EXPERIMENTAL WORKS

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

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Ca^{2+}-dependent regulation of Ca^{2+} exchange in mitochondria is carried out with participation of calmodulin. We have shown previously that calmodulin antagonists reduced the level of mitochondrial membrane polarization and induced increase of the ionized Ca concentration in both the mitochondrial matrix and cell cytoplasm. The concentration-dependent influence of trifluoperazine on the level of polarization of mitochondrial membranes has been shown in this work. The coordinates of the Hill graphs were used to calculate the constant $K_{0.5}$ and the Hill coefficient. $K_{0.5}$ was 24.4 ± 5 μM (n = 10). The Hill coefficient was 2.0 ± 0.2, indicating the presence of two centers of the trifluoperazine binding. We have also studied $[Ca^{2+}]_m$ changes, when incubating mitochondria in mediums of different composition: without ATP and ions of Mg (0-medium), in the presence of 3 mM Mg (Mg-medium) and 3 mM Mg + 3 mM ATP (Mg,ATP-medium). It was shown that the composition of the incubation medium affected the $[Ca^{2+}]_m$ values in the absence of exogenous Ca^{2+} and did not affect them in the presence of the latter. Preincubation of 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+} to the incubation medium. It was concluded, that trifluoperazine depolarized myometrial mitochondria membranes in concentration-dependent manner. However, mitochondria preincubation with 25 μM trifluoperazine accompanied by 50% decrease in membrane polarization did not affect the $[Ca^{2+}]_m$ values.

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

Intracellular Ca^{2+} concentration increase is accompanied by rapid uptake of Ca^{2+} in the mitochondria. The main pathway of mitochondrial Ca^{2+} uptake is uniporter found in the inner membrane. Ca^{2+} uptake into mitochondria requires an electrical potential across the mitochondrial inner membrane [1-4]. It was shown that Ca^{2+} uptake into mitochondria is a Ca^{2+}-activated process with a requirement for functional calmodulin [5]. Nevertheless, still little is known about how mitochondrial Ca^{2+} uptake is regulated. In previous papers, using two models – isolated mitochondria and intact myometrial cells, we have shown that calmodulin antagonists caused depolarization of the mitochondria membranes and increase of Ca^{2+} concentration in the mitochondria matrix ($[Ca^{2+}]_m$) and cell cytoplasm [6, 7]. However, experiments with mitochondria were conducted with 100 μM trifluoperazine, which caused full depolarization of mitochondrial membranes. So the aim of this work was to study the concentration-dependent influence of trifluoperazine on the polarization of mitochondrial membranes and the Ca^{2+} concentration in the mitochondria matrix. Experiments have been conducted in the incubation mediums of different composition and with or without 100 μM Ca^{2+}.

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 removed rapidly. All procedures were executed separately from other rats.

Nonpregnant rat myometrium mitochondria were isolated using differential centrifugation method [8]. 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. The 0.1% BSA was also added. Protein concentration in the mitochondria fraction was determined by Bradford assay [9].

Polarization of mitochondrial 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 spectrofluorimeter (Photon Technology International). The studies were carried out in a medium containing (mM): Hepes – 20, sucrose – 250, succinate – 5, K-phosphate – 0.1, $MgCl_2$ – 0.5. 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 QuantaMaster™ 40 spectrofluorimeter (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 following composition (mM): sucrose – 250, EGTA – 1, Hepes – 20, pH 7.4 in the presence of 0.1% BSA. 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 ionized Ca in the mitochondria matrix was calculated using the Grynkiewicz equation [10].

The statistical methods and the software for statistical processing were used from http://graphpad.com/.

In the study the following reagents were used: EGTA, Hepes, BSA (fatty acid free), trifluoperazine, 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

Two approaches are used to study the polarization of membranes.

Nonquench mode is used in experiments on flow cytometry or confocal microscopy. The concentration of the potential sensitive dyes in such experiments is less than 100 nM. These devices allow us to register the redistribution of the probe between the medium and polarized structures, in particular, the mitochondria. Probe accumulation in the polarized structures is accompanied by the increase in probe fluorescence. Membranes depolarization is accompanied by the dye release to the medium, thus lowering fluorescence level. This method we used in the previous papers [6, 7].

Quench mode is used in experiments conducted on spectrofluorimeter. The concentration of the potential sensitive dyes in such experiments is higher than 1 μM. This approach also enables to track the redistribution of the probe between the medium and polarized structures. But probe accumulation in the polarized structures is accompanied by the quenching of probe fluorescence, i.e. by the decrease in probe fluorescence. Quench mode for dye fluorescence is a sensitive mean to detect rapid changes in $\Delta\Psi$ that occur during the experiment [11].

We have studied polarization of myometrium mitochondrial membranes using quench mode and potential sensitive probe 1 μM TMRM. It was shown that addition of potential sensitive fluorescent probe TMRM to the incubation medium was accompanied by output of fluorescence intensity at a certain level (Fig. 1, *A*), the probe TMRM accumulated in mitochondria that was accompanied by a decrease in the intensity of dye fluorescence (Fig. 1, *A*). After adding protonophore CCCP the probe came out of the mitochondria into incubation medium that was accompanied by increasing of the dye fluorescence intensity. Under such conditions, the adding of 100 μM trifluoperazine did not affect the intensity of fluorescence probe. Thus, it was shown that: 1) the mitochondrial membranes were polarized, as evidenced by quen-

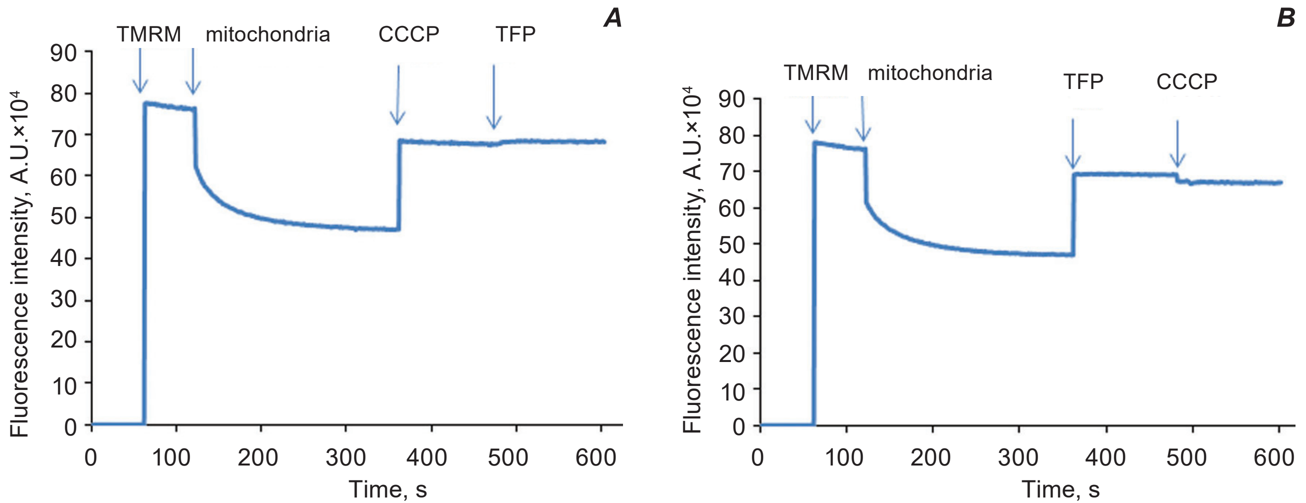

Fig. 1. Effects of CCCP (A) and trifluoperazine (B) on polarization of the mitochondria membranes. Fluorescent probe – 1 µM TMRM (tetramethylrhodamine-methyl-ester, λ_{exc} = 540 nm, λ_{em} = 590 nm)

ching of probe fluorescence. Depolarization of the mitochondrial membranes by adding protonophore CCCP was accompanied by TMRM release from mitochondria and restoration of the fluorescence level; 2) trifluoperazine addition to the depolarized mitochondria (after the incubation with CCCP) had not affected the level of dye fluorescence.

The next experiment was similar to the previous, but on the 6th min of incubation 100 µM trifluoperazine was added instead of CCCP. As it can be seen from the results shown in Fig. 1, *B*, trifluoperazine at a concentration of 100 µM was a factor of mitochondrial membrane potential dissipation. Addition of CCCP on the 8th min of the experiment did not cause any changes in the fluorescence probe intensity values. Thus, it was shown that incubation of mitochondria with trifluoperazine was accompanied by depolarization of the mitochondrial membranes.

Next experiments were conducted to study the concentration-dependent influence of trifluoperazine on the level of polarization of mitochondrial membranes. Fig. 2 presents a slice of the experiment, from 5 to 7 minute of incubation, i.e. the time when trifluoperazine was added to the incubation medium. TFP was taken at concentrations 10, 20, 30, 50, 70, 100 µM.

Initial fluorescence level of mitochondria loaded with TMRM was the reference for each experiment (n = 10). The graph presented in Fig. 3, is a delta between plateau values of probes fluorescence after the trifluoperazine addition and control values (before trifluoperazine addition). Thus the concentration-dependent depolarizing influence of trifluoperazine on the myometrium mitochondria membranes has been shown.

The coordinates of the Hill graphs were used to calculate the constant $K_{0.5}$ (half-maximum depolarization) and the Hill coefficient. $K_{0.5}$ was 24.4 ± 5 µM (n = 10). The Hill coefficient was 2.0 ± 0.2, indicating the presence of two centers of the trifluoperazine binding.

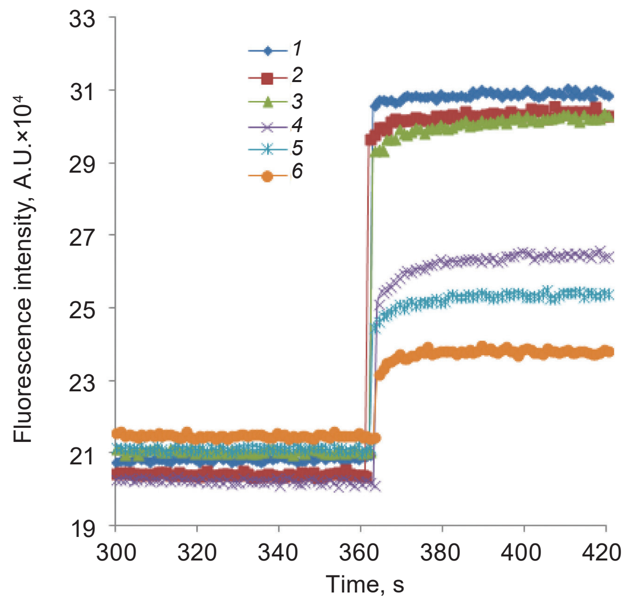

Fig. 2. Concentration-dependent trifluoperazine influence on mitochondria membranes polarization (n = 10): 1 – 100 µM TFP, 2 – 70 µM TFP, 3 – 50 µM TFP, 4 – 30 µM TFP, 5 – 20 µM TFP, 6 – 10 µM TFP. This figure represents typical results

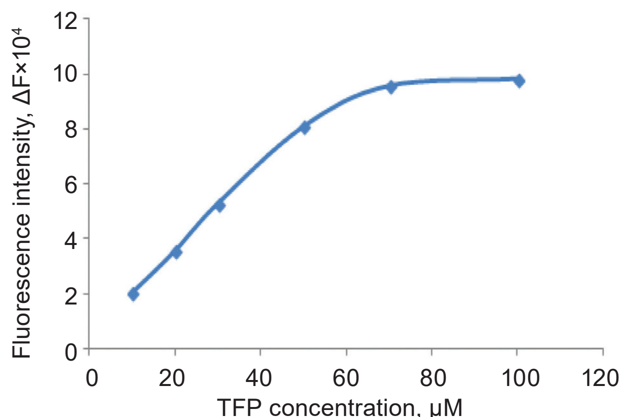

Fig. 3. Concentration-dependent trifluoperazine effects on the fluorescence intensity of research samples

Next experiments were designed to study the impact of pre-incubation of mitochondria with 25 μM trifluoperazine on $[Ca^{2+}]_m$ with incubation of mitochondria in three mediums: without ATP and Mg^{2+} (0-medium), in the presence of 3 mM Mg (Mg-medium) and 3 mM Mg + 3 mM ATP (Mg,ATP-medium). In our previous work we have investigated the total Ca^{2+} accumulation in mitochondria. For this purpose the isotope method ($^{45}Ca^{2+}$) was used. It was found that ATP presence in the incubation medium led to a significant increase of Ca^{2+} accumulation in the mitochondria matrix [12]. Fluorescence dyes are used to determine free Ca^{2+} concentration. So, first we have studied $[Ca^{2+}]_m$ changes under mitochondria incubation in mediums of different composition. As presented in Fig. 4, mitochondria incubation in the mediums of different composition resulted in different $[Ca^{2+}]_m$ values: 0-medium – 123.6 ± 9.4 nM; Mg-medium – 83.7 ± 8.9 nM; Mg,ATP-medium – 330.9 ± 13.4 nM. So the highest $[Ca^{2+}]_m$ value was registered when mytochondria were incubated in Mg,ATP-medium, the lowest – in the Mg-medium.

Subsequent 100 μM Ca^{2+} addition to the incubation medium was accompanied by an increase of $[Ca^{2+}]_m$ values. As it can be seen from the results presented in Fig. 4, the $[Ca^{2+}]_m$ values were the same in the case of mitochondria incubation in 0- and Mg,ATP-medium and somewhat lower in Mg-medium. Thus, it was concluded that the composition of the incubation medium affected the $[Ca^{2+}]_m$ values in the absence of exogenous Ca^{2+} and did not in the presence of the latter.

The following experiments were designed to study the impact of mitochondria preincubation within 5 min with 25 μM trifluoperazine on the $[Ca^{2+}]_m$ values. This concentration of TFP gives half-maximum depolarization of the myometrium mitochondrial membranes (Fig. 2, 3). Experiments were conducted in three mediums listed above.

As it can be seen from the results shown in Fig. 5 the incubation of mitochondria with 25 μM trifluoperazine did not affect the $[Ca^{2+}]_m$ values in the absence of Ca ions in the incubation medium, compared with those for the control samples. $[Ca^{2+}]_m$ values after 100 μM Ca^{2+} addition were to some ex-

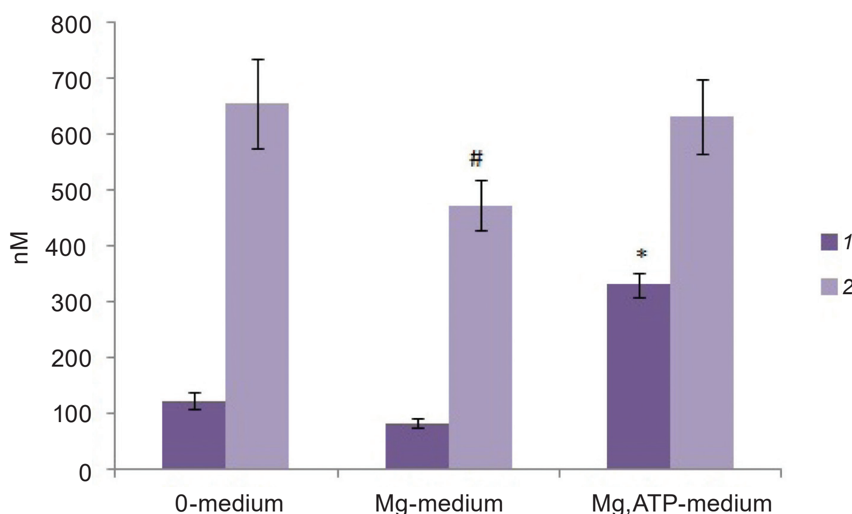

*Fig. 4. $[Ca^{2+}]_m$ values when mitochondria are incubated in mediums of different composition: 1 – 0 μM Ca^{2+} in the medium; 2 – 100 μM Ca^{2+} in the medium. M ± m, n=7. P < 0.01, * the difference relative to the 0-medium is statistically significant. P < 0.01, # the difference relative to the 0-mediumin the presence of 100 μM is statistically significant*

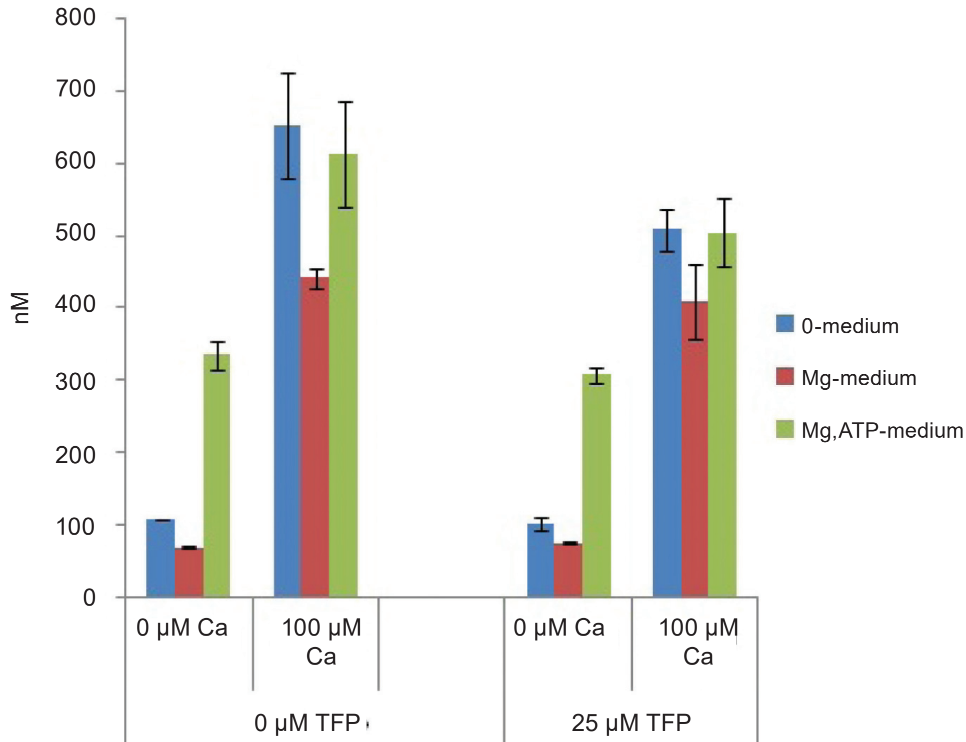

Fig. 5. $[Ca^{2+}]_m$ values under preincubation of mitochondria in mediums of different composition and presence of 25 µM trifluoperazine (M ± m, n = 4)

tent influenced by the presence of trifluoperazine in the incubation medium, but these changes were not statistically reliable. Thus, preliminary incubation of mitochondria with 25 µM trifluoperazine did not affect the $[Ca^{2+}]_m$ values both before and after the addition of 100 µM Ca^{2+} to the incubation medium.

At first glance, the lack of the effect of mitochondria preincubation with 25 µM trifluoperazine on the Ca^{2+} level surprised, since this compound reduced polarization of mitochondrial membranes to 50%. It is well known that the main system that provides the Ca^{2+} accumulation in the mitochondria is Ca^{2+}-uniporter; its activity depends on the polarization of membranes. However, it was shown that mitochondria in metabolically inhibited renal epithelial cells take up Ca^{2+} via the Na^{+}/Ca^{2+} exchanger, acting in the reverse mode [13]. In these cells the mitochondrial potential was lost, excluding mitochondrial Ca^{2+} uptake via the potential-dependent mitochondrial Ca^{2+} uniporter. Our previous results also indicate that even under conditions of 100% mitochondrial membrane depolarization (preincubation with 10 µM CCCP) Ca accumulation still took place in these organelles [7]. The question, what system provides the accumulation of Ca ions in the depolarized myometrium mitochondria, remains open.

Thus, it was shown, that trifluoperazine depolarized mitochondrial membranes of the myometrium in a concentration-dependent manner. However, mitochondria preincubation with 25 µM trifluoperazine did not affect the $[Ca^{2+}]_m$ values when mitochondria were incubated in mediums with or without ATP and in the presence or absence of 100 µM Ca.

Ca^{2+}-ЗАЛЕЖНА РЕГУЛЯЦІЯ КОНЦЕНТРАЦІЇ Ca^{2+} В МІТОХОНДРІЯХ МІОМЕТРІЯ. І. ЕФЕКТИ ТРИФЛУОПЕРАЗИНУ НА ПОЛЯРИЗАЦІЮ МІТОХОНДРІАЛЬНИХ МЕМБРАН ТА $[Ca^{2+}]_m$

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Ca^{2+}-залежна регуляція обміну Ca^{2+} в мітохондріях відбувається за участю кальмодуліну. Раніше ми показали, що

антагоністи кальмодуліну знижують рівень поляризації мітохондріальних мембран та спричинюють ріст концентрації іонізованого Са як у матриксі мітохондрій, так і в цитозолі клітин. У цій роботі вивчався залежний від концентрації вплив трифлуоперазину на рівень поляризації мітохондріальних мембран. Показано, що $K_{0,5}$ становить 24,4 ± 5,0 μМ (n = 10). Коефіцієнт Хілла дорівнює 2,0 ± 0,2, що вказує на наявність двох центрів зв'язування трифлуоперазину на мембрані мітохондрій. Також було досліджено зміни $[Ca^{2+}]_m$ за умов інкубації мітохондрій в середовищах різного складу, а саме: без АТР та іонів Mg (0-середовище), з 3 мМ Mg (Mg-середовище) та те, що містило 3 мМ Mg + 3 мМ АТР (Mg,АТР-середовище). Встановлено, що склад середовища інкубації впливав на значення $[Ca^{2+}]_m$ за відсутності екзогенного Ca^{2+} та не впливав у присутності цього катіона. Попередня інкубація мітохондрій в середовищах різного складу із 25 μМ трифлуоперазином не впливала на значення $[Ca^{2+}]_m$ як до, так і після додавання 100 μМ Ca^{2+} до інкубаційного середовища. Таким чином, трифлуоперазин спричинює деполяризацію мітохондріальних мембран і цей ефект залежить від його концентрації. Проте попередня інкубація мітохондрій з 25 μМ трифлуоперазином, яка супроводжується 50% зниженням поляризації мембран, не впливає на значення $[Ca^{2+}]_m$.

Ключові слова: міометрій, мітохондрія, трифлуоперазин, $[Ca^{2+}]_m$, Mg, АТР.

Ca^{2+}-ЗАВИСИМАЯ РЕГУЛЯЦИЯ КОНЦЕНТРАЦИИ Ca^{2+} В МИТОХОНДРИЯХ МИОМЕТРИЯ. I. ЭФФЕКТЫ ТРИФЛУОПЕРАЗИНА НА ПОЛЯРИЗАЦИЮ МИТОХОНДРИАЛЬНЫХ МЕМБРАН И $[Ca^{2+}]_m$

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Ca^{2+}-зависимая регуляция обмена Ca^{2+} в митохондриях происходит при участии кальмодулина. Ранее мы показали, что антагонисты кальмодулина снижают уровень поляризации митохондриальных мембран и индуцируют рост концентрации ионизированного Са как в матриксе митохондрий, так и в цитозоле клеток. В этой работе изучалось зависимое от концентрации влияние трифлуоперазина на уровень поляризации митохондриальных мембран. Показано, что $K_{0,5}$ составляет 24,4 ± 5,0 μМ (n = 10). Коэффициент Хилла равен 2,0 ± 0,2, что говорит о наличии двух центров связывания трифлуоперазина на мембране митохондрий. Также были исследованы изменения $[Ca^{2+}]_m$ в условиях инкубации митохондрий в средах разного состава, а именно: без АТР и ионов Mg (0-среда), с 3 мМ Mg (Mg-среда) и та, что содержала 3 мМ Mg и 3 мМ АТР (Mg,АТР-среда). Показано, что состав среды инкубации влиял на значение $[Ca^{2+}]_m$ в отсутствие экзогенно добавленного Ca^{2+} и не влиял в его присутствии. Предварительная инкубация митохондрий в средах разного состава с 25 μМ трифлуоперазином не влияла на значение $[Ca^{2+}]_m$ как до, так и после внесения 100 μМ Ca^{2+} в инкубационную среду. Таким образом, трифлуоперазин вызывает деполяризацию митохондриальных мембран и этот эффект зависит от его концентрации. Однако предварительная инкубация митохондрий в средах разного состава с 25 μМ трифлуоперазином, которая сопровождается 50% снижением поляризации мембран, не влияет на значение $[Ca^{2+}]_m$.

Ключевые слова: миометрий, митохондрия, трифлуоперазин, $[Ca^{2+}]_m$, Mg, АТР.

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