Evaluation of Intrinsic Modulation of Synaptic Transmission by ATP in Mouse Fast Twitch Muscle

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Hong, S. J. and C. C. Chang. Evaluation of intrinsic modulation of synaptic transmission by ATP in mouse fast twitch muscle. J. Neurophysiol. 80: 2550–2558, 1998. This study aims to evaluate whether endogenous ATP or adenosine modulates the neurotransmission and contractile function of mouse phrenic nerve–diaphragm. Bath application of ATP (1 mM) and α,β-methylene ATP (m-ATP, 0.1 mM) elevated muscle tones, depressed contractions (~12%), and depolarized muscle membranes (~20 mV). Adenosine (1 mM) or low concentrations of ATP (0.1 mM) had no effect. In a low Ca<sup>2+</sup> media, ATP caused prolonged inhibitions of endplate potentials (EPPs), whereas m-ATP augmented EPPs while both agents produced slight effects in normal Tyrode solution. When applied by puff ejection, ATP and m-ATP additionally elicited fast transient suppressions of EPPs in association with inhibition of high K<sup>+</sup>-evoked releases of miniature EPPs. Blockades of P<sub>2</sub><sub>1</sub> purinceptors with suramin antagonized all the effects of ATP and m-ATP except the prolonged inhibitions of EPPs induced by ATP, which were antagonized instead by 8-cyclopentyl-1,3-dipropylxanthine (CPDPX), an A<sub>1</sub> adenosine receptor antagonist. Suramin and CPDPX did not change contractions nor alter EPPs evoked by a low- or high-frequency nerve stimulation. The results indicate that exogenously applied ATP and m-ATP, via activations of distinct pre- and postsynaptic purinceptors, exert inhibitory and facilitatory pharmacological modulations on the mature neuromuscular junction. However, because of intrinsic high efficiency of the synaptic transmission under physiological conditions, endogenously released ATP and its degradation product—adenosine—do not build up to concentrations high enough to alter motor functions.

INTRODUCTION

ATP is widely distributed in biological systems and plays a fundamental role in the energy production. Another unique feature of the physiological function of ATP is copackages of the nucleotide with conventional neurotransmitters within specific exocytotic vesicles—granules (Klein and Thureson-Klein 1984; Whittaker 1984). Several lines of evidence suggest that, on exocytosis, vesicular–granular contents are discharged into extracellular compartment, and the released ATP could stimulate functionally distinct nucleotide receptors, acting as a modulator, a cotransmitter, or even as an excitatory transmitter in nervous systems (Evans et al. 1992; Jahr and Jessell 1983; Krishtal et al. 1988).

In the neuromuscular junctions, the molar ratio of ATP to acetylcholine inside synaptic vesicles is estimated to be 0.1:1 (Volkman and Zimmermann 1986). In response to an excitation of motor nerve, ATP is released into synaptic cleft not only from nerve terminals (Silinsky and Redman 1996) but also from postsynaptic muscle component (Abood et al., 1962). The released ATP might facilitate openings of acetylcholine channels (Lu and Smith 1991) and, after being degraded to adenosine by ectonucleotidases, produce reversible depressions of transmitter release (Redman and Silinsky 1994; Ribeiro and Sebastião 1987) or participate in desensitizations of nicotinic receptors (Pitchford et al. 1992). However, in view of a high safety margin of the synaptic transmission of mature neuromuscular junction, in which nerve stimulation-evoked subsynaptic response is several fold greater than the threshold for inducing muscle action potential (Hong and Chang 1989; Wood and Slater 1997), functional influences of endogenous ATP and adenosine on the contractions evoked by nerve stimulation need scrutiny. The issue was explored by studying effects of ATP and its nonhydrolyzable derivative in combination with specific blockers of adenosine–purinergic receptors on various parameters of the synaptic transmission.

METHODS

Nerve–muscle preparation

Mice (ICR strain, 2–3 mo old weighing 20–25 g) were stunned and exsanguinated. Phrenic nerve–diaphragms were isolated and bathed in 10–15 ml Tyrode solution (composition in mM: 137 NaCl, 2.8 KCl, 1.8 CaCl<sub>2</sub>, 1.1 MgCl<sub>2</sub>, 11.9 NaHCO<sub>3</sub>, 0.33 NaH<sub>2</sub>PO<sub>4</sub>, and 11.2 dextrose). Tyrode solution was maintained at 35–37°, and the pH was adjusted to 7.3–7.4 by aeration with 5% CO<sub>2</sub> in O<sub>2</sub>.

Indirect and direct contractions were evoked, respectively, by stimulation of phrenic nerve with supramaximal pulses of 0.03-ms duration and by field stimulation of diaphragm with pulses of 0.3-ms duration. The stimulation frequencies ranged from a low single pulse stimulation (0.05–0.5 Hz) to a train of high-frequency stimulation (10–300 Hz for 3–10 s). Contractile responses were recorded isometrically. Effects of various treatments on contractile responses were evaluated either in normal Tyrode solution or in a high Mg<sup>2+</sup>/low Ca<sup>2+</sup> Tyrode solution, in which the efficiency of neurotransmission was substantially reduced. The magnitudes of effects were quantified by contraction amplitude and contraction area delineated by tetanic contraction curve. For each preparation, only one single concentration (rather than cumulative application) of ATP was introduced by bath application to minimize run-downs of agonist effects consequent on prolonged stimulations of receptors. Effects of ATP were observed for periods of 15–90 min and then preparations were washed with fresh Tyrode solution for 2–6 times, separated by 10 min each.

Intracellular recording

Endplate potentials (EPPs), miniature endplate potentials (mEPPs), and membrane potentials were recorded as described previously (Hong and Chang 1995). Endplate regions were identi-
fied only if the rise time (a period spanning 10–90% amplitude) of mEPPs was ≥0.3 ms. Because of technical limitations, we applied voltage-clamp experiments (with a single electrode voltage clamp amplifier, Dagan 8100) to assess slow or small current changes only (e.g., miniature endplate currents) but not large and fast current changes (e.g., endplate currents). Glass microelectrodes were filled with 3 M KCl and had a resistance of 10–15 MΩ. Those used for voltage clamp had a low resistance (2–3 MΩ). For recordings of EPPs, preparations were paralyzed by blocking the Na⁺ channels of diaphragm muscle with μ-conotoxin (1 μM). This method of immobilization enables us to record and compare unattenuated EPPs as well as mEPPs from the same endplate simultaneously (Hong and Chang 1989). For each preparation, EPPs were taken from four to eight endplates. For each endplate, 20 EPPs evoked at 0.6 Hz were sampled. Amplitudes of EPPs—mEPPs were presented either as recorded per se or the amplitudes were corrected for nonlinear summation and then normalized to a resting membrane potential of −80 mV (cf. Chang et al. 1986). Electrical signals were DC coupled and recorded on a waveform recorder (Gould 4600). The frequency response of the unit was 30 kHz. Trains of EPPs during a high-frequency stimulation were AC coupled, and EPPs were expressed in percent relative to the amplitude of the first EPP. ATP or α,β-methylene ATP (m-ATP) was applied either by bath application or by focal pressure ejection (3 μl of 10–300 mM stock solution prepared in normal or in a high Mg²⁺/low Ca²⁺ Tyrode solution) from a point ~100 μm away from the recording electrode.

Statistics and chemicals

Data presented (means ± SE) were pooled from three to six preparations. Difference between means was considered significant by Student’s t-test with a P value < 0.05. Tracings shown in Figs. 1–3 are representatives of similar results obtained with 3–6 preparations, and those in Figs. 4–9 are representatives of 10–15 recordings.

Chemicals used were ATP, m-ATP, adenosine (Sigma, St. Louis, MO, USA), 8-cyclopentyl-1,3-dipropylxanthine (CPDPX), 2-Cl-adenosine, suramin (Research Biochemicals International, Natick, MA, USA,), and μ-conotoxin (Peptide Institute, Osaka, Japan). CPDPX was dissolved in ethanol, and the final concentration of ethanol in the organ bath was <0.1% (vol/vol).

RESULTS

Contractility experiments

EFFECTS OF ATP ANALOGS. When mouse phrenic nerve–diaphragms were incubated in normal Tyrode solution, bath application of ATP (≥100 μM) caused little, if any, change of contractilities evoked by low-frequency direct or indirect stimulations. At a high concentration (1.0 mM), ATP produced transient slight elevations of muscle tone (by 1.9 ± 0.8 mN on top of 10 mN resting tension, n = 5) and, simultaneously, short-term parallel depressions of both direct and indirect contractile forces (by 12 ± 3%, as indicated by ▲ in Fig. 1A). These effects faded within 15 min, and small increases of contractilities (±8 ± 3% over the control level, ▲) succeeded in the following 30 min. The augmented contractile forces reverted to the control level after washout of ATP. The effects of ATP on tetanic contractions evoked by high-frequency stimulations (10–100 Hz for 3–10 s) were similar to its effects on single twitches, initial depressions by 17 ± 4% (n = 5) and late augmentations by 10 ± 3% with a same time course of actions. If the preparations were not washed, a second challenge of ATP produced far less influences on contractile responses. However, these ATP-induced effects could be reproduced qualitatively and quantitatively provided that ATP was washed out.

m-ATP (30–300 μM), an ATP analog resistant to metabolic degradations, affected muscle tone and contractile force in a similar manner but more potently than did ATP (Fig. 1B). Unlike ATP and m-ATP, adenosine and 2-Cl-adenosine, a selective adenosine A₁ receptor agonist, did not change contractility nor altered muscle tone at concentrations as high as 1.0 mM.

EFFECTS OF ATP ON INDIRECT CONTRACTION. The previous results indicate that motor functions are affected by high concentrations of ATP. To further unmask possible effects of ATP on the neuromuscular transmission, the efficiency of synaptic transmission was decreased by reducing evoked acetylcholine release with a high Mg²⁺ (2.2 mM)/low Ca²⁺ (0.6–0.8 mM) Tyrode solution. This pretreatment suppressed indirect contractile forces to 10–40% of control, whereas direct twitches were not altered significantly. In these preparations with an impaired synaptic transmission, ATP at 0.3–1.0 mM produced, in addition to initial elevations of basal tone, transient depressions then augmentations (as marked by asterisk in Fig. 2A) followed by
long-lasting inhibitions (▼) of indirect twitches. Unlike ATP, however, m-ATP (30–300 μM) elicited, after similar initial transient depressions (Fig. 2B, asterisk), sustained augmentations of indirect twitches (▲), which were not followed by inhibitions. In the low neurotransmission condition, if the amplitude of indirect twitches before ATP or m-ATP challenge was taken as 100%, the ATP-induced late inhibitions ranged from 57 ± 9% (at 0.3 mM, n = 4) to 89 ± 4% (at 1 mM), and the extent of m-ATP-induced augmentations ranged from 24 ± 5% (at 30 μM, n = 5) to 104 ± 9% (at 100 μM), both effects being in a dose-dependent manner. Interestingly, when applied after ATP, m-ATP counteracted the ATP-induced late phase inhibitions (Fig. 2A, ●). The increases of indirect contractions after m-ATP, although seemingly remarkable, was susceptible to a very small increase of MgCl₂ (0.4 mM, Fig. 2B). As will be described, this elevation of Mg²⁺ level decreased quantal content of the EPPs.

**ANTAGONISM BY CPDPX AND SURAMIN.** In normal Tyrode solution, CPDPX up to 100 μM to block A₁ receptors, or suramin up to 300 μM to block P₂ receptors, affected neither direct nor indirect contractile responses evoked by low- or high-frequency stimulations.

Under an impaired transmission condition (high Mg²⁺/low Ca²⁺ solution), in which indirect contractions were reduced to 20 ± 3% of control, CPDPX (1–30 μM) produced a limited dose-dependent recovery of indirect twitches. The amplitude of indirect twitches (relative to that before CPDPX) increased 13 ± 2% at 3 μM, 28 ± 5% at 10 μM, and 47 ± 6% at 30 μM (n = 5) without changes in the contractilities evoked by direct stimulations (Fig. 3A), suggesting that the increases were due to augmented synaptic transmission. The increments by CPDPX were completely counteracted by further addition of a small amount of MgCl₂ (0.2 mM). Because CPDPX at 3 μM antagonized the synaptic depressions produced by 2-Cl-adenosine (Fig. 3B), it is conceivable that CPDPX at 30

![Diagram](https://via.placeholder.com/150)

**FIG. 2.** Inhibition and facilitation of contractile responses by ATP and αβ-methylene ATP in low Ca²⁺ media. At t, the incubation medium was changed from normal Tyrode solution to a high MgCl₂ (2.2 mM)/low Ca²⁺ (0.6–0.8 mM) Tyrode solution. A: preparation was treated with ATP 300 μM, which caused transient depressions followed by augmentations (▲) and then long-term inhibitions (▼) of contractilities. Further addition of m-ATP 100 μM caused short-term inhibitions (●) and then sustained increases of contractilities (▲); right panel: 30 min later, the preparation was treated with an extra amount of MgCl₂ (0.4 mM).

![Diagram](https://via.placeholder.com/150)

**FIG. 3.** Antagonism by 8-cyclopentyl-1,3-dipropylxanthine (CPDPX) and suramin against 2-Cl-adenosine, ATP, and αβ-methylene ATP. All traces shown are 10 min after incubation in a low Ca²⁺ solution and the indirect twitch responses depressed to a steady state. A: preparation was treated with CPDPX 30 μM followed by ATP 100 μM, which now caused late phase increases of indirect contractions (▲, vs. the ATP-elicited inhibitions in C, ▼). B: left panel: preparation was treated with 2-Cl-adenosine 100 μM followed by CPDPX 3 μM; right panel: 30 min later, the preparation was treated further with m-ATP 100 μM. C, left panel: control; right panel: 30 min after suramin 100 μM, the preparation was treated further with m-ATP 100 μM followed by ATP 100 μM; the latter agent produced suramin-insensitive inhibitions of indirect contractions (▼). Note that suramin did not change indirect contractions but inhibited the effects of m-ATP.

![Diagram](https://via.placeholder.com/150)

**FIG. 4.** Inductions of membrane depolarizations and muscle action potentials by ATP. The recording microelectrode was inserted into an extrajunctional area ~4 mm away from endplate region. Left panel: control (resting membrane potential: ~80 mV); middle panel: the same impalement 1 min after bath application of ATP 1.0 mM; right panel: 30 min after ATP.
In the absence of CPDPX (cf. Figs. 2A and 3C), ATP now produced late phase inhibitions of endplate potentials and that CPDPX antagonized the late-phase inhibitions. 

The effects of ATP changed; ATP now produced late phase inhibitions of EPPs (Fig. 3C). Suramin also inhibited the initial short-lived (*) and prolonged (▼) inhibitions of endplate potentials and that CPDPX antagonized the late-phase inhibitions.

When preparations were pretreated with CPDPX (30 μM), the effects of ATP changed; ATP now produced late phase inhibitions of endplate potentials (Fig. 3A, ▲) in contrast to inhibitions in the absence of CPDPX (cf. Figs. 2A and 3C, ▼). CPDPX, however, altered none of the effects of m-ATP (Fig. 3B, right panel). All these CPDPX-resistant effects of m-ATP, including rapid basal tone elevations as well as depressions and facilitations of direct and indirect twitches, were antagonized by suramin (100 μM) both in normal (Fig. 1C) and in the impaired transmission conditions (Fig. 3C). Suramin also inhibited the initial short-term effects (transient depressions and facilitations of indirect twitches and elevations of muscle tone) produced by high concentrations of ATP (not shown). However, unlike CPDPX, suramin did not suppress the late inhibitions of indirect twitches caused by ATP (Fig. 3C, ▼).

**Electrophysiological experiments**

**EFFECTS OF ATP ON MEMBRANE POTENTIAL.** Bath application of ATP (1.0 mM) elicits sustained membrane depolarizations (−63.1 ± 4.6 mV, n = 4, cf. Figs. 4 and 5A) and reduced membrane input resistances (561 ± 20 vs. 632 ± 24 kΩ). Spontaneous burst firings of muscle action potential occurred when the membranes were depolarized to approximately −70 mV (Fig. 4, an extrajunctional recording). Within 30 min, muscle membranes gradually repolarized and action potential firings diminished. These effects took place all over entire muscle fibers, including the endplate regions. By contrast, adenosine (1 mM) had no such actions. Despite restorations of membrane potential, a second challenge of ATP produced a far less extent of membrane depolarizations than that of the initial one. However, effects of ATP on membranes could be reproduced when the preparations were washed. These results suggest that the spontaneous restoration of membrane potential is not due simply to degradations of ATP but possibly to desensitizations.

**EFFECTS OF ATP ON EVOKED ACETYLCHOLINE RELEASE.** In μ-conotoxin paralyzed control, mEPPs had an amplitude of 1.3 ± 0.2 mV, and EPPs evoked on low-frequency stimulations (0.05–0.5 Hz) reached 30.5 ± 2.4 mV (36.8 ± 2.7 mV after correction, n = 4). The quantal content estimated by the direct method was ~30. ATP at 1.0 mM depressed EPP amplitude to 20.6 ± 2.5 mV in ~8–20 min (after corrections for the ATP-induced membrane depolarizations, the value was 30.9 ± 2.7 mV, ~85% of control, 11 endplates, n = 4, cf. Fig. 5A). Hereafter, all the statistic data of EPPs shown were after corrections. The inhibitions of EPPs com-

μM should produce an adequate blockade of A1 receptors and that the slight increments of transmission after CPDPX might be due to limited effects of endogenous adenosine. When preparations were pretreated with CPDPX (30 μM), the effects of ATP changed; ATP now produced late phase increases of indirect twitches (Fig. 3A, ▲) in contrast to inhibitions in the absence of CPDPX (cf. Figs. 2A and 3C, ▼). CPDPX, however, altered none of the effects of m-ATP (Fig. 3B, right panel). All these CPDPX-resistant effects of m-ATP, including rapid basal tone elevations as well as depressions and facilitations of direct and indirect twitches, were antagonized by suramin (100 μM) both in normal (Fig. 1C) and in the impaired transmission conditions (Fig. 3C). Suramin also inhibited the initial short-term effects (transient depressions and facilitations of indirect twitches and elevations of muscle tone) produced by high concentrations of ATP (not shown). However, unlike CPDPX, suramin did not suppress the late inhibitions of indirect twitches caused by ATP (Fig. 3C, ▼).

**FIG. 5.** Effects of bath application of ATP on membrane potentials and endplate potentials and the antagonism by CPDPX. Preparations were immobilized with μ-conotoxin 1 μM, and phrenic nerves were stimulated at 0.1 Hz to evoke endplate potentials. A: effects of a high concentration of ATP (1.0 mM) in normal Tyrode solution (control resting membrane potential: −84 mV). There was 10 min elapsed between panels. B: effects of ATP 100 μM in a high Mg2⁺ (2.2 mM)/low Ca2⁺ (0.6–0.8 mM) solution (resting membrane potential: −78 mV). C, left panel: control, in a low Ca2⁺ solution (resting membrane potential: −76 mV); right panel: 30 min after CPDPX 30 μM, the preparation was treated with ATP 100 μM. Note that ATP elicited short-lived (*) and prolonged (▼) inhibitions of endplate potentials and that CPDPX antagonized the late-phase inhibitions.

**FIG. 6.** Effects of puff application of ATP on membrane potentials and endplate potentials in normal Tyrode solution. The preparation was immobilized with μ-conotoxin, and the phrenic nerve stimulated at 0.1 Hz or with a train of high-frequency pulses (100 Hz for 3 s) to examine the profile of endplate potentials. A: puff ejection of ATP (3 μl of 100 mM stock solution, ◆) onto endplate region (control resting membrane potential: −82 mV). Note that puffed ATP produced fast suppressions of endplate potentials (*). B: traces of train of endplate potentials at times corresponding to the numeric subscripts in trace A.
FIG. 7. Inhibitions of endplate potentials by puffed ATP and effects of suramin. Preparations were immobilized with μ-conotoxin and bathed in a low Ca$^{2+}$/solution, and the phrenic nerve stimulated at 0.1 Hz. A: puff ejection of ATP (3 μl of 100 μM stock solution, †; control resting membrane potential: −78 mV). B, left panel: control (resting membrane potential: −84 mV); right panel: 30 min after bath application of suramin 100 μM, followed by puff application of ATP. Note that suramin did not change endplate potentials by itself but attenuated the ATP-induced depolarization surges and the initial transient inhibitions of endplate potentials (cf. A†). Also note the decreases of endplate potentials at 5–10 min after ATP in both A and B (●).

prised two components, an early short-lived (as marked by asterisk) and a late phase slight but prolonged inhibitions (▼). In a high Mg$^{2+}$/low Ca$^{2+}$/solution, where the EPP amplitude was reduced to ~25% that in normal Tyrode solution, ATP caused more marked inhibitions of EPPs; for example, ATP at a low concentration of 100 μM depressed EPP amplitude by ~70% (2.5 ± 0.7 vs. 7.8 ± 1.2 mV, 17 endplates, n = 5, cf. Fig. 5B, ▼) without much alteration of postsynaptic membrane potential.

EFFECTS OF ATP APPLIED BY PUFF EJECTION. Because of the topography of nerve–muscle junction, ATP released from presynaptic sites is localized around endplate regions, and the local ATP concentration created on nerve stimulations could reach high levels within a short time range (ms). Large amounts of ATP were therefore puffed onto endplate regions to simulate the effects of nerve-released ATP. With this mode of application, ATP (3 μl, 10–300 mM solution) depolarized muscle membranes in a concentration-dependent manner from a control level of −82.7 ± 1.7 mV to −71.4 ± 2.9 mV (at 30 mM), to −59.5 ± 3.6 mV (at 100 mM), and down to −49.1 ± 3.8 mV (at 300 mM, n = 5). The depolarizations subsided more rapidly than those induced by bath application probably caused by dilutions by the organ bath solution. The depolarizations, however, lasted longer than those observed in other skeletal muscles and in neurons (Dunwiddie et al. 1997; Thomas and Hume 1990b). This could be due to a higher concentration of ATP puffed in this study. At high concentrations (100–300 mM), ATP elicited short-term early membrane hyperpolarizations by ~5 mV in 5 of 12 preparations (cf. Fig. 6A, asterisk). The ATP-induced depolarizations were not changed by μ-

conotoxin, nifedipine, nor by tubocurarine, indicating that the depolarizations are not secondary to activations of Na$^{+}$/channels, Ca$^{2+}$/channels, or nicotinic receptor-ion channels.

In addition to depolarizations of muscle membranes, puff ejection of ATP (100 mM) produced fast suppressions of inward currents by ATP. The preparation was paralyzed with μ-conotoxin, and the endplate region was voltage clamped at −80 mV. ATP was applied by puff ejection (3 μl of 30 mM stock solution, †). Top panel: monitored membrane potentials of the clamped endplate region; bottom panel: changes of membrane currents. Note that the amplitude of miniature endplate currents was not altered by ATP.

FIG. 8. Inhibitions and facilitations of endplate potentials by α,β-methylene ATP and the antagonism by suramin. Preparations were incubated in a low Ca$^{2+}$/solution containing μ-conotoxin, and the phrenic nerve stimulated at 0.1 Hz. A: bath application of m-ATP 100 μM (control resting membrane potential: −80 mV). B, left panel: control (resting membrane potential: −78 mV); right panel: 30 min after bath application of suramin 100 μM, followed by bath application of m-ATP 100 μM. Note that suramin antagonized m-ATP induced membrane depolarizations as well as the late phase increases of endplate potentials (cf. A†). C: puff application of m-ATP (3 μl of 100 μM stock solution, †; control resting membrane potential: −78 mV). Note that, despite the marked membrane depolarizations, m-ATP elicited late phase increases of endplate potentials (●).

FIG. 9. Inductions of inward currents by ATP. The preparation was paralyzed with μ-conotoxin, and the endplate region was voltage clamped at −80 mV. ATP was applied by puff ejection (3 μl of 30 mM stock solution, †). Top panel: monitored membrane potentials of the clamped endplate region; bottom panel: changes of membrane currents. Note that the amplitude of miniature endplate currents was not altered by ATP.
EPPs within 1 min (Fig. 6A, asterisk). Thereafter, irrespective of the progressing depolarizations of postsynaptic side, EPPs recovered gradually, and full restorations of EPPs were accomplished as the depolarizations subsided. The changes of EPPs during high-frequency stimulations were tested at different stages of EPP suppressions. In control, trains of EPPs ran down to a steady state (76 ± 2%, with respect to the first EPP, n = 4) in ~200 ms after commencing stimulations (Fig. 6B1). After puffed ATP, when the EPP amplitude was severely depressed to <30% of control, a pulse-to-pulse run-up (facilitation) of EPPs developed (Fig. 6B2). Thereafter, as the amplitude of EPPs restored to 40–50% of control, trains of EPPs maintained a rather stable value (Fig. 6B3), and, when the EPP amplitude further restored to 60–80% of control, EPPs displayed the usual run-down profile (Fig. 6B4).

In a high Mg²⁺/low Ca²⁺ media, puffed ATP (100 mM) produced, in addition to the initial short-lived inhibitions of EPPs (Fig. 7A, asterisk), marked late phase inhibitions (▼). The EPP amplitude decreased by >50% to 2.1 ± 0.6 mV (8–10 min after ATP, 12 endplates, n = 5).

EFFECTS OF α,β-METHYLENE ATP. m-ATP depolarized muscle membranes and transiently suppressed EPPs, whether by bath or puff application, like ATP. However, unlike ATP, m-ATP did not induce late phase inhibitions of EPPs (Table 1). Instead, when the synaptic transmission was impaired with a high Mg²⁺/low Ca²⁺ solution, m-ATP produced late phase increases of EPPs; the EPP amplitude after bath application increased slightly from 7.5 ± 1.3 mV to 10.9 ± 1.7 mV (15 endplates, n = 5, cf. Fig. 8A, ▲); when an extra amount of MgCl₂ (0.4 mM) was added, the enhanced EPPs decreased back to 8.1 ± 1.4 mV. Puff ejection of m-ATP, after initial depressions, increased EPP amplitude from 8.2 ± 1.3 mV to 10.6 ± 1.6 mV (cf. Fig. 8C, ▲).

EFFECTS ON SPONTANEOUS QUANTAL RELEASE. ATP and m-ATP exerted relatively minor actions on mEPPs. There was no discernible change in the mEPP amplitude during the early times when EPPs were rapidly suppressed. The decreases of mEPP amplitude by 25 ± 6% at 5–10 min after application of ATP or m-ATP could be due to the depolarizations of endplate regions. In the mean time, slight increases of mEPP frequency were observed (Table 1). In voltage-clamped endplate regions (~80 mV), puff ejection of ATP (3 μl, 30 mM) elicited inward currents without changes in the amplitude of miniature endplate currents (Fig. 9). Similar effects were observed for m-ATP (Table 1).

We also studied effects of ATP on the spontaneous quantal discharges activated by persistent depolarizations of nerve terminals. High K⁺ (17 mM) Tyrode solution depolarized muscle membranes to approximately ~65 mV and elevated mEPP frequency to 20–30 s⁻¹. Puffed ATP or m-ATP, at high concentrations (3 μl, 100 mM), which elicited extra depolarizations of postsynaptic membranes by 3–5 mV lasting for 5–10 min, ceased the intense mEPP firings down to <1 s⁻¹ within 100 s of application. Three to 5 min later, when the postsynaptic membranes were still in the much-depolarized state, mEPP firings were restored to a level 5–15 s⁻¹ (10 endplates, n = 4). The time course of inhibitions of mEPP firings was comparable with that of the initial fast suppressions of EPPs.

ANTAGONISMS BY SURAMIN AND CPDPX. In preparations pretreated with suramin (100 μM), the initial fast EPP depressions and membrane depolarizations induced by ATP (Fig. 7, B vs. A) and by m-ATP (Fig. 8, B vs. A) were all antagonized. Suramin also antagonized the late EPP facilitations induced by m-ATP (Fig. 8, B vs. A, ▲) and the inhibitory effects of ATP and m-ATP on high K⁺-evoked mEPP firings (not shown). The late-phase inhibitions of EPPs caused by ATP were not attenuated by suramin (Fig. 7B, ▼) but were completely antagonized by CPDPX (Fig. 5, C vs. B, ▼). CPDPX, however, did not suppress other effects of ATP and m-ATP.

EFFECTS OF SURAMIN AND CPDPX ON NEUROMUSCULAR TRANSMISSION. Under normal transmission conditions, suramin (=100 μM) and CPDPX (=30 μM) did not alter the amplitudes of miniature endplate currents and EPPs (Table 1). The profile of EPP run-down on high-frequency stimulations was not significantly changed; the steady-state EPP amplitudes were 71 ± 3 versus 75 ± 2% for suramin (25 endplates, n = 4) and 80 ± 2 versus 76 ± 2% for CPDPX (31 endplates, n = 5).

When the synaptic transmission was predepressed, CPDPX caused very slight increases of EPP amplitude (Table 1), whereas suramin did not produce significant alteration.

<table>
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<tr>
<th>Treatment</th>
<th>Frequency, s⁻¹</th>
<th>Amplitude, nA</th>
<th>Normal tyrode</th>
<th>High Mg²⁺/low Ca²⁺</th>
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<tr>
<td>Control</td>
<td>1.09 ± 0.22</td>
<td>1.83 ± 0.31</td>
<td>36.8 ± 2.7</td>
<td>7.9 ± 1.2</td>
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<tr>
<td>CPDPX</td>
<td>2.18 ± 0.60</td>
<td>2.19 ± 0.23</td>
<td>39.2 ± 2.9</td>
<td>9.5 ± 1.3**</td>
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<td>Suramin</td>
<td>1.02 ± 0.34</td>
<td>1.58 ± 0.25</td>
<td>35.6 ± 3.1</td>
<td>6.8 ± 0.9</td>
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<tr>
<td>ATP</td>
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<td>1.66 ± 0.27</td>
<td>31.3 ± 2.2</td>
<td>2.5 ± 0.8**</td>
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<tr>
<td>α,β-Methylene ATP</td>
<td>6.87 ± 0.94*</td>
<td>1.75 ± 0.29</td>
<td>38.5 ± 2.5</td>
<td>10.6 ± 1.6**</td>
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Values are means ± SE. * Phrenic nerve-diaphragms were incubated in normal Tyrode solution containing μ-conotoxin (1 μM) to block muscular Na⁺ channels and endplate areas were voltage clamped at ~80 mV with a single electrode clamp amplifier. Spontaneous quantal releases were sampled for 20–30 s for each observation. Data from 35–48 endplates, n = 4–5. † Preparations were incubated in the indicated solution containing μ-conotoxin. Amplitudes of endplate potentials from each endplate were the average of 20 eps evoked at 0.5 Hz. Values of endplate potentials were after correction for nonlinear summation and normalized to 40 ± 50% for nonlinear summation and normalized to 80 mV. Data from 23–37 endplates, n = 4–6. ‡ ATP or α,β-methylene ATP (3 μl of 100 mM stock solution) was puffed onto endplate regions. miniature endplate currents and endplate potentials were sampled 5–10 min after each puff application, a time period when postsynaptic membranes were depolarized to a steady level (~65 mV). * P < 0.05, ** P < 0.05 (paired t-test).
DISCUSSION

Modulation of contractility by exogenous ATP

In mature mouse skeletal muscle, m-ATP and exogenous ATP, but not adenosine, elicited inward currents and depolarized muscle membranes at concentrations hundredfold higher than the plasma ATP level. These excitatory effects, which desensitized during prolonged ATP application, were antagonized by suramin and are similar to those observed for cells of cardiac (Friel and Bean 1988), vascular (Benham and Tsien 1987), visceral (Honore et al. 1989), and developing—cultured skeletal (Kolb and Wakelam 1983; Thomas and Hume 1990a) muscles. In these cells, extracellular ATP increases membrane permeability by opening membrane channels gated by the nucleotide. It is thus likely that mature skeletal muscles also express such ligand gated ion channels, although the concentration of ATP required is 10- to 100-fold higher than that effective on other tissues. Hence, depending on the degree of openings of sarcolemma ATP ion channels and the consequential perturbations of cellular ionic homeostasis, ATP at high concentrations may modulate the Ca\(^{2+}\) influx or the activation—inactivation of voltage-sensitive Na\(^{+}\) channels to elevate muscle tones or to alter contractile forces of the skeletal muscles. The underlying mechanism for the early short-term membrane hyperpolarizations induced by high concentrations of ATP remains to be elucidated. In cultures of chick embryonic muscle, ATP, in addition to activate excitatory currents, elicits K\(^{+}\) currents that exhibit a fast desensitization characteristic (Thomas and Hume 1990b).

Activations of mammalian skeletal muscle could release substantial amounts of ATP into extracellular space (Abood et al. 1962; Forrester 1972). However, because the contractilities of murine skeletal muscles on direct stimulations were not changed after blockades of P\(_2\) receptors nor after desensitizations of ATP receptors, it may be inferred that the amount of ATP released does not build up to critical concentrations high enough to modulate contractions.

Modulation of neuromuscular transmission by ATP

Current data revealed that exogenous ATP has two components of inhibitory actions on the quantal releases of acetylcholine, a rapid-onset transient inhibition of EPPs and mEPP frequency particularly after puff application and a slow-onset inhibition of EPPs. For the early transient inhibition, the effect was mimicked by m-ATP and was antagonized by suramin but not by CPDPX. It is inferred that ATP receptors, rather than adenosine receptors, are responsible for the transient inhibition. The short-lived effect cannot be ascribed to rapid degradations of ATP because similar transient effects were produced by m-ATP nor to rapid diffusions of ATP away from target sites because even after bath application both ATP and m-ATP still elicited short-lived inhibitions of contractile responses (Fig. 2) and EPPs (Figs. 5C and 8A). It was reported that ATP gated P\(_2\)-like purinoceptors on cholinergic nerve terminals that are liable to rapid desensitizations (Sun and Stanley 1996). During the transient inhibition, the amplitude of miniature endplate currents did not deviate significantly from the control value, and the run-down of EPPs on high-frequency stimulations was reduced or changed into a form of run-up, effects similar to the situations where the Ca\(^{2+}\) influxes into motor nerve terminals are reduced (Hong and Chang 1995; Magleby 1973; Protti et al. 1996), suggesting inhibitions of quantal releases. It may be argued that these initial effects of high concentrations of ATP—m-ATP could be caused by nonspecific actions such as chelations of Ca\(^{2+}\) or changes in pH or osmolarity. Although these possibilities cannot be totally excluded, the desensitization phenomena of ATP—m-ATP and the antagonistic activities of suramin indicate that specific receptor is definitely involved. It remains to be seen whether ATP directly inhibits the motor nerve Ca\(^{2+}\) channels. In addition, ATP—m-ATP might depolarize nerve endings via the nucleotide gated channels, similar to its postsynaptic `excitatory' actions, thereby inactivating preterminal Na\(^{+}\) channels.

As for the slow-onset inhibition, the effect was sensitive to CPDPX (but not suramin) and was more evident when the efficiency of synaptic transmission was lowered. Moreover, m-ATP did not display a slow inhibition. These results indicate that the slow-onset inhibition comes from activations of A\(_1\) adenosine receptors. Under normal transmission conditions, the maximum inhibition of EPPs obtained with 1.0 mM ATP was ~15%, close to values reported for inhibitions of \(^{[3}H\)-acetylcholine release (Correia-de-Sá et al. 1996). Nevertheless, the magnitude of the depressed EPPs is still two times in excess of the threshold amplitude (~8–10 mV, unpublished) for triggering muscle action potentials and indirect contractions. However, when the acetylcholine release was impaired, ATP did elicit substantial inhibitions of EPPs and indirect contractions. These effects are in agreement with the notion that ATP, after hydrolytic conversion to adenosine, reversibly stimulates presynaptic A\(_1\) receptors to reduce evoked quantal release (Dunwiddie et al. 1997; Hamilton and Smith 1991; Henning 1997; Nagano et al. 1992; Redman and Silinsky 1994; Ribeiro and Sebastião 1987). Several possible mechanisms were proposed for the adenosine-induced inhibitions of neurotransmitter releases: activations of K\(^{+}\) conductance (Bennett and Ho 1992), inhibitions of Ca\(^{2+}\) entry (Hamilton and Smith 1991; Umeniya and Berger 1994), decreases in the efficiency of Ca\(^{2+}\) to promote exocytosis (Redman and Silinsky 1994), and direct suppressions of release apparatus (Capogna et al. 1996).

In contrast to the presynaptic inhibitory actions via A\(_1\) receptors, adenosine could activate presynaptic A\(_2\) receptors to increase EPP amplitude and \(^{[3}H\)-acetylcholine release in rat phrenic nerve—hemidiaphragms (Correia-de-Sá et al. 1996; Silinsky et al. 1989). In the mouse preparations incubated in a low transmission condition, ATP increased EPP quantal content ~10% when A\(_2\) receptors were preoccupied by CPDPX. m-ATP also produced suramin-sensitive increments (~20%) of EPPs, suggesting an involvement of P\(_2\) receptors. However, it seems that these facilitations, via activations of A\(_2\)-P\(_2\) receptors, are redundant in view of the high safety factor of the synaptic transmission.

Modulation of motor function by endogenous ATP?

It may be concluded, on the basis of the previous discussion, that mouse phrenic nerve does have P\(_2\) and A\(_1\) (and A\(_2\)) receptors and on the diaphragm muscle P\(_2\) receptors
for modulations of the synaptic transmission as far as the pharmacological effects of high concentrations of ATP and m-ATP are concerned. A question then arises as to whether large enough amounts of endogenous ATP or adenosine are generated under normal physiological conditions to carry on the modulatory actions. Because it is difficult to measure the concentration of intrinsic ATP or adenosine at specific biophase with appropriate time resolution, A₁ and P₂ receptor antagonists appear useful tools to monitor possible actions of the endogenous modulators. Fortunately, at the selected concentrations, these antagonists display relatively good selectivity; do not cross-inhibit the other receptor, do not alter postsynaptic responses to presynaptic quantal discharges, and do not change contractions evoked by direct stimulations. Given that suramin did not induce hyperpolarization of endplate membranes and that there was no sign of cumulative depolarization of endplate areas when motor nerves were stimulated with high-frequency pulses, it is unlikely that motor nerves release ATP in sufficient quantities to regulate the synaptic transmission. A preexisting desensitization of endplates to a resting level of ATP is also unlikely because exogenous ATP still induced dramatic depolarizations of the subsynaptic membranes. In addition, blockades of A₁ and P₂ receptors did not cause a significant change of evoked quantal releases nor altered the profile of EPPs during high-frequency stimulations. Our results on mice are accordingly different from the results taken from frogs where presynaptically released ATP—adenosine is hypothesized to cause feedback inhibitions to result in influential neuromuscular depressions at normal levels of acetylcholine output (Redman and Silinski 1994). However, species differences cannot be overlooked regarding that m-ATP had little effects on the acetylcholine releases in the neuromuscular junctions of frogs (Ribeiro and Sebastião 1987) and rats (Smith and Lu 1991; cf. Henning 1997).

From the viewpoint of contractions, blockades of A₁ or P₂ receptors in mice did not augment contractilites in response to nerve stimulations. It can be inferred that pre- or postsynaptically released ATP—adenosine, which could modulate evoked quantal release, does not practically alter fidelities of motor functions. Obviously, for the mature mouse skeletal muscles the inherent high efficiency of neuromuscular transmissions overwhelms the negative modulations elicited by the released ATP and adenosine. It is probable that an analogous situation could take place in the neuromuscular junctions of the fast voluntary contracting muscles of frogs and rats (cf. Wood and Slater 1997). However, the interplay between released ATP and motilities may become evident on sustained heavy loading works, and the modulations by intrinsic ATP—adenosine may turn into important factors in the triggerings of muscle action potentials when the synaptic transmission is abnormally low, as might occur in myasthenic syndrome, myasthenia gravis, fatigue condition, etc. Furthermore, as the expression of ATP responsiveness in skeletal muscle is regulated by the status of innervation (Wells et al. 1995), ATP might play roles during stages of development.

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