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CALMODULIN ANTAGONISTS EFFECT ON Ca^{2+} LEVEL IN THE MITOCHONDRIA AND CYTOPLASM OF MYOMETRIUM CELLS

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It is known that Ca^{2+}-dependent regulation of this cation exchange in mitochondria is carried out with participation of calmodulin. We had shown in a previous work using two experimental models: isolated mitochondria and intact myometrium cells, that calmodulin antagonists reduce the level of mitochondrial membrane polarization. The aim of this work was to investigate the influence of calmodulin antagonists on the level of ionized Ca in mitochondria and cytoplasm of uterine smooth muscle cells using spectrofluorometry and confocal microscopy. It was shown that myometrium mitochondria, in the presence of ATP and $MgCl_2$ in the incubation medium, accumulate Ca ions in the matrix. Incubation of mitochondria in the presence of CCCP inhibited cation accumulation, but did not cease it. Calmodulin antagonist such as trifluoperazine (100 μM) considerably increased the level of ionized Ca in the mitochondrial matrix. Preliminary incubation of mitochondria with 100 μM Ca^{2+}, before adding trifluoperazine to the incubation medium, partly prevented influence of the latter on the cation level in the matrix. Incubation of myometrium cells (primary culture) with another calmodulin antagonist calmidazolium (10 μM) was accompanied by depolarization of mitochondrial membrane and an increase in the concentration of ionized Ca in cytoplasm. Thus, using two models, namely, isolated mitochondria and intact myometrium cells, it has been shown that calmodulin antagonists cause depolarization of mitochondrial membranes and an increase of the ionized Ca concentration in both the mitochondrial matrix and the cell cytoplasm.

K e y w o r d s: isolated mitochondria, primary culture of myometrium cells, Ca^{2+}, mitochondrial membrane potential, calmodulin antagonists, smooth muscle.

Mitochondria play a fundamental role in the control of intracellular processes particularly in providing calcium signaling [1-3]. Ca^{2+} is a universal regulatory ion that exerts its effect via binding to proteins, in particular calmodulin (CaM). CaM belongs to the superfamily of Ca^{2+}-binding proteins that contain the same Ca^{2+}-binding structural domain, so-called EF-hand [4]. Ca^{2+}-CaM complex activates a series of enzymes and ion-transporting proteins. Some enzymes contain EF-hand motif directly in their structure [5]. It has been found that some mitochondria channels and transporters contain EF hands in their structure or are activated by the binding to Ca^{2+}-CaM complex [5]. First of all it refers to the mitochondrial calcium uniporter, K^+/H^+/Ca^{2+} exchangers, glutamate/aspartate transporter and others [5]. However, a lot of questions in this area still remain unresolved. Calmodulin-dependent regulation of Ca-ions metabolism in the myometrium mitochondria has been little studied. In a previous work, we had shown that the incubation of myometrium mitochondria with calmodulin antagonists such as calmidazolium (10 μM) or trifluoperazine (100 μM) caused a mitochondria membrane depolarization [6, 7]. The aim of this work was to investigate the effect of calmodulin antagonists on the ionized calcium level in the mitochondria and in the cytoplasm of uterine smooth muscle cells by using spectrofluorometry and confocal microscopy.

Materials and Methods

Mitochondria were isolated from myometrium of nonpregnant rat using differential centrifugation [8]. The obtained preparation was suspended in a solution with the following composition: 10 mM HEPES (pH 7.4), 250 mM sucrose, 1 mM EGTA, 0.1% bovine serum albumin; at 4 °C.

Protein concentration in the mitochondria fraction was determined by Bradford assay [9]. The concentration of mitochondrial protein in the sample was 25 μg/ml.

Changes in the ionized calcium concentration in the mitochondria from rat myometrium were investigated using the QuantaMasterTM 40 spectrofluorometer (Photon Technology International) and the fluorescent probe Fluo-4 AM ($\lambda_{exc.}$ = 490 nm, $\lambda_{em.}$ = 520 nm) in a medium with the following composition: 20 mM HEPES (pH 7.4), 250 mM sucrose, 2 mM P_i (K^+-phospate buffer, pH 7.4), 5 mM sodium succinate, 3 mM $MgCl_2$, 3 mM ATP. 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 ionized Ca in the matrix was calculated using the Grynkiewicz equation [10].

The suspension of myometrium cells from non-pregnant rat was obtained by treating the tissue with collagenase 1a. Cell counting was performed using hemocytometer. Cell viability was determined using trypan blue dye. It was observed, that more than 95% of the cells had green colour in a visible light that indicate the integrity of the plasma membrane.

Primary myometrium cell culture was grown in the DMEM medium with 10% FBS to 4 passages. Culture medium was changed daily. Cells used in the experiments were removed from Petri dishes by a solution of 0.05% trypsin + 0.5 mM EDTA, then washed from the latter and transferred to confocal microscope chambers.

Previously, conditions for attaching of myocytes to the flow chamber for confocal microscopy were developed [7]. Attached to the chamber surface myocytes were loaded with Ca^{2+}-sensitive probe: 1 μM Fluo-4 AM ($\lambda_{exc.}$ = 490 nm, $\lambda_{em.}$ = 520 nm) and potential-sensitive probe: 100 nM TMRM (tetramethylrhodamine-methyl-ester, $\lambda_{exc.}$ = 540 nm, $\lambda_{em.}$ = 590 nm). Fluo-4 AM loaded for 30 min at 37 °C thereafter, the incubation medium was changed to medium containing no probe. The cells were washed from the probe, remaining outside the cells, three times. TMRM loaded for 3 min just before the experiment. The studies were carried out in a medium containing 10 mM HEPES (pH 7.4), 136.9 mM NaCl, 5.36 mM KCl, 4.5 mM $NaHCO_3$, 5.5 mM glucose, 0.26 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 0.4 mM $MgCl_2$, 0.4 mM $MgSO_4$. Registration of alterations of the fluorescent probe signals was performed using a confocal laser scanning microscope LSM 510 META Carl Zeiss with a modern system of image processing on the principle of spectral separation, on the basis of universal motorized inverted fluorescence microscope Axiovert 200M. The studies were performed on the MultiTrack platform using Plan-Apochromat lens 63x/1.4 Oil DIC. The fluorescence of TMRM probe was recorded at wavelengths above 560 nm using an emission filter LP 560 and the obtained image was stained red. The fluorescence of Fluo-4 AM was recorded at wavelength range 505-545 nm (BP 505-570, NFT 545) and the obtained image was stained green.

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

In the study the following reagents were used: collagenase 1a, trypan blue, EGTA, HEPES, BSA fatty acid free, oxytocin, trifluoperazine, calmidazolium, protonophore CCCP, D(+)-sucrose, ATP, oligomycin, trypsin-EDTA (Sigma, USA), DMEM, PBS (pH 7.2; Ca^{2+}, Mg^{2+} and pyruvate free), FBS, penicillin/streptomycin solution (Gibco, USA), Ca^{2+}-sensitive probe Fluo-4 AM, potential-sensitive probe TMRM (Invitrogen, USA) and other chemicals of domestic production of analytical or reagent grades.

Results and Discussion

The study of the changes in the ionized calcium concentration in the myometrium mitochondria under the actions of calmodulin antagonists. This series of experiments was performed on isolated myometrium mitochondria. It was shown that the ionized calcium concentration in the mitochondrial matrix, under the conditions of organelles having been incubated for 5 min in a medium as described above, was 257 ± 60 nM (Fig. 1). Introduction of 100 μM Ca^{2+} to the incubation medium was accompanied by an increase in the cation level in the mitochondria and it was 514 ± 117 nM at the 3rd min of incubation (Fig. 1). Preliminary incubation of mitochondria for 5 min in the presence of 10 μM CCCP was accompanied by partial reduction in the level of endogenous Ca^{2+} compared to the control. Following addition of 100 μM Ca^{2+} to the incubation medium was accompanied by cation accumulation in the matrix, however, its concentration was significantly less than in the control (without protonophores); the level of ionized calcium at the 3rd min of incubation was 309 ± 45 nM (Fig. 1).

In further experiments we studied the effect of 100 μM trifluoperazine on Ca^{2+} concentration in the myometrium mitochondrial matrix in the absence of

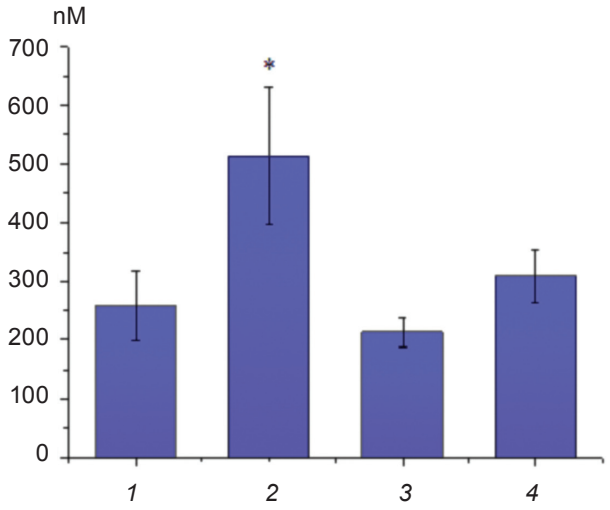

*Fig. 1. Concentration of Ca ions in myometrium mitochondria (M ± m, n = 6; P ≤ 0.05, *the difference relative to the control is statistically significant): 1 – at the 5th min of preliminary incubation in a standard medium – control; 2 – at the 3rd min after introduction of 100 μM Ca^{2+} to the control samples; 3 – at the 5th min of preliminary incubation of mitochondria in a standard medium in the presence of 10 μM CCCP; 4 – at the 3rd min after introduction of 100 μM Ca^{2+} to the samples, which incubated with 10 μM CCCP*

this cation in the incubation medium. As it is seen in Fig. 2, the preliminary incubation of mitochondria with 100 μM trifluoperazine for 5 min was accompanied by a significant increase in the level of ionized calcium in the matrix compared to control.

Next, we studied the level of ionized calcium in the mitochondrial matrix upon addition of 100 μM Ca to the incubation medium. Effect of trifluoperazine on Ca^{2+} level in the mitochondrial matrix depends on the order of introduction of an antagonist and Ca ions to the incubation medium. Thus, if mitochondria was preliminary incubated with 100μM Ca^{2+}, the following addition of 100 μM trifluoperazine to the medium was accompanied by an increase in the cation concentration in the matrix, however less significant than in case of preliminary organelle incubation with 100 μM trifluoperazine and subsequent addition of 100 μM Ca^{2+} into the incubation medium (Fig. 3).

Thus, myometrium mitochondria, in the presence of ATP and $MgCl_2$ in the incubation medium, accumulated Ca ions in the matrix. Preliminary incubation of mitochondria in the presence of CCCP inhibited the cation accumulation but did not

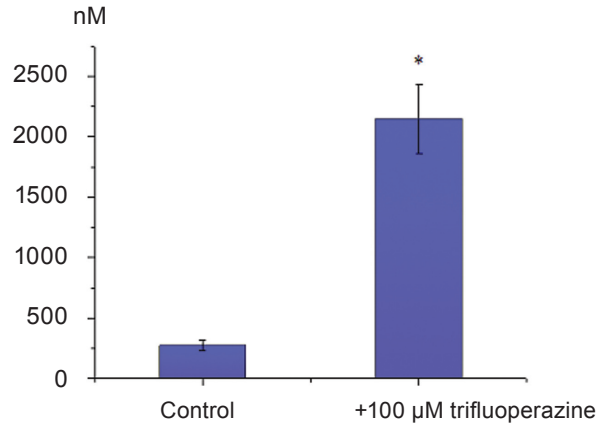

*Fig. 2. Concentration of ionized Ca in myometrium mitochondria under the action of trifluoperazine (M ± m, n = 6; P ≤ 0.05, *the difference relative to the control is statistically significant)*

cease it. Trifluoperazine significantly increased endogenous level of Ca^{2+} in the myometrium mitochondrial matrix. Preliminary incubation of mitochondria with 100 μM Ca^{2+} partially inhibited the effect of trifluoperazine on the cation level in the mitochondrial matrix.

Visualization of the impact of calmodulin antagonists on fluorescence of the calcium-sensitive probe Fluo-4 AM and potential-sensitive probe TMRM in myometrium cells. Further experiments were focused on the studies of the effect of calmodulin antagonists on the polarization of the mitochon-

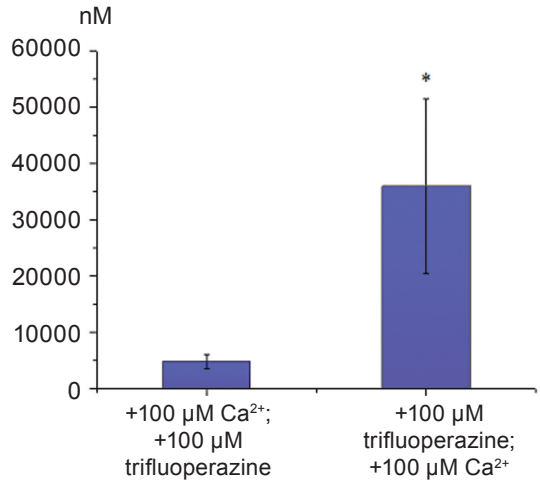

*Fig. 3. Concentration of ionized Ca in myometrium mitochondria under the action of trifluoperazine depending on the preliminary incubation conditions (M ± m; n = 6; P ≤ 0.05, *the difference relative to the control is statistically significant)*

drial membrane and the level of ionized Ca^{2+} in the cytoplasm of intact myometrium cells. The studies were performed using confocal microscope LSM-510 META and two fluorescent dyes namely, potential-sensitive probe TMRM, which is accumulated in polarized mitochondria and Ca^{2+}-sensitive probe Fluo-4 AM. The result of calmidazolium impact on the myometrial cells, which were simultaneously loaded with two fluorescent dyes, is shown in Fig. 4. At the beginning of the experiment (before adding calmidazolium) (Fig. 4, *1*), the basal Ca^{2+} concentration in the cytoplasm and the level of mitochondria polarization were measured. Incubation of the myometrium cells in the presence of 10 μM calmidazolium was accompanied by a decrease in TMRM fluorescence intensity and a simultaneous increase in Fluo-4 fluorescence intensity (Fig. 4). Decreasing of TMRM fluorescence intensity indicates its release from the mitochondria therefore, mitochondria depolarization occurs. Increased Fluo-4 fluorescence intensity indicates an increase in the ionized calcium concentration in the cell cytoplasm.

Plots of the fluorescence intensities of both probes with time on randomly selected part of cell (ROI) (marked by red on the confocal image) are shown in Fig. 5.

Thus, incubation of myometrium cells with 10 μM calmidazolium was accompanied by mitochondrial membrane depolarization and an increase in the ionized calcium concentration in the cytoplasm of myometrium cells.

Thus, using confocal microscopy, we have found that incubation of myometrium cells with calmodulin antagonists led to depolarization of the inner mitochondrial membrane. Interestingly, complete depolarization was preceded by a brief membrane hyperpolarization (Fig. 5). Literature data suggest that mitochondrial membrane hyperpolarization can be a necessary event for depolarization [11, 12].

Observed myometrium mitochondrial membrane depolarization should be accompanied by the release of Ca ions from these organelles. Indeed, an increase in the ionized calcium level in the cell cytoplasm under the influence of calmodulin antagonists which we showed on the intact myocytes is rather natural. However, the question arises: if calmodulin antagonists depolarize mitochondrial membrane how can the sharp increase in the level of ionized calcium in the organelle matrix, registered on the mitochondrial fraction, be explained? It is well known that calcium ion accumulation in mitochondria is provided mainly by Ca^{2+}-uniporter, whose activity de-

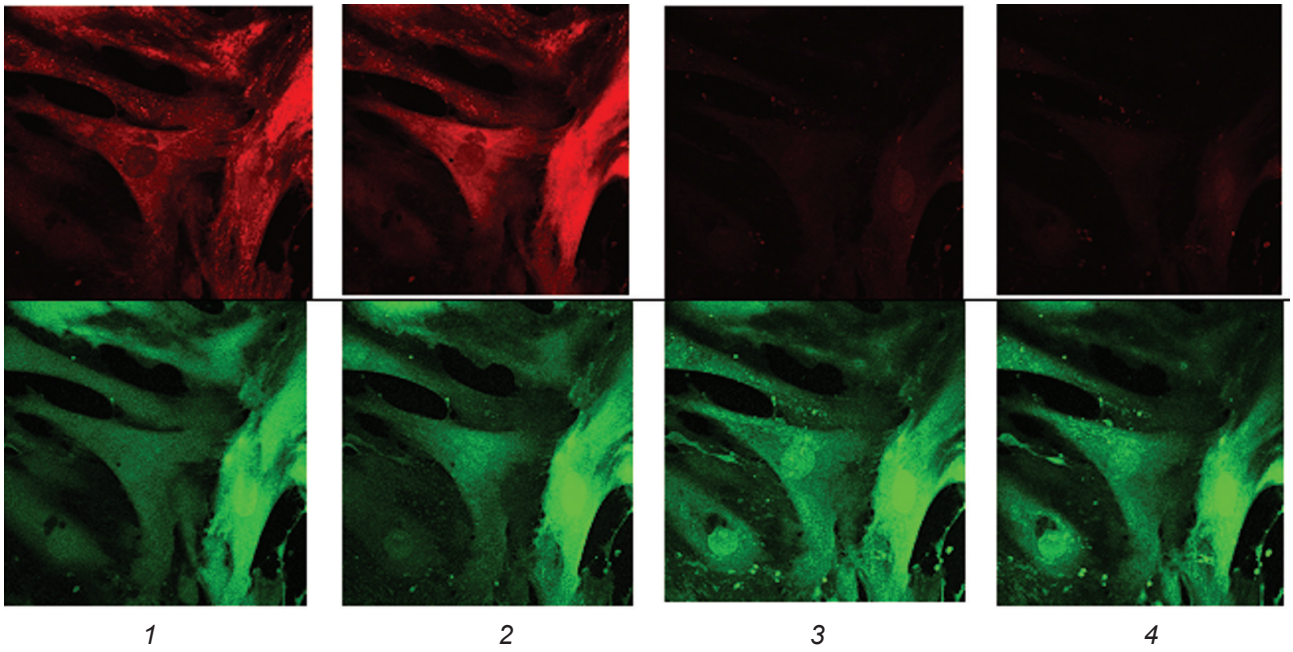

Fig. 4. Dynamics of alterations in the fluorescence intensity of potential-sensitive probe TMRM and Ca^{2+}-sensitive probe Fluo-4 AM in rat myometrium cells under the action of calmidazolium (10 μM): 1 – the beginning of the experiment; 2 – 2 sec after addition of calmidazolium; 3 – 60 sec after addition of calmidazolium; 4 – 170 sec after addition of calmidazolium

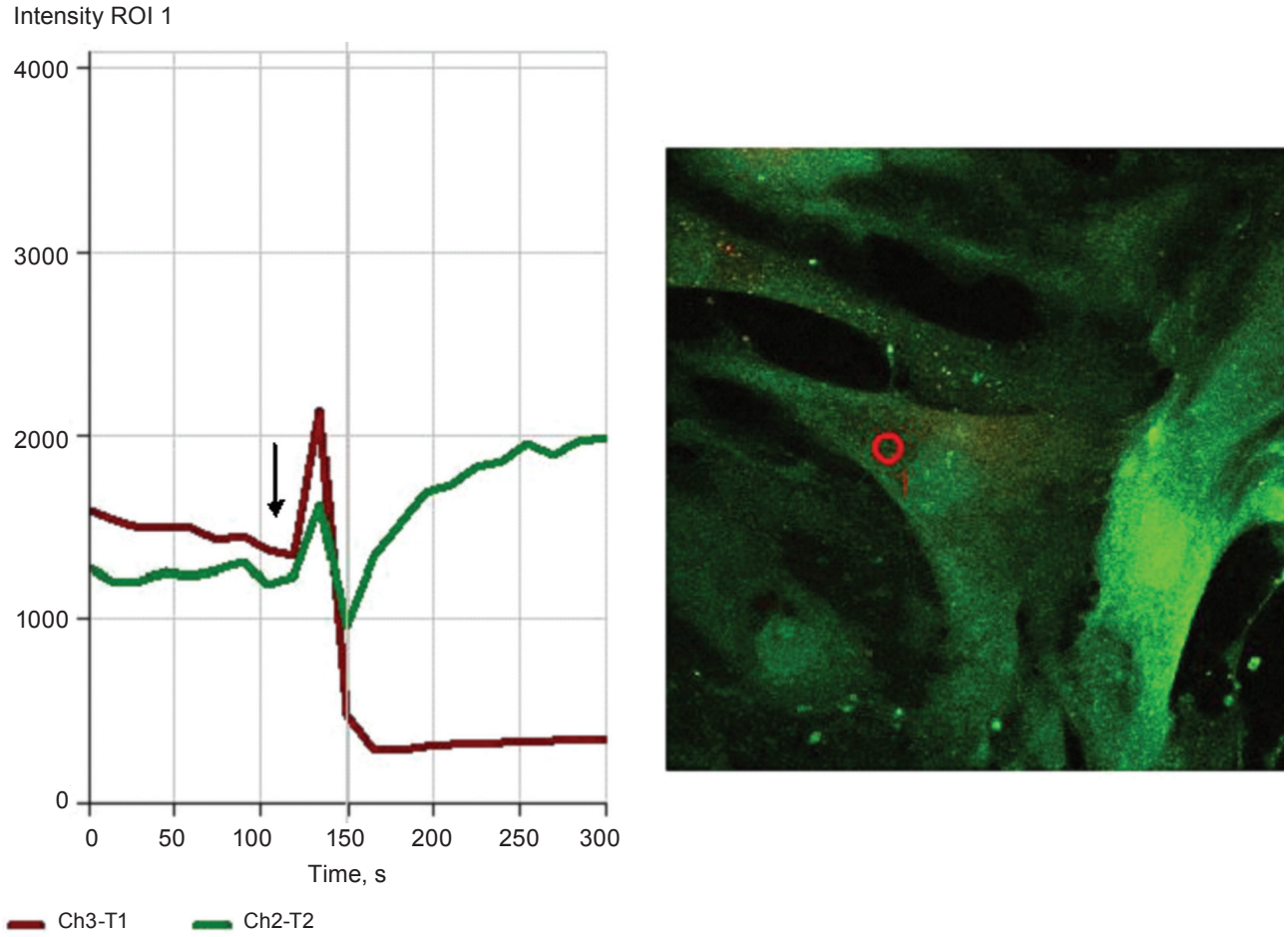

Fig. 5. The fluorescence intensity of potential-sensitive probe TMRM (curve 1) and Ca^{2+}-sensitive probe Fluo-4 AM (curve 2) in rat myometrium cells under the action of calmidazolium (10 μM) (the time of addition of calmidazolium marked with an arrow): A – plot of the fluorescence intensities of TMRM and Fluo-4 AM probes with time, (the fluorescence intensities averaged on marked region (ROI)); B – ROI (Region of Interest) (outlined by red circle), which was selected for plotting the fluorescence intensities of TMRM and Fluo-4 AM probes with time

pends on the level of membrane polarization. Then, why the level of ionized calcium in the mitochondria is increased? Considering our previously reported results [13] and the data shown in Fig. 1, we concluded that the mitochondrial membrane depolarization significantly reduces the level of Ca ions accumulation in mitochondria, but does not completely inhibit the cation transport. Under the presence of the Ca^{2+} concentration gradient directed inward mitochondria and the absence of inner membrane polarization (that is inactivation of Ca^{2+} uniporter) it is likely, that Ca^{2+}-H^{+} exchanger provides cation influx to the matrix. However, even having accepted such interpretation, the following question arises: how does one explain the increase in the ionized calcium concentration in the mitochondria under the influence of calmodulin antagonists under the absence of exogenously added cation, that is, under the absence of Ca^{2+} concentration gradient? Indeed, if we look at the results presented in Fig. 2, precisely this question arises. We suppose that this increase in the ionized calcium concentration may be the result of at least several processes. Firstly, it is well known that Ca ions in the mitochondrial matrix form complexes with the phosphate, whose formation and dissociation depends on pH of the mitochondrial matrix. Matrix acidification that occurs upon depolarization leads to the dissociation of these complexes, consequently, to an increase in the concentration of free Ca [2]. Secondly, it has been shown that the Ca^{2+}-binding sites, so-called “EF hands”, are part of the system that provide the cation metabolism in mito-

chondria [5]. Therefore, the release of the cation may occur not only from the Ca^{2+}-phosphate complexes but also from other binding sites. In case of introduction of calmodulin antagonist in the incubation medium and formation of Ca^{2+} concentration gradient directed inwards mitochondria (Fig. 3), a significant increase in non-specific mitochondrial membrane permeability occurred that led to a sharp increase in the matrix ionized calcium concentration.

Thus, it was shown using two models namely, the mitochondria suspension and intact myometrium cells, that calmodulin antagonists induced depolarization of mitochondrial membranes and the increase in the ionized calcium concentration in both the mitochondrial matrix and the cell cytoplasm. For understanding the mechanism of this phenomenon further researches are required.

ВПЛИВ АНТАГОНІСТІВ КАЛЬМОДУЛІНУ НА РІВЕНЬ Ca^{2+} В МІТОХОНДРІЯХ ТА ЦИТОПЛАЗМІ КЛІТИН МІОМЕТРІЯ

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Встановлено, що Ca^{2+}-залежна регуляція обміну цього катіона в мітохондріях відбувається за участю кальмодуліну. У попередній роботі із залученням двох експериментальних моделей – суспензії мітохондрій та інтактних клітин міометрія – ми показали, що антагоністи кальмодуліну знижують рівень поляризації мітохондріальної мембрани. Мета цієї роботи – з використанням методів спектрофлуориметрії та конфокальної мікроскопії дослідити вплив антагоністів кальмодуліну на рівень іонізованого Са в мітохондріях та цитоплазмі клітин гладенького м'яза матки. Показано, що мітохондрії міометрія у присутності в середовищі інкубації АТР та $MgCl_2$ акумулюють іони Са в матриксі. Інкубація мітохондрій у присутності протонофору СССР гальмує процес накопичення катіона, проте не припиняє його. Антагоніст кальмодуліну – трифлуоперазин (100 мкМ) – значно підвищує рівень іонізованого Са в матриксі мітохондрій. Попередня інкубація суспензії мітохондрій зі 100 мкМ Ca^{2+} перед внесенням трифлуоперазину до інкубаційного середовища частково пригнічує вплив останнього на рівень катіона в матриксі. Інкубація клітин міометрія (первинна культура) з іншим антагоністом кальмодуліну – кальмідазоліумом (10 мкМ) – супроводжується деполяризацією мітохондріальної мембрани та збільшенням концентрації іонізованого Са в цитоплазмі. Таким чином, на двох моделях: суспензії мітохондрій та інтактних клітинах міометрія показано, що антагоністи кальмодуліну спричинюють деполяризацію мембран мітохондрій та ріст концентрації іонізованого Са як у матриксі мітохондрій, так і в цитоплазмі клітин.

К л ю ч о в і с л о в а: ізольовані мітохондрії, первинна культура клітин міометрія, Ca^{2+}, мембранний потенціал мітохондрій, антагоністи кальмодуліну, гладенькі м'язи.

ВЛИЯНИЕ АНТАГОНИСТОВ КАЛЬМОДУЛИНА НА УРОВЕНЬ Ca^{2+} В МИТОХОНДРИЯХ И ЦИТОПЛАЗМЕ КЛЕТОК МИОМЕТРИЯ

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Известно, что Ca^{2+}-зависимая регуляция обмена этого катиона в митохондриях происходит при участии кальмодулина. В предыдущей работе с использованием двух экспериментальных моделей – фракции митохондрий и интактных клеток миометрия – мы показали, что антагонисты кальмодулина снижают уровень поляризации митохондриальной мембраны. Цель этого исследования – с использованием методов спектрофлуориметрии и конфокальной микроскопии протестировать влияние антагонистов кальмодулина на уровень ионизированного Са в митохондриях и цитоплазме клеток гладкой мышцы матки. Показано, что митохондрии миометрия в присутствии в среде инкубации АТР и $MgCl_2$ аккумулируют ионы Са в матриксе. Инкубация митохондрий в присутствии протонофора СССР ингибирует процесс накопления катиона, однако не останавливает его. Антагонист кальмодулина – трифлуоперазин (100 мкМ) – значи-

тельно увеличивает уровень ионизированного Са в матриксе митохондрий. Предварительная инкубация митохондрий со 100 мкМ Ca^{2+} перед внесением в среду инкубации трифлуоперазина частично снимала влияние последнего на уровень катиона в матриксе. Инкубация клеток миометрия (первичная культура) с другим антагонистом кальмодулина – кальмидазолиумом (10 мкМ) – сопровождается деполяризацией митохондриальной мембраны и увеличением концентрации ионизированного Са в цитоплазме.

Таким образом, на двух моделях: фракции митохондрий и интактных клетках миометрия показано, что антагонисты кальмодулина приводят к деполяризации мембран митохондрий и росту концентрации ионизированного Са как в митохондриях, так и в цитоплазме.

Ключевые слова: изолированные митохондрии, первичная культура клеток миометрия, Ca^{2+}, мембранный потенциал митохондрий, антагонисты кальмодулина, гладкие мышцы.

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