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Different Effects of ATP on the Contractility and Nonquantal Acetylcholine Release of Rat Tonic and Phasic Muscles

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ABSTRACT

Background: It was shown earlier that ATP plays a neuromodulatory role in the phasic and tonic skeletal muscles of amphibians and phasic skeletal muscles of mammals. However no such role was investigated so far in the tonic skeletal muscles of mammals. The purpose of this study was to compare the neuromodulatory role of ATP in the tonic and in the phasic skeletal muscles of mammals. The effects of ATP and its metabolite-adenosine, on the contractility and level of nonquantal acetylcholine secretion (H-effect) of the rat phasic (long digital extensor muscle of the leg) and tonic (lateral rectus muscle of the eye) skeletal muscle preparations were investigated. **Materials and Methods:** Contractile responses of isolated muscles were evoked by the electrical field stimulation and were isometrically recorded before and after incubation with ATP or adenosine (100 μM). The H-effect was quantified as the differences between the mean resting membrane potentials measured by glass microelectrodes in 20 or more fibers before and after the addition of (+) tubocurarin (10 μM) to the medium. **Results:** Adenosine produced an equally efficient inhibitory effect on the amplitude of long digital extensor muscle and lateral rectus muscle contractions but caused no influence on the H-effect in both muscles. In contrast to adenosine, ATP had a different effect on the studied muscles: It inhibited contractions of long digital extensor muscle and increased the ones of lateral rectus muscle. ATP also eliminated the H-effect in lateral rectus muscle, but not in long digital extensor muscle. ATP and adenosine had shown no effect on membrane potential of these muscles. The study of intracellular metabolic mechanisms revealed that the effects of ATP on contraction and nonquantal secretion on tonic muscles are coupled with protein kinase C and do not involve the activity of protein kinase A, guanylate cyclase, phospholipase A_2 and phospholipase D. **Conclusion:** It is concluded from this study that ATP plays a neuromodulatory role in tonic skeletal muscles of mammals, while different mechanisms are involved in implementation of the effects of ATP on mammalian phasic and tonic skeletal muscles.

Key words: ATP, adenosine, neuromuscular synapse, rats, tonic skeletal muscles, phasic skeletal muscles

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INTRODUCTION

The tonic neuromuscular system is well developed in the musculature of amphibia, while in highly organized vertebrates, especially mammals, the number of tonic neuromotor units relative to the total amount of skeletal muscles decreases and their significance in implementing the tonic function drops. In mammals, the tonic fibers completely disappear from the locomotor musculature but they still play an important role in some muscles that are responsible for sense organs functioning (Peachey and Huxley, 1962).

It is known that the main difference between the tonic and the phasic muscle fibers is not so much in mechanism of contraction but in the synaptic organization (Samosudova and Frank, 1962). There was no key differences found between phasic and tonic neuromuscular junction both in terms of presynaptic vesicles with the neurotransmitter acetylcholine (Page, 1965) and in activity of cholinesterase (Magazanik *et al*., 1979). It was also shown that synaptic transmission of tonic muscular system is not significantly modulated by serotonin (Cooper *et al*., 2003), GABA (Golan and Grossman, 1996) and substance P (Pfeiffer-Linn and Glantz, 1990). The modulatory role of ATP in the tonic muscular system of mammals has not been investigated so far although it is well known now that this substance is an important co-transmitter in many tissues (Burnstock, 2009), including skeletal muscles of amphibians (Grishin *et al*., 2005, 2011) and mammals (Galkin *et al*., 2001; Grishin *et al*., 2006).

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In tonic fibers the reserves of intracellular calcium are able to provide the entire contraction, i.e., there is no need for extracellular calcium to enter the cell in order to activate and support the contraction. Thus, the significant difference between the functioning of phasic and tonic fibers is in the ratio of calcium binding and release rates from the intracellular stores. It is known, that the effect of ATP as an endogenous modulator of synaptic transmission is calcium-dependent, while that is not true for adenosine (Grishin *et al*., 2005). Therefore, one can expect that ATP will modulate the functional state of phasic and tonic muscles in a different fashion.

The aim of this study was to compare the neuromodulatory role of ATP and adenosine in the tonic and in the phasic skeletal muscles of mammals.

MATERIALS AND METHODS

Contractility: Long Digital Extensor Muscle (LDEM) from the leg and Lateral Rectus Muscle (LRM) from the eye were rapidly dissected from Wistar rats (120-150 g b. wt.) killed under ether anesthesia by cervical dislocation. The muscles were suspended vertically in 10 mL organ baths for isometric recording of mechanical activity according to the previously described technique (Ziganshin *et al*., 2009). Electrical Field Stimulation (EFS) was applied using a Digitimer MultiStim D330 stimulator via two platinum wire rings 2.5 mm in diameter, 15 mm apart, through which the muscles were threaded. Contractile activity of the tissue was elicited by applying rectangular impulses at a frequency of 1 Hz, 0.5 m in length and 100 V amplitude and recorded for 10 sec. The amplitude mean of all individual twitches recorded during the 10 sec of EFS was calculated and used as a single data. Contractile responses to EFS were recorded before and after adding a single concentration of ATP or adenosine to the bathing solution. Intervals of at least 15 min were allowed between consecutive stimulations to prevent unstable responses. ATP (100 μM) or adenosine (100 μM) was directly added to the organ bath and after recording of the contractions the tissue was washed out several times with fresh Ringer-Krebs solution. In studies with suramin and blockers of the different secondary messenger metabolic pathways an additional stage of the study was performed which included a 30 min incubation of the muscles with one of the antagonists followed by addition of an agonist in to the organ bath and EFS. The effect of each agonist was calculated as a percentage of the corresponding initial contraction of the tissue.

Electrophysiology: For the electrophysiological studies, the muscles were pinned with stainless steel needles to a layer of silicone rubber placed on the bottom of a Perspex chamber and superfused with a standard Ringer-Krebs oxygenated solution. For cholinesterase inhibition, the preparations were treated with the irreversible anticholinesterase agent diethoxy-p-nitrophenyl phosphate (armin; 10 μM solution) for 30 min and then rinsed several times for 15 min with Ringer-Krebs solution (Galkin *et al*., 2001).

The nonquantal release which causes depolarization of muscle fibers at the endplate zone, was quantified by measuring membrane potentials with glass microelectrodes (tip resistance 8-12 MΩ, filled with 2.5 M KC1) in 20 or more fibers during a 5-10-min period before and 8-12 min period after the addition of 10 μM (+) tubocurarin to the medium (Teplov *et al*., 2009). The differences between the mean Resting Membrane Potentials (RMP) under these two conditions were considered to be due to the nonquantal release (H-effect) of Acetylcholine (ACh).

Drugs used: ODQ (1H-[1, 2, 4]-Oxa-diazolo-[4, 3-a]-quinoxalin-1-one) was obtained from Tocris Cookson (UK). Adenosine-3, 5-monophosphothioate (Rp-cAMP), 4-(4-Octadecylphenyl)-4-oxobutenoic acid (OBAA) and all the rest of the chemicals were purchased from Sigma (USA).

Ethics: The experimental protocol has been approved by the Ethical Committee of Kazan State Medical University (Kazan, Russia).

Statistical analysis: Nonlinear least squares data fitting by the Gauss method was applied to generate a fit curve of concentration-effect dependences. Student's t-test was used for comparison of drug effects and parametric data and the Wilcoxon test was chosen for nonparametric data analysis. A probability of less than 0.05 was considered significant. Data are presented as Mean±SEM (n is the number of muscle preparations [contractility] or synapses [electrophysiology]).

RESULTS

Contractility: Long Digital Extensor Muscle (LDEM) Electrical Field Stimulation (EFS) of rat Long Digital Extensor Muscle (LDEM) evoked consistent contractile responses (Fig. 1a) which were reproducible for 2-3 h at given conditions. ATP inhibited contractions of rat LDEM evoked by EFS (Fig. 1b). In the presence of 100 μM of ATP the contractile responses of the muscle were 79.6±3.2% (n = 6) and were significantly different from the corresponding control values which were taken as a 100% ($p<0.05$, Student's paired t-test). When adenosine (100 μM) was applied to LDEM, the contraction force decreased to 70.3±5.3% of initial contractions ($p<0.05$; n = 6). A P2 receptor antagonist suramin (100 μM) abolished the inhibitory effect of ATP

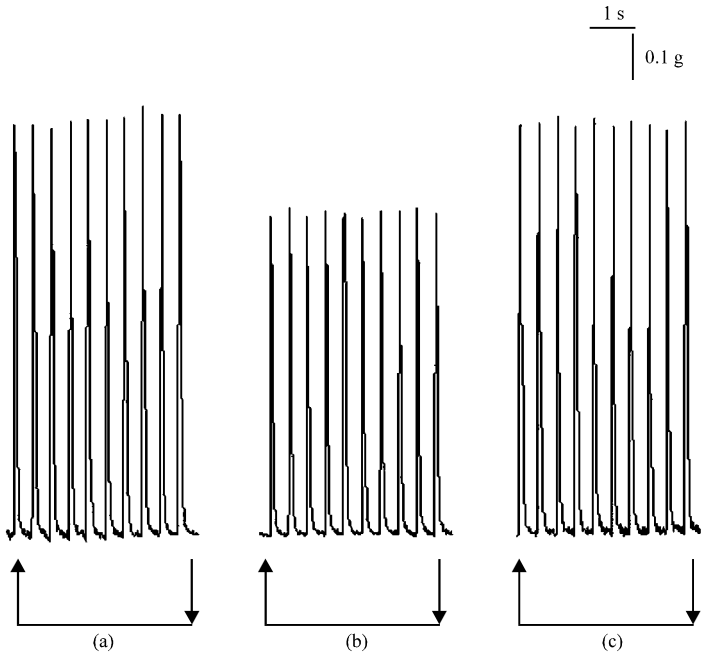

Fig. 1(a-c): Contractions of the rat long digital extensor muscle evoked by the electrical field stimulation (1 Hz, 0.5 ms, 100 V) (a) Initial contractions, (b) Effects of ATP (100 μM) and (c)Effects of ATP (100 μM) in the presence of suramin (100 μM, 30 min). Arrows indicate start and finish of the electrical field stimulation

on the contractile responses of LDEM (Fig. 1c), but did not affect the similar effect of adenosine.

Blockade of nicotinic cholinergic receptors with tubocurarin (10 μM) also abolished the inhibitory effect of ATP on the force of contraction of LDEM which was 89.9±10.2% of the initial values (n = 5, p>0.05). Adenosine (100 μM) in the presence of tubocurarine also showed no marked effect on the contractions (90.4±9.4%, n=5, p>0.05).

Lateral rectus muscle (LRM): In lateral rectus muscle (LRM), ATP at a concentration of 100 μM caused a significant increase in the muscle contraction force to 130.5±6.2% (n = 5, p<0.05) of control (Fig. 2). The presence of suramin abolished the effects of ATP (98.7±9.1%, n = 5, p>0,05). When adenosine at the same concentration (100 μM) was applied on LRM the contraction force decreased to 73.4±7.9% (p<0.05; n = 5). P2 receptor antagonist suramin (100 μM) abolished the inhibitory effect of ATP on the contractile responses of LRM but did not change the effect of adenosine (Fig. 2).

Blockade of nicotinic cholinergic receptors by tubocurarin (10 μM) abolished the inhibitory effect of ATP on the force of contraction of LRM which was 103.2±7.8% from the initial level of contraction (n=5, p>0.05). Adenosine (100 μM) in the presence of tubocurarine also showed no marked effect (98.7±5.8%, n = 5, p>0.05).

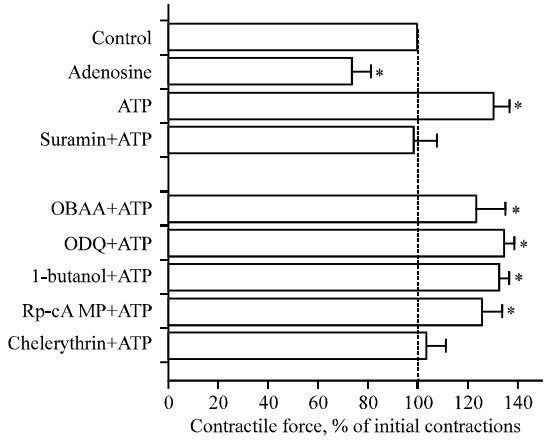

Fig. 2: Effect of various agents on the contractile force of the rat eye lateral rectus muscle evoked by electrical field stimulation. The compounds were used at the following concentrations, adenosine: 100 μM, ATP: 100 μM, suramin: 100 μM, OBAA: 20 μM, ODQ: 1 μM, l-butanol: 0,03%, Rp-cAMP: 50 μM, chelerythrin: 50 μM, n = 5; *: p<0.05 compared with control

A guanylate cyclase inhibitor, ODQ (Garthwaite *et al*., 1995), at a concentration of 1 mM neither changed the contraction force, nor blocked the action of ATP when applied concomitantly. Similarly, the contraction force remained unchanged and ATP, applied simultaneously, still had the same effect on LRM in the presence of a protein kinase A inhibitor, Rp-cAMP (100 mM) (Dostmann *et al*., 1990), a phospholipase A2 inhibitor, OBAA (20 μM) (Eintracht *et al*., 1998) and the 0.03% solution of a phospholipase D inhibitor, 1-butanol (Shin *et al*., 2001). A protein kinase C inhibitor, chelerythrin (Bull and Barnett, 2002), at a concentration of 5 μM abolished the effects of ATP on LRM (103.6±7.4%, n = 5, $p<0.05$).

Electrophysiology

LDEM: The membrane resting potential in intact LDEM was -76.4±0.51 mV (n=110). Application of 100 μM ATP did not alter the membrane potential (0.1 mV change after 15 min incubation with ATP). Incubation with the anticholinesterase agent armin at a concentration of 10 μM decreased the membrane resting potential in the field of end plates to -71.8±0.9 mV (n=110 fibers). Further addition of tubocurarine (10 μM) caused hyperpolarization -76.6±0.8 mV (n=110). Thus, the H-effect was calculated as 4.8±0.4 mV and taken as a control.

ATP and adenosine (both at 100 μM) had no appreciable effect on the H-effect-after application of ATP the level of nonquantal secretion was 4.7±0.3 mV (n=100), while after adding of adenosine the value of H-effect was 4.9±0.5 mV (n = 100).

LRM: ATP eliminated the H-effect on LRM. In the presence of 100 μM of ATP, hyperpolarization of the membrane of the LRM fibers after blocking the postsynaptic cholinergic receptors by addition of tubocurarin (10 μM) was 0.9±0.3 mV (n = 92, $p<0.001$), whereas, in the controls the H-effect was 5.0±0.4 mV (n = 95; Fig. 3). Unlike ATP, adenosine did not exert any significant effect on the level of nonquantal secretion of LRM. Therefore, after application of 100 μM of adenosine the H-effect was 5.2±0.7 mV (n = 105).

P2 receptor antagonist suramin (100 μM) completely abolished the effect of ATP on the level of nonquantal secretion, the H-effect was 4.8±0.7 (n=100, $p>0,05$). Rp-cAMP (50 μM), OBAA (20 μM), l-butanol (0.03%) and ODQ (1 μM) did not change the effect of ATP on the H-effect. The presence of cheleritrine (5 μM), eliminated the effect of ATP on the H-effect on LRM (5.1±0.5 mV, n = 89, $p>0.05$).

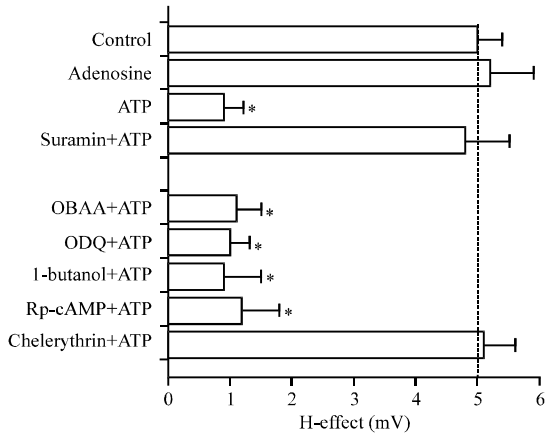

Fig. 3: Effect of various agents on the H-effect in the synapses of the rat eye lateral rectus muscle. The compounds were used at the following concentrations, ATP: 100 μM, adenosine: 100 μM, suramin: 100 μM, OBAA: 20 μM, ODQ: 1 μM, l-butanol: 0,03%, Rp-cAMP: 50 μM, chelerythrin: 50 μM, n = 92-100 (fibers); *: $p<0.05$ compared with control

DISCUSSION

It has been shown this study that ATP has opposite effects in phasic and tonic skeletal muscles due to different mechanisms that are involved.

Tonic fibers are characterized by an extensive degree of summation of the synaptic potentials in response to rhythmic stimulation which is largely due to a delay of certain potentials (Blokhina and Zefirov, 1984). Further maintenance of a high level of the summed depolarization (taking into account the fast decrease in the amplitude of discrete synaptic potentials) is most likely determined by accumulation of the mediator in the synaptic zones during this rhythmic stimulation. Low cholinesterase activity of the tonic fiber postsynaptic membrane contributes to such accumulation of the mediator. The amplitude of synaptic potentials under frequent stimulation is significantly decreased in comparison with the initial one which provides evidence for the fact that the equilibrium between mediator secretion on to the postsynaptic membrane and replenishment of its reserves at the presynaptic membrane under the rhythmic stimulation is achieved at a significantly lower level than in the case of the phasic fibers. Such explanation of less intensive replenishment of mediator reserves in the thin nerve terminals of the tonic fibers correlates well with the experimental data presented in this study. In this study, a positive

neuromuscular purinergic modulation was observed, in which ATP contributes to muscular force accumulation, instead of weakening it similarly to its effect seen in phasic muscles where the action of ATP initiates negative regulatory feedback (Sokolova *et al*., 2003; Grishin *et al*., 2005). It is suggested that the ATP target to produce the above mentioned effect is the neuromuscular synapse since a stimulation mode used triggers only nerve terminals preserved on the muscle preparation. Earlier, a positive regulatory feedback action of extracellular ATP via presynaptic P2 receptors was shown on the diaphragm muscle (Grishin *et al*., 2006) which is also characterized by polyneuronal innervation. Similarly, in the diaphragm muscle preparation ATP, but not adenosine, inhibits nonquantal acetylcholine release at the mouse neuromuscular junction (Galkin *et al*., 2001).

It should be noted that the end-stage metabolite of ATP degradation in the synaptic cleft is adenosine which possesses its own independent inhibitory action on the tonic muscle. Thus it can be concluded that the action of the two effective purines, namely ATP and adenosine, is opposite to each other in the synapses of tonic musculature and their final effect in the case of rhythmic stimulation is quite complicated to predict and requires further investigations.

A major result of this study is the finding the regulatory effect of ATP on the functional state of tonic muscles of vertebrates.

Blockade of nicotinic cholinergic receptors by tubocurarin abolished the inhibitory effects of ATP and adenosine on the force of contraction of the studies muscles suggesting that these effects are purely synaptic. According to the data obtained in this study with the H-effect, ATP eliminates nonquantal acetylcholine secretion in the synapses of the tonic muscles. Nonquantal secretion, being two fold greater than quantal secretion, traditionally is considered less significant due to its tonic noncumulative action. At the same time, a decrease in the membrane potential by 5 mV due to nonquantal acetylcholine secretion significantly affects the postsynaptic membrane activity and in particular, the sensitivity of acetylcholine receptors (Ziganshin *et al*., 2005). It is possible that ATP by eliminating nonquantal secretion in the synapse of the tonic muscles decreases the number of desensitized postsynaptic receptors, therefore increasing the number of active cholinoceptors in response to quantal release of the neuromediator. On the other hand, ATP by preventing nonquantal secretion from the presynapse increases the amount of the mediator for its quantum release. Eventually all this may lead to an increased contraction in tonic muscles. Apparently, there are no such mechanisms in the synapses of phasic muscles.

In the study with the blockers of different metabolic pathways of secondary messengers it was found that the eliminating nonquantal release effect of ATP is mediated by the activation of protein kinase C only. This correlates well with the results of earlier studies, where the neuromuscular effects of ATP was found to be mediated via protein kinase C (Galkin *et al*., 2001; Sokolova *et al*., 2003; Grishin *et al*., 2005).

The results of this study suggest that opposing effects of purinergic regulation reflect the basic differences in the functional organization of the phasic and tonic muscular systems, similar to what was previously observed in amphibians (Grishin *et al*., 2011). It is concluded that the increase in the amplitude of contraction under the influence of ATP is a mechanism that provides the maintenance of tonic muscle in a contracted state without significant energy consumption.

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