EFFECTS OF ATP AND ADENOSINE ON CONTRACTION AMPLITUDE OF RAT SOLEUS MUSCLE AT DIFFERENT TEMPERATURES

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ABSTRACT: *Introduction*: The aim of this study was to evaluate the effects of adenosine 5′-triphosphate (ATP) and adenosine on the contractility of mammalian skeletal muscle under hypothermic conditions. *Methods*: Contractions of isolated rat soleus muscle were induced by either electrical stimulation (ES) or carbachol at physiological temperatures (37°C) and hypothermic conditions (30–14°C) and recorded in the presence of ATP, adenosine, suramin, and 8-(p-sulfophenyl)-theophylline (8-SPT). *Results*: At 37°C, incubation of the muscles with ATP inhibited ES-induced contractions; the inhibitory effect of ATP disappeared at 14°C. Adenosine inhibited ES-induced contractions at all temperature levels; 8-SPT fully prevented the action of adenosine. ATP and adenosine did not significantly affect carbachol-induced contractions at 37°C, while at lower temperatures ATP potentiated them. Suramin fully prevented effects of ATP. *Conclusions*: ATP is involved in both pre- and postsynaptic regulation of rat soleus muscle contractility, and these processes are significantly more pronounced at low temperatures.

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For many years, hypothermia has been used widely in pediatric and adult cardiac surgery for cerebral protection,[1,2] in patients with traumatic brain injury for reducing intracranial pressure,[3] and in management of adult[4] and pediatric patients[5] with out-of-hospital cardiac arrest. Hypothermia significantly decreases the global cerebral metabolic rate for glucose and oxygen,[6] thereby facilitating brain protection. Topical hypothermia is also commonly used as an adjunct for myocardial protection during open-heart surgery.[7] Such widespread and increasing use of hypothermia for therapeutic purposes adds importance to investigations of its effects on various mammalian tissues, including skeletal muscle. The effect of hypothermia on receptor-based interactions in skeletal muscles is of special interest.

Previous studies on smooth muscle tissue (guinea pig urinary bladder and vas deferens) have shown that P2 receptor-mediated contractions were significantly more prominent under hypothermic conditions than at physiological temperatures.[8] Similar effects have also been established in frog sartorius muscle, in which adenosine 5′-triphosphate (ATP) inhibited the electrical stimulation (ES) -evoked contractions more effectively at low temperatures compared with physiological conditions[9] This effect of ATP was also mediated by means of P2 receptors, because it was effectively blocked by a P2 receptor antagonist.[9] However, not much is known about the effect of hypothermia on P2 receptor-mediated interactions in mammalian skeletal muscle. Although low temperatures are known to affect the contractility of mammalian skeletal muscle,[10] it is not yet known how ATP-dependent modulation of muscle contractions changes under hypothermic conditions.

P2 receptors are a class of membrane receptors that are activated by extracellular ATP and other purine and pyrimidine nucleotides and regulate important cell functions.[11] The current classification of P2 receptors includes 2 big subclasses, P2X and P2Y receptors.[12,13] P2 receptors are widely distributed in human and animal tissues, including those of the musculoskeletal system.[14,15] Extracellular ATP is released into the neuromuscular junction as a co-transmitter with acetylcholine and activates pre- and postjunctional P2 receptors. ATP then undergoes enzymatic dephosphorylation to adenosine, which in turn activates P1 adenosine receptors located in the neuromuscular junction. In skeletal muscles adenosine receptors are involved in neurotransmission,[16] microcirculation,[17] and recovery from muscle injury.[18] Several drugs commonly used in clinical practice, such as clopidogrel[19] and adenosine,[20] act by means of P2 and P1 receptors, respectively. P2 receptors in particular are exciting potential targets for development of novel drugs that target different organs and tissues.[11] However, detailed analyses of the behavior of these receptors under various physiologic and nonphysiologic conditions (such as hypothermia) and in different mammalian tissues are needed before such drugs can be developed.

Abbreviations: 8-SPT, 8-sulfophenyl theophylline; ANOVA, analysis of variance; ATP, adenosine 5′-triphosphate; EFS, electrical field stimulation; ES, electric stimulation; MEPP, miniature end-plate potentials

Key words: ATP; adenosine; P2 receptors; hypothermia; skeletal muscle; suramin

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Therefore, the aim of this study was to investigate whether the involvement of adenosine and P2 receptors in regulation of mammalian skeletal muscle contraction depends on temperature.

MATERIALS AND METHODS

Animals and General Procedures. Experiments were conducted on isolated soleus muscle preparations of male Wistar rats weighing 140–180 g, which were kept in groups of 3–5 animals with food and water *ad libitum*. A total of 45 rats were used for this study. Animals were exsanguinated under intraperitoneal anesthesia (etaminal sodium, 40 mg/kg), and soleus muscles from both hindlimbs were isolated. Muscle preparations were mounted vertically in 10-ml organ baths. One end of the muscle was fixed, while the other end was attached to a mechanical sensor using silk thread. The organ baths were filled with modified 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, $MgSO_4*7H_2O$ 1.18, glucose 11.0, pH: 7.4 ± 0.1, t: 14–37 ± 0.5°C. A Techne TE-10A (Cambridge, UK) thermostat was used to control the temperature of the bathing solution, and the bath was continuously aerated with a mixture of O_2/CO_2 (95/5%). An initial tension of 1 g was applied to the muscle preparations, which were then left to equilibrate under these conditions for 60 min; the Krebs solution was changed every 15 min.

All procedures used in the experiments were carried out with the approval of the local ethics committee of Kazan State Medical University (Kazan, Russia) and in accordance with the European Union guidelines on animal care.

Contractions Induced by ES. Stimulation of the isolated muscles was carried out by means of the nerve stump, using a suction electrode specially designed for this study. The nerve stump was sucked into a thin polymer tube (inner diameter of 0.7–0.8 mm) containing 2 silver electrodes on the inside connected to a Digitimer MultiStim D330 (Hertfordshire, UK) electric stimulator. Contractile responses were induced by rectangular electrical pulses at a frequency of 0.1 Hz, 0.5 ms duration, and voltage of 10 V for 2 min. These parameters provided stable and reproducible contractions for 3–4 h, which corresponded with the duration of our experiment. The amplitude of contractile responses was measured in grams and was calculated as a difference in the amplitude before and after ES.[8,9] Responses were recorded isometrically using a mechanical sensor Linton FSG-01 (Norfolk, UK), captured using an MP100WSW analog-to-digital converter (Biopack, Goleta, California), and displayed and stored on a personal computer. The contractions were induced and recorded for 2 min (12 peaks), and the average amplitude of all 12 contractions was analyzed as a single result.

Contractions Induced by Carbachol. First, the concentration–response relationship for carbachol was established on isolated preparations of rat soleus muscle. The concentration of carbachol that induced 70% of the maximal contraction was then calculated and was found to be 0.8 mM. This concentration provided reproducible and stable muscle contractions at 15- to 20-min intervals for up to 4 h. The amplitude of contractile responses was measured in grams and was calculated as the difference in amplitude before and after addition of carbachol.

Effects of Purinergic Agonists and Antagonists. After 1 h of equilibration, initial control contractile responses were recorded twice at 5-min intervals (for ES) or 20-min intervals (for carbachol). Then one of the purinergic agonists (ATP or adenosine, 100 μM) was added to the bath solution, and after 10 min of incubation, the contractile responses to ES or carbachol were recorded. The tissue was then washed by changing the bathing solution several times, and one of the antagonists (suramin or 8-SPT, 100 μM) was added and incubated for 20 min. This was followed by 10-min incubation with the respective agonist (ATP or adenosine, 100 μM), and contractile responses were recorded again. All contractile responses were calculated as a percentage of the initial control contractions (induced either by ES or carbachol, respectively).

Effects of Hypothermia. All initial experiments were carried out at a physiological temperature (37°C). Then, the temperature of the bath solution was gradually decreased by adding ice to the thermostat, and the contractile activity of each muscle preparation was studied again at 2 or 3 lower temperature points. Altogether, 6 temperature points were studied (34, 30, 26, 22, 18, and 14°C). At each temperature point, the effects of ATP and adenosine as well as suramin and 8-SPT were evaluated. Only one agonist (ATP or adenosine) and one antagonist (suramin or 8-SPT) was used on a given muscle preparation, on which up to 4 temperature points (including 37°C) were evaluated.

Effects of Tubocurarine. In a separate series of experiments with ES, the mechanical activity of the muscle was also evaluated in the presence of tubocurarine (an N-cholinoreceptor blocker), which was added to the bathing medium at a concentration of 10 μM 20 min before ES or addition of carbachol.

Compounds Used. Adenosine 5′-triphosphate disodium salt hydrate (PubChem CID: 16211020), adenosine (PubChem CID: 60961), suramin sodium salt

(PubChem CID: 8514), carbamoylcholine chloride (carbachol, PubChem CID: 5831), and (+)-tubocurarine chloride penthahydrate (PubChem CID: 23422) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, Missouri). 8-(p-sulfophenyl)-theophylline (8-SPT, PubChem CID: 1908) was from Tocris Cookson (Bristol, UK). Pentobarbitone (etaminal sodium, PubChem CID: 4737) was obtained from the State Research Center of Virology and Biotechnology, VECTOR (Koltsovo, Russia).

Stock solutions of ATP, adenosine, 8-SPT, and suramin at a concentration of 0.1 M were stored in a freezer (-20°C) and used within a week. The needed aliquots of these solutions were thawed on ice on the day of the experiment, kept on ice during the entire experiment and discarded at the end of the day.

Statistical Analysis of Results. Student *t*-tests and Wilcoxon tests were used to compare parametric and nonparametric data, respectively; 1-way analysis of variance (ANOVA) was used to evaluate temperature dependency. A probability of <0.05 was considered significant. Data are presented as mean ± standard error of mean (n, N), where n is the number of muscle preparations, and N is the number of animals used.

RESULTS

Effects of Different Temperatures and Purinergic Antagonists on ES-Induced Contractions. At physiological temperature (37°C), ES of the soleus muscle induced contractions with an amplitude of 2.62 ± 0.06 g (n = 18, N = 10). This value was taken as 100%, and the rest of the results with ES were calculated in relation to this initial control response. Reducing the bath temperature led to an increase in the amplitude of contractions. At 14°C, the amplitude of contraction of the soleus muscle increased by more than 50% compared with physiological temperature (Fig. 1A). At all temperature points, incubation of the tissue with 8-SPT (100 μM) did not affect the amplitude of contraction (Fig. 1A). In contrast, incubation of the tissue with suramin (100 μM) prevented the hypothermia-dependent increase in the amplitude of contraction. Thus, in the presence of suramin, the amplitude of contraction at 14°C was 105% of the initial control values at 37°C (Fig. 1A).

Effects of ATP on ES-Induced Contractions at Different Temperatures. At a temperature of 37°C, ATP (100 μM) decreased the amplitude of rat soleus muscle contractions induced by ES to 34% of the initial contraction (Fig. 1B). With decreasing temperature of the bathing medium, the inhibitory effect of ATP on the amplitude of contraction gradually decreased until it completely disappeared at 14°C. At

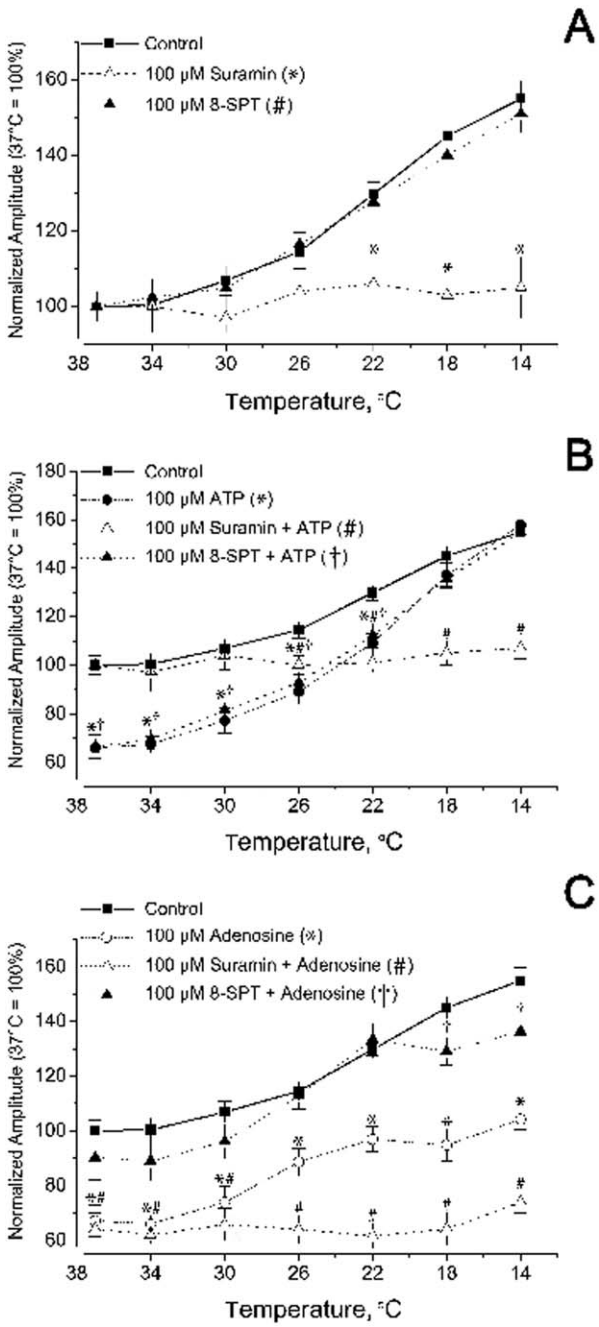

FIGURE 1. Temperature-dependency of the amplitude of rat soleus muscle contractions caused by ES (0.1 Hz frequency, 0.5 ms duration, 10 V). **(A)** Under control conditions and in the presence of 100 μM suramin or 100 μM 8-SPT (n = 8–18, N = 4–10). **(B)** The effect of ATP (100 μM) in the presence of 100 μM suramin or 100 μM 8-SPT (n = 9–20, N = 5–12). **(C)** The effect of adenosine (100 μM) in the presence of 100 μM suramin or 100 μM 8-SPT (n = 8–18, N = 5–10).*, #, and † denote statistical significance ($P < 0.05$, 1-way ANOVA) of each curve compared with the control curve.

temperatures of 18 and 14°C, the amplitudes of the contraction were not statistically different from the corresponding control figures. At all studied temperature points, 8-SPT (100 μM) did not alter the effects of ATP (Fig. 1B). However, incubation with suramin (100 μM) prevented the inhibitory action

of ATP at 37–22°C and prevented the ATP-mediated increase in contractility at 18 and 14°C.

Effects of Adenosine on ES-Induced Contractions at Different Temperatures. At all temperature points, adenosine at a concentration of 100 μM significantly reduced the amplitude of the ES-induced contractions of rat soleus muscle by approximately 25–30% (Fig. 1C). Incubation of tissue with 8-SPT (100 μM) fully prevented the inhibitory effect of adenosine on the soleus muscle contractions at most of the tested temperatures, with the exception of the 2 lowest temperature points (18 and 14°C) (Fig. 1C). In contrast, suramin (100 μM) did not significantly alter the inhibitory effect of adenosine on the contractile responses at temperatures of 37–30°C but prevented the hypothermia-dependent increase in the amplitude (in the presence of adenosine) at 26–14°C (Fig. 1C).

Effects of Different Temperatures and Purinergic Antagonists on Carbachol-Induced Contractions. At 37°C, the amplitude of the soleus muscle contraction induced by carbachol at a concentration of 0.8 mM was 0.68 ± 0.08g (n = 19, N = 10), which was taken as 100% and all of the results with carbachol-stimulated contractions were calculated as a percentage of this initial control value. As the bath temperature decreased, the amplitude of the carbachol-induced contractions increased, and at bath temperatures of 26°C and lower, it became significantly different from that at physiological temperature. At 14°C, the amplitude of contraction of the soleus muscle was 153% of the initial control values (Fig. 2A). 8-SPT (100 μM) did not have any appreciable effect on the amplitude of carbachol-induced contractions at any of the tested temperature levels (Fig. 2A). Suramin (100 μM) did not affect the amplitude of carbachol-induced contractions at 37°C but fully prevented the increase of the amplitude of contraction at low temperatures (Fig. 2A).

Effects of ATP on Carbachol-Induced Contractions at Different Temperatures. ATP at a concentration of 100 μM at 37°C did not significantly modify carbachol-induced contractions of the soleus muscle (Fig. 2B). When the bath temperature was lowered, incubation with ATP (100 μM) gradually increased the amplitude of contraction in response to carbachol. At a temperature of 14°C, the amplitude of carbachol-induced contraction in the presence of ATP was enhanced by almost 1.5 times when compared with the corresponding control values at the same temperature (Fig. 2B). Application of suramin (100 μM) abolished the modulatory effect of ATP on rat soleus muscle contractions at all investigated temperature levels (Fig. 2B).

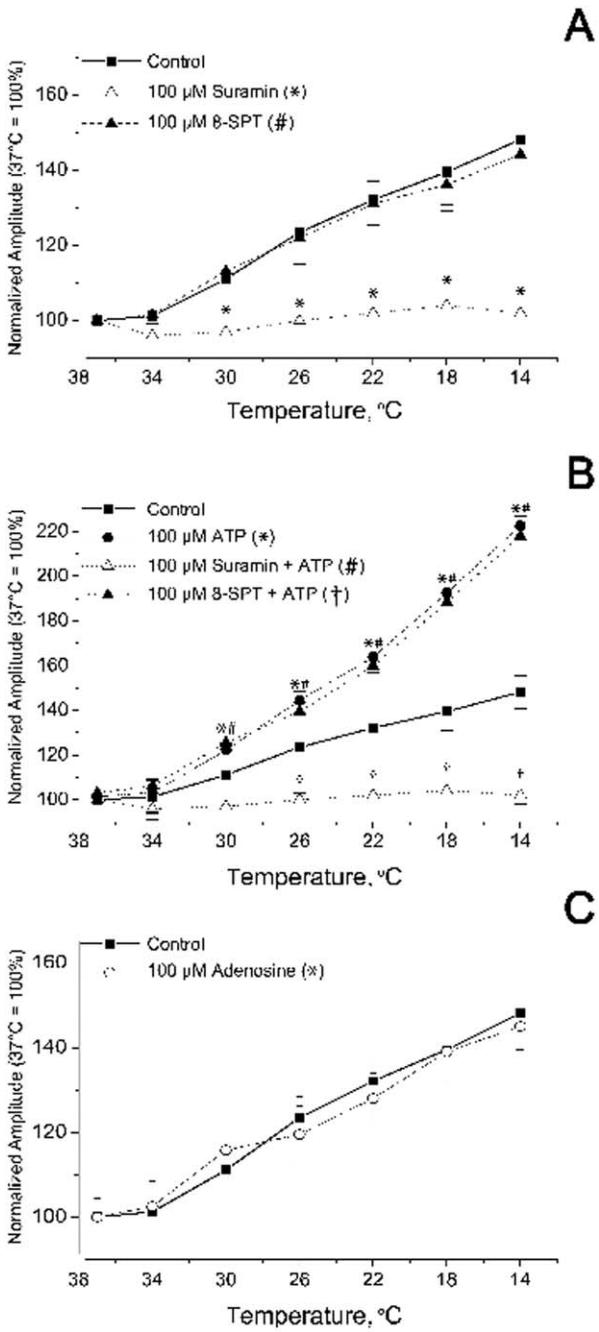

FIGURE 2. Temperature-dependency of the amplitude of carbachol-induced contractions of rat soleus muscle. **(A)** Under control conditions and in the presence of 100 μM suramin or 100 μM 8-SPT (n = 8–19, N = 5–11). **(B)** Effect of ATP (100 μM) alone and in the presence of 100 μM suramin or 100 μM 8-SPT (n = 8–18, N = 5–10). **(C)** Effect of adenosine (100 μM) (n = 8–18, N = 5–10). *, #, and † denote statistical significance ($P < 0.05$, 1-way ANOVA) of each curve compared with the control curve.

Effects of Adenosine on Carbachol-Induced Contractions at Different Temperatures. Adenosine (100 μM) had no effect on the amplitude of carbachol-induced contractions at all studied temperature levels (Fig. 2C).

Effects of Tubocurarine on ES or Carbachol-Induced Contractions. Incubation of the soleus muscle with 10 μM of tubocurarine (a nicotinic cholinergic receptor blocker) completely abolished the contractile activity of the muscle in response to both ES and carbachol at physiological and lower temperature points.

DISCUSSION

In this study, we demonstrate that the contractility of rat soleus muscle, induced either by ES of the nerve or carbachol application, is temperature-dependent. Regardless of the mechanism of contraction induction, the amplitude of contraction of rat soleus muscle increases under hypothermic conditions. Suramin, a nonselective antagonist of P2 receptors, but not 8-SPT, a nonselective antagonist of adenosine receptors, prevented this effect, which suggests involvement of P2 receptors in hypothermia-induced increased contractility.

The action of extracellular ATP on skeletal muscles was described over 40 years ago.[21] ATP is released from nerve terminals in contracting skeletal muscles[22–24] and sensitizes the postsynaptic nicotinic cholinergic receptors.[25] At the presynaptic level, ATP activates a negative feedback mechanism and inhibits neurotransmitter release by means of both presynaptic adenosine (P1) and P2Y receptors.[16,26–30] A recent study has shown that $P2Y_{12}$ receptors play a key role in the purinergic control of synaptic transmission in frog sartorius muscle.[31] In the rat neuromuscular junction, ATP is metabolized by a series of ectonucleotidases, among which ecto-AMP deaminase plays an important role in controlling the amount of produced adenosine.[24]

The temperature-dependency of P2 receptor-mediated processes has been demonstrated on smooth and skeletal muscle. On isolated preparations of guinea pig urinary bladder and vas deferens, the contractions in response to α,β-methylene ATP (a P2X receptor agonist) and in response to electrical field stimulation (EFS) were significantly more prominent at low temperatures than at physiological temperature.[8] Furthermore, the EFS-induced P2Y receptor-dependent relaxation of the guinea pig tenia ceci increased in a temperature-dependent manner with decreases in temperature.[8] Similar results were shown on isolated frog satorius preparations, in which ATP inhibited the EFS-evoked contractions more prominently at 17°C than at 22°C.[32]

Of interest, the effects of ATP were inhibited by P2 receptor antagonists, but not by the adenosine receptor antagonist (8-SPT), indicating the involvement of P2 receptors in mediating these effects. Thus, the temperature-dependency of P2 receptor-mediated responses has been shown on 3 different muscle preparations: mammalian smooth muscle,[8] amphibian skeletal muscle,[32] and mammalian skeletal muscle (this study). In all 3 different muscle tissues, we see the same consistent pattern, the efficacy of P2 receptor-involved processes is higher at low temperatures than at physiological temperature. We hypothesize that this indicates a general role of P2 receptors in muscular tissues; they are silent or masked by other regulatory systems at physiological conditions, but their role becomes increasingly evident and important under pathological or extreme conditions. This type of behavior of P2 receptors has also been shown in some prior studies.[14]

Although reducing the temperature significantly decreases the release of acetylcholine at the neuromuscular junction, it has been shown on rat nerve-hemidiaphragm preparations that the amplitude of contractions is maintained and facilitated by decreased enzymatic degradation of the transmitter and by increased sensitivity of the postjunctional membrane to acetylcholine.[10] Similarly, in our experiments, the amplitude of contractions of the sartorius muscle induced either by direct (carbachol) or indirect (nerve stimulation) activation of postjunctional acetylcholinine receptors is increased. We hypothesize that these effects are primarily due to a hypothermia-related increase in the sensitivity of nicotinic acetylcholinine receptors. Decrease of the enzyme-degrading activity is probably less important here, because carbachol is not easily hydrolyzed by cholinesterases. In our experiments, blockade of nicotinic cholinergic receptors by tubocurarine completely abolished the contractility of rat soleus muscle induced by both ES and carbachol at physiological and hypothermic conditions, which indicates that the recorded contractions are mediated by direct or indirect stimulation of postsynaptic nicotinic receptors.

In nerve stimulation-induced contractions at low temperature the co-release of ATP from nerve terminals can be decreased[22] with subsequent decrease of the presynaptic inhibitory action of ATP on acetylcholine release. Inhibitory effects of ATP on neurotransmitter release at the neuromuscular junction have been thought to be mediated by means of a type of P2Y receptor.[26,27] Recent studies suggested[33] and then provided evidence[31] that $P2Y_{12}$ receptors play the main role in mediating this effect. On the other hand, Salgado et al.[34] found that nondegradable analogues of ATP facilitate acetylcholine release by means of postulated activation of presynaptic P2X receptors. We believe that, in our experiments at low temperatures the enzymatic degradation of ATP is significantly decreased.

This is supported by data that show temperature to be an important factor affecting the kinetic and thermodynamic properties of ectoenzymes,[35]

and by data that demonstrate decreased degradation of ATP by ecto-enzymes under hypoterhmic conditions.[36] With such a decrease in enzymatic degradation of ATP, the degree of ATP-induced acetylcholine release becomes similar to that of its nondegradable analogues. We also found that at physiological temperature both ATP and adenosine decrease the amplitude of ES-induced contractions, most likely due to presynaptic inhibition of transmitter release.[26,27,37] Surprisingly, the effect of ATP gradually diminished with a decrease in temperature, while the inhibitory effect of adenosine was unchanged at all temperatures. We suggest that at low temperatures the balance between ATP-induced presynaptic inhibition of transmitter release,[16,26–30,37] on the one hand, and the P2 receptor-activated transmitter release[34] and ATP-dependent postsynaptic increase in sensitivity of acetylcholine receptors[25,38] on the other, is shifted toward the latter.

The tripanocidal drug suramin has a wide range of biological activities,[39,40] including nonspecific inhibition of P2 receptors[41,42] and inhibition of several ATP-metabolizing ecto-enzymes.[43–45] In rat diaphragm, suramin competitively reversed the action of a nondepolarizing, but not a depolarizing, skeletal muscle relaxant without affecting the ATP-sensitive receptors.[46] Another study showed that suramin can inhibit presynaptic Ca^{2+} channels[47] and decrease transmitter release. Therefore, effects of suramin on neuromuscular transmission are very complicated and most likely variable in different types of skeletal muscles. In our experiments, it not only antagonized ATP-dependent inhibition of ES- or carbachol-induced contractions, but also prevented low-temperature-dependent increase of contractility regardless of the stimulation paradigm. This might indicate that similar to other tissues,[34] there is a natural mechanism by which P2 receptors mediate the increase of transmitter release in rat soleus muscle, which becomes more prominent at low temperatures and which is fully antagonized by suramin.

The temperature dependency of adenosine receptor-mediated responses was studied previously in 4 guinea-pig smooth muscle tissues,[48] and it was found that the adenosine A1 receptor-mediated responses were more prominent at low temperatures, whereas the adenosine A2 receptors were not susceptible to temperature changes. In our experiments on rat skeletal muscles, no temperature-related effects of adenosine were detected either with ES or with carbachol application. Further experiments are required to clarify the exact type(s) of adenosine receptors that might be involved in mediating this action of adenosine.

A study of miniature end-plate potentials (MEPPs) in rat soleus muscle showed that their frequency did not change after application of carbachol at temperatures between 18°C and 34°C, but decreased by 40% at physiological temperatures of 37–38°C.[49] These data are in line with our results and are suggestive of presynaptic effects of increasing temperature. The lack of change in MEPP frequency with carbachol application at low temperatures supports our data on postsynaptic effects during hypothermia. Another report showed that as temperature increases there is a constant decline of postsynaptic responses.[50] Based on these data, we hypothesize that a purine-modulated regulation circuit of synaptic effects exists in skeletal muscles (like the soleus muscle) of warm-blooded species.

Finally, we believe that studying the effects of hypothermic conditions on mammalian tissues (such as skeletal muscles) has potentially important clinical implications. Hypothermia is widely used in clinical practice,[1–5] primarily with the purpose of cerebral protection during surgical interventions or resuscitation of critically ill patients. This underscores the importance of studying the reaction of other organs and tissues to hypothermia, and especially the effect that low temperatures have on receptor-based interactions. This study adds important information regarding the activity of P1 and P2 receptors under hypothermic conditions in mammalian skeletal muscles. Although these results are not directly transferrable to human muscle tissues, they provide important insight into how activation of human P1 and P2 receptors of skeletal muscles might behave under hypothermia and predict how effects of certain drugs might be altered by this nonphysiologic state.

We conclude from this study that, in rat soleus muscle, P2 receptors are involved in both pre- and postsynaptic regulation of contractility and that these processes are significantly more pronounced at low temperatures. We suggest that the temperature sensitive tonic effects of endogenous ATP on the amplitude of contraction underlie the phenomenological changes of P2 receptor-mediated muscle responses at low temperatures.

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