1991). Unfortunately, all of these studies were performed in the presence of significant levels of basal adenosine emanating from non-synaptic sources, thus confounding assessments of the importance of adenosine released by synaptic stimulation to prejunctional depression. Furthermore, the precise mechanisms by which exogenous or endogenous adenosine might mediate its presynaptic inhibitory effects are controversial. One plausible mechanism is that adenosine reduces Ca^{2+} entry (Hamilton & Smith, 1991). An alternative mechanism is that adenosine reduces the ability of intracellular Ca^{2+} to promote ACh release (Silinsky, 1981, 1984; Silinsky & Solsona, 1992).

It thus appears of interest to evaluate the importance of endogenous adenosine derivatives in the process of neuromuscular depression, as well as to determine if changes in Ca^{2+} entry are responsible for neuromuscular depression. To this end, we made electrophysiological measurements of evoked ACh release (i.e. endplate potentials, EPPs) and presynaptic Ca^{2+} currents at neuromuscular junctions in frog cutaneous pectoris muscle. We chose experimental conditions to minimize the basal leakage of adenosine derivatives from unstimulated preparations. Under such conditions, our results demonstrate that all of the presynaptic neuromuscular depression observed at normal levels of ACh output is due to the concomitant release of endogenous adenosine derivatives from stimulated neuromuscular junctions. Neuromuscular depression is not attributable to effects on presynaptic Ca^{2+} currents. A brief abstract of some these results has been published (Redman & Silinsky, 1992).

METHODS

Overview

Cutaneous pectoris nerve–muscle preparations with their attached nerve supply were dissected from the frog (*Rana pipiens*), following immersion anaesthesia with ether and pithing, and subsequently superfused with flowing Ringer solution. Suprathreshold electrical stimuli were applied to the proprialis pectoris nerve trunk via a suction electrode at frequencies ranging from 0·05 to 10 Hz. Evoked responses (Ca^{2+} currents and EPPs) were recorded using a conventional high-input impedance microelectrode preamplifier purchased from WP Instruments (Sarasota, FL, USA) or from Axon Instruments (Foster City, CA, USA; Axoclamp 2A). Responses were averaged using an IBM AT-compatible microcomputer, TL-1 interface and pCLAMP software (Axon Instruments). Hard copies of the digitalized traces were made on an X–Y plotter or a LaserJet Printer (Hewlett Packard Series III). Generally, ASCII files from pCLAMP were first imported to a spreadsheet program for data organization (Quatro Pro, Borland, Scotts Valley, CA, USA) before importing into Sigma Plot 4.0 (Jandel Scientific, Corte Madera, CA, USA) for hard copy.

Measurements of ACh release and Ca^{2+} currents

Intracellular recordings of EPPs and perineural recordings of Ca^{2+} currents through N-type channels were made using methods previously employed in this laboratory (Silinsky, 1984; Silinsky & Solsona, 1992). With respect to electrophysiological measurements of evoked ACh release, effects of adenosine reagents on EPP amplitudes reflect the action of these drugs on the evoked ACh release process (i.e. effects on the number of ACh quanta released by a nerve impulse) as none of the adenosine reagents used in this study affects the size of the miniature EPP (Ribeiro & Walker, 1975; Silinsky, 1980, 1984; Ribeiro & Sebastiao, 1987; E. M. Silinsky & R. S. Redman, unpublished data). With respect to measurements of prejunctional ionic currents, a recent publication provides a detailed description of the methods used in this laboratory for measuring ionic currents from motor nerve endings (Silinsky & Solsona, 1992). The following is a brief description of the method. Ca^{2+} currents were recorded from within the perineurium after blockade of a proportion of the K^+ channels using microelectrodes of 5–15 MΩ resistances filled with normal Ringer solution. The perineural electrode was positioned under visual control near small axon bundles at the termination of the myelin sheaths and within 50 μm of the intracellular recording electrode used for measuring EPPs. The perineurium was then gently penetrated; a steady 2–6 mV deflection (generally negative) was associated with a successful positioning of the electrode for recording of perineural currents. These currents generate voltage changes across the extracellular resistance, hence they are reported as the magnitude of the extracellular voltage change in the figure legends. Perineural Ca^{2+} currents, when flowing across the extracellular and perineural resistances, generally produced a 0·8–3 mV voltage change across the resistance between recording and reference electrodes. Current amplitudes were highly sensitive to electrode position, however. Indeed, in some experiments a decline or increase of both the Na^+ and Ca^{2+} currents was observed; such experiments were not included in the data reported herein. For further details of the particular current waveforms and potential sources of contamination of Ca^{2+}

currents at frog motor nerve endings, see the reports of Mallart (1984), Anderson & Harvey (1988), Molgo, Delpozo, Banos & Angaut-Petit (1991) and Silinsky & Solsona (1992).

Composition of solutions

The normal Ringer solution contained (mM): NaCl, 115; KCl, 2; $CaCl_2$, 3·6; Hepes, 2 (pH 7·2–7·4); and 4 mg l^{-1} tubocurarine chloride or 100 nM α-bungarotoxin. A modified Ringer solution (0·9 mM CaCl, 10 mM Mg^{2+}) containing K^+ channel blockers (100 μM diaminopyridine (DAP) and 250 μM TEA) was used for the experiments in which Ca^{2+} currents were measured simultaneously with EPPs ('Ca^{2+} current Ringer solution'; e.g. Figs 7–9). These specific concentrations of K^+ channel blockers enabled us to measure simultaneously changes in ACh release (i.e. EPPs) and changes in Ca^{2+} currents with minimal complications arising from opposing Ca^{2+}-activated K^+ currents observed in the absence of TEA and without the profound depletion of ACh release seen in the presence of higher concentrations of TEA (see e.g. Anderson & Harvey, 1987). These concentrations of K^+ channel blockers also allowed for the detection of both increases and decreases in Ca^{2+} currents as the extracellular Ca^{2+} concentrations were changed accordingly (Silinsky & Solsona, 1992; Redman & Silinsky, 1993*a*). In Ca^{2+} current Ringer solution, the number of ACh quanta released by a nerve impulse ranged from 344 to 569 with a mean of 457 ± 23·8 (mean ± S.E.M., $n = 22$) as calculated by the tubocurarine method (see Silinsky, 1984).

Drugs were obtained from Research Biochemicals Incorporated (adenosine receptor antagonists) or the Sigma Chemical Company (all other drugs). Either adenosine or 2-chloroadenosine was employed as an adenosine receptor agonist. 2-Chloroadenosine is a potent adenosine receptor agonist that is not a substrate for uptake or deamination at motor nerve endings (for justification, see Silinsky, 1984; Ribeiro & Sebastiao, 1987).

Conditions chosen to minimize confounding presynaptic effects during depression

We used the following procedures to minimize both the basal leakage of adenosine from non-synaptic sources (e.g. cut tissue) and the non-selective effects of older adenosine antagonists. (1) The cutaneous pectoris nerve–muscle preparation was employed in these studies. This muscle is very thin (only a few fibres thick in some regions) and can thus be removed from the frog with little extraneous cut muscle attached. The cutaneous pectoris muscle is thus more likely to be in the ionic steady state and not leaching adenosine (as a result of deep fibre anoxia) than the thick sciatic nerve–sartorius muscle used by other investigators (Ribeiro & Sebastiao, 1987). (2) Rapid dual superfusion, both by bath application and by local fast-flow pipes, was implemented prior to experimentation to limit the exposure of the preparation to basal adenosine. (3) Neuromuscular junctions were stimulated minimally prior to experimentation and only to the extent necessary to ascertain that neuromuscular transmission was intact. Low-frequency stimulation was also used whenever possible throughout the experiment. (4) Two highly selective adenosine receptor antagonists possessing the 8-cyclopentyl alkyl xanthine structure were employed to detect endogenously released purines (see Results for specific drug names). At the low concentrations used in this study, these alkyl xanthines competitively inhibit the action of adenosine on prejunctional adenosine receptors without any reported secondary effects (Sebastiao & Ribeiro, 1989; Redman & Silinsky, 1993*b*).

We also controlled for the complex effects of tubocurarine, which was used to antagonize postjunctional nicotinic receptors and thus block muscle twitches in all previously published studies of adenosine made at normal ACh outputs (see references in Introduction). However, tubocurarine also affects ACh release (Bowman, Marshall, Gibb & Harborne, 1988; Sosa & Zengel, 1992). Most notably, tubocurarine progressively decreases ACh release during repetitive high-frequency nerve stimulation (Harborne, Bowman & Marshall, 1988), thus itself causing an unphysiological prejunctional depression that is likely to skew the relative importance of adenosine as an endogenous mediator of neuromuscular depression. To eliminate such potentially confusing effects of tubocurarine (which also include direct block of the postjunctional ionic channel linked to the nicotinic receptor), we performed a number of experiments using the irreversible postjunctional nicotinic receptor antagonist, α-bungarotoxin, to reduce the EPP below threshold for muscle action potentials. α-Bungarotoxin is devoid of the prejunctional depressant effects and the postjunctional channel blocking effects of tubocurarine (Bowman *et al.* 1988). In these experiments, 100 nM α-bungarotoxin was included in the Ringer solution until all nerve evoked twitches were eliminated. The results with α-bungarotoxin were similar to those found with tubocurarine (see e.g. Fig. 5). In some experiments, we used 3·6 mM Ca^{2+} in lieu of the traditional 1·8 mM Ca^{2+}. This Ca^{2+} concentration produces near normal levels of ACh release (Silinsky, 1981), but allowed depression to be produced at low frequencies of nerve stimulation (0·1–10 Hz).

Delivery of solutions

Normal Ringer solutions were applied continuously by superfusion using a flow pump both for delivering fresh Ringer solution and removing the effluent. All drugs were dissolved in Ringer solution and applied by rapid local superfusion ('fast-flow' delivery) from a series of 300 μm diameter glass flow tubes (Yellen, 1982). The tubes were gravity-fed from a syringe reservoir. The latency from the opening of the tap to the beginning of a discernable postjunctional depolarization was 50–100 ms when tested with 100 μM ACh in the flow tube.

Statistical methods

Statistical procedures were as described previously (see Silinsky, 1984; Silinsky & Solsona, 1992). In many instances appropriate numbers of evoked responses were averaged to reduce the coefficient of variation to less than 5%. At the highest level of ACh release in Ca^{2+} current Ringer solution, secretion was high enough so that small differences between individual EPPs were statistically significant.

RESULTS

General observations on the effects of exogenous ATP in the absence of neuromuscular depression

Figures 1 and 2 illustrate the typical effects of exogenous ATP when the motor nerve is stimulated at frequencies too low to provoke prejunctional depression of ACh release (0·05–0·1 Hz). As shown in Fig. 1, the presynaptic inhibitory effect of exogenous ATP observed normally (50 μM, lower bar) does not occur when the degradation of ATP to adenosine is prevented by a selective inhibitor of

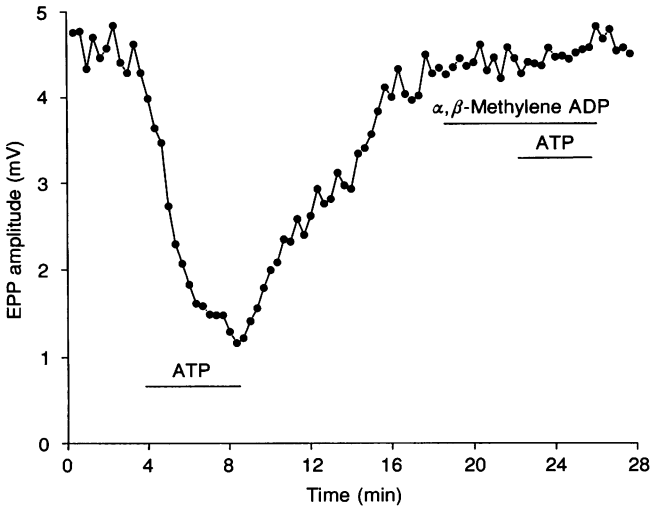

Figure 1. The inhibitory effect of exogenous ATP (50 μM) is prevented by α,β-methylene ADP (50 μM)
The nerve was stimulated at a low frequency (0·05 Hz) to prevent prejunctional neuromuscular depression. Normal Ringer solution (1·8 mM Ca^{2+}) was employed in this experiment. Indicated drugs were superfused during the times indicated by the bars. The drug effects on EPP amplitudes in this and all subsequent figures represent the effects of adenosine reagents on evoked ACh release (see Methods).

ecto-5′nucleotidase, α,β-methylene ADP (Keller & Zimmermann, 1983; Kreutzberg, Heymann & Reddington, 1986). Figure 2 shows that a highly selective adenosine receptor antagonist, 8-cyclopentyl-1,3,dipropylxanthine (DPCPX; Lohse, Klotz, Lindenborn-Fotinos, Reddington, Schwabe & Olsson, 1987), inhibits the effects of exogenous ATP. These results ($n = 5$ for each) confirm earlier suggestions that ATP must be hydrolysed to adenosine to inhibit ACh release (Silinsky, 1980; Ribeiro & Sebastiao, 1987; Smith & Lu, 1991). The effect of adenosine (25 μM) as an inhibitor of ACh release was not impaired by α,β-methylene ADP (data not shown, $n = 3$)

ACh release in the absence of prejunctional depression was not affected by either α,β-methylene ADP (Fig. 1, early part of upper bar) or DPCPX (Fig. 2, see also Figs 3 and 4). It thus appears that the basal efflux of adenosine derivatives is minimal and hence does not affect ACh release in our experiments under conditions in which neuromuscular depression does not occur.

α,β-Methylene ADP prevents prejunctional neuromuscular depression

If ATP released by synaptic stimulation is responsible for neuromuscular depression, then α,β-methylene ADP should prevent neuromuscular depression in response to repetitive high-frequency stimulation by preventing the conversion of the released ATP (which is inactive on the presynaptic adenosine receptor) into the active nucleoside adenosine. Figure 3 shows this to be the case. Figure 3*A* and *B* illustrates neuromuscular depression in normal Ringer solution. At a low frequency of stimulation (0·05 Hz), the averaged EPP is well maintained (control, panel *A*; see also Figs 1 and 2). When the frequency of motor nerve stimulation is increased to 0·5 Hz, prejunctional depression of ACh release reduces the EPP to 44 % of the control level after 20 s of stimulation (*B*, depression). After recovery of the EPP, the addition of α,β-methylene ADP (50 μM, Fig. 3*C* and *D*) whilst not affecting ACh release at the low

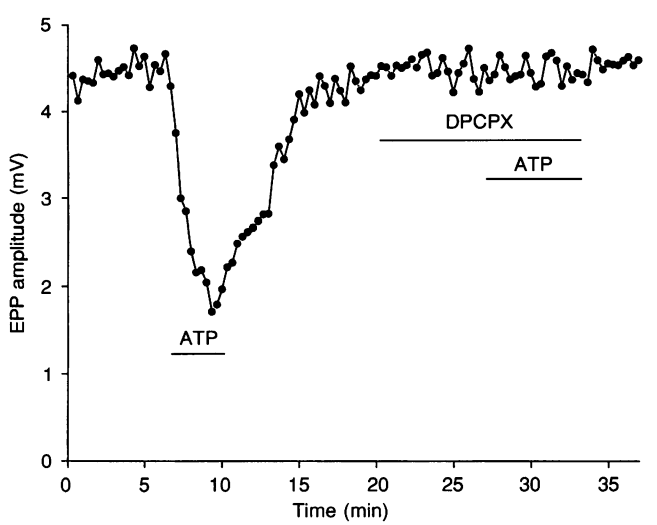

Figure 2. The inhibitory effect of exogenous ATP (50 μm) is prevented by 8-cyclopentyl-1,3,dipropylxanthine (DPCPX, 100 pM)
DPCPX is a selective adenosine receptor antagonist. Drugs were superfused during the times indicated by the bars. The motor nerve was stimulated at 0·05 Hz. Normal Ringer solution (1·8 mM Ca^{2+}) was employed in this experiment.

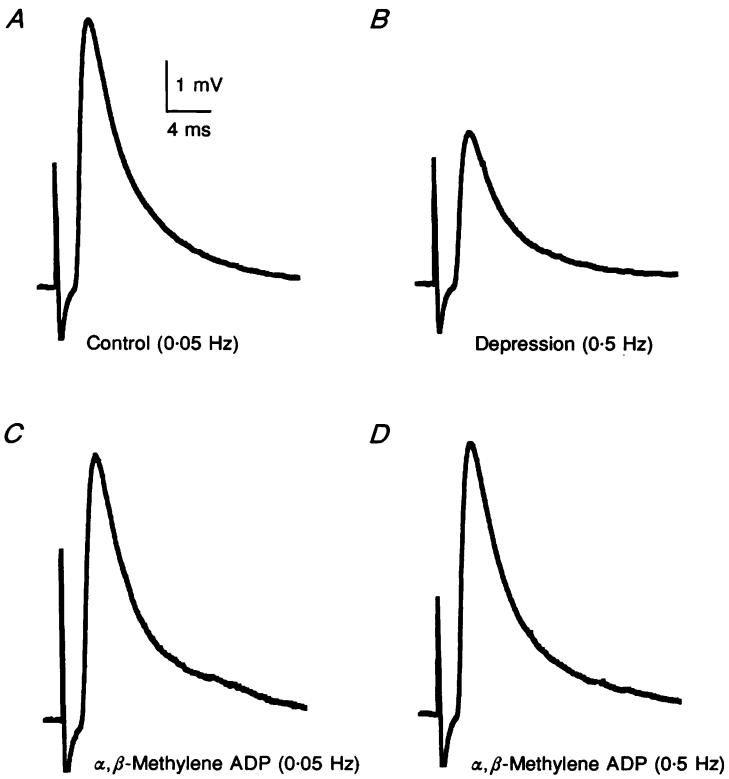

Figure 3. Blockade of neuromuscular depression by α,β-methylene ADP in response to increasing the frequency of presynaptic stimulation
Normal Ringer solution (1·8 mM Ca^{2+}) was employed in this experiment. Each EPP is the averaged response to four consecutive stimuli. *A*, control EPP in response to 0·05 Hz stimulation. This EPP was well maintained. *B*, presynaptic depression of the EPP produced 20 s after an increase in the stimulation frequency to 0·5 Hz. *C* and *D* are similar to *A* and *B* respectively except for the addition of α,β-methylene ADP (50 μM). Note the absence of effect of α,β-methylene ADP on EPPs produced by low-frequency stimulation (0·05 Hz; *C*) and the elimination of neuromuscular depression by this agent (*D*) during 0·5 Hz stimulation.

frequency of nerve stimulation (compare Fig. 3*C* with Fig. 3*A*), completely eliminated neuromuscular depression produced by 0·5 Hz stimulation (compare Fig. 3*D* with Fig. 3*B*). Similar results were observed in all four experiments.

Adenosine receptor antagonists prevent neuromuscular depression

If adenosine is the only mediator of neuromuscular depression at normal levels of ACh release, then selective adenosine receptor antagonists should fully reverse neuromuscular depression. Figure 4 shows that the adenosine receptor antagonist 8-cyclopentyltheophylline (CPT; Dunwiddie & Fredholm, 1989; Sebastiao & Ribeiro, 1989) prevents prejunctional depression. Figure 4 (Control) shows the average endplate potential (EPP) in response to low-frequency stimulation (0·1 Hz). Continuous nerve stimulation at 1·0 Hz produced a presynaptic neuromuscular depression; the EPP is reduced to approximately 50 % of control by a presynaptic effect on ACh release (Fig. 4, Prejunctional depression). During the depression, application of 1 μM CPT restored ACh release to the control level. In a total of eleven experiments, 1 μM CPT restored ACh release to 97 ± 2 % of the control level. Results similar to those shown in Fig. 4 were observed with the selective adenosine receptor antagonist DPCPX; in a total of eight

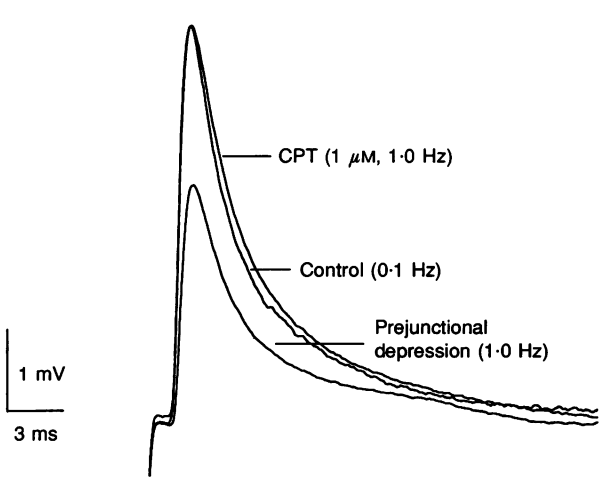

Figure 4. Elimination of presynaptic neuromuscular depression by the adenosine receptor antagonist 8-cyclopentyltheophylline (CPT)
The motor nerve trunk was stimulated for approximately 2 min at 0·1 Hz (control) before increasing the stimulation parameters to 1·0 Hz. Each of the 3 traces represent an average EPP (5–10 consecutive stimuli) from each of the experimental conditions. The 'Prejunctional depression' trace shows the depression produced 2 min after an increase in stimulation frequency to 1 Hz. While continuing to stimulate at 1·0 Hz, 1 μM CPT was applied by rapid local superfusion. Note the recovery of the EPP amplitude to the original level. Ringer solution contained 3·6 mM Ca^{2+}. Similar results were observed with other adenosine receptor antagonists (DPCPX; see text). The application of adenosine receptor blockers in a non-depressed state produced no significant effect on ACh release.

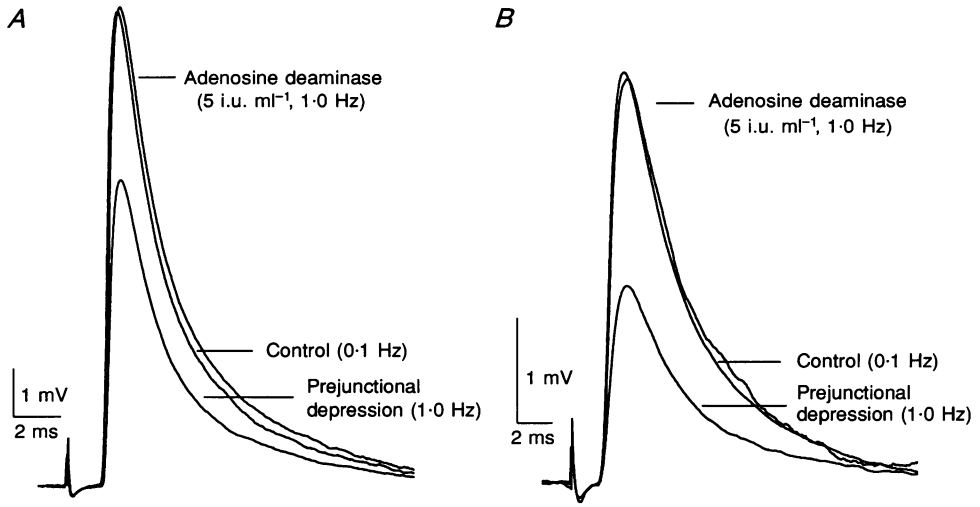

Figure 5. Elimination of presynaptic neuromuscular depression by exogenous adenosine deaminase (5 i.u. ml^{-1})
Experiments were performed as described in Fig. 4. This enzyme alleviated neuromuscular depression whether in the presence of 4 mg l^{-1} tubocurarine (*A*) or 100 μM α-bungarotoxin (*B*). Application of this drug in a non-depressed state produced no significant effect.

experiments DPCPX (100 pM) restored ACh release to 105 ± 3 % (mean ± S.E.M.) of the initial level (Redman & Silinsky, 1993*b*).

Exogenous adenosine deaminase prevents neuromuscular depression

Based on the results thus far, it might be predicted that exogenous adenosine deaminase, by hydrolysing neurally released adenosine to inosine (which is inactive on the prejunctional adenosine receptor), should alleviate neuromuscular depression. This prediction is borne out by the experimental results. Figure 5 shows typical experiments from two different preparations (*A* and *B*). (The experiment of Fig. 5*A* was made using tubocurarine and that of Fig. 5*B* using α-bungarotoxin to reduce the EPP below threshold for the generation of muscle action potentials; see Methods.)

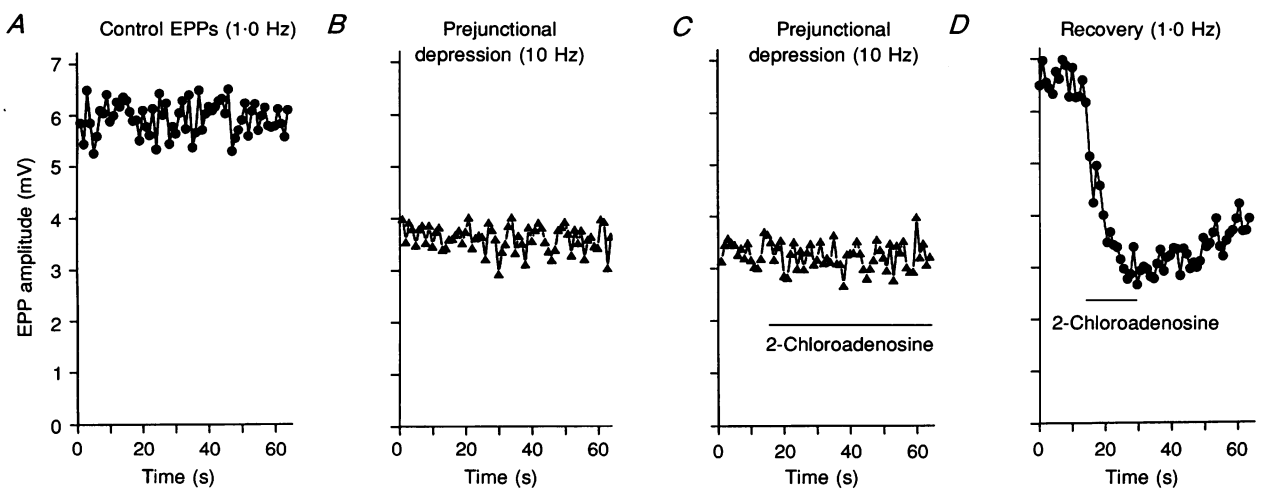

Figure 6. Occlusion of the inhibitory effects of exogenous 2-chloroadenosine during neuromuscular depression
A, the preparation was stimulated at 1·0 Hz to obtain a control value for the EPP amplitude. *B*, continuous stimulation at 10 Hz caused a 40 % depression of EPP amplitude. *C*, application of 25 μM 2-chloroadenosine during this state of depression had no effect. *D*, after wash-out of the drug and subsequent 20 min rest, stimulation at 1·0 Hz produced EPPs of the original amplitude and in this non-depressed state the application of 2-chloroadenosine produced a 45 % depression in EPP amplitude.

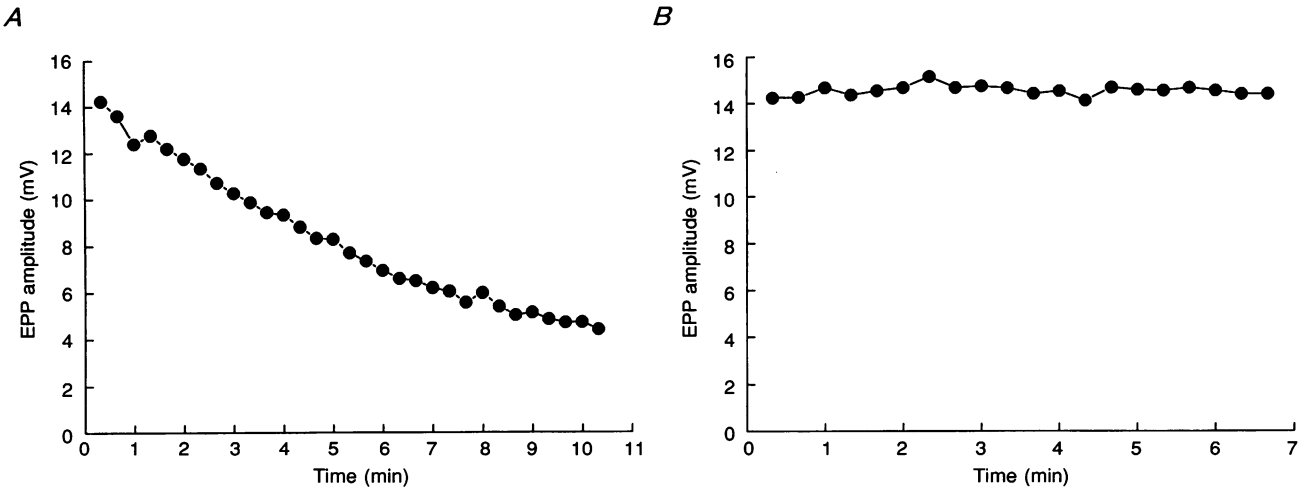

Figure 7. Presynaptic neuromuscular depression in 'Ca^{2+} current Ringer solution' (*A*) and its antagonism by α,β-methylene ADP (*B*)
For both *A* and *B*, the motor nerve was stimulated at a frequency of 0·05 Hz. Ca^{2+} current Ringer solution consisted of potassium channel blockers and modified concentrations of Ca^{2+} and Mg^{2+} to allow for simultaneous measurements of EPPs and perineural Ca^{2+} currents (see Methods and Figs 8 and 9). α,β-Methylene ADP was present during *B*.

Note that in each experiment, adenosine deaminase restored ACh release to the control level observed prior to depression. In a total of ten experiments, the addition of adenosine deaminase restored ACh release to 98·5 ± 3 % of the control level.

The effects of exogenous adenosine receptor agonists are occluded during high-frequency nerve stimulation

If adenosine is the exclusive mediator of neuromuscular depression in response to repetitive motor nerve stimulation, then during prejunctional neuromuscular depression the inhibitory effects of exogenous adenosine should be occluded. In the experiment of Fig. 6, continuous nerve stimulation (10 Hz) for 75 s depressed the EPP from the control level (6·0 mV, Fig. 6*A*, 1 Hz) to 3·6 mV (Fig. 6*B*). During this depression, 2-chloroadenosine (25 μM) did not inhibit ACh release (Fig. 6*C*). After 20 min without nerve stimulation, ACh release recovered to the control level and 2-chloroadenosine (25 μM) produced its conventional inhibitory effect (Fig. 6*D*; 1 Hz stimulation), reducing ACh release to the level seen during neuromuscular depression. Occlusion of the effects of adenosine receptor agonists was found in fifteen other experiments using 25 μM 2-chloroadenosine and in seven experiments using 25 μM adenosine.

The experiments thus far suggest that release of endogenous adenosine derivatives causes *all* of the neuromuscular depression that occurs at physiological levels of ACh release when the basal leak of adenosine from *in vitro* neuromuscular junctions is minimized.

Simultaneous measurements of ACh release and nerve terminal ionic currents during depression and the action of endogenous adenosine derivatives

The remaining experiments were made in a modified Ringer solution containing K^+ channel blockers so that Ca^{2+} currents may be measured simultaneously with EPPs and in which the time course of depression and its reversal by adenosine antagonists may be studied. Figure 7 shows the time course of a typical experiment on ACh release in this Ca^{2+} current Ringer solution (see Methods). Note that continuous low-frequency nerve stimulation (0·05 Hz) causes point by point presynaptic neuromuscular depression (Fig. 7*A*). In the same cell, after a rest period of 5 min in which ACh release was restored to the control level (Fig. 7*B*), the addition of α,β-methylene ADP completely eliminated neuromuscular depression (Fig. 7*B*).

Such results demonstrate that endogenous ATP is the mediator of neuromuscular depression in this modified Ringer solution. It would thus appear of interest to determine if neuromuscular depression and the action of endogenous adenosine are associated with changes in Ca^{2+} currents. The upper superimposed traces in Fig. 8 show the perineural Na^+ currents and Ca^{2+} currents recorded when K^+ channels are partially blocked in Ca^{2+} current Ringer solution. The lower traces show superimposed EPPs recorded simultaneously. Note that four stimuli produced a depression of ACh release (EPPs) without effects on the Ca^{2+} currents (or Na^+ currents), demonstrating that under these conditions, neuromuscular depression is not mediated

by measurable changes in ionic currents. Similar results were observed in ten other experiments.

Figure 9 shows the ability of an adenosine receptor antagonist (CPT) and adenosine deaminase to reverse completely prejunctional neuromuscular depression (*A*) under conditions in which Ca^{2+}currents may be measured (*B*). Note that in Fig. 9*A*, fifteen consecutive stimuli produced a cumulative neuromuscular depression of release to 45% of control. Before the sixteenth stimulus was delivered, local rapid superfusion of the adenosine antagonist CPT (1 μM) between nerve stimuli (see Methods) restored release to the higher level found eight stimuli earlier, an inter-impulse elimination of approximately half of the accumulated neuromuscular depression. Several stimuli later, all of the depression was abolished by CPT. After the wash-out of CPT, depression continued along the same time course observed prior to CPT application. At a later stage of the same experiment, adenosine deaminase rapidly eliminated neuromuscular depression as well (Fig. 9*A*). Fig. 9*B* shows that neither the Ca^{2+} currents recorded during neuromuscular depression nor those recorded during the reversal of depression by CPT or adenosine deaminase are associated with changes in Ca^{2+} currents (letters *a–f* illustrate corresponding time points in the graph of EPPs and the perineural currents). In contrast, either increasing or decreasing the extracellular Ca^{2+} respectively increased or decreased Ca^{2+} currents under the same experimental conditions (Redman & Silinsky, 1993*a*; R. S. Redman & E. M. Silinsky, unpublished data; see also Methods and Silinsky & Solsona, 1992).

DISCUSSION

These results suggest that endogenous ATP, after hydrolysis to adenosine, is the exclusive mediator of prejunctional neuromuscular depression in response to repetitive nerve stimulation at physiological levels of ACh release in the frog cutaneous pectoris nerve–muscle preparation. The progressive inhibition of quantal ACh secretion during repetitive nerve stimulation occurs without measurable changes in prejunctional ionic currents.

The outcome of these experiments, in addition to contributing insights into the mechanisms of neuromuscular depression, provides electrophysiological evidence in support of the earlier biochemical data demonstrating that ATP and ACh are co-released from motor nerve endings (Silinsky, 1975; Schweitzer, 1987). Briefly, when the amount of ACh release is low due to low-frequency stimulation, no release of endogenous ATP can be detected either electrophysiologically or biochemically at the neuromuscular junction (Silinsky, 1975). When the total amount of ACh release is increased by increasing the frequency of nerve stimulation, sufficient endogenous ATP is released from motor nerve endings to be detected both biochemically (Silinsky, 1975) and electrophysiologically, the latter as a

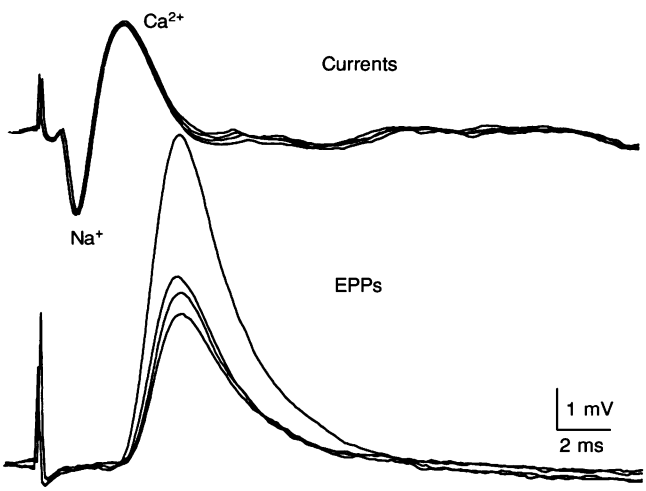

Figure 8. Simultaneous measurements of EPPs and prejunctional ionic currents during neuromuscular depression

Four superimposed currents (upper traces) and the EPPs evoked by these currents (lower traces) are shown. The Ca^{2+} current in the nerve ending is the large upward deflection at the end of the trace. The downward spike preceding the Ca^{2+} current represents the Na^+ current originating from the junction of the myelinated and non-myelinated axon (for further details, see Silinsky & Solsona, 1992). Note that during depression of the EPPs at 1 Hz, there is no change in the Na^+ or Ca^{2+} components of the perineural traces measured simultaneously. In other experiments where a comparable reduction in EPP amplitude was achieved by lowering bath [Ca^{2+}], the measured perineural Ca^{2+} current was reduced by at least 25% (see Silinsky & Solsona, 1992). The experiment was made in 'Ca^{2+} current Ringer solution'; see Methods). Vertical calibration for perineural currents reflects a voltage change of 1·5 mV produced by presynaptic currents flowing in the perineural space.

reduction in the quantal ACh output per impulse. Under the conditions of our experiments in curarized preparations, ATP is not likely to be emerging from postjunctional sources (Silinsky, 1975). It thus appears that sufficient ATP is released from stimulated motor nerve terminals to cause inhibition of ACh secretion from an individual nerve ending as assessed electrophysiologically. Previous biochemical studies on vertebrate synaptosomes indeed suggest an intimate stoichiometry between the presynaptic co-release of ATP and ACh (see Schweitzer, 1987).

Earlier results using less selective adenosine antagonists and adenosine deaminase in amphibian species have suggested a variable but generally small role for endogenous adenosine and ACh release. In the first such publication, Ribeiro & Sebastiao (1987) examined the effects of basal adenosine in frog sciatic nerve sartorius muscle preparations and found small tonic effects of adenosine, even at very low levels of release. In a comprehensive statistical study, Bennett *et al.* (1991) found that endogenous adenosine was unlikely to be regulating ACh release from its own release locus, but rather regulated neighbouring release sites. In the only study specifically addressing the phenomenon of neuromuscular depression, a careful correlative study of motor nerve ending morphology and ACh release, Meriney & Grinnell (1991) reported difficulty in finding a significant component of depression attributable to adenosine (only 4–5 % on average) unless corrections were made for the release per unit length of nerve terminal. Even with these corrections, the effects of endogenous adenosine appear to be modest. All of these studies revealed basal effects of endogenous adenosine that appeared unrelated to synaptic stimulation. In our present study we have chosen conditions to isolate the effects of adenosine derivatives to the stimulus-specific release of ATP (see Methods). The absence of substantial tonic leak of adenosine in our experiments was confirmed by the absence of effects of α,β-methylene ADP or adenosine receptor antagonists at low frequencies of nerve stimulation (Figs 1–3). Given the apparent intra-impulse regulation of release by endogenous adenosine (Fig. 9), it would appear of interest in future studies to characterize the time course of action of endogenous adenosine by examining the effects of different stimulation

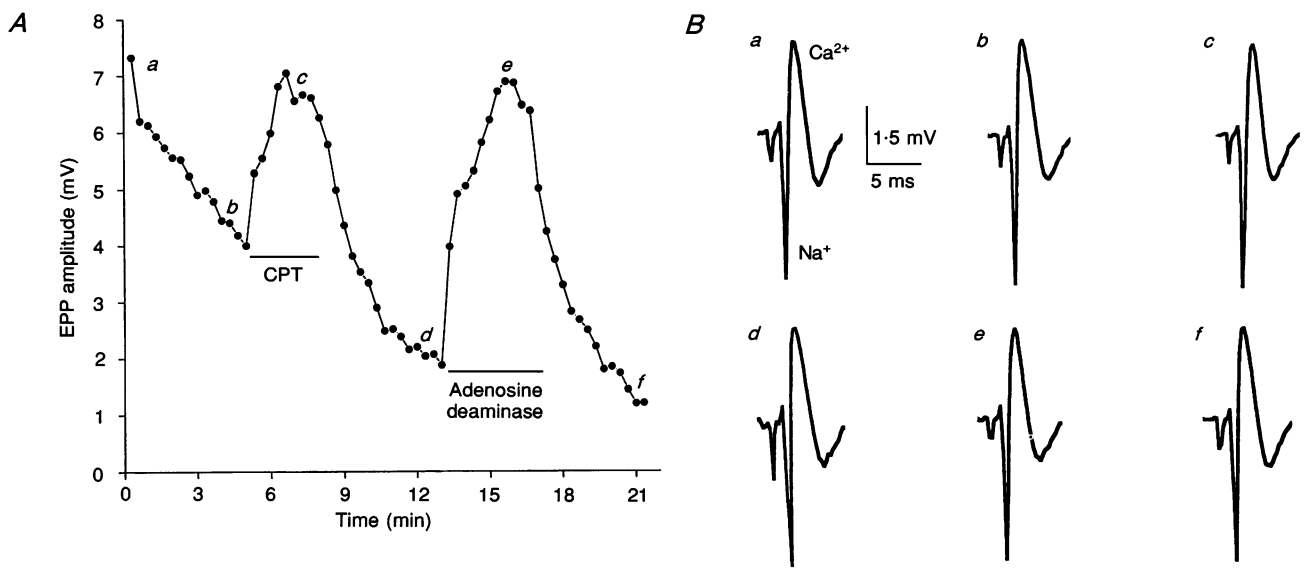

Figure 9. Adenosine antagonism during depression is not associated with changes in Ca^{2+} currents

Simultaneous measurements of ACh release (EPP amplitudes, *A*) and perineural Ca^{2+} currents (*B*) during neuromuscular depression and during the actions of the adenosine receptor antagonist CPT and adenosine deaminase. Experiments were made in Ca^{2+} current Ringer solution (see Methods). Under these conditions neuromuscular depression is produced at low frequencies of stimulation (0·05 Hz), thus eliminating the need to change stimulation parameters during the experiment (see e.g. Figs 1–3). *A*, during continuous stimulation (0·05 Hz), neuromuscular depression was eliminated by the addition of 1 μM CPT; depression resumed upon wash-out of the drug. The application of 5 i.u. ml^{-1} adenosine deaminase also completely abolished neuromuscular depression. *B*, perineural calcium currents were measured simultaneously with the EPPs above. For clarity, only 6 of the 64 current traces are shown here. They correspond to the time points *a–f* in the graph of EPP amplitudes in *A*. It is apparent that while there are profound changes in EPP amplitude, a measure of ACh release, the perineural Ca^{2+} currents remain unchanged during neuromuscular depression. Calibration bar represents a voltage change of 1·5 mV produced by current flow across the perineurial resistances. (Note, only the initial component of the current traces are shown as the currents from several experiments demonstrated repetitive firing.)

intervals on depression and on the elimination of depression by adenosine receptor antagonists.

The underlying explanation as to why effects of endogenous adenosine derivatives were so dramatic in this study when compared to earlier studies is unknown. One possibility was our effort to minimize the leak of adenosine from *in vitro* neuromuscular junctions by stimulating sparingly prior to experimentation. In several experiments, high basal levels of adenosine were apparently present at the beginning of an experiment in which the preparation had been subjected to excessive neuromuscular activity from stimulating during studies performed on a neighbouring junction, as indicated by the ability of adenosine antagonists to increase ACh release at low frequencies of nerve stimulation. In one such experiment, the efficacy of CPT in reversing prejunctional depression was examined and found to be less dramatic than the results presented here (only a 25% recovery from depression was observed). Our results cannot be attributed to presynaptic effects of the postjunctional nicotinic receptor blocker tubocurarine as results similar to those presented with adenosine deaminase, CPT and DPCPX in curarized preparations (Figs 3–9) were also found for adenosine deaminase (Fig. 5), CPT ($n = 5$, data not shown) or DPCPX ($n = 7$; Redman & Silinsky, 1993*b*) using α-bungarotoxin to block suprathreshold neuromuscular transmission. α-Bungarotoxin is devoid of presynaptic effects of tubocurarine (Bowman *et al.* 1988). For details of other conditions chosen to minimize confounding presynaptic effects, see the Methods.

Normal neuromuscular depression in the frog is not attributable to changes in Ca^{2+} entry, but is more likely to reflect a decrease in the ability of Ca^{2+} to promote the secretory process (Silinsky, 1981, 1984; Silinsky & Solsona, 1992). (For a discussion of evidence against the participation of potassium channels in the action of adenosine at motor nerve endings, see Silinsky & Solsona, 1992). The observation that *endogenous* adenosine mediates prejunctional depression by a mechanism downstream of calcium entry is consistent with other studies of *exogenous* adenosine and neurotransmitter release at frog (Silinsky & Solsona, 1992), *Torpedo* (Muller, Loctin & Dunant, 1987) and hippocampal nerve endings (Scholz & Miller, 1992; Scanziani, Capogna, Gahwiler & Thompson, 1992). Our results in frog differ from those found in rat motor nerve, however (Hamilton & Smith, 1991). These differences between results at motor nerve endings could be due to species differences or to the different types of Ca^{2+} channels (N-type channels in frog and P channels in rat; see Silinsky & Solsona, 1992 for discussion). It is also possible that in the rat, adenosine released by neuromuscular activation from both prejunctional and postjunctional loci (see Smith, 1991) acts by more than one mechanism to inhibit ACh release (see Ginsborg & Hirst, 1972). In our present studies we have chosen our conditions to study the N-type Ca^{2+} current associated with evoked ACh release in the frog (Silinsky & Solsona, 1992) and no measurable effect of adenosine on these currents could be detected.

In conclusion, these results are consistent with a model in which ATP is released from motor nerve endings in stoichiometric amounts with ACh, and after degradation to adenosine is the exclusive mediator of neuromuscular depression at normal levels of ACh output (Silinsky, 1975). The clinical implications of these findings merit some consideration. First, these results are highly relevant to mammalian preparations. In our preliminary experiments, α,β-methylene ADP eliminated neuromuscular depression in mouse phrenic nerve–diaphragm preparations ($n = 3$). If similar results occur at human motor nerve endings, then selective antagonists of adenosine accumulation or adenosine receptor activation might be useful therapeutic adjuncts to eliminate neuromuscular depression in patients with disorders associated with a low safety factor of neuromuscular transmission (e.g. myasthenia gravis, Lopate & Pestronk, 1993).

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Acknowledgements

This work was supported by grants from the US Public Health Service (R01NS12782, T32NSO7140).

Received 5 July 1993; accepted 19 October 1993.