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COMPLEX SYSTEMS BIOPHYSICS

A High Calcium Level-Based Model for Identifying Postsynaptic Effects of ATP

A. E. Khairullin[a,b,*], S. N. Grishin[a], A. Yu. Teplov[a], A. A. Eremeev[b], T. V. Baltina[b], and A. U. Ziganshin[a]

[a] *Kazan State Medical University, Kazan, 420012 Russia*
[b] *Kazan Federal University, Kazan, 420008 Russia*
**e-mail: khajrulli@yandex.ru*

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Abstract—Identification of the pre- and postsynaptic effects of ATP is a methodological challenge. In our previous study, the role of P2 receptor signaling in synaptic transmission processes was evaluated using carbachol-induced skeletal muscle contractions. The search for models that can record the postsynaptic side of purinergic signaling during the application of electrical stimulation led to the idea of controlling the presynaptic terminal of ATP-mediated modulation. In *in vitro* experiments, electromyograms and mechanomyograms during isometric contractions of isolated nerve-muscle preparations of rat soleus and extensor digitorum longus (EDL) muscles revealed postsynaptic effects of ATP in the presence of a high intracellular calcium level. Thus, the effects of ATP in the presence of increased Ca^{2+} content were seen through contraction of soleus muscles that started to contract quicker by fifty percent and inhibition of contractility of EDL muscles; this was in accord with data obtained earlier on carbachol-induced contractions. We have demonstrated an ATP-dependent processes in the postsynaptic site that may contribute significantly to adaptation mechanisms in hypothermia.

Keywords: neuromuscular synapse, hypercalcium model, ATP, suramin, postsynaptic effects
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INTRODUCTION

The important role of calcium ions in the regulation of the functional activity of almost all cells and tissues is undeniable. At rest, the concentration of free calcium in the cytoplasm is extremely low – about twenty thousand times lower than in the extracellular environment. An increase in the content of calcium ions to 10^{-6}–10^{-5} M triggers a cascade of biochemical reactions, which, for example, may result in mediator exocytosis into the synaptic cleft from the nerve ending.

It has been unconditionally proven that ATP in the neuromuscular junction modulates the amplitude of multiquantum currents by activating presynaptic P2Y receptors [1–3].

The inhibitory effect of ATP on the amplitude of presynaptic currents may be due to a change in the activity of calcium channels, the entry of calcium through which triggers the process of exocytosis of synaptic vesicles. Indeed, ATP reversibly reduced the Ca^{2+} current in the perisynaptic axon [4] and decreased the amplitude of the Ca^{2+} transient recorded in different parts of the frog nerve terminal [5]. The change in the amplitude of the Ca^{2+} transient reflects the change in the concentration of free calcium ions inside the terminal [6], and its change under the action of ATP may indicate the effect of this purine on the activity of presynaptic calcium channels. Several types of voltage-gated calcium channels operate at the nerve terminal [7].

In the myoneural synapses of warm-blooded animals, not only a presynaptic, but also a postsynaptic effect of ATP has been revealed [8–10]. At the same time, in the fast-twitch muscle synapse, the effect is similar in sign to the negative postsynaptic effect, while in the slow motor units, on the contrary, it is potentiating. Is the postsynaptic modulating action of ATP in the myoneural synapses of warm-blooded animals also Ca^{2+}-dependent?

MATERIALS AND METHODS

The studies were carried out on muscle preparations of white laboratory male rats weighing 140–180 g, which were kept in groups of three to five individuals with water and food *ad libitum*. Animals were placed under anesthesia by intraperitoneal injection of sodium ethaminal at a dose of 40 mg/kg, bled and the soleus and EDL muscles of the hind limbs were isolated. The isolated muscles were fixed vertically by attaching one end to a mechanical activity sensor and

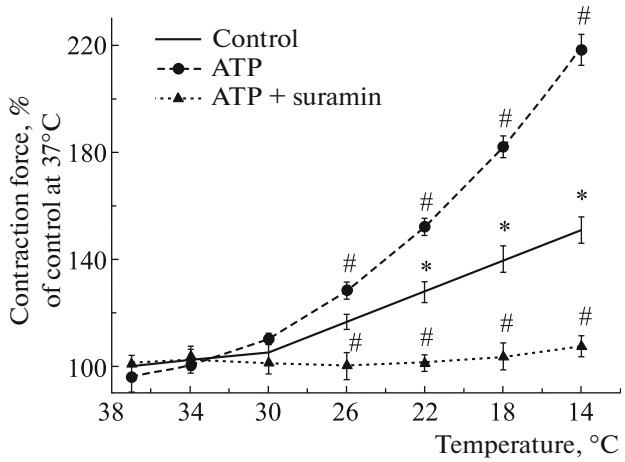

Fig. 1. Effects of ATP at elevated Ca^{2+} concentration (7.2 mM) on the force of m. soleus contractions induced by electric current in control and with the application of suramin at a concentration of 100 μM at various temperature conditions; $n = 8–14$; $*p < 0.05$ of the effect at 37°C; $\#p < 0.05$ from control.

immersed in 10 mL organ baths filled with a Krebs solution of the following composition (in mM): NaCl, 118.0; KCl, 4.75; $CaCl_2$, 2.5; $NaHCO_3$, 24.8; KH_2PO_4, 1.18; 37.0 ± 0.5°C. A thermostat maintained the set temperature value. The muscles were stretched with an initial load of 1 g, then they were left alone for 30 min to acclimatize.

The contractions were recorded first in a normal Krebs solution, and then in a solution with an increased content of calcium ions (7.2 mM).

Electrical stimulation was performed by stimulating the nerve stump, which was placed in the original design of the suction electrode. For stimulation, a MultiStim D330 stimulator (Digitimer Ltd, United Kingdom) was used. Muscle contractions were induced by stimulation with rectangular pulses with a frequency of 0.1 Hz, a length of 0.5 ms, and an amplitude of 10 V for 2 min. The force of contractions was recorded with an FSG-01 isometric mechanical activity sensor (Linton, United Kingdom), the analog signal was digitized and processed using a Biopack MP100WSW data acquisition system (Biopack, United States).

The average values of all contractions received within 2 min (12 responses) were processed as one result [11]. Contractile responses were calculated as a percentage relative to the initial results obtained at the beginning of the experiment.

Thirty minutes after the fixation of the tissue, control muscle stimulation was performed twice with an interval of 5 minutes, after confirming the stability of contractile responses, experimental procedures were started.

100 μM ATP was added to the organ bath with Krebs solution and the contractile responses of the muscle were evaluated after 10 min. Then, the tissue was incubated with the nonselective P2 receptor antagonist suramin (100 μM) for 20 min, followed by the addition of ATP, and contractile responses were recorded again.

All data were calculated as a percentage relative to the original results obtained at the beginning of the experiment at 37°C.

The effect of temperature on the contractile activity of the soleus and EDL muscles was evaluated in the experiments with ATP and suramin, contraction was initiated both by an electric field and by application of carbacholine at a temperature of 37°C. Then, the temperature was successively reduced to 34, 30, 26, 22, 18, and 14°C. At each temperature, ATP was added and the contractile responses of the muscle were evaluated 10 minutes after application. After this, the tissue was incubated with suramin for 20 min, followed by the addition of ATP. The temperature of the solution was controlled using a TE-8A water pump (Techne, United Kingdom), the rapid decrease in the temperature of the liquid in the water pump was performed by adding ice.

The results of mechanomyographic experiments on the soleus and EDL muscles were analyzed using the ANOVA method. A significance level of less than 0.05 was taken as statistically significant.

Experimental data are presented as the arithmetic mean ± standard error of the mean (n is the number of neuromuscular preparations for mechano-myographic experiments).

RESULTS

With an increase in the concentration of extracellular calcium to 7.2 mM, the force of contraction of the slow and fast motor units did not change significantly. Thus, at 37°C, the contraction amplitude was 102 ± 8.2% ($n = 11$, $p > 0.05$) for soleus muscle and 104 ± 3.9% ($n = 11$, $p > 0.05$) for m. EDL relative to contractions at normal calcium levels [12, 13].

ATP at a concentration of 100 μM at 37°C did not significantly modify the contraction force of the soleus muscle (95.8 ± 5.1% of the initial values before the agent was applied, $n = 11$, $p > 0.05$).

With a decrease in the temperature of the solution washing the soleus muscle, the effect of exogenous ATP at a concentration of 100 μM manifested in an exponential increase in the contraction force up to a one and a half increase at 14°C. Thus, at this temperature, at the 10th minute, the contraction force was 214.6 ± 5.7% ($n = 12$, $p < 0.05$) of the value of this parameter before ATP addition at 37°C (Fig. 1).

The non-selective P2 receptor antagonist suramin at a concentration of 100 μM abolished not only the potentiating effect of exogenous ATP, but also the hypothermia-associated changes in the contractility of soleus muscle.

With an increase in the concentration of extracellular calcium to 7.2 mM, ATP at a concentration of 100 μM at 37°C inhibited the force of contraction of the extensor digitorum longus muscle (85.2 ± 5.6% of initial values before ATP supply, $n = 11$, $p > 0.05$).

With a decrease in the temperature of the solution washing the studied muscle, an increase in reduction of the inhibitory effect of exogenous ATP at a concentration of 100 μM was manifested. Thus, at this temperature, at the 10th minute, the contraction force was 43.1 ± 4.6% ($n = 12$, $p < 0.05$) of the value of this parameter before ATP supply (Fig. 2).

Suramin (100 μM) in the incubation medium prevented not only the effect of ATP on the muscle, but also partially prevented the inhibitory effect of hypothermia on contractions of EDL muscle.

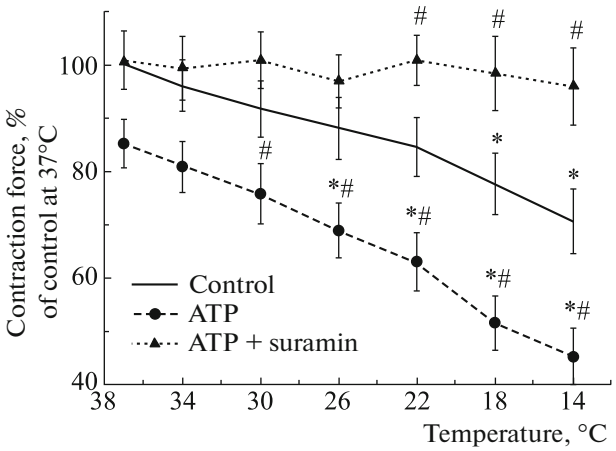

Fig. 2. Effects of ATP at elevated Ca^{2+} (7.2 mM) concentration on the force of EDL muscle contractions induced by electric current in control and with the application of suramin at a concentration of 100 μM at various temperature conditions; $n = 8–14$; $^{*}p < 0.05$ of the effect at 37°C; $^{\#}p < 0.05$ from control.

DISCUSSION

The release of the neurotransmitter from the nerve endings of vertebrates is modulated by purines, acting via purine receptors, they change the quantum composition [2–6]. However, it is well known that both the quantum composition and the kinetics of neurotransmitter secretion directly depend on the level of $[Ca^{2+}]_i$ in presynaptic terminals [14, 15].

We have shown in the perineural process that it is ATP, and not its metabolite adenosine (as previously thought), that inhibits calcium entry into the motor neuron terminal [2–4]. ATP has a presynaptic inhibitory effect on the quantum composition both through the activation of phospholipase A_2 and through the synthesis of hydrogen peroxide [2]. In our experiments, the successive activation products of phospholipase A_2, arachidonic acid and prostaglandin E_2, as well as ATP, reduced the amplitude of the calcium component by one sixth.

This work work shows the possibility of detecting postsynaptic effects of ATP under conditions of indirect electrical stimulation against the background of a hypercalcium environment. A differentiated assessment of the degree of participation of ATP-dependent mechanisms in various parts of the myoneural junction is given. The experimental model used allowed the identification of predominantly postsynaptic effects of purines, which become more pronounced under conditions of hypothermia [16].

There is evidence that hypothermia inhibits calcium current through L-type channels [17]. It is known that an increase in the concentration of extracellular calcium leads to a number of presynaptic effects, including the elimination of the presynaptic inhibitory effect of ATP [2, 8, 18]. On the other hand, no significant effect of Ca^{2+} on postsynaptic cholinergic receptors was found [19].

In our experiments, the potentiating effect of ATP was reproduced with an increased content of extracellular calcium under normal electrical stimulation, which can be observed with a basic perfusion solution only for carbachol-induced contractile responses [12, 13]. This indirectly confirms the postsynaptic nature of the action of this purine.

As is known, ATP, in addition to playing the role of an energy carrying molecule in the body, is also a mediator of synaptic transmission [4].

During normothermia ATP does not have a significant effect on the force of contraction of the slow-twitch muscle, but hypothermia leads to a significant increase in the force of contraction and to the potentiating effect of ATP.

The difference in the dynamics of indicators characterizing the postsynaptic link demonstrates a complex picture of the participation of purines in the adaptation of the myoneural junction to hypothermia.

CONCLUSIONS

The data presented in this article show that the hypercalcium model can be used to isolate the postsynaptic effects of ATP in studies on neuromuscular preparations. The verification of this model provides evidence that the postsynaptic effects of ATP, both potentiating in the slow-twitch muscle and inhibitory in the fast-twitch muscle, are calcium-independent.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflicts of interest.

Statement on the welfare of animals. All manipulations with experimental animals were carried out in accordance with the European Convention for the Protection of Vertebrate Animals used in scientific research.

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