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The influence of hypothermia on P2 receptor-mediated responses of frog skeletal muscle

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Abstract

The contractile responses of isolated *Rana ridibunda* frog sartorius muscle contractions evoked by electrical field stimulation (EFS) were studied at three temperature conditions of 17, 22 and 27 °C. Temperature-dependent increase of muscle contractility was found. ATP (10–100 μM) concentration dependently inhibited the electrical field stimulation-evoked contractions of sartorius muscle at all three temperatures; this effect was significantly more prominent at a temperature of 17 °C than at other two temperatures. Adenosine (100 μM) also caused inhibition of electrical field stimulation-evoked contractions which was statistically identical at all three temperature conditions tested. A P2 receptor antagonist, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS, 10 μM) reduced the inhibitory effect of ATP at all three temperatures but did not affect inhibitory action of adenosine. In contrast, 8-(*p*-sulfophenyl)theophylline (8-SPT, 100 μM), a nonselective P1 receptor antagonist, abolished inhibitory effects of adenosine at all three temperature conditions but did not antagonize inhibition caused by ATP. In electrophysiological experiments, ATP (100 μM) and adenosine (100 μM) temperature dependently reduced end-plate currents recorded in sartorius neuromuscular junction by voltage-clamp technique. The inhibitory effects of both agonists were enhanced with the decrease of temperature. 8-SPT (100 μM) abolished the inhibitory effect of adenosine but not ATP on end-plate currents. Suramin (100 μM), a nonselective P2 receptor antagonist, inhibited the action of ATP but not adenosine, while PPADS (10 μM) had no influence on the effects of either ATP or adenosine. It is concluded from this study that the effectiveness of P2 receptor-mediated inhibition of frog skeletal muscle contraction in contrast to that of adenosine is dependent on the temperature conditions.

Keywords: Skeletal muscle, frog; P2 receptor; P1 receptor; Temperature dependency

1. Introduction

It is widely accepted that extracellular ATP and other purine and pyrimidine nucleotides regulate many important cell functions acting via specific P2 receptors (Burnstock, 2002). According to the current classification, P2 receptors are divided into two families, P2X and P2Y receptors, P2X receptors being a ligand-gated ion channels while P2Y receptors are G-protein coupled (Abbrachio and Burnstock, 1994; Ralevic and Burnstock, 1998; Abbracchio and Williams, 2001). Both families consist of several subtypes, which were numbered after their molecular structure was identified (Burnstock and King, 1996; Fredholm et al., 1994; Burnstock, 2003).

In smooth muscles, stimulation of P2X receptors causes contractile responses, while stimulation of P2Y receptors usually leads to relaxant effects (Hoyle, 1992). In contrast, in adult skeletal muscles, it has been established that, while stimulation of P2 receptors does not cause either contraction or relaxation, it significantly inhibits transmitter release at

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the neuromuscular junction (Giniatullin and Sokolova, 1998; Galkin et al., 2001).

Although most experiments on P2 receptors were carried out on normal temperature conditions, we have shown recently that in guinea-pig smooth muscles, the responses mediated by both P2X and P2Y receptors are more prominent at a low temperature than at normal or high temperatures (Ziganshin et al., 2002). We concluded from that study that the effectiveness of P2 receptor-mediated responses increases with decrease of temperature, which may have some clinical importance. We also suggested that supersensitivity of P2 receptor-mediated responses at low temperatures is likely to be even more important in cold-blooded animals. The aim of the present study was to investigate whether the P2 receptor-mediated inhibition of frog skeletal muscle contraction depends on bath temperature conditions.

2. Methods

2.1. Miophysiology

Frogs *Rana ridibunda* (10–25 g) were killed by decapitation and destruction of spinal brain. The sartorius muscles were dissected free and suspended vertically in 10-ml organ baths for isometric recording of mechanical activity. An initial load of 1 g was applied to the muscles, which were then allowed to equilibrate for bath conditions for 15 min. During preparation and experiment, the muscles were immersed in the Ringer solution with the following composition (mM): NaCl 113, KCl 2.5, $CaCl_2$ 1.8, pH adjusted to 7.3 with $NaHCO_3$.

Electrical field stimulation (EFS) was applied using a Grass S9 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 ms length and 100 V amplitude and recorded for 20 s. Responses of the tissue were registered isometrically by a Linton FSG-01 (UK) force displacement transducer acquired by Biopack MP100WSW Data Acquisition System displayed on a PC screen. The mean of the amplitude of all the individual twitches recorded during 20 s was calculated and used as a single data (see Fig. 1). Contractile responses to electrical field stimulation were recorded before and after addition of a single concentration of an agonist (ATP or adenosine). Intervals of at least 15 min were allowed between consecutive stimulations to prevent unstable responses. ATP (10–100 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 solution. In experiments with pyridoxalphosphate-6-azophenyl-2′ ,4′ -disulphonic acid (PPADS, 10 μM) and 8-(*p*-sulfophenyl)theophylline (8-SPT, 100 μM), one more stage including 30-min incubation with a given antagonist (PPADS or 8-SPT) followed by addition of the agonist and electrical field stimulation was performed. Contractile responses were calculated as percentages of the contraction evoked by the KCl solution at a concentration of 240 mM being added at the very end of the experiment to produce the maximal contractile response of the given tissue. The effect of an agonist was calculated as a percent of corresponding initial contractions of the tissue.

2.2. Temperature dependency

Bath temperature was regulated by a TE-8A (Techne, UK) water pump. In all experiments, initial procedures of preparation and equilibration were carried out at a temperature of 22±0.5 °C. After recording of the results of the initial stimulation, agonist (ATP or adenosine) was added, and the responses of the tissue to electrical field stimulation were recorded again. After that, all procedures were repeated consecutively at the temperatures of 17±0.5 and 27±0.5 °C.

2.3. Electrophysiology

The frog sartorius muscle was dissected, and tissue fibers were cut transversely to prevent contractions. The muscle strips were pinned with needles to layer of silicone rubber on the bottom of a Lucite chamber (2.5 ml) and superfused with Ringer solution.

End-plate currents were evoked every 30 s by a single supramaximal nerve stimulation. Synaptic currents recorded

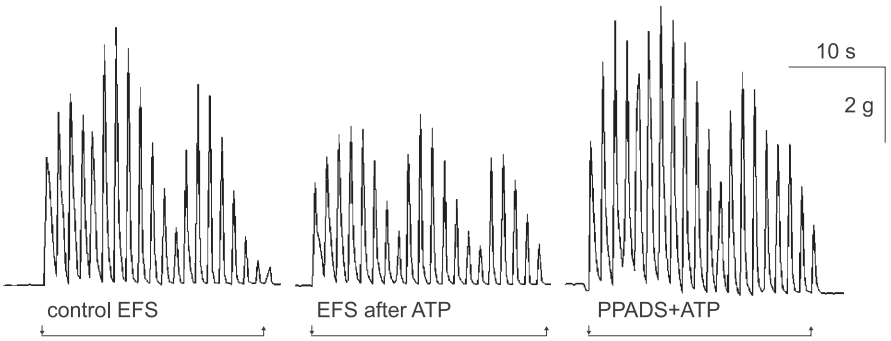

Fig. 1. Sample mechanograms of the contractions of frog sartorius muscle evoked by electrical field stimulation (EFS) before and after incubation with ATP (30 μM) and PPADS (10 μM) at a temperature of 22 °C.

using the standard two-electrode voltage clamp technique at −40 mV holding potential. Intracellular electrodes had 3–5 MΩ resistance when filled with 2.5 M KCl. Nerve action potentials and extracellular end-plate potentials were recorded using focal Ringer-filled extracellular pipettes with tip diameter of 1–2 μm and 1–3 MΩ resistance. Extracellular pipettes were positioned under visual control at the proximal region of a nerve terminal (Sokolova et al., 2003). All drugs were applied to a muscle via superfusion system (2 ml/min). Data were normalized with respect to control end-plate currents taken as 100%.

2.4. Drugs used

Adenosine 5'-triphosphate sodium salt (ATP) and adenosine were obtained from Sigma. PPADS tetrasodium salt and suramin were provided by Tocris Cookson. 8-(*p*-sulfophenyl)-theophylline (8-SPT) was supplied by RBI.

2.5. Analysis of results

Nonlinear least squares fitting by Gauss was applied to generate a fit curve of concentration–effect dependences. Student's *t*-test (one and two population) was employed for comparison of drugs influence and parametric data, Wilcoxon's test suited for nonparametric data; One-way analysis of variance (ANOVA) was additionally used to study temperature dependency. A probability of less than 0.05 was considered significant. Data are presented as mean±S.E.M. (*n* is the number of muscle preparations [organ bath] or synapses [electrophysiology]).

2.6. Ethics

The experimental protocol has been approved by the Ethical Committee of Kazan State Medical University.

3. Results

3.1. Miophysiology

At a temperature of 22 °C, frog sartorius muscle responded with transient contractions to electrical field stimulation which were 85.5±3.5% (*n*=42) relative to the contraction evoked by KCl at a concentration of 240 mM. Lowering of the temperature to 17 °C led to decrease of the electrical field stimulation-evoked contractile response 62.3±4.3% (*n*=36), while the increase of the bath temperature to 27 °C enhanced the muscle contractions to 135.2±7.9% (*n*=42); the last two values are significantly different from that at 22 °C.

3.1.1. P2 receptor-modulated contractions

ATP concentration dependently inhibited contractions of frog sartorius muscles evoked by electrical field stimulation at all three temperature conditions (Fig. 1). In the presence of the highest ATP concentration tested (100 μM), the contractile responses of the tissue to electrical field stimulation were 66.8±2.6% (*n*=12), 77.8±2.4% (*n*=14) and 84.2±0.8% (*n*=14) at temperatures of 17, 22 and 27 °C, respectively, and all these data were significantly different from the corresponding control values, which were taken as a 100% ($P<0.05$, Student's paired *t*-test). Moreover, at all concentrations of ATP, there was a temperature dependency of its inhibitory effect—the lower the bath temperature, the more prominent was ATP effect registered. ANOVA test revealed significant differences between concentration–effect curves for ATP taken at three temperature conditions tested.

3.1.2. P1 receptor-modulated contractions

Adenosine (100 μM) caused modification of the contractions of frog sartorius muscle. At a temperature of 22 °C, the agonist decreased contraction level of the tissue to 81.1±2.4% (*n*=22) relatively to controls taken as a 100% ($P<0.05$). When temperature increased to 27 °C or lowered to 17 °C, this did not lead to a significant change in the inhibitory effect of adenosine—contractions were 82.9±5.7% (*n*=22) and 78.0±2.8% (*n*=22), correspondingly. There was no statistical differences between effects of adenosine at three temperature conditions ($P>0.05$).

3.1.3. Effects of PPADS

A P2 receptor antagonist PPADS (10 μM) at 22 °C abolished inhibitory effect of ATP on contractile responses of sartorius muscle—contractions became 97.7±4.3%

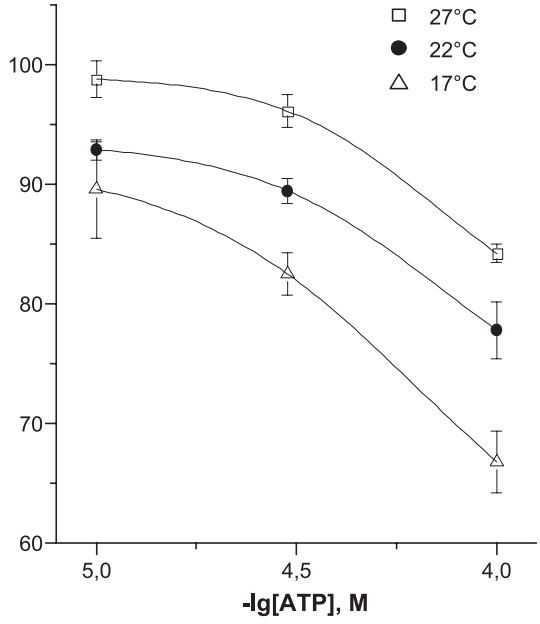

Fig. 2. Effects of ATP at temperatures of 17, 22 and 27 °C on contractile responses of isolated frog sartorius muscles evoked by electrical field stimulation. Data are presented as a percentage of initial tissue responses (before adding ATP). Data shown are means and vertical bars indicate S.E.M., *n*=12–14.

(n=10) of initial contractions (without ATP) taken as a 100% (Fig. 2). Similar results were obtained with PPADS at two other temperatures—inhibitory effect of ATP was completely reversed and muscle contraction became 92.5±2.9% (n=10) and 93.1±5.1% (n=10) of initial response at temperatures of 17 and 27 °C, respectively. According to the statistical analysis performed, at all three temperature conditions, significant differences between responses modulated with ATP in the presence and absence of PPADS revealed (P<0.05). Meanwhile, effects of PPADS were not significantly different at the three temperature conditions tested (P>0.05).

PPADS did not significantly affect the adenosine influence on tissue contractility. At all three temperatures tested, depressant effects of adenosine on electrical field stimulation-evoked contraction of frog skeletal muscle in the presence and absence of the antagonist was statistically similar (Fig. 3).

3.1.4. Effects of 8-SPT

A P1 receptor antagonist 8-SPT (100 μM) did not significantly influence on inhibitory effect of ATP on contractile responses of sartorius muscle at all three temperatures (Fig. 2).

The inhibitory effect of adenosine on tissue contractions was prevented by 8-SPT (Fig. 4). Statistical analysis revealed significant differences between responses modulated with adenosine in the presence and absence of antagonist at all three temperature conditions (P<0.05). For instance, at a temperature of 22 °C, the effect of adenosine measured at 81.1±2.4% (n=22) was antagonized by 8-SPT to 100.5±2.7% (n=6). Similarly, at 17 °C, contraction reached 94.4±2.2% (n=6), and at 27 °C, they were 100.0±4.5% (n=22; Fig. 3).

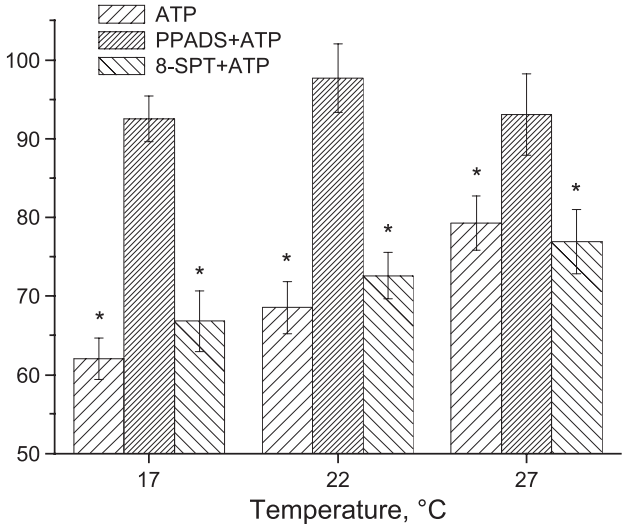

Fig. 3. Effects of PPADS (10 μM) and 8-SPT (100 μM) on ATP-induced (100 μM) inhibition of contractile responses of isolated frog sartorius muscles evoked by electrical field stimulation at temperatures of 17, 22 and 27 °C. Data are presented as a percentage of initial tissue responses (before adding ATP, controls). Data shown are means and vertical bars indicate S.E.M., n=10–22. *P<0.05 from corresponding controls.

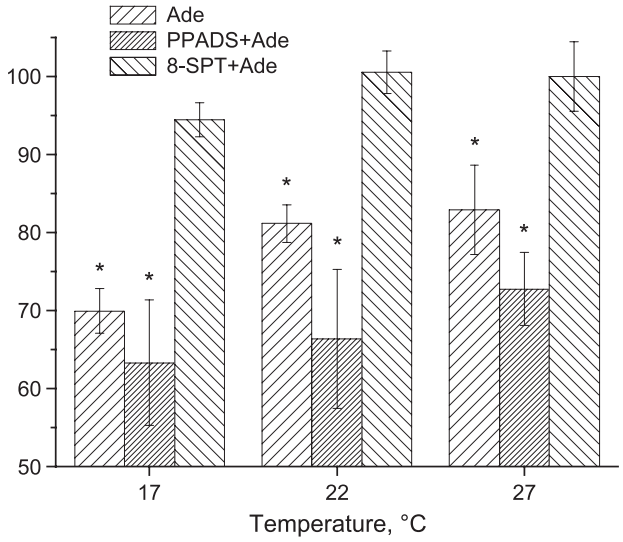

Fig. 4. Effects of PPADS (10 μM) and 8-SPT (100 μM) on adenosine-induced inhibition (Ade, 100 μM) of contractile responses of isolated frog sartorius muscles evoked by electrical field stimulation at temperatures of 17, 22 and 27 °C. Data are presented as a percentage of initial tissue responses (before adding ATP, controls). Data shown are means and vertical bars indicate S.E.M., n=10–22. *P<0.05 from corresponding controls.

3.2. Electrophysiology

At a temperature of 22 °C, motor nerve stimulation of frog sartorius muscle evoked end-plate currents with an amplitude of 138±25 nA (n=25) and with exponential decay. Temperature increment to 27 °C led to enhancement of the amplitudes of end-plate currents to 162±26 nA (n=10), while lowering the bath temperature down to 17 °C decreased amplitudes of end-plate currents by 96±28 nA (n=12).

3.2.1. P2 and P1 receptor-modulated quantal release

At a temperature of 22 °C, ATP (100 μM) reduced amplitude of end-plate currents in a reversible way to 66.0±2.5% (n=25; Fig. 5). The similar degree of depression (70.0±3.3%, n=26) was observed after adenosine (100 μM) application. At a temperature of 27 °C, the inhibitory effect of ATP was significantly lower than that at a temperature of 22 °C establishing at 74.0±3.5% (n=8; P<0.05). The lower

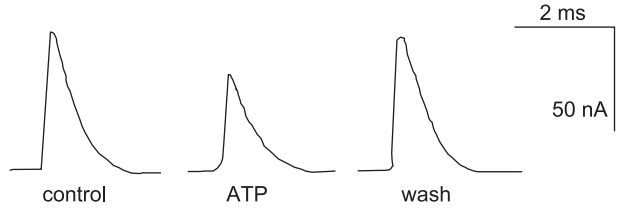

Fig. 5. Representative multiquantal end-plate currents of isolated frog sartorius muscles at a temperature of 22 °C before and after addition of ATP (100 μM) and after washout of agonist.

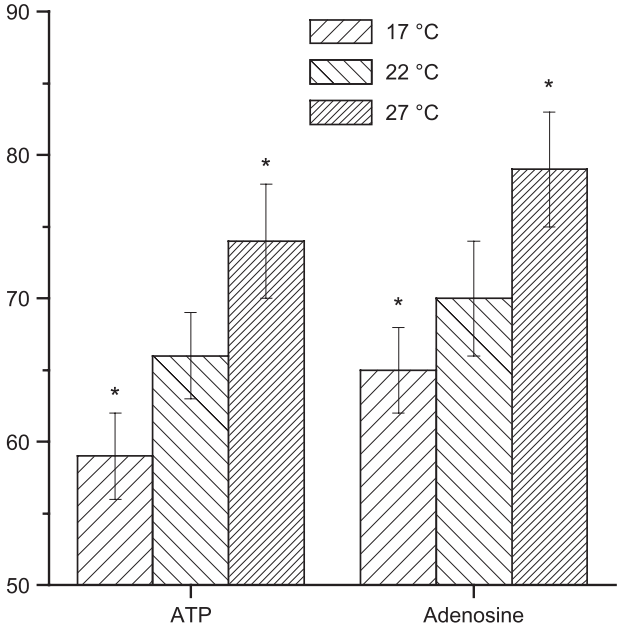

Fig. 6. Effects of ATP (100 μM) and adenosine (100 μM) on amplitude of end-plate currents of isolated frog sartorius muscles recorded at temperatures 17, 22 and 27°C. Data are presented as a percentage of control quantal release (before addition of agonists). Data shown are means and vertical bars indicate S.E.M., n=6–26. *P<0.05 from results obtained at 22 °C.

end-plate currents were obtained in the presence of ATP at a temperature of 17 °C measuring 58.6±2.6% (n=11). Similarly, end-plate currents modulated by adenosine were statistically diverse at temperatures of 17 and 27 °C establishing on 64.9±2.8% (n=7) and 79.3±3.8% (n=6), respectively (P<0.05; Fig. 6).

3.2.2. Effects of suramin, PPADS and 8-SPT

Suramin (100 μM), a nonselective P2 receptor antagonist, abolished the inhibitory action of ATP on end-plate currents but did not affect the action of adenosine. In contrast, PPADS (10 μM) did not antagonize the effects of either ATP or adenosine on end-plate currents.

A P1 receptor antagonist 8-SPT (100 μM) at a temperature of 22 °C did not significantly modify the inhibitory effect of ATP on end-plate currents (Fig. 7), while it completely reversed the inhibitory action of adenosine.

4. Discussion

In the present study, we had demonstrated that presynaptic P2 receptor-mediated inhibition of the frog skeletal muscle contractions produced by nerve stimulation have a clear temperature-dependent feature—lowering the temperature lead to increase of P2 receptor-mediated inhibition.

The depressant effect of exogenous ATP on neuromuscular transmission was demonstrated for the first time almost 30 years ago (Ribeiro and Walker, 1975), although for a long time, it was believed that inhibitory action of ATP is indirect and depends on degradation to adenosine (Ribeiro and Sebastio, 1987; Redman and Selinsky, 1994). In mammalian tissues, the existence of presynaptic P2 receptors at neuromuscular junction was suggested by immunohistochemical analysis (Parson et al., 2000), and electrophysiologically, it was established that ATP but not adenosine inhibited nonquantal release of acetylcholine (Galkin et al., 2001). At frog neuromuscular junction, it was shown that ATP inhibited transmitter release via presynaptic P2 receptors (Giniatullin and Sokolova, 1998), and it was proposed that ATP produces its effect via $P2Y_2$-like receptors coupled to multiple intracellular cascades (Sokolova et al., 2003).

In this study, we have shown that contractility of frog skeletal muscle depends on temperature; compared to normal room temperature (22 °C), at a higher temperature (27 °C), contractility increased, while at a low temperature (17 °C), muscle contractility decreased. Temperature dependency of skeletal muscle contractility is a known phenomenon. It has been shown that this phenomenon has an endothermic nature, and raising the temperature increases the force and the strain of the myosin heads attached in the isometric contraction (Piazzesi et al., 2003).The decrease of contractile force at lower temperature could be due to attenuation of metabolic enzyme activities (Cowan and Storey, 2001; MacDonald and Storey, 2002) or processes of energy production and transfer (Boutilier and St-Pierre, 2002; Mantovani et al., 2001).

Earlier temperature dependency of P1 receptor-mediated responses has been studied on some mammalian tissues. It

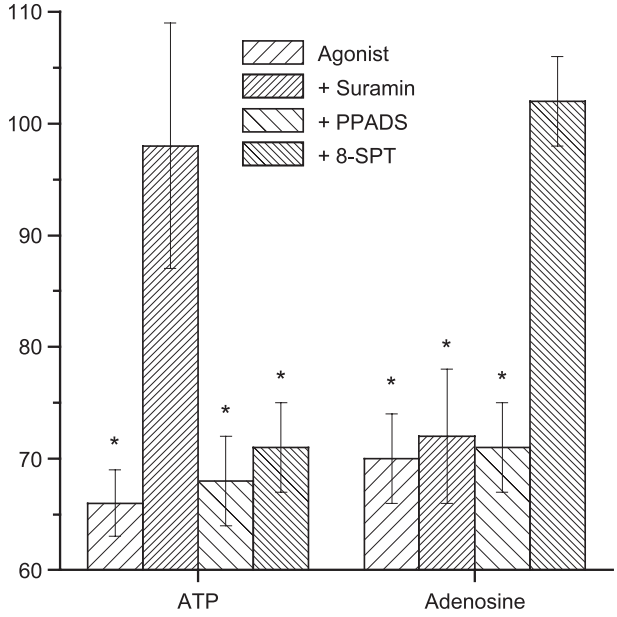

Fig. 7. Effects of suramin (100 μM), PPADS (10 μM) and 8-SPT (100 μM) on the inhibitory action of ATP (100 μM) and adenosine (100 μM) on amplitude of end-plate currents of isolated frog sartorius muscles at a temperature of 22 °C. Data are presented as a percentage of control quantal release taken as 100%. Data shown are means and vertical bars indicate S.E.M., n=5–7. *P<0.05 from controls.

was shown that in guinea-pig tissues, adenosine A_1 receptor-mediated inhibition of cholinergic and adrenergic transmissions increased with decrease of the bath temperature conditions, while adenosine A_2 receptor-mediated responses were not sensitive to temperature changes (Broadley et al., 1985). The authors showed that the low temperature-induced supersensitivity of adenosine A_1 receptors is not due to reduction of adenosine breakdown by adenosine-deaminases or its tissue uptake. They suggested that there were fundamental temperature-dependent differences between two subtypes of P1 receptors.

Recently, we have shown the temperature dependency of P2 receptor-mediated responses in guinea-pig smooth muscle tissues (Ziganshin et al., 2002). We established that P2 receptor-mediated responses of the guinea-pig urinary bladder and vas deferens were significantly larger at hypothermia (30 °C) than at normal (37 °C) or high (42 °C)-temperature conditions. Similar results were obtained for P2Y mediated relaxation of taenia caeci caused by electrical field stimulation, although relaxation of this tissue caused by ATP was not significantly affected by the temperature.

It has been proposed that presynaptic P1 receptors in frog neuromuscular junction belong to A_1 subtype since 1,3-dipropyl-8-cyclopentylxantine (DPCPX), a selective adenosine A_1 receptor antagonist, abolishes the inhibitory action of adenosine on transmitter release (Giniatullin and Sokolova, 1998). In our study, we found that adenosine-induced inhibition of muscle contractility increased at lower temperature, while the increase of the temperature did not affect the action of adenosine. However, in electrophysiological experiments, we found that there was a gradual increase of adenosine-induced inhibition of end-plate currents with the decrease of the temperature, and thus, in the presence of adenosine, the amplitude of end-plate currents at 17 °C was significantly different from that of at 27 °C. Thus, although the effect of adenosine on end-plate currents has some temperature dependency, it does not show a similar relationship to muscle contractility.

In contrast to adenosine, we have found that the effect of ATP on neuromuscular transmission was temperature-dependent in functional experiments. Lowering the temperature caused the increase of ATP-induced inhibition of electrical field stimulation-evoked contractions, and this effect was highly sensitive to P2 receptor antagonist PPADS and nonsensitive to 8-SPT, a P1-antagonist. These differences between two purines are thought to be coupled at their action mechanism. Both ATP and adenosine reduce quantal release of acetylcholine (Giniatullin and Sokolova, 1998), thereby decreasing amplitude of postsynaptic end-plate currents (this study). However, the temperature-mediated effect of ATP is more prominent and can achieve corresponding to amplitude of end-plate current reduction of the muscle contraction.

To find the nature of receptors involved, we used PPADS, a P2 receptor antagonist with a preferential effect on P2X receptors in functional whole-tissue experiments (Ziganshin et al., 1993, 1994), and found that ATP-evoked inhibition of muscle contraction was highly sensitive to this antagonist, while in electrophysiological study, it failed to affect responses to ATP. However, another nonselective P2 receptor antagonist suramin (Hoyle et al., 1990) significantly reduced ATP-induced inhibition. Although both PPADS and suramin are considered as nonselective P2 receptor antagonists, it has been shown that suramin, comparing to PPADS, has a more broad P2 receptor antagonist activity, affecting most of P2X and P2Y receptor subtypes (Burnstock, 2001). For instance, it has been shown that recombinant $P2Y_2$ receptors are sensitive to antagonistic effect of suramin but not of PPADS (Charlton et al., 1996). In addition, in organ bath pharmacological experiments, PPADS tends to antagonize mostly P2X receptor subtypes (Ziganshin et al., 1993, 1994; McLaren et al., 1994), blocking P2Y receptor-mediated processes only at higher concentrations (Windscheif et al., 1995). Neither PPADS nor suramin affect inhibition caused by adenosine. These results support the view that ATP inhibited the electrical field stimulation-evoked contractions of frog skeletal muscle by acting on presynaptic P2 receptors. It is most likely that these receptors belong to P2Y family, but involving some subtypes of P2X receptors cannot be ruled out at present.

It has been proposed that purine nucleotides and nucleosides were among the first neurotransmitters in the evolution and development of the living cells (Trams, 1981; Burnstock, 1996). Thus, it is possible that, in phylogenically older animals, which organism is functioning in low-temperature conditions, the transmitter role of purine nucleosides and nucleotides in cell-to-cell communications is as important as well-known intracellular metabolic actions of purines (production of energy, involvement in synthesis of nucleic acids). Thus, we suggest that supersensitivity of P2 receptor-mediated responses at lower temperature, which we have demonstrated in mammal (Ziganshin et al., 2002) and amphibian tissues (this study), is a fundamental feature of these receptors which could be a reflection of their past role in the early stage of evolution. Further experiments with a wide range of animals are necessary to carry out to find a stronger support to explore this hypothesis.

In conclusion, in this study, we have demonstrated that the effectiveness of P2 receptor-mediated inhibition of frog skeletal muscle contraction, in contrast to that of adenosine, is dependent on the temperature conditions. The physiological meaning of this phenomenon is yet to be understood.

Acknowledgments

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