ELECTROCHEMICAL POTENTIAL OF THE INNER MITOCHONDRIAL MEMBRANE AND Ca^{2+} HOMEOSTASIS OF MYOMETRIUM CELLS

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*We demonstrated using Ca^{2+} -sensitive fluorescent probe, mitochondria binding dyes, and confocal laser scanning microscopy, that elimination of electrochemical potential of uterus myocytes' inner mitochondrial membrane by a protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (10 μM), and by a respiratory chain complex IV inhibitor sodium azide (1 mM) is associated with substantial increase of Ca^{2+} concentration in myoplasm in the case of the protonophore effect only, but not in the case of the azide effect. In particular, with the use of nonyl acridine orange, a mitochondria-specific dye, and 9-aminoacridine, an agent that binds to membrane compartments in the presence of proton gradient, we showed that both the protonophore and the respiratory chain inhibitor cause the proton gradient on mitochondrial inner membrane to dissipate when introduced into incubation medium. We also proved with the help of 3,3'-dihexyloxacarbocyanine, a potential-sensitive carbocyanine-derived fluorescent probe, that the application of these substances results in dissipation of the membrane's electrical potential. The elimination of mitochondrial electrochemical potential by carbonyl cyanide *m*-chlorophenyl hydrazone causes substantial increase in fluorescence of Ca^{2+} -sensitive Fluo-4 AM dye in myoplasm of smooth muscle cells. The results obtained were qualitatively confirmed with flow cytometry of mitochondria isolated through differential centrifugation and loaded with Fluo-4 AM. Particularly, Ca^{2+} matrix influx induced by addition of the exogenous cation is totally inhibited by carbonyl cyanide *m*-chlorophenyl hydrazone. Therefore, using two independent fluorometric methods, namely confocal laser scanning microscopy and flow cytometry, with Ca^{2+} -sensitive Fluo-4 AM fluorescent probe, we proved on the models of freshly isolated myocytes and uterus smooth muscle mitochondria isolated by differential centrifugation sedimentation that the electrochemical gradient of inner membrane is an important component of mechanisms that regulate Ca^{2+} homeostasis in myometrium cells.*

Key words: mitochondria, calcium, electrochemical potential of mitochondrial membrane, calcium homeostasis, nitric oxide.

Mitochondrial Ca^{2+} transport plays a major role in maintaining Ca^{2+} homeostasis in smooth muscle cells, as it provides for post-transient energy-dependent Ca^{2+} uptake from myoplasm. Certain authors have expressed an opinion that mitochondria may provide for a decrease in Ca^{2+} cytosol concentration within physiologically sound timeframe, which is a requirement for relaxation, and that they also protect the cell against the cation's cytotoxic effects under its extracellularly induced overflow [1-3].

Energy dependent Ca^{2+} mitochondrial influx is performed by Ca^{2+} uniporter, and its driving force is the potential difference on the organelle's inner membrane reaching values -160 to -180 mV (nega-

tive on the matrix side). Thus, Ca^{2+} accumulation by energized mitochondria is conducted primarily via electrophoretic mechanism [1-4]. The reverse process of release of accumulated Ca^{2+} by mitochondria is mainly dependent on $\text{Ca}^{2+}/\text{Na}^{+}$ and $\text{Ca}^{2+}/\text{H}^{+}$ exchangers of the inner membrane and, possibly by permeability transition pore and Ca^{2+} uniporter (in the latter case by reverse mechanism under mitochondria unenergized state) [3-7]. It has been proven that in unexcitable tissues and smooth muscle cells the $\text{Ca}^{2+}/\text{H}^{+}$ exchanger plays the main role in the maintaining of the optimal matrix concentrations of Ca^{2+} [6, 7].

The electrochemical potential on the inner membrane of mitochondria ($\Delta\mu_{\text{H}^{+}}$) is generated

through the functioning of electron-transport chain and is the direct result of maintenance of transmembrane proton gradient in intact organelles. It is composed of two components: chemical (ΔpH) and electrical ($\Delta\phi$) [2, 8]. The Ca^{2+} uniporter functioning depends on efficiency of proton and electron transport on the inner mitochondrial membrane and, accordingly, on proton gradient. A substantial decrease in the membrane's electrochemical potential (a depolarization) causes inhibition of electrophoretic accumulation of Ca^{2+} , and it can be surmised that Ca^{2+} release from matrix into cytosol would become the prevailing transporting process under such conditions due to $\text{Ca}^{2+}/\text{H}^{+}$ exchanger activity [2-4, 6]. The $\Delta\mu_{\text{H}^{+}}$ modifiers such as respiration inhibitors and protonophores may be expected to affect both mitochondrial and myoplasm concentrations of Ca^{2+} .

To estimate the effect of mitochondria energization levels on myoplasm Ca^{2+} concentrations in myocytes it is appropriate to employ the confocal laser microscopy, which allows for visualization of fluorescent probes' distribution within cells.

The aim of the present work was to investigate the effect of sodium azide (NaN_3), a conventional inhibitor of complex IV of mitochondrial electron transport chain, and carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a protonophore, on the electrochemical potential of the inner mitochondrial membrane and myoplasm Ca^{2+} concentration in uterus smooth muscle (myometrium) cells.

Materials and Methods

Experiments on uterus smooth muscle cells suspension. The myocytes were isolated from the uterus of non-pregnant laboratory rats with collagenase and soybean trypsin inhibitor after Mollard [9]. The animals were anesthetized with diethyl ether inhalation, and then decapitated. The experiments were conducted in accordance with guidelines for work with laboratory animals (International Convention, Strasbourg, 1986).

The cells immobilization for confocal microscopy, washing off the unadherent myocytes, and all experimental manipulations were performed in Hanks' balanced salt solution containing (mM): NaCl – 136.9; KCl – 5.36; KH_2PO_4 – 0.44; NaHCO_3 – 0.26; Na_2HPO_4 – 0.26; CaCl_2 – 0.03; MgCl_2 – 0.4; MgSO_4 – 0.4; glucose – 5.5; HEPES (pH 7.4 at 37 °C) – 10 [10]. Digitonin was added to the medium at 0.1% concentration in experiments on permeabilized myocytes.

The intracellular spatial distribution of fluorescent dyes was examined with LSM 510 META confocal laser scanning microscope (Carl Zeiss, Germany) using myocytes immobilized on poly-L-lysine. 1 μM 10-nonyl acridine orange (NAO), a fluorescent probe, was used to visualize mitochondria, and 50 μM Hoechst 33342 to visualize nuclei. 10 μM 9-aminoacridine (9-AA) was used as a fluorescent dye able to bind to membrane compartments having a proton gradient. Changes in transmembrane potential were registered with 0.5 mM $\text{DiOC}_6(3)$ (3,3'-dihexyloxycarbocyanine), a potential-sensitive fluorescent probe, and Ca^{2+} myoplasm concentrations with 10 μM Fluo 4-AM. The probes were loaded for 15 min at 24 °C. The readings were performed in Multi Track mode of the confocal microscope [10].

9-AA and Hoechst 33342 fluorescence was excited with laser set at 420-480 nm and the signal was detected at 405 nm setting of BP filter. NAO, $\text{DiOC}_6(3)$ and Fluo 4-AM fluorescence was excited at 488 nm, and the emission was registered at 505 to 530 nm (BP 505-530 filter setting).

We choose elongated cells well adhered to the substrate for analysis.

Experiments with mitochondrial suspension. The mitochondrial fraction was isolated from rats' myometrium by differential centrifugation as described earlier [3].

Changes of ionized Ca content in matrix of the isolated mitochondria were identified with Fluo-4 AM (2 μM). The mitochondria were loaded with the probe in a buffer medium containing 10 mM HEPES (pH 7.4, 37 °C), 250 mM sucrose, 1 mM EGTA, 0.1% bovine serum albumin, for 30 min at 37 °C. The probe was mixed with Pluronic F-127 (0.02%) to facilitate loading [3, 6, 11].

Changes of ionized Ca in mitochondrial matrix were investigated by flow cytometry on COULTER EPICS XL™ flow cytometer (Beckman Coulter, USA) with SYSTEM II software (Beckman Coulter, USA). The virtual levels of Ca^{2+} in matrix were measured with Fluo-4 AM at 2 μM ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 520 \text{ nm}$) in medium containing (in mM): 20 HEPES (pH 7.4 at 37 °C), 250 sucrose, 2 potassium phosphate buffer (pH 7.4 at 37 °C), 3 MgCl_2 , 3 ATP, 5 sodium succinate, and Ca^{2+} concentration was 80 μM . We used for sample analysis a working protocol developed specifically to characterize fractions of mitochondria isolated from myometrium. The events for analysis were selected by introducing logical restriction for lateral and direct

scattering (SS and FS) in flow cytometer protocol. Sample analysis was terminated at 10000th event within the selected gatiug [3, 11].

The statistical analysis of the data was performed with standard IBM PC software using conventional methods and Student's *t*-test [12].

The following reagents were used in the study: HEPES, glucose, sucrose, digitonin, sodium succinate, bovine serum albumin, poly-L-lysine, collagenase type IA, ATP, Pluronic F-27, EGTA, NAO, 9-AA, CaCl₂ (Sigma, USA), DiOC₆(3), Hoechst 33342, soybean trypsin inhibitor (Fluka, Switzerland), Fluo-4 AM (Invitrogen, USA). Other reagents were of local manufacture and analysis-grade purity.

Results and Discussion

We prove in this study the identical subcellular localization of 9-AA and NAO fluorescent probes in myocytes (Fig. 1). As NAO interacts specifically with cardiolipin, which is abundant in mitochondrial membrane [13], and 9-AA interacts with subcellular membrane structures bearing Δ pH [14], we can assert that we have positively identified energized mitochondria with proton gradient on their inner membrane.

It has been demonstrated that CCCP, a protonophore, in concentration of 10 μ M (Fig. 2) and NaN₃, a mitochondrial respiration inhibitor, in concentration of 1 mM [2, 3, 10] caused substantial decrease in 9-AA fluorescence for 5 min, if added to intact myocytes. The concentrations of the effector substances

used in our experiment are well established in mitochondrial studies. As Hoechst 33342 and 9-AA have similar excitation and emission wavelength characteristics, the violet coloration of myoplasm is quenched in the presence of the protonophore, while the similar coloration of the nucleus does not change with time (Fig. 2, top panel).

The kinetics of intracellular distribution of the fluorescent dyes was characterized in Time Series mode, and quantified via ROI (Region of Interest) function that produces graphical representation of temporal changes in fluorescence intensity, averaged over selected area (Fig. 2, bottom panel). The analytical method allows for quantification of level of discoloration of 9-AA and for corresponding calculations. Particularly, CCCP caused 62% decrease in probe fluorescence on the average, and NaN₃ caused 87% decrease (Fig. 3). These results may be interpreted as inner membrane proton gradient dissipation under the effect of protonophore and inactivation of its restoration under respiratory chain inhibition by sodium azide. Therefore, the investigated compounds partially remove the chemical component of electrochemical potential of mitochondria (Δ pH).

We had established in previous studies the feasibility of determining the changes in polarization of mitochondrial membranes from smooth cells of rat uterus with DiOC₆(3) potential-sensitive fluorescent probe [10]. In order to prove the direct interaction of DiOC₆(3) and mitochondria we used a mitochondria-

Fig. 1. Distribution of mitochondrial membrane binding (NAO) and membrane proton gradient sensitive (9-AA) fluorescent probes in myocytes. Confocal microscopy data

Fig. 2. Extinction of 9-AA fluorescence under effect of protonophore (top panel) and digital analysis of its ROI function (bottom panel). Confocal microscopy data

specific marker, the MitoTracker Orange CM-H₂T-MRos fluorescent probe, that is accumulated by the organelles depending on their potential, and interacts with matrix proteins. The dyes colocalize if applied simultaneously, which is proved by their identical profiling in distribution of corresponding fluorescent probes. The data obtained allows us to assume that DiOC₆(3) accumulation in myometrium cells is mostly dependent on mitochondria [10]. This substance is a carbocyanine dye and a lipophilic cation that is accumulated in a potential-dependent manner inside cellular compartments. The increase in mem-

branes' negative potential causes its accumulation and consequent increase in fluorescence [15, 16]. The preferential accumulation of the probe by mitochondria may be attributed to their high inner membrane potential, up to -180 mV. DiOC₆(3) may thus be used to investigate the effects of compounds that affect mitochondrial transmembrane potential.

In our previous studies we had demonstrated using spectrofluorometry, flow cytometry and confocal laser scanning microscopy that CCCP and NaN₃ permeate myocytes' plasma membrane and cause mitochondrial membrane depolarization [10, 17].

Fig. 3. 9-AA fluorescence quenching in myocytes in the presence of the protonophore and the mitochondrial respiratory chain inhibitor, in comparison to control (100%); $M \pm m$, $n = 3-4$. The probe fluorescence in cells without the effectors is taken as 100% (ordinate axis). Confocal microscopy data

It is as yet unclear, if this effect is directly dependent on mitochondria, or whether it is mediated by plasmalemma and cytosol processes. In order to facilitate access of the modulators of mitochondrial potential to their target, we increased the unspecific permeability of plasma membrane with 0.1% digitonin. The procedure is reportedly non-disruptive for intracellular membrane structures [18].

It was established (Fig. 4), that the decrease in DiOC₆(3) fluorescence intensity due to probe fluorescence quenching under laser irradiation of the corresponding wavelength (10-20% of maximum power) reaches 20% in 5 min in control (14 cells were used for quantitative analysis). The degree of photobleaching is variable and depends primarily on its concentration, laser power, and exposition. Sodium azide (1 mM) caused substantial decrease (about 60%, Fig. 4) in fluorescence intensity of myocytes (5 cells were used). The residual fluorescence levels are evidently caused by unspecific potential-independent sorption of DiOC₆(3) by membrane structures. In certain cases we observed nearly total elimination of DiOC₆(3) fluorescence in the presence of sodium azide (Fig. 5).

We have demonstrated previously that probe bleaching under effects of sodium azide in intact myocytes is 40% only even under much higher concentration of the affecting substance (4 mM), according to confocal microscopy data. This may be explained by barrier function of plasma membrane towards exogenous substances [10].

The permeabilized cells responded adequately to CCCP treatment (Fig. 4). Addition of 10 μM CCCP led to a marked decrease in DiOC₆(3) fluorescence, which may indicate a partial depolarization of inner mitochondrial membrane under effect of the protonophore. The quantitative analysis of the effects demonstrates a 40% bleaching of the probe (6 cells analyzed). The fairly modest depolarizing effects of CCCP may be explained by inadvertent effects of increase in DiOC₆(3) fluorescence in the presence of ethanol from the protonophore's stock solution.

It is hence possible to use laser scanning confocal microscopy and DiOC₆(3) probe to study polarization of the inner mitochondrial membrane on permeabilized myocytes as well as on intact cells. We observed decreased electric potential of mitochondrial membrane of intact and permeabilized cells, which was evidently caused directly by the effect of the investigated substances on mitochondria. The effect of sodium azide was more pronounced than that of CCCP. In our opinion, this difference was caused not by the difference in concentrations of the compounds, but by NaN₃ degradation and consequent production of reactive nitrogen species [19] and mitochondrial catalase and cytochrome-*c*-oxidase inhibition by azide, which is associated with increased reactive oxygen species generation [20, 21]. Hence, both the protonophore and the respiratory chain in-

Fig. 4. Decrease in fluorescence intensity (increase in level of bleaching) of DiOC₆(3) at 5 min exposition in control and under effect of membrane depolarizing agents in myocytes permeabilized with 0.1% digitonin; $M \pm m$, changes are statistically significant in comparison to control ($P \leq 0.05$), 5-14 cells were used in independent experiments. The control bar represents bleaching levels of the fluorescent probe exposed to laser radiation in the absence of the effectors. Confocal microscopy data

Fig. 5. Quenching of fluorescence of DiOC₆(3) in digitonin permeabilized myocytes for 2 min under exposition to 1 mM sodium azide. Confocal microscopy data

hibitor partially eliminate the electrical component of mitochondrial electrochemical potential.

As can be seen from the mentioned parameters of dissipation of electrical and chemical potentials on the mitochondrial membrane, they generally do not drop to zero value during 5-min exposure to CCCP and sodium azide, with some exceptions. At the same time, the concentrations we used are commonly employed for guaranteed dissipation of $\Delta\mu_{H^+}$ on various experimental subjects. Our results can be explained by assuming the existence of unspecific sorption of fluorescent dyes by membrane structures and macromolecular complexes of cytoplasm, and this phenomenon is difficult or impossible to avoid. One of possible solutions may be the use of comparative studies of other probes of various chemical compositions in order to verify the results.

The dissipation of the electrochemical potential of mitochondrial inner membrane affects functioning of Ca²⁺-transporting complexes of mitochondria, which may result in changes in Ca²⁺ concentrations of myoplasm. Various types of synthetic fluorescent probes are currently employed to perform high-quality readily available rapid evaluations of cellular concentrations of ionized Ca, such as bioluminescent aquarines, fluorescent proteins, and Ca²⁺-fluorescent indicators with low molecular mass. Of the latter,

Fluo-4 AM has been widely used in biochemical studies, which has a higher quantum fluorescent output than its predecessors, permeates the biological membranes more easily and consequently accumulates faster within cells [11, 22].

We found noticeable increase in fluorescence of Fluo-4 AM, a Ca²⁺-sensitive probe, under the effect of 10 μ M CCCP without addition of exogenous Ca²⁺, which may signify an increase of Ca ions concentration in myoplasm of myometrium cells due to its release from intracellular stores (Fig. 6).

According to our calculations, the cation's concentration nearly doubles (Fig. 7). This result is explained by the disabling of mitochondrial Ca²⁺ uniporter due to elimination of electrochemical proton gradient on their inner membrane by the protonophore. Therefore, the mitochondria cannot accumulate Ca ions from myoplasm under such conditions. On the contrary, Ca²⁺/H⁺ exchanger may function efficiently under the experimental conditions, thus ensuring Ca²⁺ release from the mitochondrial matrix into the myoplasm.

Although the deenergizing effect of sodium azide on mitochondria is even more pronounced than that of CCCP, its effect on myoplasm concentration of Ca ions was not strongly marked. This result may be explained by the presence of products of

Fig. 6. Visualization with Fluo-4 AM of the increase in Ca^{2+} myoplasm concentration under effect of the $10\mu\text{M}$ protonophore (top panel) and digital analysis of its ROI function (bottom panel). Confocal microscopy data

Fig. 7. Increase in myoplasm Ca^{2+} concentration in intact cells under the effect of CCCP protonophore (control values are taken as 100%); $M \pm m$, 8 cells from independent experiments were used for the analysis. Fluo-4 AM fluorescence in cells without the effectors is taken as 100% (ordinate axis). Confocal microscopy data

NaN_3 degradation, which may efficiently modulate activity of Ca^{2+} -transporting systems in myocytes. Reactive nitrogen species, NO and its derivatives in particular, induce relaxation of smooth muscle cells via decrease in myoplasm Ca^{2+} concentration. Their biochemical targets may include energy-dependent transport systems of the cation on plasma membrane and sarcoplasmic reticulum. There is data concern-

Fig. 8. Effects of Ca^{2+} -ionophore and mitochondria-deenergizing compounds on matrix Ca^{2+} content (the cation had been accumulated in energy-dependent manner); F_0 – Fluo-4 AM fluorescent signal without exogenous Ca^{2+} addition (endogenous ionized Ca, ‘starting’ level), F – Fluo-4 AM fluorescent signal with addition of exogenous Ca^{2+} and the investigated compounds, ($M \pm m$, $n = 5$). Flow cytometry data

ing properties of sodium azide as an indirect donor of NO in biological systems [23].

In parallel to these experiments, we investigated with flow cytometry the effect of the protonophore and sodium azide on matrix content of Ca ions in mitochondria isolated by differential centrifugation from rat myometrium. Addition of exogenous Ca^{2+} to mitochondria suspension was associated with the increase in fluorescence of Fluo-4 AM that had been loaded into them in advance (Fig. 8, bar 1), which indicates increased matrix Ca^{2+} concentration. The cation was accumulated in the presence of Mg-ATP²⁻ and succinate for 5 min., at which moment the stable level of Ca^{2+} accumulation was achieved. We ascertained the barrier function of mitochondrial inner membrane towards Ca ions by addition of A-23187 Ca^{2+} -ionophore to suspension. This was associated with rapid release of the accumulated cation (Fig. 8, bar 2). For the correct understanding of these results it is important to note that the working principle of flow cytometer allows for detection of changes in Ca ions concentration inside mitochondria, disregarding the extramitochondrial medium. The protonophore and sodium azide caused efficient release of accumulated Ca^{2+} from mitochondria. The CCCP effect was somewhat more marked than that of azide (Fig. 8, bars 3 and 4).

On the other hand, 5-min preincubation of mitochondria with the protonophore without exogenous Ca^{2+} led to decreased matrix cation levels by 20% on the average (data not shown). Sodium azide had no effect in these experimental conditions. Thus, sodium azide facilitated Ca^{2+} release from mitochondria that had been loaded with cation, and had no effect on ‘starting’ level of Ca^{2+} in the organelles. We assume that mitochondrial Ca^{2+} content in non-activated intact myocytes with low extracellular Ca^{2+} concentration is lower than that during its energy-dependent accumulation by isolated mitochondria. This can be an additional explanation for the absence or near absence of effect of sodium azide on myoplasm Ca^{2+} concentration in intact cells.

Therefore, we demonstrate using laser scanning confocal microscopy, flow cytometry, and fluorescent probes’ colocalization methodology that elimination of electrochemical proton gradient on the inner membrane of mitochondria is associated with an increase in myoplasm Ca ion concentration of uterus smooth muscle cells. The fact that sodium azide, a mitochondrial electron-transport chain inhibitor, had no effect on myoplasm Ca^{2+} concentration may

be explained by degradation of the compound with consequent production of reactive nitrogen species, which modulate Ca^{2+} -transport systems of myocytes.

ЕЛЕКТРОХІМІЧНИЙ ПОТЕНЦІАЛ ВНУТРІШНЬОЇ МЕМБРАНИ МИТОХОНДРІЙ ТА Ca^{2+} -ГОМЕОСТАЗ У КЛІТИНАХ МІОМЕТРИЯ

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Із використанням Ca^{2+} -чутливого флуоресцентного зонда, барвників, які взаємодіють з мітохондріями, та методу лазерної скануючої конфокальної мікроскопії продемонстровано, що руйнування електрохімічного потенціалу на внутрішній мітохондріальній мембрані міоцитів матки протонофором carbonyl cyanide m-chlorophenyl hydrazone, СССР (10 мкМ) та інгібітором IV комплексу дихального ланцюга азидом натрію (1 мМ) супроводжується істотним зростанням концентрації Ca^{2+} в міоплазмі лише у разі дії протонофору, але не азиду натрію.

Зокрема, застосування специфічного щодо мітохондрій барвника nonyl acridine orange (NAO) та 9-аміноакридину, який зв'язується із мембранними компартментами за наявності градієнта протонів, показало, що введення протонофору та інгібітора дихального ланцюга спричинює дисипацію градієнта протонів на внутрішній мітохондріальній мембрані. За допомогою потенціалчутливого флуоресцентного зонда карбоціанінового ряду 3,3'-дигексилоксакарбоціаніну доведено також дисипацію електричного потенціалу мембрани в умовах дії зазначених сполук. Руйнування електрохімічного потенціалу мітохондрій carbonyl cyanide m-chlorophenyl hydrazone спричинює значне зростання флуоресценції Ca^{2+} -чутливого барвника Fluo-4 AM в міоплазмі клітин гладенького м'яза.

Одержані результати чітко підтверджено методом протокової цитофлуориметрії на ізольованих диференційним центрифугуванням мітохондріях, навантажених Fluo-4 AM. Зокрема, ініційоване додаванням екзогенного Ca^{2+} зростання концентрації катіона в матриксі повністю

пригнічується carbonyl cyanide m-chlorophenyl hydrazone.

Отже, із застосуванням двох незалежних спектрофлуориметричних методичних підходів, а саме лазерної конфокальної мікроскопії та протокової цитофлуориметрії, із використанням Ca^{2+} -чутливого флуоресцентного зонда Fluo-4 AM на моделях свіжовиділених міоцитів та ізольованих диференційним центрифугуванням мітохондріях гладенького м'яза матки підтверджено важливу роль електрохімічного градієнта внутрішньої мембрани цих органел в механізмах підтримання Ca^{2+} -гомеостазу клітин міометрія.

Ключові слова: мітохондрії, кальцій, електрохімічний потенціал мембрани мітохондрій, кальцієвий гомеостаз, оксид азоту.

ЭЛЕКТРОХИМИЧЕСКИЙ ПОТЕНЦИАЛ ВНУТРЕННЕЙ МЕМБРАНЫ МИТОХОНДРИЙ И Ca^{2+} -ГОМЕОСТАЗ В КЛЕТКАХ МИОМЕТРИЯ

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С использованием Ca^{2+} -чувствительного флуоресцентного зонда, красителей, которые взаимодействуют с митохондриями, и метода лазерной сканирующей конфокальной микроскопии продемонстрировано, что разрушение электрохимического потенциала на внутренней митохондриальной мембране миоцитов матки протонофором carbonyl cyanide m-chlorophenyl hydrazone, СССР (10 мкМ) и ингибитором IV комплекса дыхательной цепи азидом натрия (1 мМ) сопровождается существенным ростом концентрации Ca^{2+} в миоплазме только в случае действия протонофора, но не азидата натрия.

В частности, применение специфического для митохондрий красителя nonyl acridine orange (NAO) и 9-аминоакридина, который связывается с мембранными компартментами при наличии градиента протонов, показало, что введение протонофора и ингибитора дыхательной цепи вызывает рассеивание градиента протонов

на внутренней митохондриальной мембране. С помощью потенциалчувствительного флуоресцентного зонда карбоцианинового ряда 3,3'-дигексилоксакарбоцианина доказана диссипация электрического потенциала мембраны в условиях действия указанных соединений. Разрушение электрохимического потенциала митохондрий carbonyl cyanide m-chlorophenyl hydrazone вызывает существенный рост флуоресценции Ca^{2+} -чувствительного красителя Fluo-4 АМ в миоплазме клеток гладкой мышцы.

Полученные результаты качественно подтверждаются методом проточной цитофлуориметрии на изолированных дифференциальным центрифугированием митохондриях, нагруженных Fluo-4 АМ. В частности, инициированный добавлением экзогенного Ca^{2+} рост концентрации катиона в матриксе полностью подавляется carbonyl cyanide m-chlorophenyl hydrazone.

Итак, с применением двух независимых спектрофлуориметрических методических подходов, а именно лазерной конфокальной микроскопии и проточной цитофлуориметрии, с использованием Ca^{2+} -чувствительного флуоресцентного зонда Fluo-4 АМ, на моделях интактных миоцитов и изолированных дифференциальным центрифугированием митохондриях гладкой мышцы матки подтверждено важную роль электрохимического градиента внутренней мембраны этих органелл в механизмах поддержания Ca^{2+} -гомеостаза клеток миометрии.

Ключевые слова: митохондрии, кальций, электрохимический потенциал мембраны митохондрий, кальциевый гомеостаз, оксид азота.

References

1. Kosterin S. A., Burdyga Th. V., Fomin V. P. et al. "Mechanism of Ca^{2+} transport in myometrium". Control of Uterine Contractility, Chap. 6, Garfield R. and Tabb T. (eds.), CRC Press Boca Raton, N. Y., 1994: 129-153.
2. Kostyuk P. G., Kostyuk O. P., Lukyanets E. A. Intracellular calcium signaling: structures and functions. Kiev: Naukova dumka, 2010. 175 p. (In Ukrainian).
3. Burdyga T., Poul R. J. Calcium homeostasis and signaling in smooth muscle. Muscle: Fundamental Biology and Mechanisms of Disease: chapter 86. 2012:1155-1172 c.
4. Csordás G., Várnai P., Golenár T., Sheu S. S., Hajnóczky G. Calcium transport across the inner mitochondrial membrane: molecular mechanisms and pharmacology. *Mol. Cell Endocrinol.* 2012;353(1-2):109-113.
5. Kosterin S. O. Calcium transport in smooth muscle. Kiev: Naukova dumka, 1990. 216 p. (In Russian).
6. Kolomiets O. V., Danylovyh Yu. V., Danylovyh H. V., Kosterin S. O. $\text{Ca}^{2+}/\text{H}^{+}$ -exchange in myometrium mitochondria. *Ukr. Biochem. J.* 2014;86(3):41-48. (In Ukrainian).
7. Vovkanyh L. S., Dubytsky L. O. Kinetic properties of the H^{+} -stimulated rat liver mitochondria Ca^{2+} efflux. *Exp. Clin. Physiol. Biochem.* 2001;3(5):34-37. (In Ukrainian).
8. Kucherenko M. E., Voytsitsky V. M. Bioenergy. Kiyv, Vyscha shkola, 1982. 272 p. (In Russian).
9. Mollard P., Mironneau J., Amedee T., Mironneau C. Electrophysiological characterization of single pregnant rat myometrial cells in short-term primary culture. *Am. J. Physiol. Cell Physiology.* 1986;250(1):C47-C54.
10. Danylovyh Yu. V., Danylovyh H. V., Kolomiets O. V., Kosterin S. O., Karakhim S. A., Chunikhin A. Yu. Investigation of nitrosative compounds influence on polarization of the mitochondrial inner membrane in the rat uterus myocytes using potential sensitive fluorescent probe $\text{DiOC}_6(3)$. *Ukr. Biochem. J.* 2014;86(1):42-55. (In Ukrainian).
11. Kolomiets O. V., Danylovyh Yu. V., Danylovyh H. V., Kosterin S. O. Ca^{2+} accumulation study in isolated smooth muscle mitochondria using Fluo-4 АМ. *Ukr. Biokhim. Zhurn.* 2013;85(4):30-39. (In Ukrainian).
12. Kucherenko M. E., Babenuk Yu. D., Voytsitsky V. M. Modern methods of biochemical studies: tutorials. Kiyv: Fitosotsiotsentr, 2001. 424 p. (In Russian).
13. Garcia Fernandez M. I., Ceccarelli D., Muscatello U. Use of the fluorescent dye 10-N-nonyl acridine orange in quantitative and location assays of cardiolipin: a study on different experimental models. *Anal. Biochem.* 2004;328(2):174-80.
14. Evron Y., McCarty R. E. Simultaneous measurement of ΔpH and electron transport in chloroplast thylakoids by 9-aminoacridine fluorescence. *Plant Physiol.* 2000;124:407-414.

15. Marchetti C., Jouy N., Leroy-Martin B., Defosse A., Formstecher P., Marchetti P. Comparison of four fluorochromes for the detection of the inner mitochondrial membrane potential in human spermatozoa and their correlation with sperm motility. *Hum. Reprod.* 2004;12(10):2267-2276.
16. Kalbocova M., Vrbacky M., Drahota Z., Melková Z. Comparison of the effect of mitochondrial inhibitors on mitochondrial membrane potential in two different cell lines using flow cytometry and spectrofluorometry. *Cytometry.* 2003 52A(2):110-116.
17. Danylovych H. V., Danylovych Yu. V., Gorchev V. F. Comparative investigation by spectrofluorimetry and flow cytometry of plasma and inner mitochondrial membranes polarization in smooth muscle cell using potential-sensitive probe DiOC₆(3). *Ukr. Biokhim. Zhurn.* 2011;83(3):99-105. (In Ukrainian).
18. Babich L. G., Shlykov S. G., Borisova L. A., Kosterin S.A. Energy-dependent transport of Ca²⁺ in the intracellular structures of the smooth muscle. *Biochemistry (Mosc.)*. 1994;59(8):1218-1229. (In Russian).
19. Karapetyants M. H., Drakyn S. I. General and nonorganic chemistry. Moscow: Chemistry, 1981. 630 p. (In Russian).
20. Chang S., Lamm S.H. Human health effects of sodium azide exposure: a literature review and analysis. *Int. J. Toxicol.* 2003;22(3):175-186.
21. Ji D., Kamalden T. A., del Olmo-Aguado S., Osborne N. N. Light- and sodium azide-induced death of RGC-5 cells in culture occurs via different mechanisms. *Apoptosis.* 2011;16(4):425-437.
22. Gee K. R., Brown K. A., Chen W-N. H., Bishop-Stewart J., Gray D., Johnson I. Chemical and physiological characterization of fluo-4 Ca(2+)-indicator dyes. *Cell Calcium.* 2000;27(2):97-106.
23. Iakovenko I. N., Zhirnov V. V. Sodium azide as indirect nitric oxide donor: researches on the rat aorta isolated segments. *Ukr. Biokhim. Zhurn.* 2005;77(4):120-123. (In Russian).

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