EXPERIMENTAL WORKS

UDC 577.352.4

doi: https://doi.org/10.15407/ubj89.03.017

Ca^{2+}-DEPENDENT REGULATION OF THE Ca^{2+} CONCENTRATION IN THE MYOMETRIUM MITOCHONDRIA. II. Ca^{2+} EFFECTS ON MITOCHONDRIA MEMBRANES POLARIZATION AND $[Ca^{2+}]_m$

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It is known that Ca^{2+} accumulation in the mitochondria undergoes complex regulation by Ca^{2+} itself. But the mechanisms of such regulation are still discussed. In this paper we have shown that Ca ions directly or indirectly regulate the level of myometrium mitochondria membranes polarization. The additions of 100 µM Ca^{2+} were accompanied by depolarization of the mitochondria membranes. The following experiments were designed to study the impact of Ca^{2+} on the myometrium mitochondria $[Ca^{2+}]_m$. Isolated myometrium mitochondria were preincubated without or with 10 µM Ca^{2+} followed by 100 µM Ca^{2+} addition. Experiments were conducted in three mediums: without ATP and Mg^{2+} (0-medium), in the presence of 3 mM Mg^{2+} (Mg-medium) and 3 mM Mg^{2+} + 3 mM ATP (Mg,ATP-medium). It was shown that the effects of 10 µM Ca^{2+} addition were different in different mediums, namely in 0- and Mg-medium the $[Ca^{2+}]_m$ values increased, whereas in Mg,ATP-medium statistically reliable changes were not registered. Preincubation of mitochondria with 10 µM Ca^{2+} did not affect the $[Ca^{2+}]_m$ value after the addition of 100 µM Ca^{2+}. The $[Ca^{2+}]$m values after 100 µM Ca^{2+} addition were the same in 0- and Mg,ATP-mediums and somewhat lower in Mg-medium. Preliminary incubation of mitochondria with 10 µM Ca^{2+} in 0- and Mg-mediums reduced changes of Fluo 4 normalized fluorescence values that were induced by 100 µM Ca^{2+} additions, but in Mg,ATP-medium such differences were not recorded. It is concluded that Ca^{2+} exchange in myometrium mitochondria is regulated by the concentration of Ca ions as in the external medium, so in the matrix of mitochondria. The medium composition had a significant impact on the $[Ca^{2+}]_m$ values in the absence of exogenous cation. It is suggested that light increase of $[Ca^{2+}]_m$ before the addition of 100 µM Ca^{2+} may have a positive effect on the functional activity of the mitochondria.

K e y w o r d s: myometrium, mitochondria, $[Ca^{2+}]_m$, Mg^{2+}, ATP.

It is well known that Ca^{2+} activates several key enzymes in the mitochondrial matrix to enhance ATP production [1, 2]. So Ca^{2+} accumulation in mitochondria is a vital process for functional activity of these organelles [3-6]. At the same time Moreau et al. reveal that the process of Ca^{2+} accumulation undergoes complex regulation by Ca^{2+} itself [7, 8]. We have shown previously that calmodulin antagonists caused depolarization of mitochondrial membranes and an increase of the ionized Ca concentration in both the mitochondrial matrix and the cell cytoplasm [9]. At the first part of this paper we have shown the concentration-dependent influence of calmodulin antagonist trifluoperazine on the level of mitochondrial membranes polarization with $K_{0.5}$ 24.4 ± 5.0 µM and the Hill coefficient 2.0 ± 0.2 [10]. But it was also shown that preincubation of isolated mitochondria in mediums of different composition with 25 µM trifluoperazine did not affect the $[Ca^{2+}]_m$ values both before and after the addition of 100 µM Ca^{2+}. This paper was aimed to study the effects of Ca^{2+} on myometrium mitochondria membrane polarization and Ca^{2+} concentration ($[Ca^{2+}]_m$) in these organelles.

Materials and Methods

All manipulations with animals were carried out according to European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and law of Ukraine "On protection of animals from cruelty". 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. The uterus was eliminated rapidly. All procedures were executed separately from other rats.

Myometrium mitochondria of a nonregnant rat was isolated using differential centrifugation method [11]. The obtained preparation was suspended in a solution (at 4 °C) with the following composition (mM): sucrose – 250, EGTA – 1, Hepes – 20; pH 7.4. 0.1% bovine serum albumin fatty acid free was also added. Protein concentration of the mitochondria fraction was determined by Bradford assay [12].

Polarization of mitochondria membranes were investigated using potential sensitive probe 1 µM TMRM (tetramethylrhodamine-methyl-ester, λ_{exc} = 540 nm, λ_{em} = 590 nm) and the QuantaMaster™ 40 spectrofluorometer (Photon Technology International). The studies were carried out in a medium containing (mM): Hepes – 20, sucrose – 250, succinate – 5, K-phosphate buffer – 0.1, $MgCl_2$ – 0.5; pH 7.4. The concentration of mitochondrial protein in the sample was 25 µg/ml.

Changes in Ca^{2+} concentration in the mitochondria from the rat myometrium were investigated using the QuantaMasterTM 40 spectrofluorometer (Photon Technology International) and the fluorescent probe Fluo 4AM (λ_{exc} = 490 nm, λ_{em} = 520 nm). Myometrium mitochondria were loaded with 2 µM Fluo 4AM for 30 min at 37 °C in a medium with the following composition (mM): sucrose – 250, EGTA – 1, Hepes – 20, pH 7.4 in the presence of 0.1% BSA (fatty acid free). Thereafter, the suspension of mitochondria was diluted (1:10) by the same medium containing no fluorescence probe followed by centrifugation. The pellet was resuspended in the same medium containing no fluorescence probe. The studies were carried out in a medium containing (mM): sucrose – 250, K^+-phospate buffer – 2, sodium succinate – 5, ±$MgCl_2$ – 3, ±ATP – 3, ±$CaCl_2$ – 0.1, Hepes – 20; pH 7.4. The concentration of mitochondrial protein in the sample was 25 µg/ml. The testing of each sample was completed by adding 0.1% Triton X-100 and, in 1 min, 5 mM EGTA (fluorescence intensities F_{max} and F_{min}, respectively). The concentration of Ca^{2+} in the mitochondria matrix was calculated using the Grynkiewicz equation [13].

The statistical methods used in this study and the software for statistical processing can be found on http://graphpad.com/.

In the study the following reagents were used: EGTA, Hepes, BSA fatty acid free, protonophore CCCP, D(+)-sucrose, ATP, (Sigma, USA), Ca^{2+}-sensitive probe Fluo 4AM, potential-sensitive probe TMRM (Invitrogen, USA) and other chemicals of domestic production of analytical or reagent grades.

Results and Discussion

Polarization of myometrium mitochondria membranes was studied using quench mode and potential sensitive probe 1 µM TMRM. The quench mode for dye fluorescence is a sensitive mean to detect rapid changes in $\Delta\Psi$ that occur during the experiment [14]. It was shown that mitochondria membranes were polarized, as evidenced by quenching of TMRM fluorescence. The addition of 100 µM Ca^{2+} was accompanied by the increase of TMRM fluorescence and restoration of the fluorescence level. It means that TMRM released from mitochondria so depolarization of the mitochondria membranes have been registered (Fig. 1). Protonophore CCCP addition to the medium (after the incubation with 100 µM Ca^{2+}) had not affected the level of dye fluorescence. It was concluded, that Ca ions directly or indirectly regulate the level of myometrium mitochondria membranes polarization.

It was shown that the process of Ca^{2+} accumulation in the mitochondria undergoes complex regulation by Ca^{2+} itself. Moreau and colleagues [7] in experiments on permeabilized RBL-1 cells had compared the rate and extent of the mitochondrial Ca^{2+} rise after stimulation with a high concentration of cytoplasmic Ca^{2+} (100 µM) with and without a preceding brief Ca^{2+} pulse. Whereas a robust mitochondrial Ca^{2+} rise was seen in response to 100 µM Ca^{2+}, the response was dramatically reduced after a 60 s pre-pulse of 10 µM Ca^{2+} and subsequent perfusion with Ca^{2+}-free solution for several minutes. It was concluded that Ca^{2+} uptake in mitochondria is a Ca^{2+}-activated process with a requirement for functional calmodulin. However, cytosolic Ca^{2+} subsequently inactivates the uniporter, preventing further Ca^{2+} uptake [7, 8]. Thus biphasic control of mitochondrial Ca^{2+} uptake by Ca^{2+} was shown in these experiments.

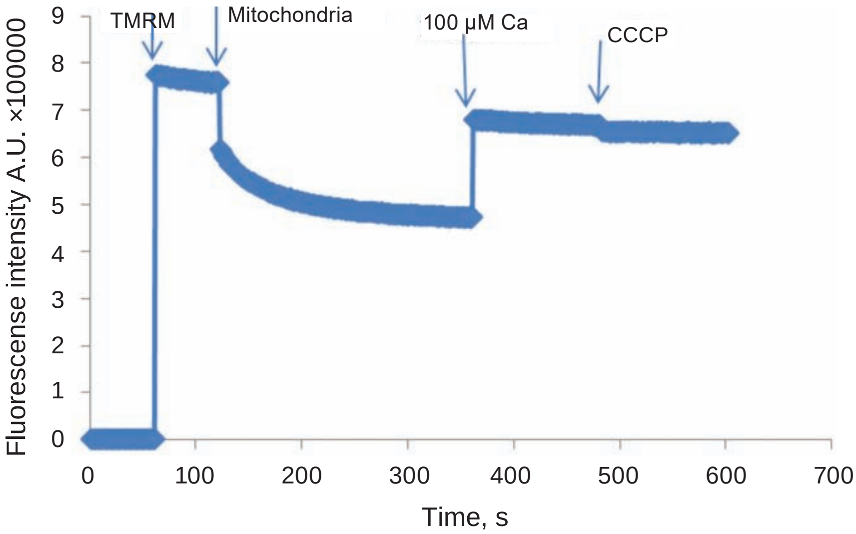

Fig. 1. Effect of 100 μM Ca^{2+} on the mitochondria membranes polarization. Fluorescent probe – 1 μM TMRM (tetramethylrhodamine-methyl-ester, λ_{ex} = 540 nm, λ_{em} = 590 nm), n = 3

Today still little is known about the mechanisms underlying regulation of myometrium mitochondria $[Ca^{2+}]_m$. The following experiments were designed to study the impact of Ca^{2+} on the myometrium mitochondria $[Ca^{2+}]_m$. Isolated myometrium mitochondria were preincubated (5 min) without or with 10 μM Ca^{2+} in three mediums: without ATP and Mg^{2+} (0-medium), in the presence of 3 mM Mg^{2+} (Mg-medium) and 3 mM Mg^{2+} + 3 mM ATP (Mg,ATP-medium). We have shown previously that the incubation of mitochondria in Mg,ATP-medium resulted in high level of total Ca^{2+} accumulation, id est to be functionally active, while in Mg-medium, the level of total Ca^{2+} accumulation was low, that mean subzero activity of organelles. Total Ca^{2+} accumulation was studied using $^{45}Ca^{2+}$ as radioactive tracer [15].

As can be seen from Fig. 2, effects of 10 μM Ca^{2+} addition were different in different mediums, namely in 0- and Mg-medium the $[Ca^{2+}]_m$ values increased, whereas in Mg,ATP-medium statistically reliable changes were not registered.

Next experiments were conducted to study the effect of mitochondria preincubation without or with 10 μM Ca^{2+} on the $[Ca^{2+}]_m$ values after 100 μM Ca^{2+} additions. As can be seen from the results shown in Fig. 3, the $[Ca^{2+}]_m$ values were the same in 0- and Mg,ATP-medium and somewhat lower in Mg-medium. Noteworthy is the fact that mitochondria preincubation with 10 μM Ca^{2+} did not affect the $[Ca^{2+}]_m$ values after 100 μM Ca^{2+} addition.

Thus, it was shown that: 1) the level of endogenous $[Ca^{2+}]_m$ in the myometrium mitochondria matrix was determined by the composition of the incubation medium; 2) preincubation of mitochondria with 10 μM Ca^{2+} did not affect the $[Ca^{2+}]_m$ values after the addition of 100 μM Ca^{2+}; 3) the $[Ca^{2+}]_m$ values after 100 μM Ca^{2+} addition did not depend on the incubation medium composition.

We have shown previously that changes of Fluo 4 normalized fluorescence in response to the exogenous Ca^{2+} addition can be used as a test of the mitochondrial functional activity: lower changes – higher functional activity (unpublished results). So let us take a look at the kinetic of Fluo 4 normali-

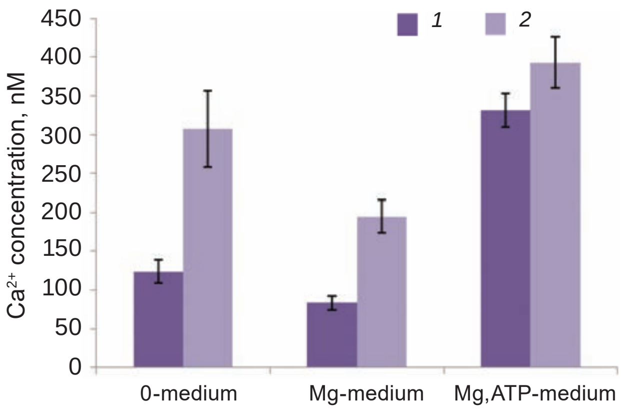

Fig. 2. $[Ca^{2+}]_m$ values at the 5 min incubation of mitochondria in different mediums with 0 (1) or 10 μM (2) Ca^{2+} (M ± m, n = 7)

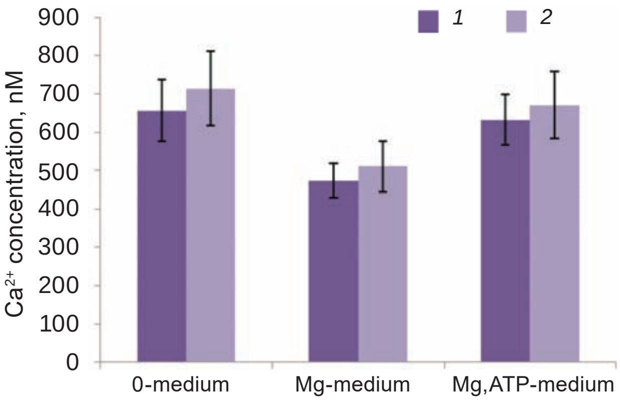

Fig. 3. $[Ca^{2+}]_m$ values at the addition of 100 μM Ca^{2+}. Mitochondria were preincubated (5 min) in mediums of different composition with 0 (1) or 10 μM (2) Ca^{2+}, (M ± m, n = 7)

zed fluorescence changes induced by the 100 μM Ca^{2+}addition in the case of different incubation mediums composition. As can be seen from the results shown in Fig. 4, the lowest changes were in the Mg,ATP-medium, the highest – in the Mg-medium.

Next question, what is happening with Fluo 4 normalized fluorescence changes in response to the 100 μM Ca^{2+} addition under the mitochondria preincubation with 10 μM Ca^{2+} in different mediums. As can be seen from the results presented in Fig. 5, incubation of myometrium mitochondria in 0-medium was accompanied by significant (up to 2.4 arbitrary units) increase of Fluo 4 normalized fluorescence in response to the 100 μM Ca^{2+} addition (Fig. 5, curve *1*). Mitochondria preincubation with 10 μM Ca^{2+} in 0-medium leads to a decrease of Fluo 4 normalized fluorescence changes in response to the 100 μM Ca^{2+} addition (Fig. 5, curve *2*).

Ca^{2+}-induced changes of Fluo 4 normalized fluorescence at mitochondria incubation in Mg-medium are represented on Fig. 6. As one can see, incubation of myometrium mitochondria in Mg-medium was accompanied by a significant (up to 2.5 arbitrary units) increase of Fluo 4 normalized fluorescence changes in response to the 100 μM Ca^{2+} addition (Fig. 6, curve *1*). Mitochondria preincubation with 10 μM Ca^{2+} in Mg-medium leads to a decrease of Fluo 4 normalized fluorescence changes in response to the 100 μM Ca^{2+} addition (Fig. 6, curve *2*).

Ca^{2+}-induced changes of Fluo 4 normalized fluorescence at mitochondria incubation in Mg,ATP-medium are represented on Fig. 7. Incubation of myometrium mitochondria in Mg,ATP-medium was accompanied by small (up to 0.3 arbitrary units) increase of Fluo 4 normalized fluorescence changes in response to the 100 μM Ca^{2+} addition (Fig. 7, curve *1*). Mitochondria preincubation with 10 μM Ca^{2+} in Mg,ATP-medium did not cause any changes of Fluo 4 normalized fluorescence in response to the 100 μM Ca^{2+} addition (Fig. 7, curve *2*).

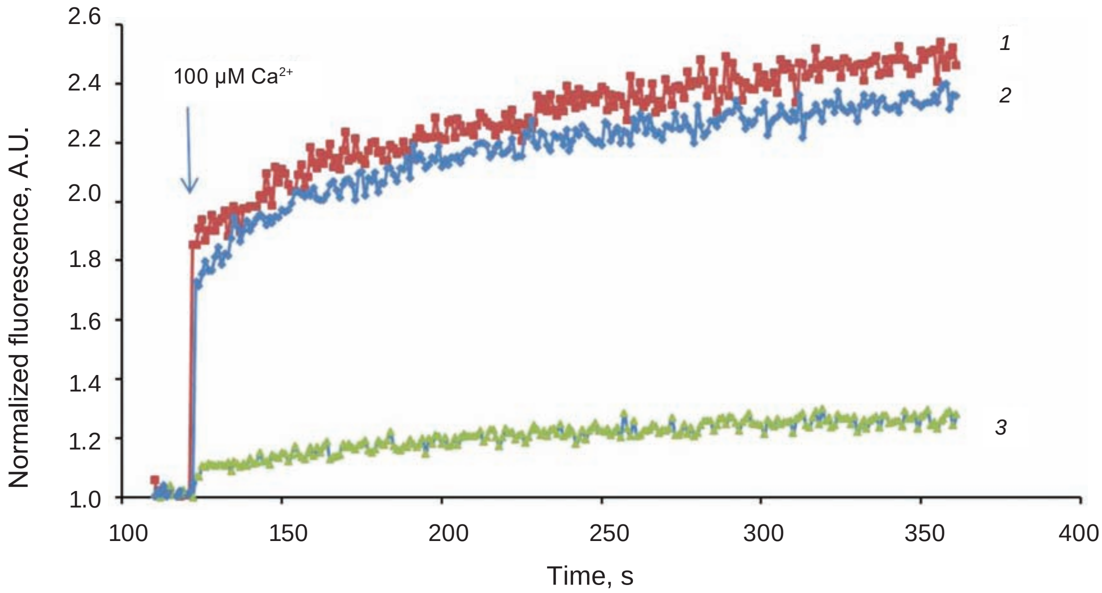

Fig. 4. Ca^{2+}-induced changes of Fluo 4 normalized fluorescence at mitochondria incubation in three incubation mediums: 1 – Mg-medium (in the presence of 3 mM Mg), 2 – 0-medium (without ATP and Mg^{2+}) and 3 – Mg,ATP-medium (in the presence of 3 mM Mg^{2+} + 3 mM ATP). 100 μM Ca^{2+} additions were made at the times indicated by the arrow. This figure represent a typical result, n = 7

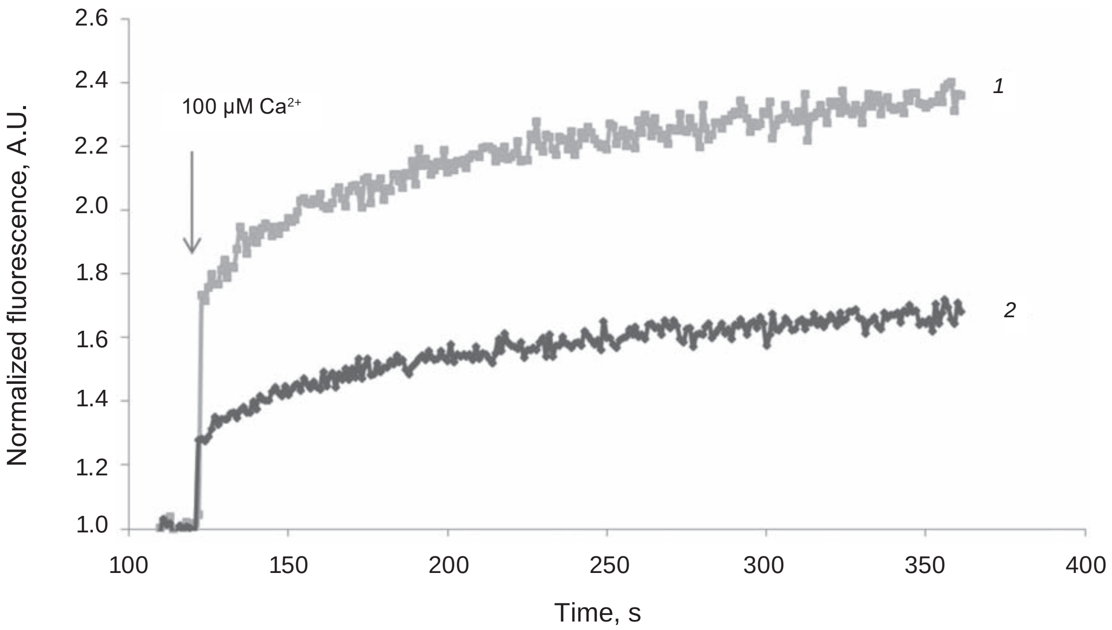

Fig. 5. Ca^{2+}-induced changes of Fluo 4 normalized fluorescence at mitochondria incubation in 0-medium without (curve 1) or with 10 μM Ca^{2+} (curve 2). The additions of 100 μM Ca^{2+} were made at the times indicated by the arrow. This figure represent a typical result, n = 7

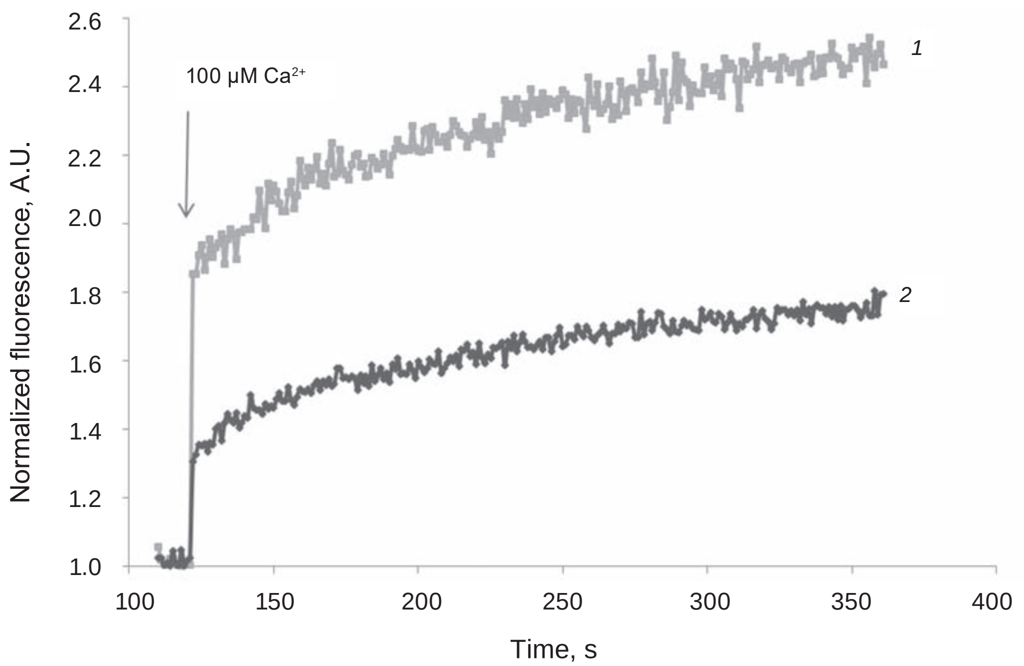

Fig. 6. Ca^{2+}-induced changes of Fluo 4 normalized fluorescence at mitochondria incubation in Mg-medium without (curve 1) or with 10 μM Ca^{2+} (curve 2). Additions of 100 μM Ca^{2+} were made at the times indicated by the arrow. This figure represent a typical result, n = 7

Thus, it was shown that preliminary incubation of mitochondria with 10 μM Ca^{2+} in 0- and Mg-mediums reduced changes of Fluo 4 normalized fluorescence values that were induced by 100 μM Ca^{2+} additions, but in Mg,ATP-medium such differences were not recorded.

Therefore, Ca^{2+} exchange in myometrium mitochondria is regulated by the concentration of Ca ions

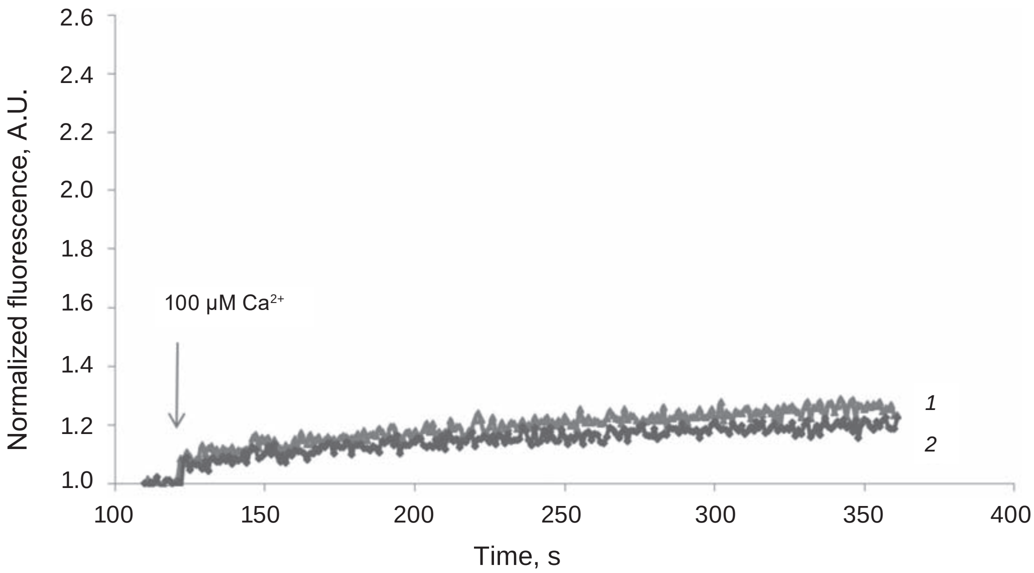

Fig. 7. Ca^{2+}-induced changes of Fluo 4 normalized fluorescence at mitochondria incubation in Mg,ATP-medium without (curve 1) or with 10 µM Ca^{2+} (curve 2). Additions of 100 µM Ca^{2+} were made at the times indicated by the arrow. This figure represent a typical result, n = 7

as in the external medium, so in the matrix of mitochondria. The medium composition had a significant impact on the $[Ca^{2+}]_m$ values in the absence of exogenous cation. It is suggested that light increase of $[Ca^{2+}]_m$ before the addition of 100 µM Ca^{2+} may have a positive effect on the functional activity of the mitochondria.

Ca^{2+}-ЗАЛЕЖНА РЕГУЛЯЦІЯ КОНЦЕНТРАЦІЇ Ca^{2+} В МІТОХОНДРІЯХ МІОМЕТРІЯ. II. ВПЛИВ Ca^{2+} НА ПОЛЯРИЗАЦІЮ МЕМБРАН МІТОХОНДРІЙ ТА $[Ca^{2+}]_m$

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Відомо, що іони Са регулюють акумуляцію їх у мітохондріях. Проте механізм цього явища й досі є предметом дискусій. У цій роботі ми показали, що іони Са безпосередньо чи опосередковано регулюють рівень поляризації мембран мітохондрій міометрія. Зокрема, внесення 100 мкМ Ca^{2+} до середовища інкубації супроводжується деполяризацією мітохондріальних мембран. Також було досліджено вплив Ca^{2+} на $[Ca^{2+}]_m$. Ізольовані мітохондрії міометрія попередньо інкубували за відсутності або у присутності 10 мкМ Ca^{2+}, після чого в інкубаційне середовище додавали 100 мкМ Ca^{2+}. Досліди проводили в трьох середовищах, а саме, без АТР та Mg^{2+} (0-середовище), у присутності 3 мМ Mg^{2+} (Mg-середовище) та 3 мМ Mg^{2+} + 3 мМ АТР (Mg,АТР-середовище). Показано, що ефект 10 мкМ Ca^{2+} був різним за різних умов, а саме, у 0- та Mg-середовищі значення $[Ca^{2+}]_m$ збільшувались, тоді як у Mg,АТР-середовищі статистично вірогідні зміни не було зареєстровано. Попередня інкубація мітохондрій з 10 мкМ Ca^{2+} не впливала на значення $[Ca^{2+}]_m$ після внесення в середовище інкубації 100 мкМ Ca^{2+}. Значення $[Ca^{2+}]_m$ після внесення 100 мкМ Ca^{2+} були однаковими за інкубації мітохондрій в 0- та Mg,АТР-середовищі та дещо менше у Mg-середовищі. Також встановлено, що попередня інкубація мітохондрій з 10 мкМ Ca^{2+} у 0- та Mg-середовищах зменшувала індуковані додаванням 100 мкМ Ca^{2+} зміни величини нормованої флуоресценції Fluo, проте у Mg,АТР-середовищі такі зміни зареєстровано не було. Дійшли висновку про те, що обмін Ca^{2+} в мітохондріях міометрія регулюється його концентрацією як у зовнішньому середовищі, так і в матриксі. Склад середовища інкубації має істотний вплив на $[Ca^{2+}]_m$ за відсутності катіона в зовнішньому середовищі. Припускається, що незначне збільшення $[Ca^{2+}]_m$ перед додаванням

100 мкМ Ca^{2+} може позитивно позначитись на функціональній активності мітохондрій.

Ключові слова: міометрій, мітохондрії, $[Ca^{2+}]_m$, Mg^{2+}, ATP.

Ca^{2+}-ЗАВИСИМАЯ РЕГУЛЯЦИЯ КОНЦЕНТРАЦИИ Ca^{2+} В МИТОХОНДРИЯХ МИОМЕТРИЯ. II. ВЛИЯНИЕ Ca^{2+} НА ПОЛЯРИЗАЦИЮ МЕМБРАН МИТОХОНДРИЙ И $[Ca^{2+}]_m$

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Известно, что ионы Са регулируют аккумуляцию их в митохондриях. Однако механизм этого явления и сегодня обсуждается. В этой работе мы показали, что ионы Са прямо или опосредовано регулируют уровень поляризации мембран митохондрий миометрия. Так, внесение 100 мкМ Ca^{2+} в среду инкубации сопровождалось деполяризацией митохондриальных мембран. Исследовалось также влияние Ca^{2+} на $[Ca^{2+}]_m$. Изолированные митохондрии миометрия предварительно инкубировали в отсутствие или в присутствии 10 мкМ Ca^{2+}, после чего в инкубационную среду вносили 100 мкМ Ca^{2+}. Опыты проводили в средах разного состава, а именно: без АТР и Mg^{2+} (0-среда), в присутствии 3 мМ Mg^{2+} (Mg-среда) и 3 мМ Mg^{2+} + 3 мМ АТР (Mg,АТР-среда). Показано, что эффект 10 мкМ Ca^{2+} был разным в различных условиях: в 0- и Mg-средах значения $[Ca^{2+}]_m$ увеличивались, тогда как в Mg,АТР-среде статистически достоверные изменения не были зарегистрированы. Предварительная инкубация митохондрий с 10 мкМ Ca^{2+} не повлияла на значения $[Ca^{2+}]_m$ после внесения в среду инкубации100 мкМ Ca^{2+}. Значения $[Ca^{2+}]_m$ после внесения 100 мкМ Ca^{2+} были одинаковыми, при условии, что митохондрии инкубировали в 0- и Mg,АТР-средах и несколько ниже в Mg-среде. Показано, что предварительная инкубация митохондрий с 10 мкМ Ca^{2+} в 0- и Mg-средах снижала индуцированное добавлением 100 мкМ Ca^{2+} изменение величины нормированной флуоресценции Fluo, однако в Mg,АТР-среде такие отличия не регистрировались. Сделан вывод о том, что обмен Ca^{2+} в митохондриях миометрия регулируется его концентрацией как во внешней среде, так и в матриксе. Состав среды инкубации существенно влияет на $[Ca^{2+}]_m$ при отсутствии катиона во внешней среде. Предполагается, что небольшое увеличение $[Ca^{2+}]_m$ перед внесением 100 мкМ Ca^{2+} может позитивно сказаться на функциональной активности митохондрий.

Ключевые слова: миометрий, митохондрии, $[Ca^{2+}]_m$, Mg^{2+}, ATP.

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Received 01.02.2017