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ATP and adenosine inhibit transmitter release at the frog neuromuscular junction through distinct presynaptic receptors

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1 The effects of exogenous ATP or adenosine on end-plate currents (e.p.cs; evoked by simultaneous action of a few hundred quanta of ACh) or on miniature e.p.cs (m.e.p.cs) were studied under voltage clamp conditions on frog sartorius muscle fibres.

2 ATP or adenosine (100 μM – 1 mM) reduced the e.p.c. amplitude but did not affect m.e.p.c. amplitude, decay time constant and voltage-dependence of m.e.p.c., suggesting that e.p.c. depression induced by these purines had presynaptic origin only.

3 The action of ATP, unlike that of adenosine, was prevented by the P2-purinoceptor antagonist suramin (100 μM). The stable ATP analogue α,β-methylene ATP (100 μM), known to be desensitizing agent on P2X receptors, also abolished the depressant effect of ATP while sparing the action of adenosine. Concanavalin A, an inhibitor of ecto-5′-nucleotidase, did not affect the presynaptic action of exogenously applied ATP.

4 The presynaptic action of adenosine was prevented by theophylline (1 mM), a blocker of adenosine receptors, while the effect of ATP was not changed under these conditions. The selective blocker of A_1 adenosine receptors, 8-cyclopentyl-1,3,dipropylxanthine (DPCPX; 0.1 μM), abolished the presynaptic action of adenosine but did not prevent the depressant effect of ATP.

5 The effects of ATP and adenosine (at nearly saturating concentration) were additive suggesting that these purines activated not only distinct receptors but also different intracellular signalling mechanisms.

6 In contrast to the hypothesis that at the neuromuscular junction ATP reduces transmitter release via enzymatic degradation to presynaptically active adenosine, our data suggest that ATP (through its own presynaptic receptors) directly inhibits ACh release. Thus, ATP and adenosine might be almost equipotent as endogenous prejunctional neuromodulators at the neuromuscular junction.

Keywords: ATP; adenosine; acetylcholine; secretion; voltage clamp

Introduction

Adenosine 5′-triphosphate (ATP) which is co-released with the neurotransmitter acetylcholine (ACh) at the neuromuscular junction (Silinsky & Redman, 1996) might serve as modulator of synaptic transmission (Ribeiro, 1996). In fact, at the neuromuscular junction exogenous ATP has been shown to depress synaptic transmission (Ribeiro & Walker, 1975). However, the mechanism for this depressant action of ATP is thought to be indirect via degradation to the presynaptically active agent adenosine (Ribeiro & Sebastiao, 1987; Meriney & Grinnell, 1991; Redman & Silinsky, 1994) which has been shown to inhibit spontaneous and evoked transmitter release (Ginsborg & Hirst, 1972; Ribeiro & Sebastiao, 1987; Redman & Silinsky, 1994). It has been proposed that endogenous adenosine mediates synaptic depression during rhythmic motor nerve stimulation (Meriney & Grinnell, 1991; Redman & Silinsky, 1994).

On the other hand, recent investigations have revealed the wide distribution of different ligand-gated (P2X) or G-protein coupled (P2Y) ATP receptors throughout body tissues including the neuromuscular system (Burnstock & Kennedy, 1985; Chen *et al*., 1995; Collo *et al*., 1996), raising the possibility of a direct effect of ATP on the neuromuscular junction. Furthermore, a recent study of the action of endogenous ATP and adenosine released from motor nerve endings on perisynaptic glial cells (Robitaille, 1995) has suggested that the rate of endogenous ATP degradation to adenosine might be overestimated. A direct presynaptic action of ATP via ligand-gated P2X purinoceptors on presynaptic nerve terminals has been established at the chick ciliary ganglion (Sun & Stanley, 1996).

For these reasons, in the present study the inhibitory action of ATP on ACh release was compared with that of adenosine at the neuromuscular junction. Our study revealed that ATP exerted its inhibitory effect via its own receptor class distinct from presynaptic receptors for adenosine. Thus, ATP might be a potent neuromodulator even before its breakdown to its physiologically active metabolite adenosine.

Methods

Experiments were carried out on frog sartorius muscle at room temperature (see Giniatullin *et al*., 1993; 1997). A muscle preparation was continuously superfused with physiological solution (in mM): NaCl 113, KCl 2.5, $CaCl_2$ 1.8, $NaHCO_3$ 2.4, pH = 7.3. In order to prevent muscle contraction and to observe physiologically high levels of transmitter release muscle fibres were cut (for details see Barstad & Lilleheilh, 1968; Giniatullin *et al*., 1993) and continuously superfused for about 40 min before the recording of end-plate currents (e.p.c.) evoked by motor nerve stimulation under two-electrode voltage clamp conditions. Spontaneous miniature end-plate currents (m.e.p.c.) were recorded from uncut muscle for better signal/noise resolution. The resistance of the electrodes (filled with 2.5 M KCl) was in the range of 3–5 MΩ. E.p.cs and

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m.e.p.cs were sampled at 10 kHz and stored on the hard disk of an IBM computer for further analysis. Amplitude, rise-time (10–90%) and exponential decay time constant were measured throughout the experiment. Between 10–20 multiquantal e.p.cs and 250–400 m.e.p.cs were usually averaged to obtain mean values.

ATP (disodium salt), adenosine, α,β-methylene ATP, theophylline, concanavalin A and 8-cyclopentyl-1,3,dipropylxanthine (DPCPX) were purchased from Sigma. Suramin was obtained from Research Biochemicals Inc. All drugs were applied to the muscle by changing the control bath solution to one containing the drug to be studied. No pharmacological agent was used to abolish muscle contraction. Data are presented as mean $\pm$ s.e. Student's t test (for comparison of the agent action with control) and non-parametric Wilcoxon test (when transformed data on the action of agent before and after application of antagonist were compared) were used for evaluating statistical significance of experimental differences.

Results

ATP and adenosine were equipotent at inhibiting ACh release

On cut sartorius muscle fibres held at -40 mV (20°C) motor nerve stimulation (0.03 Hz) evoked e.p.cs with amplitude of 155 ± 34 nA and decay time constant ($\tau_{e.p.c.}$) of 1.36 ± 0.08 ms ($n = 19$ synapses). Either ATP or adenosine in the 100 μM–1 mM concentration range reversibly reduced the amplitude of such multiquantal e.p.cs (Figure 1A for example of responses to 100 μM). At 100 μM ATP reduced the amplitude of the e.p.c. to 67 ± 6% of control ($n = 11$; $P < 0.05$) while adenosine (100 μM) induced a similar depression, lowering e.p.cs to 70 ± 3% ($n = 9$; $P < 0.05$; Figure 1B). At 1 mM ATP and adenosine reduced the amplitude of e.p.cs to 43 ± 9% ($n = 10$; $P < 0.05$) and to 62 ± 3% ($n = 11$; $P < 0.05$), respectively. Thus, both purines exerted a comparable inhibitory action on synaptic currents at the neuromuscular junction.

On uncut muscles (-70 mV holding potential) neither ATP ($n = 8$) nor adenosine ($n = 11$) at 100 μM changed the parameters of m.e.p.cs. The control value for the m.e.p.c. amplitude was 2.97 ± 0.05 nA with decay time constant ($\tau_{m.e.p.c.}$) of 1.01 ± 0.05 ms ($n = 10$). As shown in Figure 2, these parameters as well as the H-coefficient (i.e., the shift in membrane potential necessary for e-fold change of $\tau_{m.e.p.c.}$) were not affected by either purine, indicating that the inhibitory action of ATP or adenosine had presynaptic origin and was not dependent on the holding potential of postsynaptic membrane.

On cut muscle ATP or adenosine (100 μM) increased presynaptic facilitation induced by paired-pulse stimulation (interstimulus interval = 17 ms). Facilitation, expressed as ratio

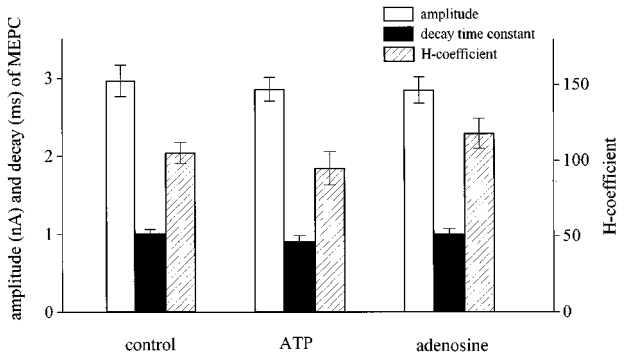

Figure 2 Effect of ATP or adenosine on m.e.p.cs. Amplitude of m.e.p.cs, m.e.p.c. decay time constant and H-coefficient (shift in membrane potential necessary for e-fold change of m.e.p.c. decay time constant) in control ($n = 10$), in the presence of 100 μM adenosine ($n = 11$) or 100 μM ATP ($n = 8$). $P > 0.05$ in all cases by t test.

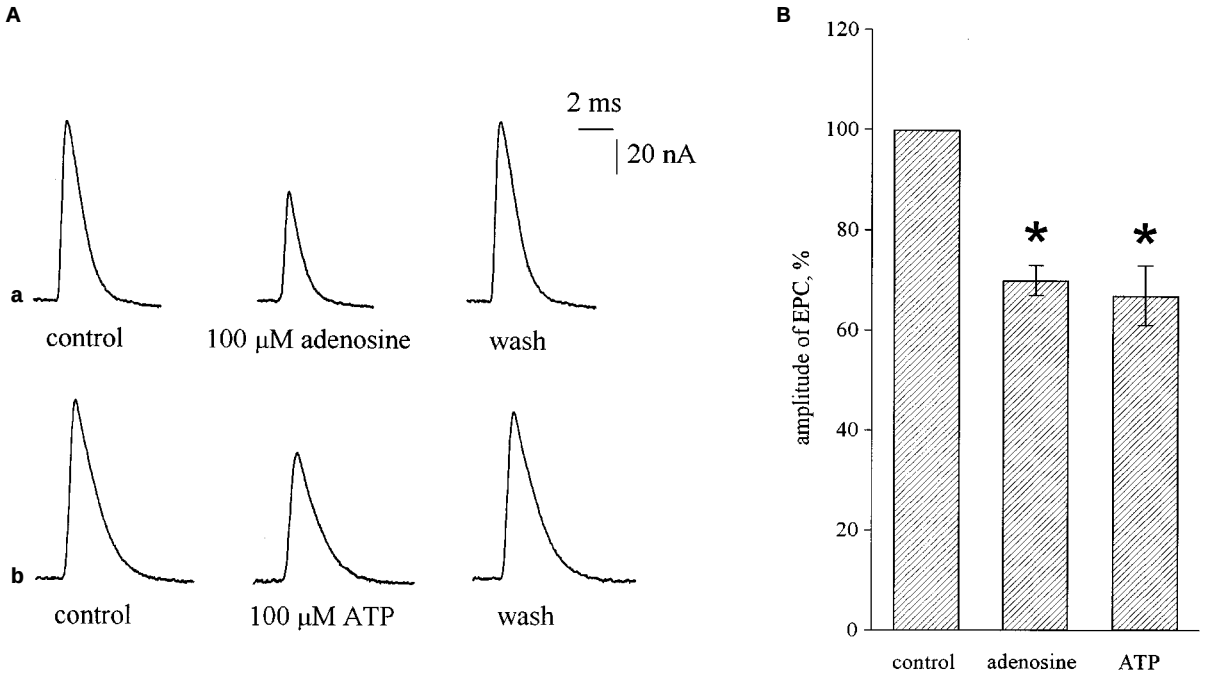

Figure 1 Effect of adenosine or ATP on multiquantal e.p.c. (A) Multiquantal e.p.c. in control, in the presence and after washout of 100 μM adenosine (Aa) or 100 μM ATP (Ab). (B) Effect of ATP or adenosine (both 100 μM) on the amplitude of e.p.cs in a sample of nine synapses for adenosine and eleven synapses for ATP. *$P < 0.05$; t test. Holding potential -40 mV.

between amplitude of the two e.p.cs, was 1.18 ± 0.05 ($n=9$) in control solution and 1.28 ± 0.05 ($n=4$; $P<0.05$) in the presence of 100 μM adenosine; the corresponding value for 100 μM ATP was 1.27 ± 0.06 ($n=5$; $P<0.05$).

Even if the concentration of adenosine (100 μM) was nearly saturating (see also Ribeiro & Walker, 1975), when 100 μM ATP was applied in the presence of 100 μM adenosine, ATP reduced the e.p.c. amplitude almost to the same level (56%; $n=3$) as in control (67%) solution (see Figure 3a for time profile of this phenomenon and Figure 3b for pooled data). Such a clear, additive effect of the two purines provided evidence for the presence of distinct presynaptic receptors inhibiting transmitter release.

In order to test this hypothesis further the action of ATP and adenosine on e.p.cs was compared in the presence of adenosine receptor (P1) and ATP receptor (P2) ligands.

Effects of P_1 receptor ligands

First, the action of the non-selective P1 receptor antagonist theophylline was studied. Theophylline (1 mM) slightly increased (by $23 \pm 8\%$; $n=5$) the amplitude of e.p.cs in agreement with previous observations (Ginsborg & Hirst, 1972; Ribeiro & Sebastiao, 1987). This agent prevented the depressant action of 100 μM adenosine on e.p.c. amplitude while it did not change the inhibitory action of 100 μM ATP.

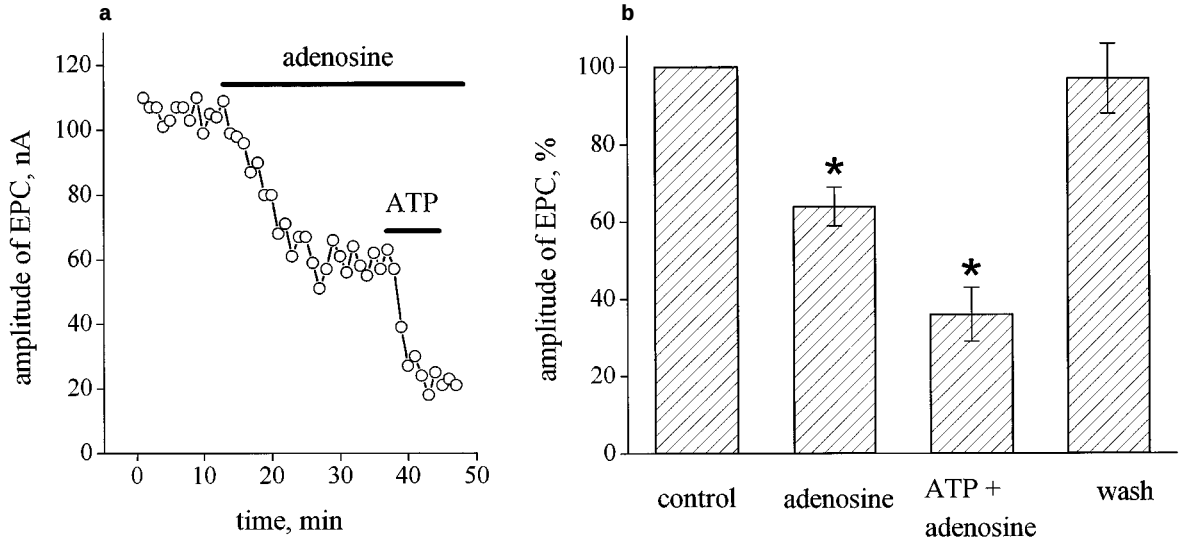

Figure 3 Additive effect of ATP and adenosine on multiquantal e.p.cs. (a) Time-course of the action of 100 μM adenosine or adenosine plus 100 μM ATP on amplitude of multiquantal e.p.c. (b) Averaged data for the action of adenosine or adenosine plus ATP on a sample of three synapses. $*P<0.05$; t test. Holding potential -40 mV.

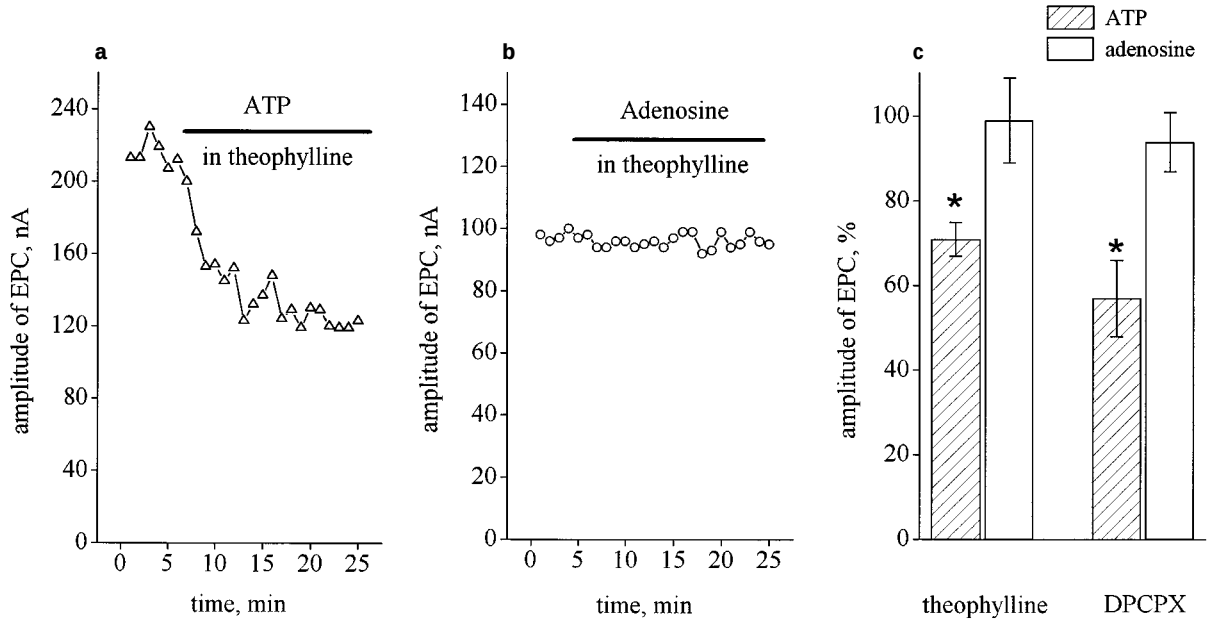

Figure 4 Effect of ATP and adenosine on multiquantal e.p.cs in the presence of 1 mM theophylline or 100 nM DPCPX. (a) Depressant effect observed with 100 μM ATP in the presence of theophylline. (b) Lack of effect of 100 μM adenosine in the presence of theophylline (different synapse from a). (c) Averaged data for the effect of adenosine or ATP in the presence of theophylline ($n=5$) or DPCPX ($n=4$). $*P<0.05$; Wilcoxon test. Holding potential -40 mV.

Thus, in the presence of theophylline (see experiment shown in Figure 4a,b) the e.p.c. amplitude after application of adenosine was like the control one (101%; $n=5$; $P>0.05$), whereas it remained fully sensitive to the inhibitory action of ATP ($n=5$; $P<0.05$).

Since theophylline is a non-selective blocker of adenosine receptors, we studied the ATP action also in the presence of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), a selective antagonist of the A_1 subtype of P1 receptors (Lohse *et al.*, 1987). Like theophylline, DPCPX (0.1 μM) slightly increased (by $14\pm7\%$; $n=8$) the e.p.c. amplitude. This blocker abolished the presynaptic action of adenosine ($94\pm7\%$ of control amplitude; $n=4$; $P>0.05$; Figure 4c) but left essentially unchanged the reduction in e.p.c. amplitude (to $57\pm9\%$ of control; $n=4$; $P<0.05$; Figure 4c) by ATP.

Effects of P2 receptor ligands

Suramin (100 μM), which antagonizes the ATP action on many subtypes of the P2 receptor (Dunn & Blakely, 1988), prevented the inhibitory action of ATP. Thus, after the addition of 100 μM ATP in the continuous presence of suramin the e.p.c. amplitude was $98\pm11\%$ of control ($n=7$; $P>0.05$; Figure 5b) suggesting the existence of distinct ATP receptors responsible for the inhibitory action of this agent on transmitter release. Conversely, adenosine in the presence of suramin reduced the amplitude of e.p.c. to $78\pm5\%$ of control ($n=4$; $P<0.05$).

The stable ATP analogue α,β-methylene ATP, which is not susceptible to hydrolysis by eco-ATPase (Khakh *et al.*, 1995), did not significantly change the amplitude of multiquantal e.p.cs when applied at 100 μM (e.p.c. amplitude was $87\pm7\%$ of control; $n=7$; $P>0.05$) in agreement with the observation of Ribeiro & Sebastiao (1987). Subsequent application of 100 μM ATP in the continuous presence of its analogue did not reduce the amplitude of the e.p.c. (Figure 5a). Thus, in the presence of ATP plus α,β-methylene ATP the amplitude of e.p.c. was $91\pm11\%$ of the value before ATP application ($n=6$; $P>0.05$). This result was significantly different from the control reduction of e.p.c. produced by ATP to $67\pm6\%$ ($n=11$; $P<0.05$). Conversely, the inhibitory action of adenosine (100 μM) was not changed by α,β-methylene ATP. One typical experiment is shown in Figure 5a and similar results were obtained from a sample of 4 synapses.

The final step in the conversion of extracellular ATP to adenosine is regulated by ecto-5′-nucleotidase (Smith, 1991). Concanavalin A, known as an inhibitor of ecto-5′-nucleotidase (Kreutzberg *et al.*, 1986), did not affect the presynaptic action of exogenous ATP in our experiments. After 20 min exposure of the muscle to 0.1 mg ml^{-1} concanavalin A (providing inhibition of ecto-5′-nucleotidase activity by 76% according to Kreutzberg *et al.*, 1986), ATP still significantly reduced e.p.c. to $75\pm3\%$ of control ($n=5$; $P<0.05$) and this effect was

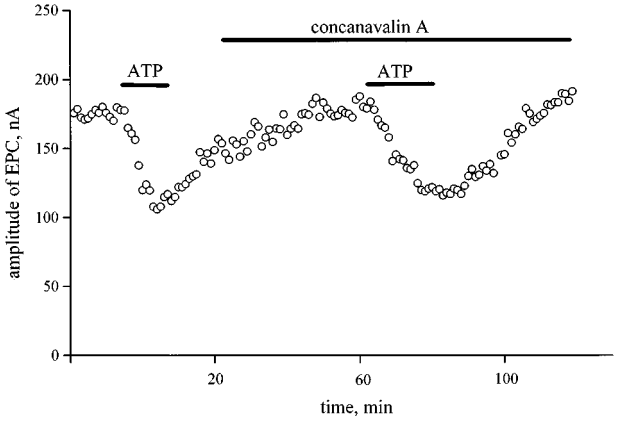

Figure 6 Effect of ATP (100 μM) on multiquantal e.p.cs at the same synapse in control and in the presence of 0.1 mg ml^{-1} concanavalin A. Holding potential -40 mV.

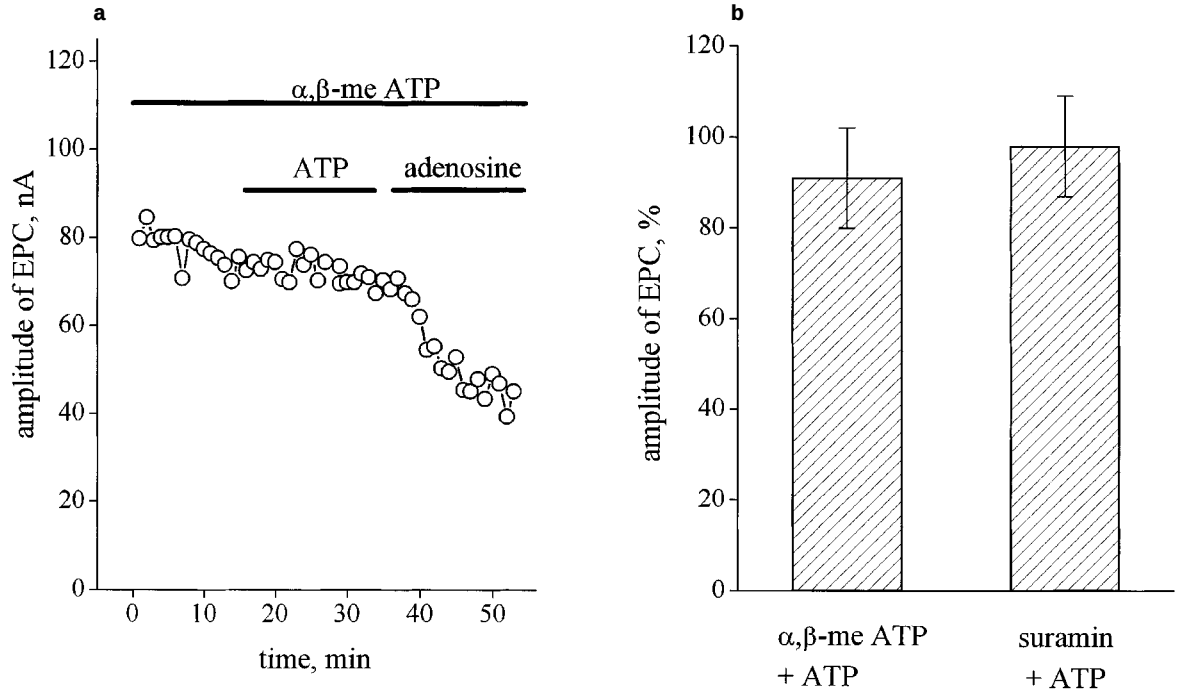

Figure 5 Effect of ATP or adenosine on multiquantal e.p.cs in the presence of α,β-methylene ATP or suramin. (a) Effect of 100 μM ATP or 100 μM adenosine on e.p.c. amplitude in the continuous presence of 100 μM α,β-methylene ATP. (b) Averaged data for the effect of ATP on e.p.cs in the presence of α,β-methylene ATP ($n=6$) or suramin ($n=7$). $P>0.05$ in both cases, t test. Holding potential -40 mV.

similar ($P>0.05$) to the effect of ATP in control solution (Figure 6).

Discussion

In keeping with data from previous studies (Ribeiro & Walker, 1975; Ribeiro & Sebastiao, 1987; Redman & Silinsky, 1994) we also observed an inhibitory action of ATP on frog neuromuscular transmission. However, in contrast to the hypothesis that ATP reduces transmitter release at the neuromuscular junction exclusively via its degradation to presynaptically active adenosine (Ribeiro & Sebastiao, 1987; Meriney & Grinell, 1991; Hamilton & Smith, 1991; Redman & Silinsky, 1994), our results support the idea that ATP acts through its own presynaptic receptors different from those for adenosine.

Both ATP and adenosine reduced the amplitude of the e.p.c. and did not affect the amplitude, decay time constant or voltage-dependence of decay of m.e.p.cs, suggesting that the depression of e.p.c. induced by these purines had presynaptic origin only and could be observed regardless of the value of holding potential of the postsynaptic membrane. The latter finding means also that neither ATP nor adenosine exert open channel blocking action on postsynaptic membrane. All these findings thus indicate a presynaptic site of action, as further confirmed by the increased presynaptic facilitation studied with a standard paired-pulse protocol. Such enhanced facilitation accords with the reduction in the e.p.c. quantal content by both purines (Zucker, 1989).

Even if these tests did not allow us to distinguish between a direct or indirect (via degradation to presynaptically active adenosine) action of ATP on e.p.cs, important differences between ATP and adenosine emerged when their action was compared in the presence of P1 and P2 receptor antagonists.

Sensitivity of ATP and adenosine effects to α,β-methylene ATP and concanavalin A

The stable ATP analogue α,β-methylene ATP, which can act as a desensitizing agent on P2 receptors (Burnstock & Kennedy, 1985; Chen *et al*., 1995) selectively abolished the depressant action of ATP on e.p.cs while not affecting that of adenosine. Similar results were produced by suramin, which is a broad spectrum antagonist of P2 receptors (Dunn & Blakely, 1988) ineffective in distinguishing between P2X and P2Y subtypes (Hoyle, 1990). In some neurones α,β-methylene ATP is able to attenuate ATP action without its own agonist activity (Khakh *et al*., 1995). Interestingly, according to Ribeiro & Sebastiao (1987) and Redman & Silinsky (1994), the structural analogue α,β-methylene ADP, a blocker of 5′-nucleotidase (Ribeiro & Sebastiao, 1987), can also prevent the inhibitory action of ATP. The later finding is apparently in support of the view that the ATP-induced inhibition of ACh release is due to hydrolysis of ATP to adenosine, which would actually be the active substance that modulates transmitter release (Ribeiro & Sebastiao, 1987; Redman & Silinsky, 1994). However, there is an alternative explanation, i.e. that α,β-methylene ADP (like the action of its analogue α,β-methylene ATP at super cervical neurones according to Khakh *et al*., 1995) prevents activation of P2 purinoceptors by ATP. In the present study we observed that when using another pharmacological tool, namely concanavalin A to inhibit 5′-nucleotidase (Kreutzberg *et al*., 1986), the presynaptic action of exogenous ATP persisted. This enzyme is responsible for the final step in the conversion of ATP to adenosine and, if the action of ATP on e.p.cs was mediated by formation of adenosine, we should have found that concanavalin A prevented the inhibitory action of ATP on transmitter release, contrary to the present data.

Is the action of ATP direct or mediated by its breakdown product, adenosine?

The crucial factor for the hypothesis that the effect of ATP is due to adenosine-mediated presynaptic depression is the rapid degradation of ATP to adenosine (Smith, 1991). Recent direct observations have revealed that at the neuromuscular junction co-released ATP and ACh open their respective channels approximately on the same time-scale (Silinsky & Redman, 1996). That is why it is of interest to compare in intact tissue the still relatively obscure rate of ATP hydrolysis with that of ACh, which is a well known process. An estimate of ATP hydrolysis by skeletal muscle indicates that exogenous ATP (50 μM) would be broken down to adenosine in about 2 min (Smith, 1991). A more precise analysis of the role of synaptic ecto-ATPase and 5′-nucleotidase requires not yet available data on the density of ATPase and 5′-nucleotidase sites and their turnover. For synaptic acetylcholinesterase these values are 600 sites per μm^2 and 9.500 s^{-1}, respectively (Anglister *et al*., 1994). In view of the high specialization of the neuromuscular junction for ACh, the turnover of ACh is presumably higher than the corresponding values for ATP/ATPase/5′-nucleotidase system. Thus, the rate of endogenous ATP degradation to adenosine might have been overestimated and a re-appraisal of the direct action of ATP should take place. This conclusion is supported by a recent investigation monitoring changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) induced by either ATP or adenosine in perisynaptic Schwann cells (Robitaille, 1995). A rise in $[Ca^{2+}]_i$ is evoked by both exogenous ATP and adenosine; however, only endogenous ATP (not adenosine) participates in Ca^{2+} signalling of perisynaptic glial cells after the release from motor nerve endings.

Our results with P1 receptor antagonists were in good agreement with the hypothesis of a direct action of ATP on motor nerve endings. Thus, the presynaptic action of adenosine was prevented by theophylline, a blocker of adenosine receptors (Ginsborg & Hirst, 1972; Meriney & Grinnell, 1991; Ribeiro & Sebastiao, 1987) while the ATP action was unchanged. Likewise, the selective blocker of A_1 adenosine receptors DPCPX (Lohse *et al*., 1987) abolished presynaptic action of adenosine but did not prevent the depressant action of ATP on e.p.cs. This observation differs from the results by Redman and Silinsky (1994), probably because we used higher concentrations of ATP.

Finally, additional evidence supporting distinct presynaptic actions of ATP and adenosine was obtained with combined application of these purines. The actions of ATP (100 μM) and adenosine (100 μM) close to their saturating concentration (see Ribeiro & Walker, 1975) were additive, supporting the idea that these purines activate not only distinct receptors but perhaps also different intracellular signalling mechanisms. The depressant action of adenosine has been proposed to be exerted via a pertussis toxin-sensitive G-protein (Hamilton & Smith, 1991; Ribeiro, 1996). The cellular mechanisms of ATP action on transmitter release might be brought about via ligand-gated (P2X) or metabotropic (P2Y) purinoceptors coupled with different G-proteins or intracellular signalling systems. Further experiments are necessary to clarify this issue. The wide distribution of at least seven subtypes of P2X receptors has been shown recently (Fredholm *et al*., 1997).

There is no direct demonstration of ATP receptors on motor nerve endings, although the presence of ATP-activated ligand-gated P2X purinoceptors has been established on presynaptic nerve terminals of the chick-ciliary ganglion (Sun & Stanley, 1996). It is also known that spinal motoneurones express mRNAs for five subtypes of P2X purinoceptors with the exception of $P2X_3$ ones (Collo *et al*., 1996).

Thus, our data support the idea that ATP might be almost equipotent as adenosine in eliciting presynaptic modulation through distinct receptors. Accumulation of ATP in the synaptic cleft during multiquantal release and subsequent tetanic activity might reach a level sufficient to inhibit transmitter release from motor nerve endings. The direct action of ATP on transmitter release via presynaptic P2 receptors, opens new perspectives in modulation of transmitter release and mechanisms mediating fatigue in the peripheral and central nervous system.

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