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Extramitochondrial ATP as $[Ca^{2+}]_m$ and cardiolipin content regulator

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ABSTRACT

An ATP-induced increase of $[Ca^{2+}]_m$ in myometrium mitochondria matrix at the absence of exogenous Ca^{2+} was shown. An ATP-induced increase of Ca^{2+} efflux from mitochondria ($[Ca^{2+}]_o$) has also been shown. Mitochondria membranes were polarized upon incubation in both Mg^{2+}- and Mg^{2+},ATP-medium. Cardiolipin (CL) content in mitochondria membranes decreased upon incubation of organelles in Mg^{2+},ATP-medium as compared to Mg^{2+}-medium. It was suggested that ATP could play the role of a signaling molecule regulating the Ca^{2+} exchange in the mitochondria.

1. Introduction

Mitochondria, long viewed solely in the context of bioenergetics, are increasingly emerging as critical hubs for intracellular signaling [1]. The cytosolic Ca^{2+} signal propagates into mitochondria, enhancing its respiratory rate, H^+ extrusion and ATP synthesis [1]. Adenosine 5′-triphosphate (ATP), the universal energy currency, sustains all cellular functions and responses, and at the same time is also an extracellular messenger involved in cell-to-cell communication in virtually every tissue [2] [3]. Virtually all cells are equipped with plasma membrane receptors for extracellular ATP, the P2 receptors (P2Rs) [4] [3]. However, it was also shown P2X7R localization to the mitochondria in different cell types [3]. The only physiological agonist of the P2X7R is ATP [3]. The discovery that the P2X7R is present on the mitochondria adds further complexity to the intracellular physiology of P2X7R [3]. The regulation of ion channels and transporters by glycolytically derived ATP has been described for a variety of tissues [5]. It was about the regulation of the cation exchange systems activity in the plasma membrane and intracellular organelles [5]. More recently, it has been proven that mitochondrial consumption of cytosolic ATP is a pathological compensation in different mitochondrial disease models [6]. However, the role of glycolytically derived ATP in the regulation of Ca^{2+} exchange in mitochondria has not been considered. We have shown previously that ionized Ca^{2+} concentration in mitochondria matrix ($[Ca^{2+}]_m$) at the absence of exogenous Ca^{2+} is regulated by ATP: in Mg^{2+},ATP-medium this number is 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. Nevertheless, total calcium accumulation was 30 times higher in the presence of ATP than in its absence [7]. 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 activation constant for ATP - 0.97 ± 0.07 mM [8].

The aim of the present work was to study ATP effects on Ca^{2+} exchange in mitochondria and to evaluate possible role of CL in this process.

2. Materials and methods

2.1. 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" [31] and the Law of Ukraine "On protection of animals from cruelty". Female non-pregnant rats were used. Experiments were performed using white female rats (weight 170–240 g). Rats were kept under the stationary vivarium conditions at constant temperature and basic allowance. Animals were narcotized with chloroform and then sacrificed using cervical dislocation.

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2.2. Isolation of myometrium mitochondria

Mitochondria from myometrium of non-pregnant rats were isolated using differential centrifugation method [9]. 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 [10]. The concentration of mitochondrial protein in the sample was 25 μg/mL.

2.3. Determination of ionized calcium concentration in the mitochondria matrix

$[Ca^{2+}]_m$ 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), (K_d for Ca^{2+} = 345 nM). Myometrium mitochondria were loaded with 2 μM Fluo-4, AM for 30 min at 37 °C in a medium the composition of which is 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 $[Ca^{2+}]_m$ 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, ±0.1 mM $CaCl_2$, 20 mM Hepes, pH 7.4. To exclude the possibility of the medium acidification by adenosine triphosphate addition, we normalized the pH of 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 (at the presence of 100 μM $CaCl_2$) and, in 1 min, 5 mM EGTA (fluorescence intensities F_{max} and F_{min}, respectively). $[Ca^{2+}]_m$ was calculated using the Grynkiewicz equation[11].

2.4. Determination of ionized calcium concentration in the incubation medium

Ionized Ca concentration in the incubation medium ($[Ca^{2+}]_o$) was determined using the QuantaMaster™ 40 spectrofluorometer (Photon Technology International) and the fluorescent probe 1 μM Fluo-4, Pentapotassium Salt, cell impermeant (λ_{exc} = 490 nm, λ_{em} = 520 nm), (K_d for Ca^{2+} = 335 nM). The $[Ca^{2+}]_o$ 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 the medium acidification by adenosine triphosphate addition, we normalized the pH of ATP stock solution with 1 M tris. The calibration of the Fluo-4, Pentapotassium Salt fluorescence was performed at the end of each testing probe by adding 5 mM EGTA and, in 1 min, 5 mM $CaCl_2$ (fluorescence intensities F_{min} and F_{max}, respectively). $[Ca^{2+}]_o$ was calculated using the Grynkiewicz equation [11].

Fluo 5F, Pentapotassium salt, (λ_{exc} = 490 nm, λ_{em} = 520 nm), (K_d for Ca^{2+} = 2.3 μM) was used to test the possible effects of the ATP solution and buffer pH on the fluorescence spectrum of the probe.

2.5. Measurements of mitochondria membrane polarization and CL content

To record mitochondria membrane polarization and CL content a COULTER EPICS XL™ (Beckman Coulter, United States) flow cytometer with an argon laser (λ_{ex} 488 nm) was used. Experimental data was analyzed using the SYSTEM II™ Software (Beckman Coulter).

Relative values of mitochondria membrane potential (Δψ) were assayed using a fluorescent probe 100 nM TMRM (λ_{ex} 488 nm, λ_{em} 590 nm) in the medium of the same composition as for Ca^{2+} concentration determination. Mitochondria suspension (25 μg/mL) was loaded with 100 nM TMRM in 1 mL incubation medium for 5 min and then immediately analyzed on the flow cytometer at a wavelength of 590 nm. Each measurement was represented as the average of fluorescent intensity of 10,000 events and expressed in relative units: average fluorescence intensity value of a sample minus average fluorescence intensity value of a sample upon 10 μM CCCP addition.

Relative values of mitochondria CL content were assayed using fluorescent probe 100 nM NAO (λ_{ex} 488 nm, λ_{em} 520 nm) in the medium of the same composition as for Ca^{2+} concentration determination. Mitochondria suspension (25 μg/mL) was loaded with 100 nM NAO in 1 mL incubation medium for 5 min and then immediately analyzed on the flow cytometer at a wavelength of 520 nm. Each measurement is represented as the average of fluorescent intensity of 10,000 events.

2.6. Measurements of mitochondria CL and other phospholipids

The content of mitochondria CL and other phospholipids was estimated by thin layer chromatography. Mitochondria suspension were incubated in Mg^{2+}- and Mg^{2+},ATP-medium for 5 min followed by centrifugation. The resulting precipitates were used to determine total lipids. The total lipids from the mitochondria suspension were extracted by Bligh & Dyer method [12]. The lipid extracts were stored in a small volume of chloroform at −20 °C. After that lipid extracts were separated by thin-layer chromatography on standard plates (Sorbfil, Russia), using solvent systems for the first dimension chloroform (65): methanol (30): ammonia (6): benzene (10), and the second dimension chloroform (5): methanol (1): acetic acid (1): water (0.5): acetone (2) [13]. The specific reagents and standards were used for individual phospholipid identification [14]. The individual phospholipid content was determined by Vaskovskiy and Kostetskiy method [15].

2.7. 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.

2.8. Chemicals and reagents

In the study the following reagents were used: EGTA, Hepes, D-(+)-sucrose, sodium succinate, oligomycin, cyclosporine A, trifluoperazine, protonophore CCCP, acridine orange 10-nonyl bromide (NAO), methanol, acetic acid, hydrazinium chloride, hydrochloric acid, sulphuric acid, sodium molybdate, acetone, ammonia and phospholipid standards – by "Sigma-Aldrich"; Fluo 4 AM, Fluo-4, Pentapotassium salt, Fluo 5F, Pentapotassium salt, TMRM – by "Invitrogen"; ATP, ruthenium red – by "Fluka"; chloroform and benzene – by "Supelco".

3. Results

3.1. ATP induced mitochondria matrix ionized Ca concentration increase

Myometrium mitochondria, loaded with fluorescent probe Fluo 4 AM, were incubated in Mg^{2+}- and Mg^{2+},ATP- medium at the absence of exogenous Ca^{2+}. As can be seen from the Fig. 1 results, ATP induced >3 times increase of $[Ca^{2+}]_m$ Fig. 1a, control).

To elucidate the possible mechanisms that are involved in ATP-induced $[Ca^{2+}]_m$ increasing 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; trifluoperazine - calmodulin antagonist. It was shown that $[Ca^{2+}]_m$ was not affected by 5 μM CsA, 10 μM RuR or 1 μg/mL oligomycin addition (Fig. 1a). Approximately 2 times elevation of $[Ca^{2+}]_m$ were recorded upon the incubation of mitochondria with 50 μM TFP in the Mg^{2+}-medium without statistically significant changes in the Mg^{2+},ATP-medium (Fig. 1b).

To exclude the possibility of the medium acidification by adenosine triphosphate addition, the pH of ATP stock solution was normalized with

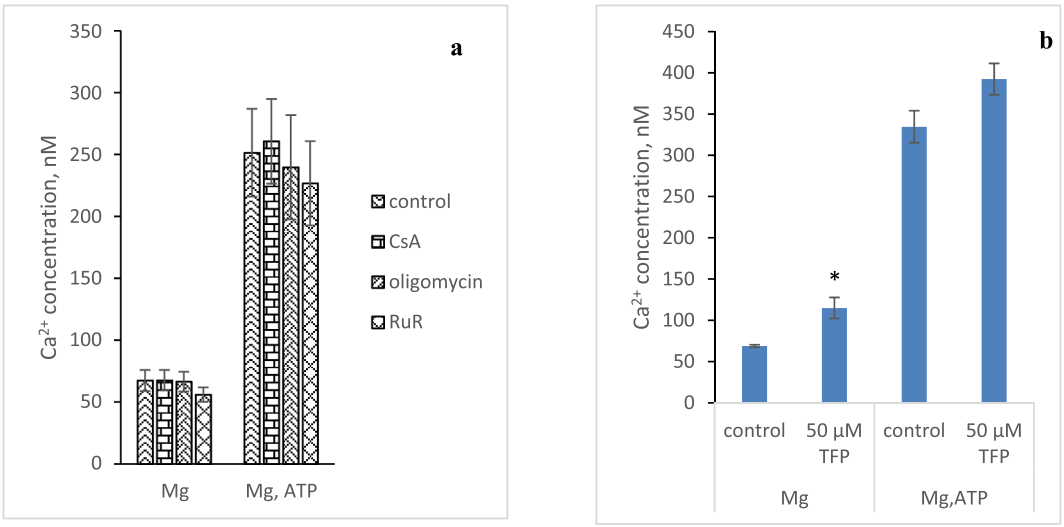


Fig. 1. Mitochondrial matrix Ca^{2+} concentration: a) 5 μM cyclosporine A (CsA), 10 μM ruthenium red (RuR) or 1 μg/mL oligomycin showed no effect on $[Ca^{2+}]_m$. Means ± SEM, $n = 4–5$; b) 50 μM trifluoperazine (TFP) in the Mg^{2+}-medium induced $[Ca^{2+}]_m$ increase without statistically significant changes in the Mg^{2+},ATP-medium. Means ± SEM, n = 4–6, * - $P < 0.05$ (as compared to control). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1 M tris. Nevertheless, the ATP solution effects on the Fluo 5F pentapotassium salt fluorescence (a water-soluble analog of fluo 4 AM) was studied. As can be seen from the results presented in Fig. 2.a, under 100 μM Ca^{2+} addition the fluorescence spectrum of the Fluo 5F pentapotassium salt did not change significantly when aliquot of 30 mM ATP (pH 7.4) was added.

Thus, an ATP solution adjusted to pH 7.4 does not affect the probe fluorescence intensity and, therefore, cannot cause an ATP-dependent increase of $[Ca^{2+}]_m$.

Next, it is known that mitochondrial ATP synthase (F_1F_0ATPase) switches from being an ATP producer to an ATP consumer. Evidence has recently been presented that ATP hydrolysis occurs in healthy mitochondria [6]. Therefore, it cannot be ruled out the possibility that the ATP-dependent increase in Fluo4 fluorescence intensity observed in Fig. 1 is influenced not by calcium ions but by ATP-induced changes in the pH of the matrix. To verify this assumption the fluorescence spectra of Fluo 5F, pentapotassium salt, at different pH values were studied. As can be seen from the obtained results (Fig. 2.b), pH values affect the Fluo 5F pentapotassium salt fluorescence spectra in the presence of 100 μM Ca^{2+}. The acidification of the incubation medium is accompanied by the partial quenching of the fluorescence intensity. However, as noted above [6], the addition of ATP is accompanied by the proton release from the matrix, i.e., it can be assumed that the matrix is alkalized. Ca^{2+} chelating with EGTA led to a decrease in the probe fluorescence at all tested pH values. However, it should be noted that at acidic pH, the addition of 1 mM EGTA was not accompanied by complete quenching of the probe fluorescence compared to other pH values. Therefore, acidification of the incubation medium does affect the probe fluorescence reducing Fmax and increasing Fmin. In our case, there is an increase in the fluorescence intensity, which cannot be explained by the direct effect of the ATP solution or the pH value of the incubation medium on the probe fluorescence.

One more question. Functional interaction between mitochondria and sarcoplasmic reticulum can impact mitochondrial Ca^{2+} signal. We have performed experiments on intact mitochondrial fraction in the presence of 1 μM thapsigargin or 1 μM cyclopiazonic acid to avoid

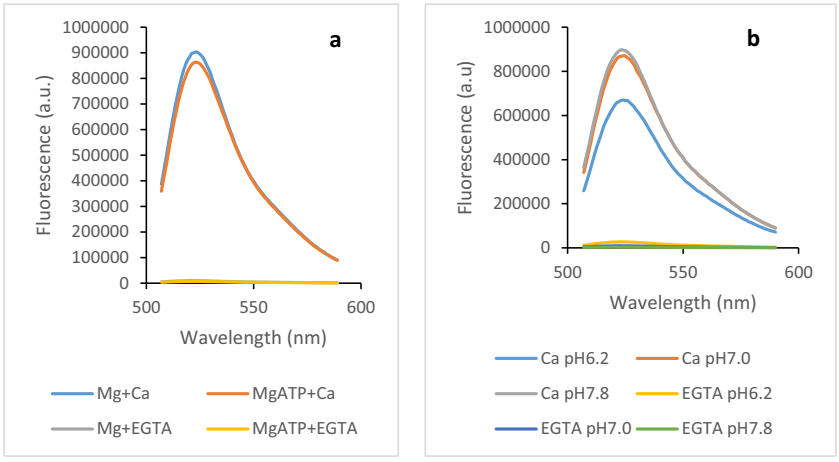


Fig. 2. Fluorescence spectra of the Fluo 5F pentapotassium salt: a) Testing was carried out in Mg- and MgATP-medium in the presence of 100 μM Ca^{2+} followed by the addition of 1 mM EGTA; b) 50 mM imidazole-HCl buffer was used with pH values of 6.2, 7.0 and 7.8. Testing was carried out in the presence of 100 μM Ca^{2+} followed by the addition of 1 mM EGTA.

contamination of sarcoplasmic reticulum fragments, attached to mitochondria. These compounds did not affect the $[Ca^{2+}]_m$ tested in both media (data not shown).

Thus, it was concluded that ATP affects precisely the Ca^{2+} concentration in the mitochondrial matrix.

3.2. ATP induced increase of Ca^{2+} efflux from mitochondria

Cell impermeant fluorescent probe Fluo-4, Pentapotassium Salt, was used to evaluate Ca^{2+} efflux from mitochondria ($[Ca^{2+}]_o$) (Fig. 3). 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 got under the presence of 25 μM EGTA. The addition of 125 μM EGTA was accompanied by a sharp decrease of the $[Ca^{2+}]_o$ (Fig. 3.a).

The fact that the fluorescence intensity of the Ca^{2+}-sensitive probe (Fluo-4, Pentapotassium Salt) decreases with an increase in the Ca^{2+}-chelating agent (EGTA) concentration is additional evidence that ATP induces changes in the $[Ca^{2+}]_o$.

Incubation of mitochondria with 5 μM CsA, 10 μM RuR or 1 μg/mL oligomycin did not affect the Ca^{2+} efflux from mitochondria that was evaluated in the Mg^{2+}- and Mg^{2+},ATP-medium (data not shown). Approximately 1.5 times elevation of $[Ca^{2+}]_o$ was recorded upon the incubation of mitochondria with 50 μM TFP in the Mg^{2+},ATP-medium (Fig. 3b).

Furthermore the ATP concentration-dependent increase of $[Ca^{2+}]_o$ in the absence of exogenous Ca^{2+} was shown. The Hill coefficient equals 3.69 ± 0.46 and activation constant for ATP - 2.94 ± 0.68 mM (Fig. 4).

3.3. ATP and polarization of myometrium mitochondria membranes

Flow cytometry and a voltage sensitive probe (100 nM TMRM) were used to test the polarization of mitochondrial membranes. TMRM accumulates in polarized mitochondria, depolarization of mitochondria membranes leads to the probe efflux, which may be assessed by the decrease in the intensity of its fluorescence. The results obtained indicate the polarization of mitochondrial membranes in both mediums (Fig. 5). However, the level of mitochondrial membranes polarization was higher in Mg^{2+}-medium. Calculated CCCP-sensitive component of the TMRM fluorescence intensity confirm this conclusion (Fig. 5c).

It was shown previously that calmodulin antagonist TFP depolarized myometrial mitochondria membranes in concentration-dependent manner ($K_{0.5}$ = 24.4 ± 5 μM) [16]. To confirm the polarization of myometrium mitochondria membranes in both Mg^{2+}- and Mg^{2+},ATP-medium, the effect of TFP was also studied. Addition of 50 μM TFP was accompanied by a decrease of the TMRM fluorescence intensity (Fig. 6).

It was concluded that mitochondria membranes were polarized in both Mg^{2+}- and Mg^{2+},ATP-medium.

3.4. ATP induced decrease of CL content in the mitochondria membranes

Mitochondrial CL content was determined using flow cytometry and 100 nM NAO. Nonyl acridine orange (NAO) is a fluorescent probe for CL which can be used to quantify the CL in isolated mitochondria. It was shown that the content of CL in mitochondria membranes decreased upon incubation of organelles in Mg^{2+},ATP-medium as compared to Mg^{2+}-medium (Fig. 7).

Thin layer chromatography was also used to test the CL and other phospholipids content in mitochondria (Table). Incubation of mitochondria in Mg^{2+},ATP-medium led to profound changes in the phospholipid composition of mitochondria: decrease in phosphatidylcholine, CL, sphingomyelin, phosphatidylinositol content and increase in lysophosphatidylcholine level compared to the phospholipids composition of mitochondria which were incubated in Mg^{2+}-medium.

4. Discussion

Intracellular calcium ion is the key secondary messenger system of the cellular processes in smooth muscle cells [17]. Mitochondrial Ca^{2+} regulation is crucial for bioenergetics and cellular signaling [18]. The kinetic properties for the uptake, storage and release of Ca^{2+} from isolated mitochondria accurately predict the behavior of the organelles within the intact cell [19]. Indeed, any experimental model has both positive and negative sides. However, isolated mitochondria make it possible to test the effects of ATP in the absence of exogenous Ca.

We have shown previously that ionized Ca^{2+} concentration in mitochondria matrix ($[Ca^{2+}]_m$) at the absence of exogenous Ca^{2+} is regulated by ATP: in Mg^{2+},ATP- medium this number is 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. Nevertheless, under addition of exogenous Ca^{2+} total calcium accumulation, which was tested using isotope techniques ($^{45}Ca^{2+}$) and is the sum of the free and bound cation, was 30 times higher in the presence of ATP than in its absence [7]. It was 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 activation constant for ATP - 0.97 ± 0.07 mM [8]. Continuing these studies, we

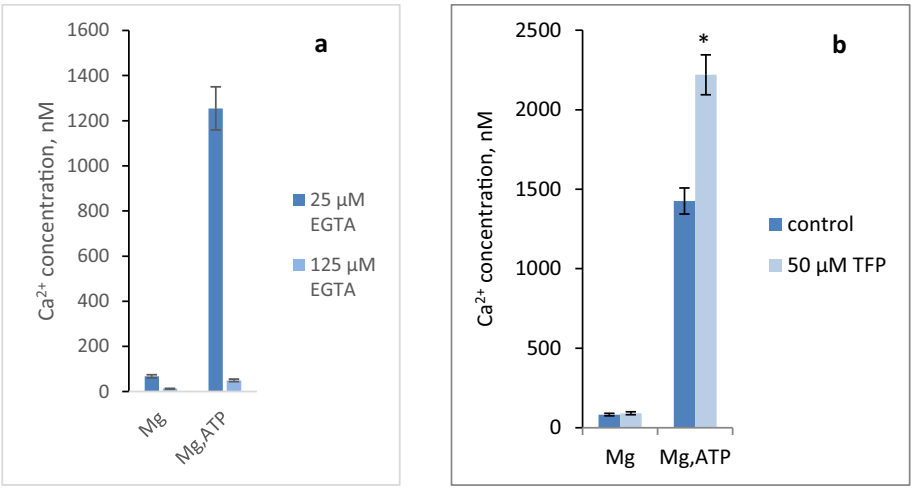


Fig. 3. Ca^{2+} efflux from mitochondria: a) $[Ca^{2+}]_o$ in the Mg^{2+}- and Mg^{2+},ATP- medium at the presence of 25 and 125 μM EGTA. Means ± SEM, n = 3; b) 50 μM trifluoperazine induced additional increase of the Ca^{2+} efflux from mitochondria in the Mg^{2+},ATP-medium. Means ± SEM, n = 5–6, * - $P < 0.05$ (as compared to control).

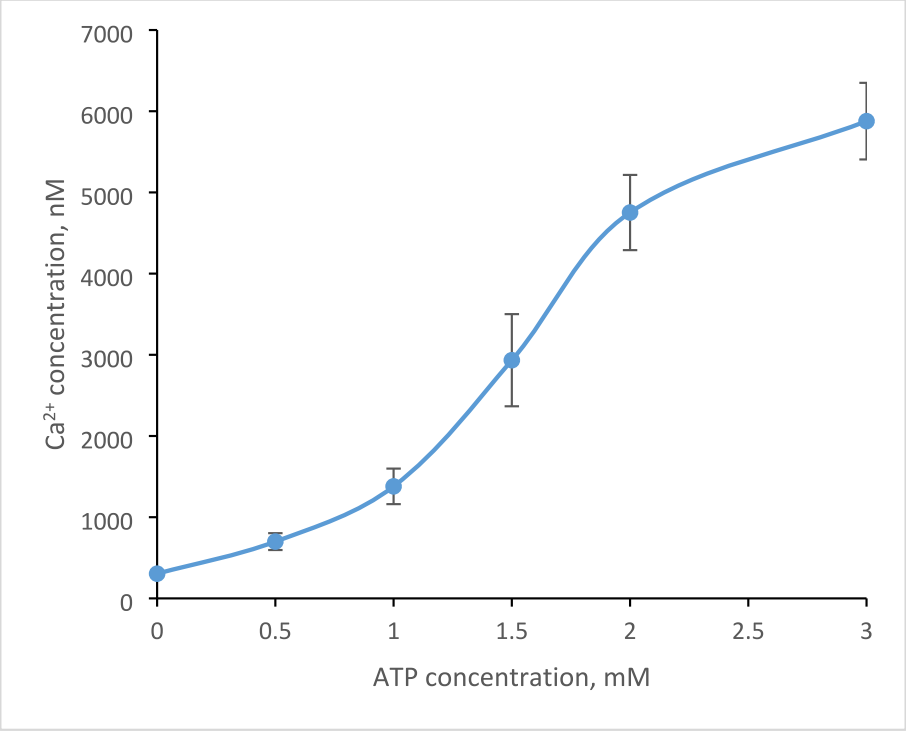


Fig. 4. ATP concentration-dependent increase of $[Ca^{2+}]_o$, M ± m, $n = 6$.

showed that at the absence of exogenous Ca^{2+} ATP addition induced both an increase of $[Ca^{2+}]_m$ and $[Ca^{2+}]_o$. Both of these phenomena were not regulated by 5 μM CsA, 10 μM RuR or 1 μg/mL oligomycin. Sarcoplasmic reticulum Ca^{2+}-pump inhibitors, 1 μM thapsigargin or 1 μM cyclopiazonic acid, did not affect the $[Ca^{2+}]_m$ tested in both media. $[Ca^{2+}]_o$ sharply decreased with an increase of the EGTA concentration, a well-known Ca^{2+} chelator. The ATP concentration-dependent increase of $[Ca^{2+}]_o$ in the absence of exogenous Ca^{2+} was also shown. The Hill coefficient equals 3.69 ± 0.46 and activation constant for ATP - 2.94 ± 0.68 mM.

The first question that may arise is whether acidification of the incubation medium occurs when ATP is added. To exclude the possibility of the medium acidification by adenosine triphosphate addition, the pH of ATP stock solution was normalized with 1 M Tris. Nevertheless, the ATP solution effects on the Fluo 5F pentapotassium salt fluorescence (a water-soluble analog of fluo 4 AM) was studied. The fluorescence spectrum of the Fluo 5F pentapotassium salt did not change significantly when aliquot of 30 mM ATP (pH 7.4) was added.

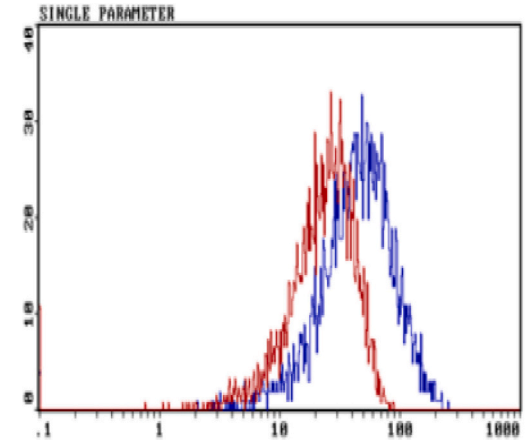
Second, a few words about Tris. We used Tris to bring the pH of the stock ATP solution to neutral. The final Tris concentration in the incubation medium was 2.5 mM. As a rule, this buffer is used at much higher concentrations.

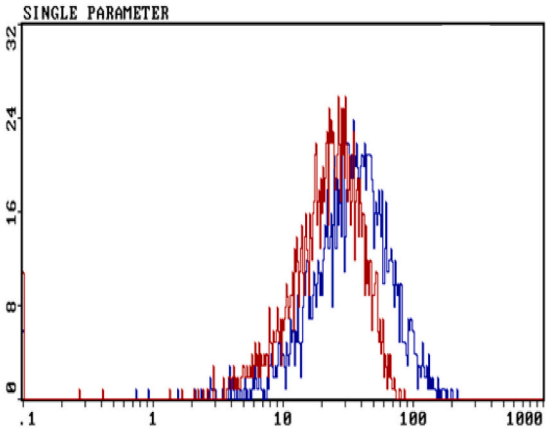
Next, it is known that mitochondrial ATP synthase (F_1F_0ATPase) switches from being an ATP producer to an ATP consumer. Evidence has recently been presented that ATP hydrolysis occurs in healthy mitochondria [6]. Using isolated intact mitochondria from mouse heart it was shown that the addition of ATP resulted in a sharp increase in proton release [6]. Therefore, it cannot be ruled out the possibility that the ATP-dependent increase in Fluo4 fluorescence intensity is influenced not by calcium ions but by ATP-induced changes in the pH of the matrix. To verify this assumption the fluorescence spectra of Fluo 5F, pentapotassium salt, at different pH values were studied. It was shown that pH values affect the Fluo 5F pentapotassium salt fluorescence spectra in the presence of 100 μM Ca^{2+}. The acidification of the incubation medium is accompanied by the partial quenching of the fluorescence intensity. However, the addition of ATP is accompanied by the proton release from the matrix [6], i.e., it can be assumed that the matrix is alkalized. In our case, there is an increase in the fluorescence intensity, which cannot be explained by the direct effect of the ATP solution or the pH value of the incubation medium on the probe fluorescence.

In other words, ATP added to the incubation medium can enter mitochondria. But, in this case, as the authors write [6], the release of protons is enhanced, which suggests alkalization of the matrix. As our experiments showed, alkaline pH values do not affect the fluorescence intensity of the probe we used.

Myometrium mitochondria membranes were polarized in both Mg^{2+}- and Mg^{2+},ATP-medium. However, the level of mitochondrial membranes polarization was higher in Mg^{2+}-medium. TFP has been used as an additional factor allowing us to confirm mitochondrial membrane polarization in both Mg^{2+}- and Mg^{2+},ATP-medium. Previously calmodulin antagonists effects on mitochondrial membrane potential have been investigated using confocal microscopy and fluorescent potential-sensitive probes TMRM and MTG. Incubation of myometrium cells with 100 μM TFP was accompanied by almost complete decrease of the “red” fluorescence (TMRM) while the “green” fluorescence (MTG) remained unchanged [20]. We have also studied polarization of myometrium mitochondrial membranes using quench mode and 1 μM TMRM. It was shown that isolated mitochondria incubation with TFP was accompanied by depolarization of the mitochondria membranes, $K_{0.5}$ was 24.4 ± 5 μM and Hill coefficient - 2.0 ± 0.2 [16]. It was concluded, that TFP depolarized myometrium mitochondria membranes in concentration-dependent manner. This time it was shown that addition of 50 μM TFP was accompanied by a decrease of the TMRM fluorescence intensity during incubation of mitochondria in both Mg^{2+}- and Mg^{2+},ATP-medium. It should be noted that the effects of TFP may be independent of calmodulin inhibition.

It should be clarified also that the TMRM fluorescence intensity was recorded 5 min after adding the probe. By this time, the mitochondrial membrane potential in Mg^{2+},ATP-medium was lower than in Mg^{2+} – one. It has been suggested that hyperpolarization of mitochondria membrane is known to be a first stage on the way to depolarization [21]. Thus, it can be assumed that the ATP-induced decrease in the





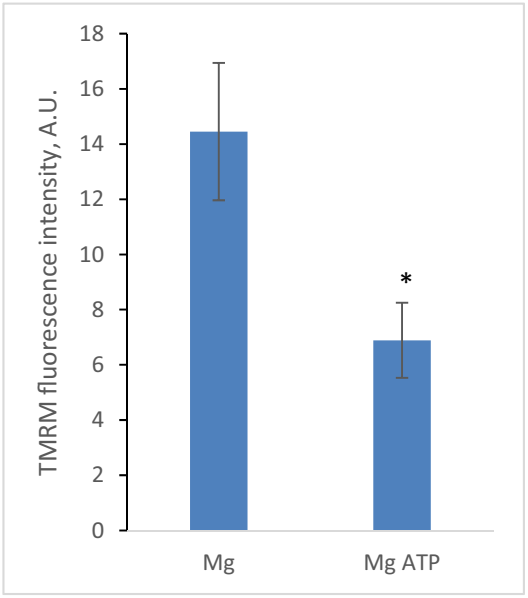


Fig. 5. Flow cytometry analyses of mitochondria membrane polarization upon incubation in Mg^{2+}- (a) and Mg^{2+},ATP-medium (b): blue graph – TMRM loaded mitochondria; red graph - TMRM loaded mitochondria +10 μM CCCP. The results of a typical experiment are presented ($n = 4$); c) CCCP-sensitive component of TMRM fluorescence intensity. Means±SEM, n = 4, * - $P < 0.05$ (as compared to Mg^{2+}-medium). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mitochondrial membrane potential after 5 min of incubation may be the result of ATP-induced hyperpolarization at the beginning of incubation.

All of the above suggests that the mitochondrial permeability transition pore is not involved in the studied ATP effects. Nevertheless, permeability transition in mitochondria can be triggered also by the formation of transient, CsA-insensitive pores (PA-mPT pores), which are fundamentally different from the classical Ca^{2+}-induced CsA-sensitive mPTP [22]. The accumulation of Ca^{2+} in the mitochondrial matrix results in the activation of PLAs and raises the content of free fatty acids in the mitochondrial inner membrane. Then, the anions of long-chain saturated fatty acids form complexes with Ca^{2+} on the matrix side of the inner mitochondrial membrane and when their content reaches a certain threshold, they segregate into solid domains, which is accompanied by the formation of lipid pores [22]. However, the identities and role of lipid regulators remained elusive until recently [23].

Phospholipids are the main building blocks of mitochondrial membranes. Mitochondrial membranes are made up of different phospholipids: phosphatidylcholine, phosphatidylethanolamine, phosphati dylinositol, phosphatidylserine, phosphatidic acid, and CL. CLs are specific phospholipids of the mitochondria comprising about 20 % of the inner mitochondria membrane phospholipids mass [24]. CL plays a pivotal role in multiple mitochondrial bioenergetic processes, including respiration and energy conversion, in mitochondrial morphology and dynamics as well as in several steps of the apoptotic process [25] [26] [27] [28]. Ca^{2+}-overload contributes to the oxidation of mitochondrial membrane lipids and associated events such as the permeability transition pore opening [29].

Using flow cytometry and thin layer chromatography it was shown that ATP induced decrease of CL content in the mitochondria membranes. Furthermore, we have observed the decreasing of PC content and increasing of LPC content in mitochondria under the incubation in Mg^{2+},ATP-medium. This indicates Ca^{2+} – induced activation of phospholipases A. Thus, the decrease in CL content may also be due to Ca^{2+}-induced activation of PLA. Phospholipases type A (PLA1 and PLA2) are known to hydrolyze acyl residues of phospholipids in the sn-1 and sn-2 position, which leads to an increase in free fatty acids content. Under the high $[Ca^{2+}]_m$ in the mitochondrial matrix free long-chain saturated fatty acids can cause the lipid pores formation. A decrease in the content of CL and an increase in the content of lysophospholipids, along with the above data, give us grounds to assume the participation of the lipid pore

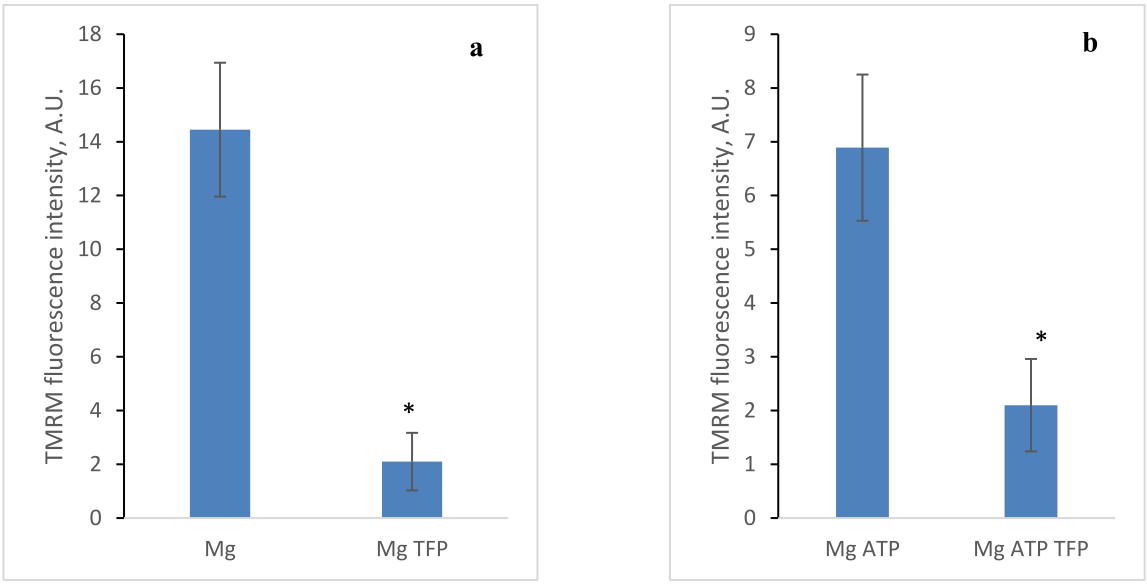


Fig. 6. 50 μM TFP effects on CCCP-sensitive component of TMRM fluorescence intensity upon mitochondria incubation in Mg^{2+}- (a) and Mg^{2+},ATP-medium (b). Means ± SEM, n = 4, * - $P < 0.05$ (as compared to control without TFP).

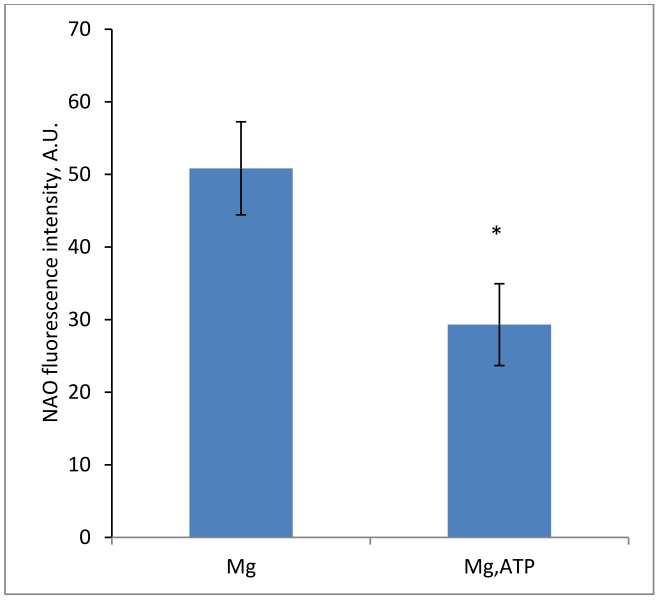


Fig. 7. The content of CL in mitochondria membranes upon incubation of organelles in Mg^{2+}- and Mg^{2+},ATP-medium. Means ± SEM, $n = 5$, * - $P < 0.05$ (as compared to Mg^{2+}-medium).

in the ATP effects on the Ca^{2+} exchange in mitochondria.

Adenosine 5′-triphosphate (ATP), the universal energy currency, sustains all cellular functions and responses, and at the same time is also an extracellular messenger involved in cell-to-cell communication in virtually every tissue [2] [3]. Virtually all cells are equipped with plasma membrane receptors for extracellular ATP, the P2 receptors (P2Rs) [4] [3]. However, 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 [3]. The only physiological agonist of the P2X7R is ATP [3]. The discovery that the P2X7R is present on the mitochondria adds further complexity to the intracellular physiology of P2X7R [3]. It should be mentioned also that there are some major differences in metabolism in smooth muscle compared to other cell types [30]. One of the most striking is the degree

Table
The content of phospholipids in mitochondria after 5 min incubation of organelles in Mg^{2+}- and Mg^{2+},ATP-medium. Means ± SEM, $n = 3$, * - $P < 0.05$ (as compared to Mg^{2+}-medium).

	Mg^{2+}-medium	Mg^{2+}, ATP-medium
	μg Pi/mg lipids	
Phosphatidylcholine (PC)	5,62 ± 0,29	4,37 ± 0,10*↓
Phosphatidylethanolamine (PE)	4,08 ± 0,36	4,27 ± 0,05
Cardiolipin (CL) or Diphosphatidylglycerol (DPG)	1,54 ± 0,06	0,72 ± 0,29*↓
Sphingomyelin (SM)	1,59 ± 0,36	0,55 ± 0,07*↓
Phosphatidylinositol (PI)	1,54 ± 0,12	1,13 ± 0,03*↓
Phosphatidylserine (PS)	1,44 ± 0,31	0,77 ± 0,102
Lysophosphatidylcholine (LPC)	0,27 ± 0,05	0,67 ± 0,16*↑
Start line (Start)	0,17 ± 0,09	0,07 ± 0,04

of metabolic compartmentalization. Oxidative phosphorylation directly supports contractile activity, while ionic regulation is supported by the ATP generated from anaerobic metabolism [5] [30].

The results obtained, as well as literature data, suggest that in the case of uterine smooth muscles, where glycolysis plays a significant role, glycolytically derived ATP can play the role of a signaling molecule that regulates the exchange of Ca^{2+} ions in mitochondria.

5. Conclusions

1. ATP induced >3 times increase of $[Ca^{2+}]_m$ in the absence of exogenous Ca^{2+}.
2. An increase of $[Ca^{2+}]_m$ is followed by an increase of the Ca^{2+} efflux from mitochondria. Ca^{2+} concentration in the Mg^{2+},ATP-medium is approximately 19 times higher than in the Mg^{2+}-medium. An increase of the EGTA concentration was accompanied by a sharp decrease of the Ca^{2+} concentration in the incubation medium.
3. Cyclosporine A, ruthenium red or oligomycin did not affect either the Ca^{2+} concentration in the matrix or the cation efflux from mitochondria.
4. The polarization of mitochondrial membranes is preserved in both Mg^{2+}- and Mg^{2+},ATP-medium. This conclusion is indirectly confirmed by the depolarizing effect of TFP.
5. ATP induced decrease of CL content and profound changes in the phospholipid composition of mitochondria membranes.

We hypothesized the possible involvement of the lipid pore in the ATP effects on the Ca^{2+} exchange in mitochondria. Taken together, our data support the critical role of extramitochondrial ATP in $[Ca^{2+}]_m$, $[Ca^{2+}]_o$ and cardiolipin content regulation.

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CRediT authorship contribution statement

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Declaration of competing interest

Authors declare no conflict of interest.

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