EXPERIMENTAL WORKS

UDC 577.352.4 **doi:** https://doi.org/10.15407/ubj98.01.030

EXTRAMITOCHONDRIAL ATP MODULATES Ca^{2+} SIGNALING IN MYOMETRIAL MITOCHONDRIA

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Received: 16 September 2025; **Revised:** 21 October 2025; **Accepted**: 30 January 2026

It was postulated that mitochondria are sensors and effectors of ATP synthesis. Our results suggest that ATP may play a role as an intracellular signaling molecule. We have shown that the baseline Ca^{2+} concentration in the mitochondrial matrix increased in the presence of ATP or MgATP in the incubation media of isolated mitochondria. Activation or inhibition of both the respiration and Ca^{2+} uniporter activity, as well as the removal of Mg^{2+} from the incubation medium, or addition of A438079, an antagonist of plasma membrane P2X7 receptors, followed by the addition of ATP did not affect the ATP-induced increase of matrix baseline Ca^{2+} concentration. These results showed that extramitochondrial ATP modulates Ca^{2+} signaling in mitochondria independently of the Ca^{2+} uniporter and the respiratory chain activity. In the presence of UTP or MgUTP instead of ATP or MgATP, an increase of the matrix baseline Ca^{2+} concentration was not observed indicating that the studied effects are selective for ATP.

K e y w o r d s: mitochondria, matrix, Ca^{2+} concentration, Mg^{2+}, ATP, UTP, A438079, Ru360, spermine.

Adenine nucleotides (ATP, ADP, and AMP) are key organic molecules that supply immediate energy, mediate signaling, and serve as metabolic intermediates [1, 2]. Using MCF7 (breast cancer cells) and S288C (*Saccharomyces cerevisiae*) cells, it was shown that cytosolic ATP was transported into the mitochondria under conditions of reduced electron transport chain activity. This ATP was likely utilized in the reverse mode of the H^+/ATPase to maintain mitochondrial membrane potential, which contributed to the avoidance of programmed cell death [3].

We have previously shown an ATP-induced increase of ionized Ca^{2+} concentration in myometrium mitochondria matrix in the absence of exogenous Ca^{2+}. Cyclosporine A, ruthenium red or oligomycin did not affect either Ca^{2+} concentration in the mitochondrial matrix $[Ca^{2+}]_m$ or Ca^{2+} concentration in the incubation medium $[Ca^{2+}]_o$. Cardiolipin (CL) content in mitochondria membranes decreased upon incubation of organelles in Mg^{2+},ATP-medium as compared to Mg^{2+}-medium [4]. It has been proven that inflammation is a significant risk factor for preterm birth. Inflammation enhances glycolytic processes in various cell types and contributes to the development of myometrial contractions [5]. It is important to consider Crabtree's hypothesis, which posits that the introduction of glycolytic ATP into a system that normally relies on OxPhos triggers a homeostatic response [2]. In the case of myometrial mitochondria, the homeostatic response to the ATP addition may consist, in particular, in the acceleration of cardiolipin oxidation [4]. It has been suggested that glycolytically derived ATP can function as an intracellular signaling molecule that regulates mitochondrial Ca^{2+} exchange [4, 6].

This study aimed to test the ATP-induced increase of baseline Ca^{2+} concentration in the mitochondrial matrix (without added Ca^{2+}, $[Ca^{2+}]_b$) at activation or inhibition of the respiratory chain, activation or inhibition of the Ca^{2+} uniporter, effects of Mg^{2+}, UTP instead of ATP and selective inhibitor of P2X7 receptors A438079.

Materials and Methods

Animals. The treatment of the lab animals was carried out according to "European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes" (Strasbourg, 1986) and the Law of Ukraine "On protection of animals from cruelty". All manipulations with laboratory animals were approved by the Bioethics Commission of the Palladin Institute of Biochemistry (Order No. 15 of September 5, 2025). Female non-pregnant rats were used. Experiments were performed using white female rats (weight 170-240 g). Rats were kept under stationary vivarium conditions at a constant temperature and with a basic allowance. Animals were narcotized with chloroform and then sacrificed using cervical dislocation.

Isolation of myometrium mitochondria. Mitochondria from the myometrium of non-pregnant rats were isolated using the differential centrifugation method [7]. The mitochondria were suspended in a medium with the following composition: 250 mM sucrose, 1 mM EGTA, 20 mM Hepes, and buffered pH 7.4 at 4°C. Protein concentration of the mitochondrial fraction was determined by Bradford assay [8]. The concentration of mitochondrial protein in the sample was 25 µg/ml.

Determination of ionized calcium concentration in the mitochondria matrix. $[Ca^{2+}]_b$ was determined using the QuantaMaster™ 40 spectrofluorometer (Photon Technology International) and the fluorescent probe Fluo-4, AM (λ_{exc} = 490 nm, λ_{em} = 520 nm). Myometrium mitochondria were loaded with 2 µM Fluo-4, AM for 30 min at 37°C in a medium of the composition indicated above. Thereafter, the suspension of mitochondria was diluted (1:10) in the same medium containing no fluorescence probe, followed by centrifugation. The pellet was resuspended in the same medium containing no fluorescence probe. The $[Ca^{2+}]_b$ was measured in a medium containing: 250 mM sucrose, 2 mM K^+-phosphate buffer, 5 mM sodium succinate, ±3 mM $MgCl_2$, ±3 mM ATP, 20 mM Hepes, pH 7.4. To exclude the possibility of medium acidification due to adenosine triphosphate addition, we normalized the pH of the ATP stock solution with 1 M Tris. The calibration of the Fluo-4 fluorescence was performed at the end of each testing probe by adding 0.1% Triton X-100 (in the presence of 100 µM $CaCl_2$) and, in 1 min, 5 mM EGTA (fluorescence intensities F_{max} and F_{min}, respectively). $[Ca^{2+}]_b$ was calculated using the Grynkiewicz equation [9], K_d for Ca^{2+} in buffer: ~345 nM.

Statistical analysis. Results are reported as means ± SEM of 3-6 independent experiments (biological replicates). Statistical analysis was performed using paired Student's *t*-test; $P < 0.05$ was taken as the level of significance.

Chemicals and reagents. In the study, the following reagents were used: EGTA, Hepes, D-(+)-sucrose, sodium succinate, oligomycin, rotenone, antimycin A, cyclosporine A, spermine, FCCP, UTP, A438079, Ru360 (Sigma-Aldrich); Fluo 4AM – (Invitrogen); ATP (Fluka).

Results

Mg^{2+}, ATP and Mg^{2+}ATP effects on $[Ca^{2+}]_b$. Our results showed that 3 mM Mg ions slightly reduced, while the 3 mM Mg^{2+}ATP complex significantly in-

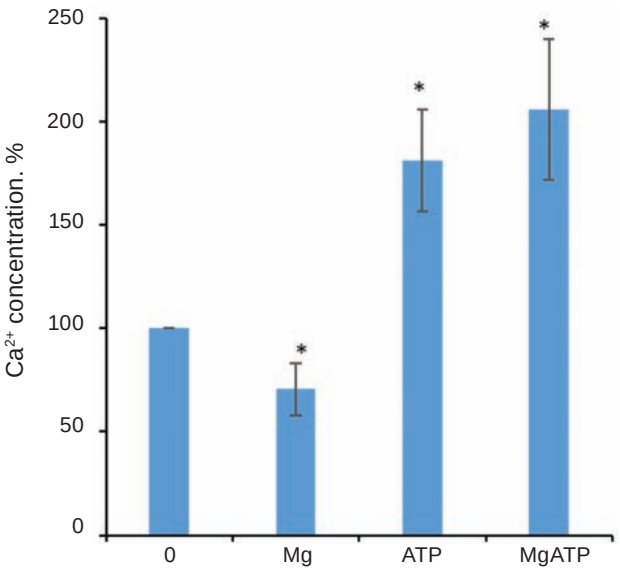

Fig. 1. Effects of 3 mM Mg^{2+}, 3 mM ATP or 3 mM Mg^{2+}ATP on $[Ca^{2+}]_b$. The $[Ca^{2+}]_b$ was measured in a medium containing: 250 mM sucrose, 2 mM K^+-phosphate buffer, 5 mM sodium succinate, ±3 mM $MgCl_2$, ±3 mM ATP, 20 mM Hepes, pH 7.4. The calibration of the Fluo-4 fluorescence was performed at the end of each testing probe by adding 0.1% Triton X-100 (in the presence of 100 µM $CaCl_2$) and, in 1 min, 5 mM EGTA (fluorescence intensities F_{max} and F_{min}, respectively). Calculations using the Grynkiewicz equation were performed at the end of 10 min of incubation (using the average value of the last 10 data points). Means ± SEM, n = 5, $P < 0.05$ compared to control

creased $[Ca^{2+}]_b$ upon incubation in sucrose medium. The effect of 3 mM ATP is maintained even in the absence of Mg ions (Fig. 1).

$[Ca^{2+}]_b$ at activation or inhibition of the respiratory chain. To test the possible contribution of respiration to the ATP-induced $[Ca^{2+}]_b$ increase, respiration was stimulated using 1 μM FCCP or inhibited using 1 μM rotenone (a complex I inhibitor) and 2 μg/ml antimycin A (an inhibitor of complex III). Experiments were conducted in the presence or absence of 1 μg/ml oligomycin (an inhibitor of F1-ATPase activity). Activation or inhibition of the respiratory chain did not affect the ATP-induced $[Ca^{2+}]_b$ increase (Fig. 2).

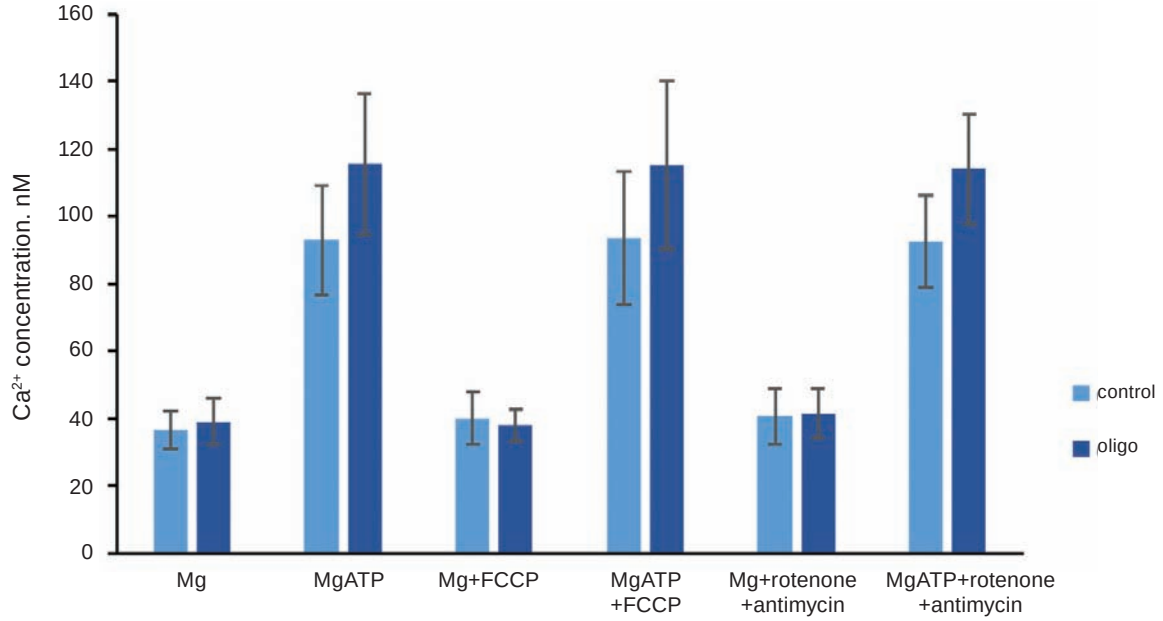

Fig. 2. $[Ca^{2+}]_b$ upon activation (1 μM FCCP) or inhibition (1 μM rotenone + 2 μg/ml antimycin A) of the respiratory chain. Testing was carried out in the absence (control) or presence of 1 μg/ml oligomycin (oligo). Means ± SEM, n = 4

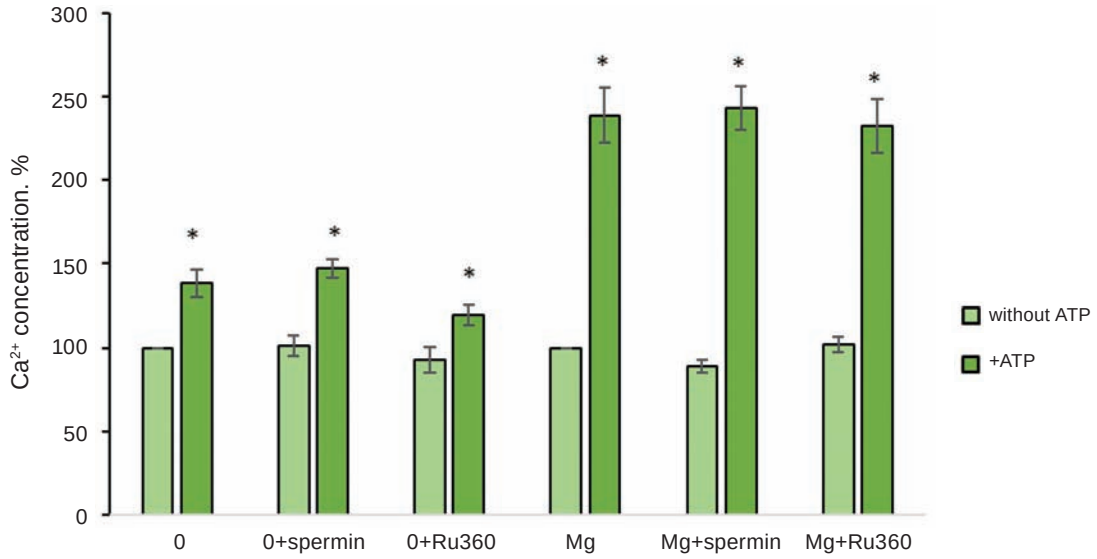

Fig. 3. $[Ca^{2+}]_b$ at activation (1 mM spermine) or inhibition (10 μM Ru360) of the Ca^{2+} uniporter. Means ± SEM, n = 4, $P < 0.05$ compared to the probe without ATP

$[Ca^{2+}]_b$ at activation or inhibition of the Ca^{2+} uniporter. To test the possible role of Ca^{2+} uniporter in the ATP-induced $[Ca^{2+}]_b$ increase, uniporter activity was stimulated using 1mM spermine and inhibited using 10μM Ru360. Activation or inhibition of the Ca^{2+} uniporter did not affect the ATP-induced $[Ca^{2+}]_b$ increase (Fig. 3).

Mg^{2+} and $[Ca^{2+}]_b$. In the next series of experiments, we changed the time of adding Mg^{2+} or ATP to the incubation medium. Mitochondria were incubated in a 3 mM ATP-containing medium without Mg^{2+} for 5 min, followed by the 3 mM Mg^{2+} addition (next 5 min incubation) or in a Mg^{2+}-containing medium without ATP for 5 min, followed by the ATP addition (next 5 min incubation). The studied ATP effects were also recorded (Fig. 4).

It was concluded that the effect of ATP on $[Ca^{2+}]_b$ is maintained even in the absence of Mg ions (Fig. 4). Changing the order of Mg^{2+} and ATP addition did not cancel the ATP-induced increase of the baseline Ca^{2+} concentration in the mitochondrial matrix.

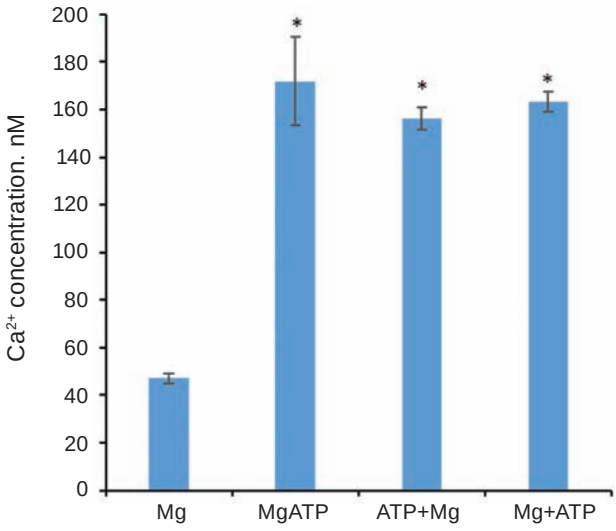

Fig. 4. $[Ca^{2+}]_b$ and the time of Mg^{2+} or ATP addition: Mg – 3 mM Mg^{2+} was added at the start of testing, incubation 10 min; MgATP – 3 mM Mg^{2+}ATP was added at the start of testing, incubation 10 min; ATP+Mg – 3 mM ATP was added at the start of testing, incubation 5 min followed by 3 mM Mg^{2+} addition and next 5 min incubation; Mg+ATP – 3 mM Mg^{2+} was added at the start of testing, incubation 5 min followed by 3 mM ATP addition and next 5 min incubation. Baseline Ca^{2+} concentration was calculated at the end of 10 min incubation. Means ± SEM, n = 4, P < 0.05 compared to control (Mg)

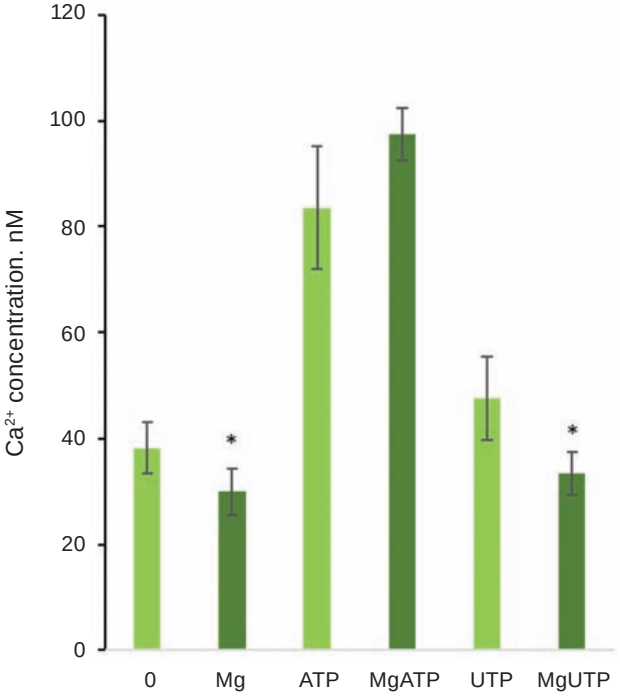

Fig. 5 Comparison of the ATP and UTP effects $[Ca^{2+}]_b$. Means ± SEM; n = 6. P < 0.05 compared to the probe without Mg^{2+}

UTP instead of ATP and $[Ca^{2+}]_b$. To determine the selectivity of ATP effects, we tested the effect of UTP (Fig. 5). ATP is a purine nucleotide, while UTP (uridine triphosphate) is a pyrimidine nucleotide. An increase of $[Ca^{2+}]_b$ was not observed when 3 mM ATP was replaced by 3 mM UTP. That is, the studied effects are ATP selective.

Selective inhibitor of P2X7 receptors A438079 and $[Ca^{2+}]_b$. It could be assumed that P2X7 receptors, the presence of which was shown in mitochondria [10], are related to the ATP-induced increase of $[Ca^{2+}]_b$. To test this assumption, a selective inhibitor of P2X7 receptors A438079 was used. 10 μM A438079 did not affect the ATP effects upon its prior incubation with mitochondria for 5 min, followed by the ATP addition and continued incubation for 5 min (Fig. 6).

Discussion

The regulation of myometrial Ca^{2+}_i is complex. Understanding the mechanisms involved may lead to the design of tocolytics that target multiple pathways and achieve improved suppression of premature labor [11]. It was shown that matrix baseline Ca^{2+} concentration $[Ca^{2+}]_b$ (in the absence of added Ca^{2+}) increased in the presence of ATP or MgATP complex.

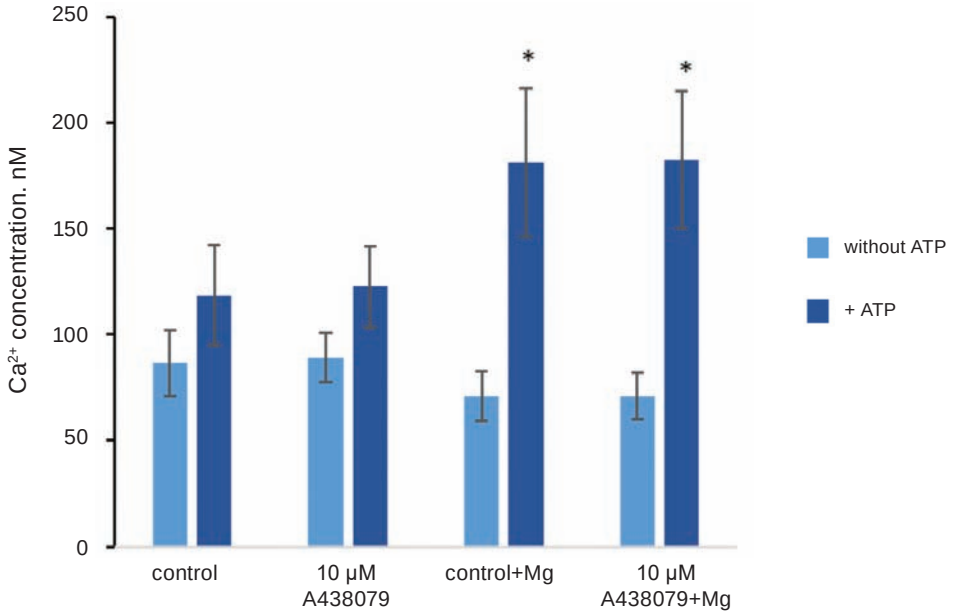

Fig. 6. A438079 effects on $[Ca^{2+}]_b$. Means ± SEM; n = 4. P < 0.05 compared to the probe without Mg^{2+}

Activation or inhibition of both the respiration and Ca^{2+} uniporter activity did not affect the $[Ca^{2+}]_b$.

Magnesium ions are involved in a wide variety of biochemical reactions, and numerous physiological functions are known to require Mg^{2+}. The major intracellular Mg^{2+} store is the mitochondria, and basic mitochondrial functions, including ATP synthesis, electron transport chain complex subunits, and oxygen detoxification, are affected by intracellular Mg^{2+} [12]. Magnesium ions have been known for many years to affect cation-transporting pathways in mitochondria [13].

The results presented in Fig. 4 indicate that ATP, either in the presence or in the absence of Mg ions, causes an increase in the baseline Ca^{2+} concentration. Removal of Mg^{2+} from the incubation medium in the presence of ATP or prior incubation of mitochondria in the presence of Mg^{2+}, followed by the addition of ATP, did not inhibit the ATP effects. It can be assumed that the removal of Mg^{2+} from the medium did not remove Mg^{2+} from the matrix, so that Mg^{2+}, in a certain way, could take part in the effects of ATP.

It has been 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 [14, 15], supporting the earlier findings of Urabe et al. [16]. Later, it was also shown P2X7R localization to the mitochondria in different cell types, and its lack impairs OxPhos, affects cardiac performance, and decreases physical fitness [10]. The only physiological agonist of the P2X7R is ATP [10]. To determine the selectivity of ATP action, we tested the effect of UTP. ATP is a purine nucleotide, while UTP (uridine triphosphate) is a pyrimidine nucleotide. An increase of $[Ca^{2+}]_b$ was not observed when ATP was replaced by UTP. Thus, it can be stated that the studied effects are selective for ATP. Does this mean that the effects of ATP are mediated by P2X7 receptors? To test this assumption, a selective inhibitor of P2X7 receptors, A438079, was used. A-438079 hydrochloride hydrate is a selective antagonist of the plasma membrane P2X7 receptor [17]. It was shown that 10 μM A438079 did not affect the ATP effects on the $[Ca^{2+}]_b$ upon its prior incubation for 5 min, followed by the ATP addition. However, it is known that many intracellular signaling pathways are regulated by P2X7R. For example, P2X7R is known to activate several phospholipases [18]. We have previously shown that extramitochondrial ATP accelerates cardiolipin oxidation [4].

It was shown that Mg^{2+} blocked the P2X7 receptor-mediated contraction in tocolysis [15, 19]. At the mitochondrial level, we showed that Mg^{2+} did not block the effects of ATP, and that the presence of Mg ions in the incubation medium enhanced the effects of ATP compared to its absence. It should be emphasized that the properties of the mitochondrial P2X7 receptor have not yet been fully studied. It can be

assumed that the properties of the plasma membrane and mitochondria receptors may differ.

It was postulated that mitochondria are sensors and effectors of ATP synthesis [1]. Our results also suggest that ATP may play a role as an intracellular signaling molecule. An increase in ATP concentration in the incubation medium is accompanied by an increase in the $[Ca^{2+}]_b$, and Mg^{2+} enhances the ATP effects.

Conclusions. It was shown that $[Ca^{2+}]_b$ increased in the presence of ATP or MgATP complex. Activation or inhibition of both the respiration and Ca^{2+} uniporter activity did not affect the ATP-induced increase of $[Ca^{2+}]_b$. Removal of Mg^{2+} from the incubation medium in the presence of ATP or prior incubation of mitochondria in the presence of Mg^{2+}, followed by the addition of ATP, did not inhibit the effects of ATP. 10 μM A438079, an antagonist of plasma membrane P2X7 receptors, did not affect the ATP effects under prior incubation for 5 min followed by the addition of ATP. However, in the presence of UTP or MgUTP complex (instead of ATP or MgATP), an increase in the baseline Ca^{2+} concentration was not observed. Thus, it was proven that extramitochondrial ATP modulates Ca^{2+} signaling in mitochondria independently of the Ca^{2+} uniporter and respiratory chain activity but an increase in $[Ca^{2+}]_b$ was not observed when ATP was replaced with UTP, so it can be concluded that the studied effects are selective for ATP.

Conflct of interest. 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.

Funding. The present study was supported by the National Academy of Sciences of Ukraine (Grants No 0124U000224, 0125U000792).

АТР ТА Ca^{2+} СИГНАЛЮВАННЯ У МІТОХОНДРІЯХ МІОМЕТРІЯ

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Було постульовано, що мітохондрії є сенсорами та ефекторами синтезу АТР. Наші результати свідчать про те, що АТР може відігравати роль внутрішньоклітинної сигнальної молекули. Показано, що базова концентрація Ca^{2+} у матриксі збільшувалась в присутності АТР або комплексу MgATP. Активація або пригнічення як дихання, так і активності Ca^{2+} уніпортера не впливала на індуковане АТР збільшення базової концентрації Ca^{2+} у мітохондріальному матриксі. Видалення Mg^{2+} з інкубаційного середовища але у присутності АТР або попередня інкубація мітохондрій у присутності Mg^{2+} з подальшим додаванням АТР не пригнічувало ефекти АТР. 10 мкМ A438079, антагоніст рецепторів P2X7 плазматичної мембрани, не впливав на ефекти АТР за попередньої інкубації мітохондрій протягом 5 хв з подальшим додаванням АТР. Однак у присутності UTP або комплексу MgUTP (замість АТР або MgATP) збільшення базової концентрації Ca^{2+} не спостерігалося. Таким чином, було показано, що екстрамітохондріальна АТР модулює Ca^{2+} сигналювання у мітохондріях незалежно від активності Ca^{2+} уніпортеру та дихального ланцюга, але збільшення $[Ca^{2+}]_b$ не спостерігалося при заміні АТР на UTP, тому можна зробити висновок, що досліджувані ефекти є селективними для АТР.

Ключові слова: міометрій, мітохондрія, Ca^{2+}, Mg^{2+}, ATP, UTP, A438079, Ru360, спермін.

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