REVIEW

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ATP AS A SIGNALING MOLECULE

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The review considers the effects of extracellular ATP mediated by plasma membrane purinoreceptors in the cells of different tissues, in particular, myometrium. Recently published results suggest that cytosolic ATP may also play a role of signaling molecule, as indicated by the detection of the ATP receptor not only in the plasma membrane, but also in mitochondria. The authors have shown that ionized Ca^{2+} concentration in the rat myometrium mitochondria matrix is regulated by ATP at the absence of exogenous Ca^{2+}. ATP concentration-dependent increase of $[Ca^{2+}]_m$ was not affected in the presence of the mitochondrial Ca^{2+}-uniporter blocker ruthenium red, the mitochondrial pore blocker cyclosporine A, or ATP synthase inhibitor oligomycin. It is assumed that cytosolic ATP could be a signaling molecule that regulates at least the Ca^{2+} ions exchange in mitochondria.

K e y w o r d s: extracellular and cytosolic ATP, P2X7 receptors, mitochondria, Ca^{2+} ions exchange.

It is well known that human cells are energy expensive and an average human needs around 65 kg of adenosine 5′-triphosphate (ATP) per day which is supplied partially through glycolysis (cytoplasm) and mainly through OXPHOS (in mitochondria) [1]. The regulation of ion channels and transporters by glycolytically derived ATP has been described for a variety of tissues [2]. It was about the regulation of the cation exchange systems activity in the plasma membrane and intracellular organelles [2]. More recently, it has been proven that mitochondrial consumption of cytosolic ATP is a pathological compensation in different mitochondrial disease models [3]. In isolated mitochondria, synthetic and reverse hydrolytic activities of ATP synthase were measured in the same preparation and showed that under different states of respiration and membrane potential, heterogeneous function of ATP synthase can co-exist in coupled mitochondria. It was suggested that ATP hydrolysis can occur heterogeneously in the cell and even within individual mitochondrion simultaneously with ATP synthesis [3].

Besides, ATP is also an extracellular messenger involved in cell-to-cell communication in virtually every tissue [4, 5]. Purine and its derivatives, exemplified by adenosine 5′-triphosphate and adenosine, have garnered increasing attention among various biological molecules [6]. Purine molecules within cells play crucial roles in regulating energy metabolism and other cellular processes, while extracellular purines transmit signals through specific purinergic receptors [6]. The first evidence that nucleotides participate in cell-to-cell communication as intercellular messengers was provided in the peripheral nervous system by Geoffrey Burnstock, who first coined the term “purinergic signaling” to describe this novel type of intercellular communication [7]. Despite widespread initial scepticism, it is now clear that extracellular nucleotides, notably ATP, are ubiquitous signaling molecules, mainly released during neurotransmission, but also in response to a variety of cell stressors such as mechanical distortion, osmotic swelling, hypoxia, and cytotoxic agents [8]. ATP is now recognized to play major roles in the central nervous system as a neuromodulator, neurotransmitter and gliotransmitter. Physiologically, extracellular ATP concentrations are relatively low (micromolar range) but can increase dramatically into the millimolar range during pathological conditions [9].

Normally, there is a delicate balance of ATP concentrations between the intracellular range (3–10 mM) and the extracellular range (20–50 nM) [10]. Therefore, the release of ATP at high levels serves as a danger signal when it is released by damaged or stressed cells, and P2X7 then detects these dramatic increases in extracellular ATP [10].

Purinergic receptors of the plasma membrane

Purinergic signaling is provided by the presence of appropriate receptors in the cell plasma membrane. Based on their structural and functional characteristics, purinergic receptors are classified into two major categories: P1 receptors (P1Rs) and P2 receptors (P2Rs). P1Rs refer to a class of G protein-coupled receptors (GPCRs) that are primarily activated by the ATP metabolite adenosine. Virtually all cells are equipped with plasma membrane receptors for extracellular ATP, the P2 receptors (P2Rs) [5, 11]. The nucleotide receptor family (P2 receptors, P2Rs) is comprised of two subfamilies: G protein-coupled metabotropic P2Y (P2YR) and ligand (ATP)-gated ionotropic P2X (P2XR) receptors [12]. The P2YR and the P2XR subfamilies consist of eight (P2Y1, 2, 4, 6, 11–14) and seven (P2X1–7) members, respectively [12]. P2YRs have a rather mixed ligand nucleotide selectivity [13]. In contrast, the preferred agonist for P2XRs is ATP, whereas all other nucleotides are inactive [13]. The only physiological agonist of the P2X7R is ATP [5]. Importantly, P2X7 receptor is different from other subtypes in that it has a low affinity for ATP and can be activated by requires ATP in the millimolar levels (>100 μM) [14].

A mechanism for channel opening (‘‘gating’’) has been suggested. ATP binding triggers a large conformational rearrangement. The gating process allows the rapid inward flux of Na^+ and Ca^{2+} and the outward flux of K^+ [12]. Both inward and outward ion fluxes are fundamental in P2X7R-mediated responses [12]. It should be noted that long-term stimulation of P2X7R with ATP causes the opening of a non-selective pore that allows the passage of molecules whose molecular weight is lower than 900 Da [15]. An interesting property of the P2X7R-dependent plasma membrane permeability increase is its reversibility. Removal of ATP within a few minutes from its addition allows full resealing of the plasma membrane [12].

Role of extracellular ATP

In addition to being a universal energy currency in the biological realm, ATP is also an important extracellular signaling molecule [6]. Numerous studies have demonstrated a strong association between dysfunction of the purine/purinergic system and the onset of various diseases [6]. The association of the P2X7R with inflammation and immunity is longstanding [12]. Most mouse and human cells express the P2X7R. Nevertheless, immune cells express this receptor to a high level and it is in the immune system that its function is best understood [12]. The role of extracellular ATP in inflammation and immunity is supported by the direct in vivo demonstration that inflammatory sites contain high (hundred micromolar) extracellular ATP concentrations, as opposed to the interstitium of healthy tissues where the ATP concentration is in the low nanomolar range [12]. The cascade spread of inflammatory response and tissue injury can release a large amount of ATP and activate P2X receptors in immune cells, produce a positive circuit phenomenon and expand inflammation. Therefore, the inhibition of P2X receptor activity has a good inhibitory effect on inflammatory response [14].

The abnormal metabolism of extracellular ATP not only represents a timely response to various cellular stressors such as inflammation and tissue damage, but also exerts critical effects on the development and immune regulation of the tumor microenvironment [6]. In the process of tumorigenesis, a large amount of intracellular ATP is released into the extracellular matrix, which leads to a significant increase in the concentration of ATP in the tumor microenvironment. By interacting with other molecular substances (such as P2X purinergic receptors), it can regulate the progression and drug resistance of tumor cells [16]. P2X7 receptor is expected to become a new pharmacological target for cancer treatment [16].

The purinergic receptor P2X7 is the main sensor of high concentrations of ATP, and P2X7 has been shown to be upregulated in the brains of Alzheimer’s disease patients, contributing to the disease’s pathological processes [10].

Human wild-type P2X7R (P2X7A) was shown to be expressed in different samples of human osteoblasts, chondrocytes and intervertebral disc cells [17]. P2X7R has attracted attention from the very

beginning as a cytotoxic receptor [18]. However, studies in human lymphoid cells made it clear that while its expression sensitizes cells to enhanced ambient ATP concentrations, under less harsh conditions P2X7R expression affords a growth advantage [19]. It has been proven that P2X7R is a potent activator of cell death that may occur via different mechanisms, depending on the cell type, agonist concentration and duration of the challenge [12]. Depending on its activation state, P2X7R can either drive cell survival and proliferation, or induce cell death [20].

The discovery of the P2X7 receptor in immune cells raised great hopes for the development of novel and more potent anti-inflammatory medicaments. Unfortunately, such hopes were partially deluded by the unsatisfactory results of most early clinical trials. However, recent findings ushered in a second life for the P2X7R in diagnostic medicine. New P2X7R radioligands proved to be very reliable tools for the diagnosis of neuroinflammation in preclinical and clinical studies, and the detection and measurement of free P2X7 receptor in human blood suggested its potential use as a circulating marker of inflammation [21].

Despite overwhelming data supporting the role of P2X7R in immune cell responses and tumor growth and progression, a complete picture of the pathophysiological functions of P2X7R, especially when expressed by non-immune cells, is lacking [17].

Extracellular ATP and muscles

Muscular dystrophies are inherited neuromuscular diseases, resulting in progressive disability and often affecting life expectancy. An acute muscle injury causes the release of large quantities of ATP, which acts as a damage-associated molecular pattern (DAMP). DAMPs trigger inflammation that clears dead tissues and initiates regeneration that eventually restores normal muscle function. However, in dystrophic muscles, the loss of ecto-ATPase activity, that normally curtails this extracellular ATP-evoked stimulation, causes exceedingly high eATP levels. Thus, in dystrophic muscles, the acute inflammation becomes chronic and damaging [22]. Studies in mouse models of dystrophino- and sarcoglycanopathies have demonstrated that P2X7 blockade reduces damaging inflammation while promoting the pro-regenerative arm of the inflammatory response [22].

Control of Ca^{2+}_i and myometrial contractions is exceedingly complex [23]. Contraction of the myometrium is tightly regulated by the concentration of intracellular calcium $[Ca^{2+}]_i$, which can be released from the sarcoplasmic reticulum or can enter the cell via the voltage-dependent Ca^{2+} channels and/or non-selective cation channels [24]. There are multiple roles for purinergic signaling in female reproductive organs. ATP, released as a cotransmitter with noradrenaline from sympathetic nerves, contracts smooth muscle via P2X1 receptors in vas deferens, seminal vesicles, prostate and uterus, as well as in blood vessels [25]. It has been shown in different species that ATP is an important extracellular nucleotide and can be released directly from smooth muscles and endothelium into the extracellular milieu [26, 27]. Once released, ATP can cause contraction in almost all types of smooth muscles directly by binding to its P2 receptors that are found on smooth muscle cell membranes [28].

It has been claimed that extracellular ATP is essential for the initiation of contractions and control of their frequency (but not contractile force) and may be involved in the pacemaking mechanism for the generation of uterine contractions [25]. It was also shown that ATP induced ion currents and contractions via P2X7 receptors in freshly isolated myometrial cells from pregnant rats and that P2X7 receptor mRNA was localized in these cells [25, 29, 30]. These authors showed further that Mg^{2+} blocked the P2X7 receptor-mediated contraction in tocolysis and suggested that targeting P2X7 receptors could lead to novel treatments for the prevention of uterine contractions in preterm deliveries [25]. It was shown that ATP-induced currents in pregnant rat myometrial cells were carried by P2X7 receptors [29].

It was also found that the P2X4 and P2X7 receptors were strongly expressed in the myometrium at the time of delivery. The expression levels of these receptors gradually increased toward the time of labor and reached a maximum at the time of delivery. In the inflammation model with LPS treatment, both the mRNA and the protein expression levels of these receptors were enhanced dramatically and were much higher than those seen either during normal delivery or in the mifepristone-induced preterm model [31].

Later it was shown that P2X7Rs were expressed in nonpregnant uterine tissues, progressively increased throughout pregnancy, and markedly peaked during postpartum involution. ATP significantly in-

creased the force of spontaneous contraction in all uterine strips from different gestational stages with a marked increase during labor and postpartum. ATP could not maintain the force when external Ca^{2+} was removed. In addition, ATP was able to cause tonic transient contraction in the absence of external Ca^{2+}. The clear effects of ATP on contractility suggest its physiological requirement for successful labor and postpartum involution [28].

Taken together, these studies support the role of extracellular ATP as a signaling molecule in various tissues.

Cytosolic ATP as a signaling molecule

It is well known that purines inside the cell are primarily involved in the regulation of energy metabolism. Manipulating ATP production by mitochondria is an important therapeutic strategy in the field of mitochondrial medicine. Inhibiting ATP production could be a strategy to treat cancer while increasing ATP production could improve pathologies such as mitochondrial myopathies, neurodegeneration, diabetes, and cardiovascular disease [32]. However, there is scattered evidence that ATP stimulation (likely binding to P2X7R) causes cytochrome c release and caspase-3, -8, and -9 cleavage [33]. It was also shown that tonic stimulation of the P2X7R (plasma membrane, ligand-gated channel) increased the Ca^{2+} concentration of the mitochondrial matrix, support mitochondrial metabolism, and increased intracellular ATP content [5, 34]. On the other hand, sustained stimulation of P2X7R causes a $\Delta\psi_{mt}$ drop, a large increase in $[Ca^{2+}]_{mt}$, mitochondrial fragmentation, and cell death. These observations help understand the mechanism of the dual effect of P2X7R stimulation on cell growth [34].

Stimulation of the P2X7R has been shown to enhance energy metabolism in mice. It was suggested that the potentiation of P2X7 function in adult mice might contribute to restore a proper balance in either aged or pathologic carbohydrate-driven energy metabolism, by forcing the balance toward a prevalent oxidation of fatty acids and enhancement of metabolic rate [35].

In other words, the binding of ATP to plasma membrane receptors also leads to changes in the mitochondria functioning.

It should be emphasized that the subcellular localization of P2X7R has not yet been sufficiently studied. However, the subcellular location of the P2X7 receptor has recently been shown [5]. Identifying the subcellular localization of a protein within a cell is often an essential step in understanding its function [17]. Recently it was shown P2X7R localization to the mitochondria in different cell types. The P2X7R localizes to mitochondria, and its lack (1) decreases basal respiratory rate, ATP-coupled respiration, maximal uncoupled respiration, resting mitochondrial potential, mitochondrial matrix Ca^{2+} level, (2) modifies expression pattern of oxidative phosphorylation enzymes, and (3) severely affects cardiac performance. It was concluded that P2X7R is a key modulator of mitochondrial energy metabolism and a determinant of physical fitness [5].

P2X7R localization at mitochondria-associated-membranes has been shown in macrophages. The purinergic P2X7 receptor is one of the most potent activators of the NLRP3 inflammasome [36].

P2X7R mitochondrial immunoreactivity was detected in osteoblasts and intervertebral disc cells. Evidence of subcellular localization of P2X7R may help to i. understand the participation of P2X7R in as yet unidentified signaling pathways, ii. identify pathologies associated with P2X7R mislocalization and iii. design specific targeted therapies [17].

We studied Ca^{2+} exchange in the uterus smooth muscle cells, in particular, the exchange of this cation in mitochondria. Using the isotope method, it was shown that in an Mg^{2+},ATP-medium, the level of Ca^{2+} accumulation in mitochondria is several times higher than in Mg^{2+}-one. However, when determining the Ca^{2+} concentration in an Mg^{2+},ATP-medium (fluorescent probes), we obtained only a slight increase in the fluorescence intensity upon the addition of exogenous Ca^{2+}. As has been shown, incubation of mitochondria in an Mg^{2+},ATP-medium, in the absence of exogenous Ca^{2+}, is accompanied by an increase in matrix Ca^{2+} concentration compared to that in Mg^{2+}-one. A little more detail below.

We have shown that ionized Ca^{2+} concentration in mitochondria matrix ($[Ca^{2+}]_m$) in the absence of exogenous Ca^{2+} is regulated by ATP: in Mg^{2+},ATP-medium this number is a couple of times higher than in Mg^{2+}- one. Addition of exogenous Ca^{2+} to Mg^{2+}- and Mg^{2+},ATP-medium resulted in an increase of $[Ca^{2+}]_m$ to approximately equal numbers. We have also shown the ATP concentration-dependent increase of $[Ca^{2+}]_m$ in the absence of exogenous Ca^{2+}. The Hill coefficient equals 3.18 ± 0.27 and the activation constant for ATP – 0.97 ± 0.07 mM [37]. We also used cell impermeant fluorescent probe Fluo-4, Pentapotassium Salt, to evaluate Ca^{2+} efflux from mi-

tochondria ($[Ca^{2+}]_o$). ATP concentration-dependent increase of ionized Ca concentration in the incubation medium ($[Ca^{2+}]_o$) in the absence of exogenous Ca^{2+} was also shown. The Hill coefficient equals 3.69 ± 0.46 and the activation constant for ATP – 2.94 ± 0.68 mM [38].

Nevertheless, total calcium accumulation (using $^{45}Ca^{2+}$ as radioactive tracer) was 30 times higher in the presence of ATP than in its absence [39]. It was shown that upon addition of 10 μM $^{45}Ca^{2+}$ myometrium mitochondria accumulated 149 ± 18 and 5 ± 2 nmol Ca^{2+}/mg of protein/5 min in Mg^{2+},ATP- and Mg^{2+}-containing medium, respectively. These results were obtained with isolated mitochondria. We also used another experimental model – a suspension of myometrial myocytes treated with digitonin (0.01%) to study the total level of Ca^{2+} accumulation in mitochondria. Ca^{2+} accumulation in mitochondria was tested as such that was not sensitive to thapsigargin (100 nM). The cells permeabilized with digitonin were preincubated for 5 min in Mg^{2+}- and Mg^{2+},ATP-containing media. Then 3 mM ATP was added to the Mg^{2+}-containing medium and the Ca^{2+} transport was started by the addition of 10 μM $^{45}Ca^{2+}$ to both media. It resulted in the Ca accumulation of 459 ± 32 pmol/10^6 cells/5 min in Mg^{2+}-containing medium and 1933 ± 182 pmol/10^6 cells/5 min in Mg^{2+},ATP-containing medium, respectively. Thus, it was shown that short-term myometrium mitochondria preincubation in the absence of ATP, Ca^{2+} accumulation did not reach the level of one at initial ATP presence in the incubation medium [39].

To elucidate the possible mechanisms that were involved in ATP-induced $[Ca^{2+}]_m$ increasing in the absence of exogenous Ca^{2+}, different mitochondria effectors were used: cyclosporine A – mitochondrial permeability transition pore inhibitor; ruthenium red – mitochondrial Ca^{2+}-uniporter inhibitor; oligomycin – inhibitor of F_1F_0-ATPase complex. As can be seen from the results presented in Fig. 1, the incubation of mitochondria in Mg^{2+},ATP-medium is accompanied by an several times increase in $[Ca^{2+}]_m$ compared to Mg^{2+}-medium. But $[Ca^{2+}]_m$ was not affected by 5 μM CsA, 10 μM RuR or 1 μg/ml oligomycin addition (Fig. 1, *a*).

Ca^{2+} efflux from mitochondria ($[Ca^{2+}]_o$) was also studied (Fig. 1, *b*). It was shown, that $[Ca^{2+}]_o$ at mitochondria incubation in Mg^{2+}-medium was 67.5 ± 6.9 and in Mg^{2+},ATP-medium – 1254.7 ± 95.5 nM. These data were obtained in the presence of 25 μM EGTA. The addition of 125 μM EGTA was accompanied by a sharp decrease of the $[Ca^{2+}]_o$ (Fig. 1, *b*).

Flow cytometry and a voltage sensitive probe (100 nM TMRM) were used to test the polarization of mitochondrial membranes under incubation in the Mg^{2+}- and Mg^{2+},ATP-medium. The results obtained indicate the polarization of mitochondrial membranes in both mediums (Fig. 2). However, the level of mitochondrial membranes polarization was higher in Mg^{2+}-medium.

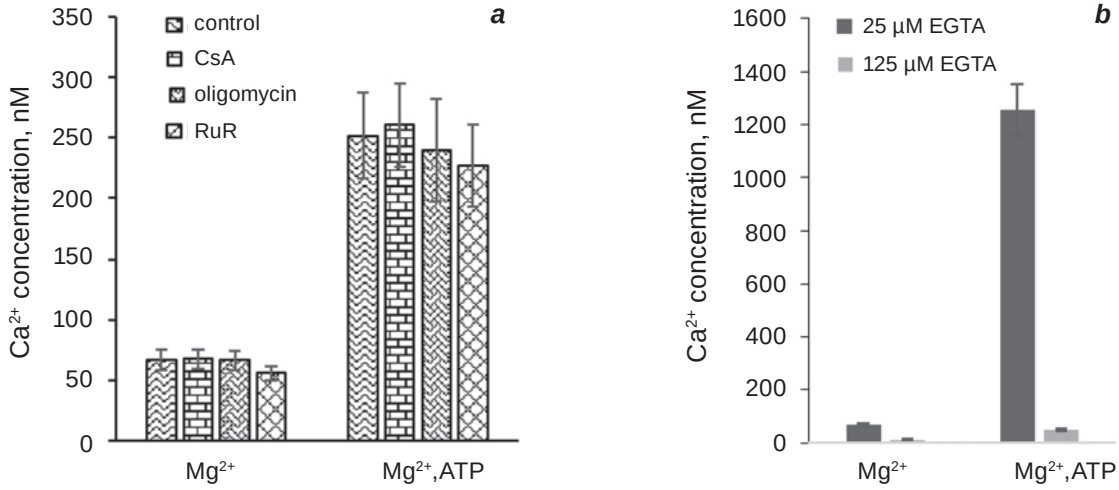

Fig. 1. ***a*** *– Mitochondrial matrix Ca^{2+} concentration: 5 μM cyclosporine A (CsA), 10 μM ruthenium red (RuR) or 1 μg/ml oligomycin showed no effect on $[Ca^{2+}]_m$. Fluorescent probe Fluo-4 AM was used (means ± SEM, n = 4-5).* ***b*** *– Ca^{2+} efflux from mitochondria: $[Ca^{2+}]_o$ in the Mg^{2+}- and Mg^{2+},ATP-medium in the presence of 25 and 125 μM EGTA. Cell impermeant fluorescent probe Fluo-4, Pentapotassium Salt, was used (means ± SEM, n = 3)*

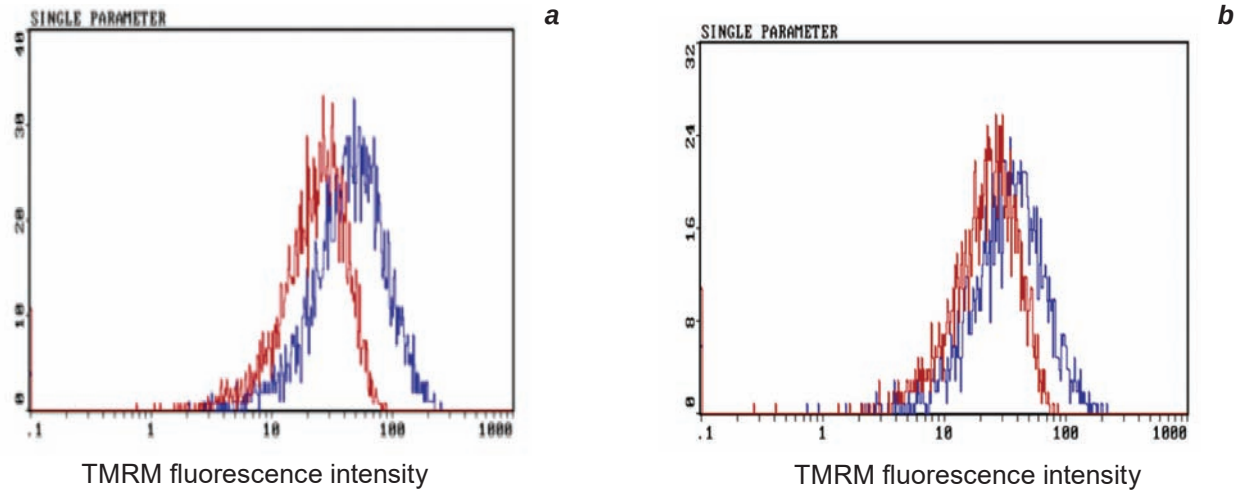

*Fig. 2. Flow cytometry analyses of mitochondria membrane polarization upon incubation in Mg^{2+}- (**a**) and Mg^{2+},ATP-medium (**b**): blue graph – TMRM loaded mitochondria; red graph – TMRM loaded mitochondria + 10 μM CCCP. The results of a typical experiment are presented (n = 4)*

The discovery that the P2X7R is present on the mitochondria adds further complexity to the intracellular physiology of P2X7R [5]. However, the presence of P2X7R receptors in mitochondrial membranes is a reason to assume that cytosolic ATP can also be a signaling molecule that regulates at least the Ca^{2+} ions exchange in mitochondria. Our current understanding of the potential role of cytosolic ATP as a signaling molecule undoubtedly requires further research.

Conflict of interest. The authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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АТР ЯК СИГНАЛЬНА МОЛЕКУЛА

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В огляді розглядаються ефекти позаклітинного АТР, що опосередковані пуринорецепторами плазматичної мембрани, в клітинах різних тканин, зокрема, міометрія. Згідно з опублікованими раніше результатами, цитозольний АТР також може відігравати роль сигнальної молекули, на що вказує наявність рецептора АТР не тільки в плазматичній мембрані, але і в мітохондріях. Автори показали, що концентрація іонізованого Ca^{2+} в матриксі мітохондрій міометрія щурів регулюється АТР за відсутності екзогенного Ca^{2+}. АТР-залежне збільшення $[Ca^{2+}]_m$ не змінювалося за наявності блокатора Ca^{2+}-уніпортера рутенієвого червоного, блокатора мітохондріальної пори циклоспорину А або інгібітора АТР-синтази олігоміцину. Припускається, що цитозольний АТР може бути сигнальною молекулою, яка регулює принаймні обмін іонів Ca^{2+} у мітохондріях.

Ключові слова: позаклітинний та цитозольний АТР, рецептори Р2Х7, мітохондрії, обмін іонів Ca^{2+}.

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