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Biochemical properties of H^+-Ca^{2+}-exchanger in the myometrium mitochondria

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ABSTRACT

Some biochemical properties of the H^+-Ca^{2+}-exchanger in uterine smooth muscle mitochondria have been described. The experiments were performed on a suspension of isolated mitochondria from the myometrium of rats. Methods of confocal microscopy, spectrofluorimetry and photon correlation spectroscopy were used. Fluo-4 probe was used to record changes in ionized Ca^{2+} in the matrix and cytosol; pH changes in the matrix were evaluated with BCECF. It was experimentally proved that in the myometrium instead of Na^+-Ca^{2+}-exchanger the H^+-Ca^{2+}-exchanger functions. It was activated at a physiological pH value, was carried out in stoichiometry 1: 1 and was electrogenic. The transport system was modulated by magnesium ions and the diuretic amiloride, but was not sensitive to changes in the concentration of extra-mitochondrial potassium ions. H^+-Ca^{2+}-exchanger was suppressed by antibodies against the LETM1 protein. Calmodulin may act as a regulator of H^+-Ca^{2+}-exchanger by inhibiting it.

1. Introduction

The relevant interest in the myometrium is associated with its unique function in women, namely the fetal development during pregnancy and providing timely labor. Regulation of myometrial function during different physiological peroids is the central focus of reproductive science research. This complex problem involves elucidating cellular and molecular mechanisms of uterine smooth muscle contractile activity. In the vast majority of cases, the molecular basis for the regulation of the myometrium contractile activity is the changes in the level of ionized Ca in the myoplasm. Ca^{2+}-transport systems of subcellular structures, in particular mitochondria, are a target for substances that modulate the contractile function of myocytes [Wray and Prendergast, 2019].

Mitochondria play the leading role in the physiology of eukaryotic cells. They provide metabolic processes with energy, determining and controlling their behavior, and play a major role in the mechanisms of cell death. Mitochondrial dysfunction underlies the emergence of numerous pathological processes – from disorders of the cardiovascular system to the emergence of tumors. In recent years, such definition as "mitochondrial disease" has been used [Wang and Wei, 2017; Alston et al., 2017; Bravo-Sagua et al., 2017; Anderson et al., 2019; Yeh et al., 2020; Kuznetsov et al., 2021].

Calcium ions play an important role in the functioning of mitochondria. On the one hand, changes in the concentration of Ca^{2+} in the matrix regulate the metabolic activity of their own organelles [Wang and Wei, 2017; Bravo-Sagua et al., 2017; Anderson et al., 2019]. On the other hand, the mitochondrial calcium transport systems largely determine the termination of calcium transient in cells [Wang and Wei, 2017]. Increased concentrations of Ca^{2+} in the mitochondrial matrix activate the synthesis of ATP and enzymes of tricarboxylic acid cycle [McCormack and Denton, 1987; Cao et al., 2019; Gellerich et al., 2013], while organelles' overload with the cation induces cellular death [Anderson et al., 2019; Naumova and Šachl, 2020]. The regulation of Ca^{2+} concentration in the mitochondria is based on coordinated functioning of the systems that provide energy-dependent accumulation of the cation and its release from the matrix to the cytosol [Anderson et al., 2019; Cao et al., 2019; Alevriadou et al., 2021].

The basic system that carries out Ca^{2+} accumulation in energized mitochondria is calcium uniporter in their inner membrane that provides storage of the cation in accordance with electrophoretic mechanism (MCU, mitochondrial Ca^{2+} uniporter) [Pallafacchina et al., 2021; Alevriadou et al., 2021]. This transport system is modulated by mitochondrial Ca^{2+} uptake regulatory proteins of the MICU's family (MICU1, MICU2, MICU3), and it is inhibited relatively specifically by RuR/Ru360

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[Marchi and Pinton, 2014; Santo-Domingo et al., 2015; Tarasova et al., 2019]. In the inner mitochondrial membrane, Na^+-Ca^{2+}- and H^+-Ca^{2+}-exchangers operate, providing Ca^{2+} release from the matrix and are sensitive (or insensitive) to ruthenium red [Marchi and Pinton, 2014; Anderson et al., 2019; Naumova and Šachl, 2020; Alevriadou et al., 2021]. Depending on the pH and the ratio of Ca^{2+} concentration in the cytosol and the matrix H^+-Ca^{2+}-exchanger can function in either direct or reverse mode [Nowikovsky et al., 2012; McCarron et al., 2013]. In the latter it provides Ca^{2+} accumulation from the cytosol at relatively low concentrations of the cation (sub-micromolar), unlike low-affinity MCU. Overloading with calcium ions and deactivation of the mitochondria result in mitochondrial permeability transition pore opening, through which cation transport may also be executed [Santo-Domingo et al., 2015; Cao et al., 2019; Naumova and Šachl, 2020; Bock and Tait, 2020].

In previous studies performed on digitonin permeabilized rat uterine myocytes using isotope techniques ($^{45}Ca^{2+}$) have shown that acidification of the medium leads to inhibition of ruthenium-dependent incorporation of Ca^{2+} in the mitochondria, and isotonic replacement of K^+ by Na^+ is ineffective [Shinlova et al., 1996]. These data indicate the primary role of H^+-Ca^{2+}-exchanger in the mitochondria of smooth muscle cells, including the myometrium. A similar transport system also operates in the cells of the liver and kidneys [Villa et al., 1998; Gunter and Pfeiffer, 1990]. However, the question of identification and biochemical properties of H^+-Ca^{2+} - exchanger in the mitochondria of uterine myocytes remain unclear.

The aim of the study was to investigate some properties of H^+-Ca^{2+}-exchanger in isolated from the rat myometrium mitochondria.

2. Materials and methods

Experiments were performed on white wild-type non-pregnant rats weighing 150–180 g. All manipulations with animals were carried out according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes [European Convention, 1999], and the Law of Ukraine “On protection of animals from cruelty”. Rats were anesthetized by chloroform inhalation and decapitated.

Experiments on uterus smooth muscle cells suspension. The myocytes were isolated from the uterus using Mollard method with same modifications [Mollard et al., 1986]. The removed uterus, freed from fat and connective tissue, was chopped into pieces (2 × 2 mm) and washed from blood and high concentrations of Ca ions (3 times for 5 min) in 5 ml of Hanks’ solution (136.9 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4, 0.26 mM $NaHCO_3$, 0.26 mM Na_2HPO_4, 0.03 $CaCl_2$, 5.5 mM glucose, 10 mM HEPES (pH, 7.4; 37 °C). After that, the tissue pieces were incubated for 20 min (37 °C, constant stirring) in 2 ml of tissue dissociation medium, which is Hanks’ solution with 0.1% collagenase type IA, 0.1% bovine serum albumin, and 0.01% soybean trypsin inhibitor. After 20 min, the tissue dissociation medium was removed, and the tissue was transferred to Hanks’ solution free from the enzyme preparation. The tissue preparation was carefully pipetted 15–20 times using a glass pipette for 1–2 min to accelerate cell separation. The solution with dissociated cells was collected, and the tissue preparation was again transferred to the tissue dissociation medium. This procedure was repeated 5–6 times. The first two portions of the solution with dissociated cells were discarded, due to the fact that they contained tissue fragments and damaged cells. The next 3–4 portions were collected, combined and centrifuged for 10 min at 80 g. The resulting cell pellet was washed with solution B and centrifuged again.

The cells immobilization for confocal microscopy, washing off the unadherent myocytes, and all experimental manipulations were performed in Hanks’ balanced salt solution. The intracellular spatial distribution of fluorescent dye was examined with LSM 510 META confocal laser scanning microscope (Carl Zeiss, Germany) using myocytes immobilized on poly-L-lysine. 50 μM Hoechst 33342 and 10 μM Fluo-4 AM were used to visualize nuclei and Ca^{2+} myoplasm. The probes were loaded for 15 min at 24 °C. The readings were performed in Multi Track mode of the confocal microscope. Hoechst 33342 fluorescence was excited with laser set at 405 nm and the signal was detected at 420–480 nm setting of BP filter. Fluo 4 fluorescence was excited at 488 nm, and the emission was registered at 505–530 nm (BP 505–530 filter setting).

Isolation of mitochondria from the uterine smooth muscle. The mitochondrial fraction was isolated from the myometrium using differential centrifugation. After uterus removal and its purification from adipose and connective tissue the preparation was kept in 0.9% NaCl solution. Myometrium was chopped with scissors into pieces (roughly 2 × 2 mm), which were put into the working solution with the following composition: 10 mM HEPES (pH 7.4), 250 mM sucrose, 1 mM EGTA at 4 °C. Tissue was homogenized with a homogenizer Heidolph Silent Crusher M (Germany) thrice for 20 s each time with cooling on ice for 1 min. The tissue:working solution ratio was 1:10. Homogenate was centrifuged at 1000 g for 15 min at 4 °C. Supernatant was centrifuged at 12000 g for 15 min at 4 °C. The pellet was resuspended in the working solution 2 (10 mM HEPES (pH 7.4), 250 mM sucrose) and again centrifuged at 12000 g for 15 min at 4 °C. The obtained isolated mitochondria were kept on ice during the experiment. The protein content in the mitochondrial fraction was determined by Bradford assey [Bradford, 1976]. Its average value in sample was 50 μg/ml.

Examining of the content of ionized Ca in mitochondria using the method of flow cytometry. The loading of mitochondria with the Fluo-4 AM probe at a concentration of 2 μM was carried out in a medium containing 10 mM HEPES (pH 7.4, 37 °C), 250 mM sucrose, 0.1% bovine serum albumin for 30 min at 37 °C. To improve the process, the dye was mixed with Pluronic F-127 (0.02%). The registration of relative Ca^{2+} level in the mitochondrial matrix, loaded with Fluo-4, was performed using flow cytometry on COULTER EPICS XLTM (Beckman Coulter, USA) with an argon laser (λex = 488 nm, λflu = 520 nm, channel FL1) (software SYSTEM IITM Software (“Beckman Coulter”, USA)). The medium from which the energy-dependent accumulation of Ca^{2+} by the mitochondria was carried out was small (volume of 2 ml): 20 mM HEPES (pH 7.4, 37 C), 250 mM sucrose, 2 mM KH_2PO_4/K_2HPO_4 (potassium phosphate buffer) (pH 7.4, 37 C), 3 mM $MgCl_2$, 3 mM ATP, 5 mM sodium succinate, 5 μM cyclosporin A, concentration of Ca^{2+} was 80 μM. Energy-dependent accumulation of Ca^{2+} was carried out for 5 min. The value of the fluorescent response was given in relative units as F/F_o, where F_o is the initial level of fluorescence, F is the fluorescence signal after the introduction of active substances.

Assessment of the content of ionized calcium and H^+ in the mitochondria. Loading of the mitochondria with the 2 μM Fluo-4 AM or 5 μM BCECF-AM was carried out in the medium that contained: 10 mM HEPES (pH 7.4, 37 °C), 250 mM sucrose, and 0.1% bovine serum albumin, 0.02% Pluronic F-127 for 30 min at 25 °C. The relative values of Ca^{2+} content in the matrix of mitochondria, loaded with Fluo-4 ($\lambda_{ex} = 490$ nm, $\lambda_{fl} = 520$ nm) or BCECF ($\lambda_{ex} = 510$ nm, $\lambda_{fl} = 535$ nm) was investigated using the fluorometric method on spectrofluorometer Quanta Master PTI 40 (Canada) with software FelixGX 4.1.0.3096. The medium, from which energy-dependent accumulation of Ca^{2+} was carried out by the mitochondria, had a composition: 20 mM HEPES (pH 7.4, 37 °C), 250 mM sucrose, 2 mM potassium phosphate buffer (pH 7.4, 37 °C), 3 mM $MgCl_2$, 3 mM ATP, 5 mM sodium succinate, concentration of Ca^{2+} – 80 μM. Energy-dependent Ca^{2+} accumulation was performed for 5 min, after which the suspension aliquot (100 μl) was diluted in the medium for Ca^{2+} release (2 ml) of the following composition: 20 mM HEPES (pH 6.5, 37 °C), 250 mM sucrose, 2 mM potassium phosphate buffer (pH 6.5, 37 °C), 5 mM sodium succinate, 5 μM cyclosporin A. The concentration of Ca^{2+}-free in this solution is 2.25 μM (calculated considering presence in Ca^{2+}-accumulation medium of 3 mM $MgATP^{2-}$). The gradients of monovalent cations Na^+, K^+, Li^+ were created under conditions of isotonic replacement of sucrose with 20 mM NaCl/KCl/LiCl +130 mM choline chloride or 150 mM choline chloride.

The activation constant of H^+-Ca^{2+}-exchanger in mitochondria for

H^+ and Hill coefficient were evaluated by Hill method [Keleti, 1986]. These constants were calculated in Hill coordinates {lg[($V_{o,max}$ – V_o)]/V_o: lg[H^+]}, where V_o – initial velocity, $V_{o,max}$ – maximum velocity of the Ca^{2+}-transport from mitochondria; $n = 5$. Correlation coefficient formulas were used to find the strength of relationship between the data. The formulas return a value between −1 and 1. Curves with the correlation coefficient R2 > 0.9 were taken into account. The data is presented as mean ± SE. The basic kinetic parameters (V_0, $\tau_{1/2}$) of the Ca^{2+} transport were calculated as described [Danylovych et al., 2018].

Study of mitochondrial membrane polarization using potential-sensitive probes DiOC$_6$(3) and JC-1. Changes in the electric potential of mitochondria were recorded according to the fluorescence intensity 10 nM 3,3′-dihexyloxacarbocyanine ($DiOC_6(3)$) and 1 μM JC-1 [Perry et al., 2011; Sivandzade et al., 2019]. Changes in the fluorescence response of this probes were investigated using a spectrofluorometry method on the Quanta Master 40 PTI (Canada) spectrometer: $\lambda_{ex} = 485$ nm, $\lambda_{fl} = 505$ nm for $DiOC_6(3)$ and $\lambda_{ex} = 488$ nm, $\lambda_{fl} = 535$ nm (monomer), $\lambda_{fl} = 595$ nm (J-agregate) for JC-1. Fluorescent probes were introduced directly into the incubation medium. The incubation medium had the following composition: 20 mM HEPES (pH 7.4, 37 °C), 2 mM potassium phosphate buffer (pH 7.4, 37 °C), 125 mM KCl, 25 mM NaCl.

Estimation of mitochondrial hydrodynamic diameter. To assess changes in the mitochondrial volume, we used the dynamic light scattering method, which allowed us to determine their sizes (average hydrodynamic diameter). The volume of the particles in suspension was analyzed using the correlation spectrometer ZetaSizer-3 (Malvern Instruments, UK) equipped with He–Ne laser LGN-111 (P = 25 mW, λ = 633 nm). Its operation principle is based on the analysis of time-dependent fluctuations in the scattering intensity when a laser ray passes through a medium with the mitochondria. The temporal intensity changes are converted into the mean translational diffusion coefficient (D) [Merkus, 2009]. The translational diffusion coefficient is related to the duration of the correlation τ_c with the following ratio:

$$Dq^2 = 1/\tau_c \quad (1)$$

The wave vector of the concentration fluctuations (q) is described by the following expression:

$$q = \frac{4\pi n}{\lambda_o} sin(\frac{\theta}{2}) \quad (2)$$

where n is the refractive index of the medium (liquid), λ_0 is the wavelength, and θ is the scattering angle.

Using the Stokes–Einstein equation that connects the particle size, the translational diffusion coefficient, and the viscosity of the liquid, we could calculate the size (diameter) d(H) of the spherical particles as follows:

$$d(H) = \frac{k_B \cdot T}{3\pi\eta D} \quad (3)$$

where k_B is the Boltzmann constant; T is the absolute temperature, K; η is the shear viscosity of the medium in which the particles are suspended; and D is the translational diffusion coefficient.

The recording and statistical processing of the changes in the scattering intensity in the mitochondria water suspension ($n = 1.33$) were performed 10 times for 10 min at +22 °C, at a scattering angle of 90°. The obtained results were processed using the PCS-Size mode v1.61 software.

The incubation medium contained: 20 mM HEPES (pH 7.4, 37 °C) 120 mM KCl, 2 mM potassium phosphate buffer (pH 7.4, 37 °C), 5 mM pyruvate, and 5 mM succinate.

Data is presented as means ± SE of the numbers of determinations. Differences between data sets of fluorometric experiments were analyzed using unpaired Student's *t*-tests in Microsoft Excel.

In the work the following reagents were used: HEPES, glucose, sucrose, sodium succinate, sodium pyruvate, bovine serum albumin, poly-L-lysine, collagenase type IA, ATP, Pluronic F-27, EGTA, $CaCl_2$, cyclosporin A, oligomycin, rotenone, antimycin A, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), sodium azide (Sigma, USA); BCECF-AM, Hoechst 33342, soybean trypsin inhibitor, $DiOC_6(3)$, JC-1 (Fluka, Switzerland); Fluo-4 AM (Invitrogen, USA). Any other reagents were produced in Ukraine.

The solutions were prepared on bidistilled water, which had a specific electrical conductivity of not more than 2.0 μcm/cm. The electrical conductivity of the water was recorded using a conductometer OK-102/1 (Hungary).

3. Results

Calcium accumulation by energized mitochondria is conducted primarily via electrophoretic mechanism [McCarron et al., 2013]. The Ca^{2+}-uniporter function depends on proton gradient. We demonstrated, that elimination of electrochemical potential of uterus myocytes' inner mitochondrial membrane by 10 μM protonophore CCCP is associated with substantial increase of Ca^{2+} concentration in myoplasm (Fig. 1, A and B). The increase in Fluo-4 fluorescence was observed without addition of exogenous Ca^{2+}, which may signify an increase of Ca ions concentration due to its release from intracellular stores. The role of mitochondria (namely, maintaining the potential of their inner membrane) in the exchange of Ca ions is proved by experiments on isolated organelles. The introduction of exogenous Ca^{2+} into the mitochondrial suspension was accompanied by an increase in the fluorescence of Fluo-4 with which they were preloaded (Fig. 1, C), which indicates an increase in the concentration of Ca^{2+} in the matrix. The preservation of the barrier to Ca ions by the function of the inner membrane of mitochondria is indicated by experiments with the introduction of Ca^{2+}-ionophore A-23187, which causes the rapid release of pre-accumulated cation. The protonophore of the CCCP caused an effective release of pre-accumulated Ca^{2+} from mitochondria (Fig. 1, C).

The next series of studies was aimed at identifying H^+-Ca^{2+}-exchange in the mitochondria of the myometrium. A release of Ca^{2+} from mitochondria, previously accumulated in the energy-dependent process, in the case of extramitochondrial pH 7.5 and lower has been shown. It was dependent on the value of extramitochondrial pH (Fig. 2, A). At the same time, the matrix was acidified, as evidenced by the decrease in the fluorescent response of the BCECF (Fig. 2, B). Gradients of the significant monovalent cations Na^+ and K^+, as well as Li^+ (under isotonic conditions) did not stimulate Ca^{2+} transport (Fig. 2, C). It has been revealed that 100 μM tetraphenylphosphonium, an inhibitor of Na^+-Ca^{2+}-exchanger in the mitochondria [Yang et al., 2021], does not affect ΔpH-induced Ca^{2+} transport too (graphical data are not given).

The next task was to study main kinetic parameters of H^+-Ca^{2+}-exchanger in the myometrial mitochondria. The curve of the Ca^{2+} release rate from isolated mitochondria on the concentration of protons tends to saturation (Fig. 3), and the calculated pH of activation (pH_a) is 6.9 ± 0.1. The value of the Hill coefficient (n_H), calculated using the appropriate coordinates, indicates the stoichiometry of H^+-Ca^{2+}-exchange 1:1 and the electrogenicity of the transport system. The consecutive step was to confirm or refute the electrogenic nature of the studied transport system using membrane electrical potential sensitive fluorescent probes.

Prior to the study, we found that the fluorescence intensity of the $DiOC_6(3)$ did not depend on the pH of the experimental medium. It was shown (Fig. 4) increase in the fluorescence intensity of the probe with a subsequent decrease under conditions ΔpH-induced Ca^{2+} release from the matrix. So, functioning of H^+-Ca^{2+}-exchange is accompanied an increase in electric potential with a subsequent depolarization mitochondrial membrane. For the results verified, we applied another widely used potential-sensitive fluorescent probe JC-1 in the next series of experiments [Perry et al., 2011; Wolken and Arriaga, 2014]. Changes in the fluorescent response of JC-1 under the action of electrical potential modulators on the inner membrane of mitochondria were investigated.

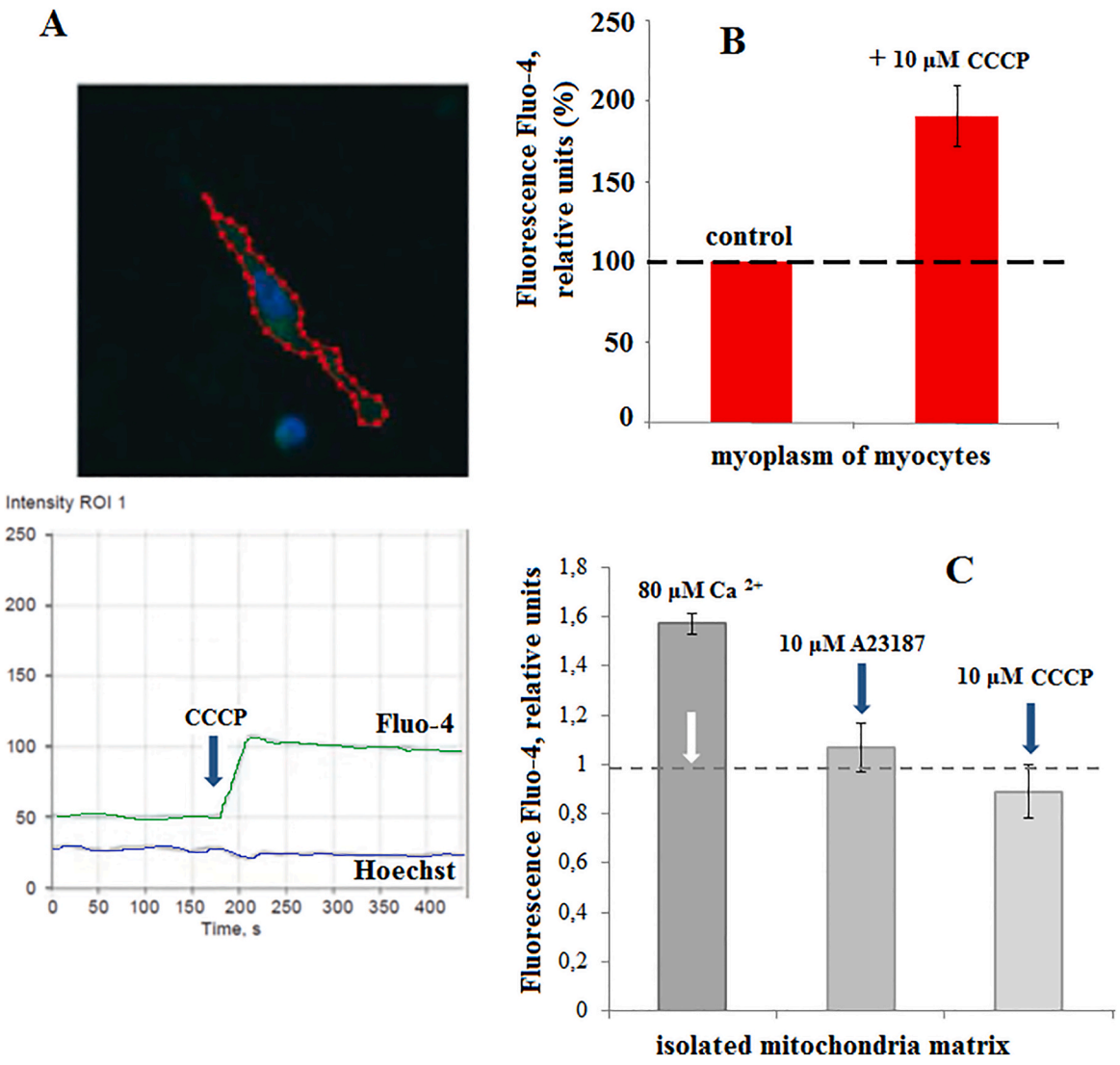

Fig. 1. Changes in the content of ionized Ca^{2+} under influence of protonophore CCCP in the myoplasm (A, B) and matrix isolated mitochondria of uterus myocytes. A - Visualization with Fluo-4 of the increase in Ca^{2+} myoplasm concentration under effect of the 10 μM protonophore, confocal microscopy data. B – Quantitative analysis of confocal microscopy data, Data are means ± SE, n = 8. C – The effect of Ca^{2+}-ionophore A23187 and protonophore CCCP on the content of ionized Ca^{2+} in the matrix isolated mitochondria, Data are means ± SE, n = 5, flow cytometry data.

The introduction of respiratory substrates 5 mM succinate and pyruvate was accompanied by an increase in electrical potential on the inner mitochondrial membrane and JC-1 fluorescence intensification (Fig. 5). Addition of 2.5 μM oligomycin, which causes artificial hyperpolarization of mitochondria due to inhibition of proton transport through the subunit of F_o ATP-synthase, resulted in JC-1 fluorescence increase (Fig. 5, A). The introduction of 3 mM $MgATP^{2-}$ into the mitochondrial suspension, which causes the generation of an electrochemical proton gradient due to the reversal of ATP-synthase activity, also caused an increase in the fluorescent response (Fig. 5, B). The protonophore CCCP (10 μM) and the inhibitor of the respiratory chain complex *III* antimycin A (1 μg/ml) caused a decrease in the fluorescent signal (Fig. 5, C and D) due to the expected depolarization of the mitochondrial membrane. The addition of 5 mM NaN_3 or 1 mM NaCN (widely used inhibitors of the respiratory chain) (Fig. 5, E and F) was accompanied by a decrease in the fluorescence of JC-1. Therefore, the fluorescent signal from JC-1 adequately reflects changes in the electrical potential of mitochondria. The fluorescent response of JC-1 at extra-mitochondrial pH 6.0, i.e. under the conditions of H^+-Ca^{2+}-exchanger functioning, indicates the initial polarization of the inner mitochondrial membrane with a subsequent decrease in electrical potential (Fig. 6).

These data are qualitatively consistent with previous experiments with $DiOC_6(3)$. This effect was not observed in the absence of ΔpH-dependent release of Ca^{2+} (in a medium with pH 7.5) (Fig. 6).

Changes in the ion transport systems activity in the inner membrane (H^+-Ca^{2+}-exchanger functioning) results in changes in the osmobalance and volume of isolated mitochondria. Photon correlation (laser correlation) spectroscopy is a convenient, fast, and relatively simple method for analyzing changes in the volume of objects [Merkus, 2009]. It was found that the value of the average hydrodynamic diameter of mitochondria was 418 ± 28 nm which corresponds to the results of electron microscopic studies [Kolomiets et al., 2014]. The changes in ion transport in the inner membrane leads to changes in mitochondrial volume [Kaasik et al., 2007; Nowikovsky et al., 2009]. The energy-dependent accumulation of 80 μM Ca^{2+} by mitochondria was accompanied by an approximately twofold increase in the value of the hydrodynamic diameter. Under the conditions of cation release (functioning of H^+-Ca^{2+}-exchanger), the acidification of the mitochondrial incubation medium from 7.5 to 6.5 was accompanied by a significant decrease in the value of the hydrodynamic diameter from 811 ± 15 nm to 727 ± 23 nm (Fig. 7). Further acidification of the medium (up to 6.0) was not accompanied by a change in the studied parameter relative to the pH

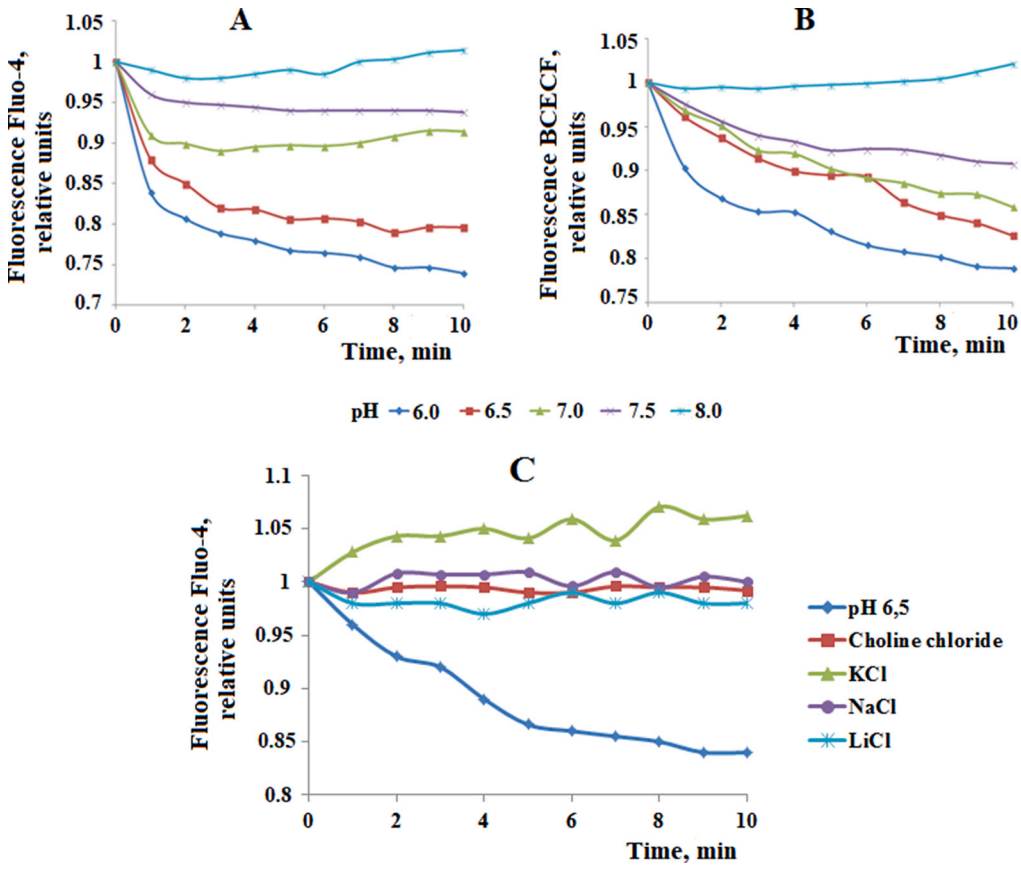

Fig. 2. Simultaneous decrease of Ca^{2+} (A) and H^+ increase (B) in mitochondrial matrix under the condition of H^+-Ca^{2+}-exchanger functioning. Effect of gradients of the monovalent cations Na^+, K^+, Li^+ and choline chloride (under isotonic conditions) on Ca^{2+} transport. The result of a typical experiment.

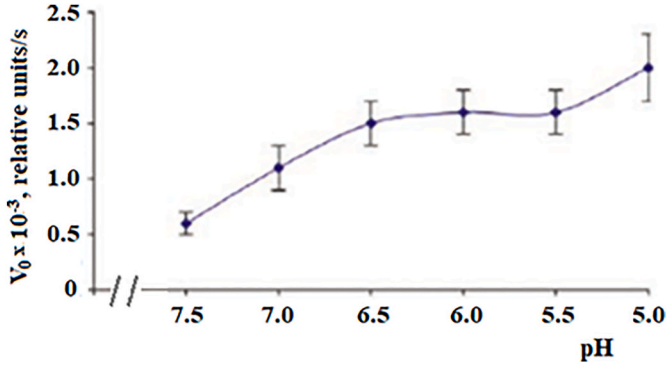

Fig. 3. The dependence of the initial velocity (V_0) of Ca^{2+} release from the mitochondria of the myometrium on pH. Data are means ± SE, n = 5.

value 7.5 (Fig. 7).

It has been reported that Ca^{2+} transport in the mitochondrial inner membrane is modulated by Mg^{2+} and diuretics (amiloride) [Gunter and Pfeiffer, 1990; Weiss et al., 1990]. In our study, Mg^{2+} inhibited (0.950 ± 0.019 r.u. *vs.* 0.900 ± 0.009 r.u. in control) and amiloride stimulated (0.870 ± 0.009 r.u. *vs.* control) H^+-Ca^{2+}-exchanger (Fig. 8).

According to modern concepts, molecular structure that ensures the functioning of H^+-Ca^{2+}-exchanger in the inner mitochondrial membrane is protein LETM1 (Leucine zipper and EF-hand containing transmembrane protein 1) [Nowikovsky et al., 2012; Lin and Stathopulos, 2019; Natarajan et al., 2021]. Therefore, to identify the molecular structure of the H^+-Ca^{2+}-exchanger, we used antibodies to the LETM1 protein. Anti-LETM1 (2.5 μg antibody to 100 μg mitochondrial protein) suppressed ΔpH-induced Ca^{2+} release from the mitochondrial matrix (Fig. 9). In case of the preincubation with antibody for 5 min, the effect was more pronounced.

LETM1 is also considered as a part of the molecular structure that is involved in the maintenance of potassium homeostasis of cellular organelles [Nowikovsky et al., 2012; Csordás et al., 2012; Natarajan et al., 2021]. It was found that the introduction of K^+ in the incubation medium at high physiological concentrations did not affect the H^+-Ca^{2+}-exchanger (Fig. 10).

It is possible that the transport enzymes of the inner mitochondrial membrane are subject to regulatory action of the Ca^{2+}-calmodulin complex (ubiquitous EF-hand containing Ca^{2+}-binding protein). Calmodulin antagonist calmidazolium at a concentration of 10 μM was found to almost completely block the ΔpH-dependent Ca^{2+} release from isolated mitochondria (Fig. 11, A). Another calmodulin antagonist 100 μM trifluoperazine, which has a different mechanism of action for Ca^{2+}-calmodulin dependent enzymes than calmidazolium, caused a faster initial release of Ca^{2+} from the matrix, but did not affect the plateau fluorescence level of Fluo-4 (Fig. 11, A).

4. DISSCUSION

Contractions of the smooth muscles are largely supported by the processes of oxidative metabolism, which are accompanied by an oxidation of fatty acids and acetoacetate. In the latter, an important role plays tricarboxylic acid cycle to provide energy for contractile activity [Anderson et al., 2019]. This fact points to the significant role of mitochondria in smooth muscle functioning, and importance of the regulation of calcium homeostasis in organelles. Energy dependent Ca^{2+} mitochondrial influx is performed by Ca^{2+}-uniporter, and its driving force is the electric potential difference on the inner membrane of the

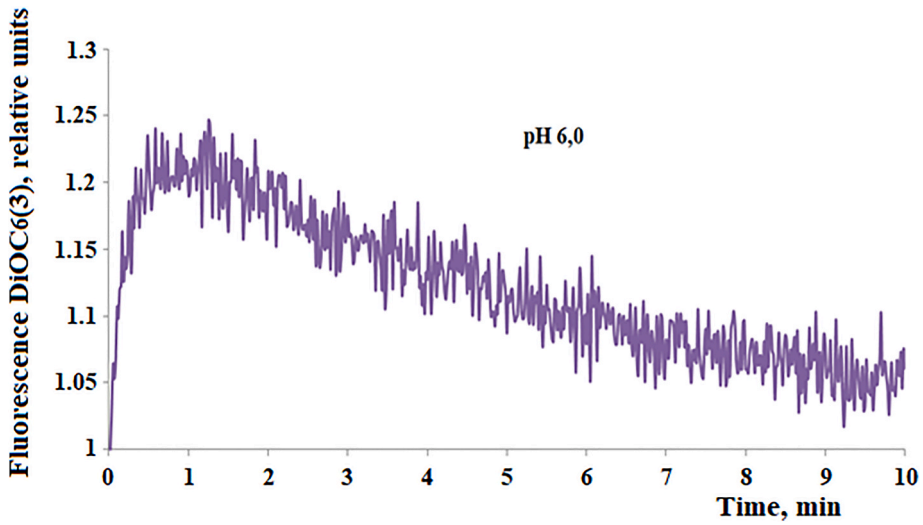

Fig. 4. Changes in the fluorescence of potential sensitive probe $DiOC_6(3)$ under the conditions of H^+-Ca^{2+}-exchanger functioning (pH 6.0). The result of a typical experiment.

organnelle reaching values −180 mV [Tarasova et al., 2019; Alevriadou et al., 2021]. A substantial decrease in the membrane potential causes inhibition of electrophoretic Ca^{2+} accumulation. It can be assumed that Ca^{2+} release from matrix into cytosol can become the prevailing transporting process under such conditions [Burdyga and Poul, 2012; Csordás et al., 2012; Kolomiets et al., 2014]. We found noticeable changes in fluorescence of Fluo-4 under the effect of CCCP (Fig. 1). This result can be explained by the disabling of mitochondrial Ca^{2+}-uniporter due to elimination of electrochemical potential on their inner membrane by the protonophore. Thus, exchangers or permeability transition pore may function efficiently ensuring Ca^{2+} release from the mitochondrial matrix into the myoplasm. These preliminary results confirm the role of the electrochemical proton gradient (Δp) on the inner mitochondrial membrane in maintaining Ca^{2+} homeostasis in the myometrium cells. Therefore, it is important to study the ΔpH-dependent component of Ca^{2+} transport in the mitochondria and its possible electrogenicity.

The procedure of studying the ΔpH-dependent transport of Ca^{2+} from isolated mitochondria was preceded by the energy-dependent accumulation of this cation. It has been shown previously that freshly isolated from rat myometrium mitochondria effectively accumulate cation in the presence of a succinate as respiratory substrate. This is accompanied by a significant increase in the level of Fluo-4 fluorescence [Kolomiets et al., 2014]. The introduction of 3 mM Mg-ATP^{2-} into the incubation medium improved the accumulation process due to the creation of conditions for the reverse functioning of ATP-synthase (F_o, F_1 - ATPase) and an increase in the electrochemical potential on the inner membrane (Fig. 5, B). A release of Ca^{2+} from mitochondria in the case of lowered extramitochondrial pH has been shown (Fig. 2, A). The presence of cyclosporin A in the medium for Ca^{2+} release makes it impossible for the permeability transition pore to contribute to the transport process. The release of Ca^{2+} from mitochondria was significantly increased in case of acidification of the incubation medium, which indicates the existence of the ΔpH-dependent component of Ca^{2+} transport from mitochondria and corresponds to the idea of H^+-Ca^{2+}-exchanger in the inner mitochondrial membrane in smooth muscle. Simultaneously, the matrix was acidified (Fig. 2, B). Physiologically significant monovalent cation Na^+ (Na^+ gradient) (Fig. 2, C) did not stimulate Ca^{2+} transport and the inhibitor of Na^+-Ca^{2+}-exchanger in the mitochondria tetraphenylphosphonium did not inhibit Ca^{2+} transport in our experiments. These observations correspond to the idea of insignificant role of Na^+-Ca^{2+}-exchanger in smooth muscle mitochondria, in particular the myometrium.

An important step in the biochemical study of the transport system is to determine its kinetic parameters (Fig. 3). The value of the proton activation constant of the transport process (pH_a about 6.9) indicates that the exchanger is able to work at a pH close to physiological. The value of the Hill coefficient indicates the electrogenicity of H^+-Ca^{2+}-exchange, which is carried out in stoichiometry 1:1. However, according to the literature data, there is evidence of the functioning of this transporter in the inner membrane of the liver mitochondria, which carries out antiport exchange of one Ca^{2+} for two H^+ [Takeuchi et al., 2015; Jiang et al., 2009, Tsai et al., 2014, Shao et al., 2016]. We used fluorescent probes sensitive to changes in membrane potential in order to confirm the electrogenicity of the studied exchanger.

The $DiOC_6(3)$ probe is a positively charged lipophilic cation, the accumulation of which inside a limited membrane space (liposomes, mitochondria, cells) is potential-dependent [Marchetti et al., 2004; Kalbocova et al., 2003]. A biphasic change in the fluorescence intensity of the probe was observed (Fig. 4) under the conditions of H^+-Ca^{2+}-exchanger functioning, namely ΔpH-initiated release of Ca^{2+} from mitochondria (pH 6.0 of the extramitochondrial medium). The first phase was accompanied by an increase in the fluorescent signal and corresponded to an increase in electric potential. This time interval, from our point of view, corresponds to the stage of activation of the electrogenic H^+-Ca^{2+}-exchanger. Subsequently, there was a decrease in the electric potential on the inner membrane of mitochondria due to the entry of protons into the matrix.

The results with the use of fluorescent probes should be verified due to the possibility of the fluorescent response dependence on the concentration of the probe, as well as the impact on the functioning of the electron transport chain. Therefore, we applied another widely used potential-sensitive fluorescent probe JC-1 in the next series of experiments to study changes in the membrane potential of the mitochondria [Perry et al., 2011; Wolken and Arriaga, 2014; Sivandzade et al., 2019]. JC-1 exists predominantly as a monomer and fluoresces in the green region (525–535 nm) at concentrations lower than 100 nM. JC-1 forms aggregates (J-aggregates) with a shift of fluorescence intensity to the red region (595 nm) at higher concentrations. The membrane potential causes the accumulation of positively charged JC-1 in the membrane compartments. Polarized mitochondria have a higher fluorescence intensity in the red region from J-aggregates than in the green region from the monomeric form of the probe. The ratio of red/green fluorescence intensities (595/535) is used as an indicator of the membrane potential, the value of which does not depend on the probe concentration [Perry et al., 2011; Wolken and Arriaga, 2014; Sivandzade et al., 2019].

In the experiments, we observed an adequate fluorescent response of

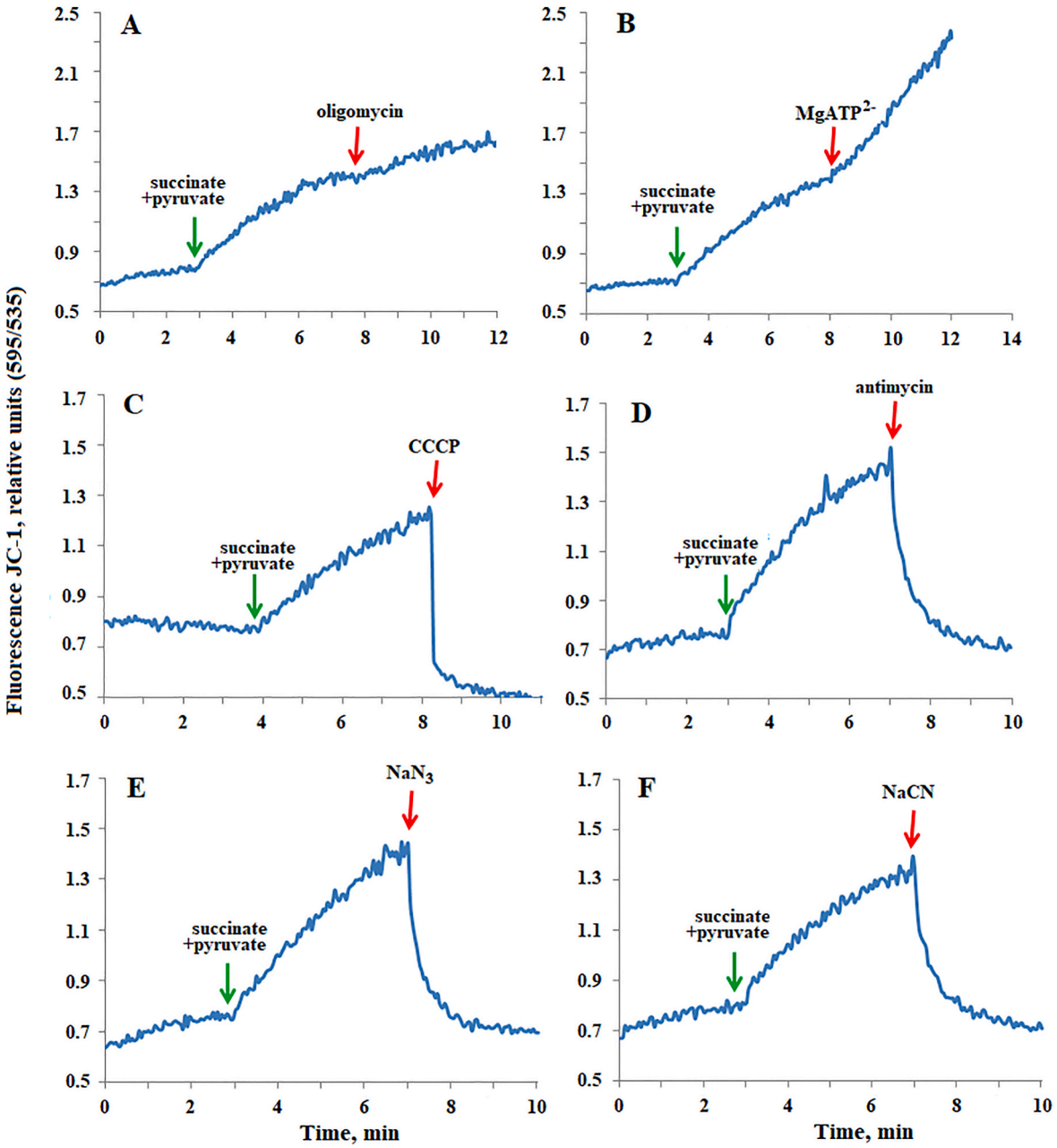

Fig. 5. Changes of the mitochondria inner membrane electrical potential under the action of its modulators determined by the fluorescence of JC-1: 5 mM succinate + pyruvate, 2.5 μM oligomycin, 3 mM $MgATP^{2-}$, 10 μM CCCP, 1 μg/ml antimycin, 5 mM NaN_3, 1 mM NaCN. The result of a typical experiment.

the probe to the introduction of compounds that are expected to change the electrical potential of the inner mitochondrial membrane in one direction or another (Fig. 5). In particular, substances that increase electrical potential, namely respiratory substrates, oligomycin and $MgATP^{2-}$ caused an increase in the fluorescent response of JC-1. At the same time, compounds that cause depolarization of the inner mitochondrial membrane (electron transport chain inhibitors, protonophore) contributed to the bleaching of fluorescence. These data prove that JC-1 can be correctly used to study changes in membrane potential in our system of isolated myometrial mitochondria. Experiments with the use of the potential-sensitive JC-1 probe confirmed the biphasic changes of mitochondrial potential under the conditions of H^+-Ca^{2+}-exchanger functioning, as well as the dependence of the phase of electric potential growth and subsequent depolarization on the pH (Fig. 6).

Thus, it was demonstrated that the intense release of Ca^{2+} from mitochondria is accompanied by an increase in the polarization of their inner membrane with the functional activity of the H^+-Ca^{2+}-exchanger. The proton input leads to further decrease electric potential. The obtained results qualitatively confirm the electrogenicity of H^+-Ca^{2+}-exchanger in the mitochondria of the myometrium.

One of the possible manifestations of the H^+-Ca^{2+}-exchanger functioning may be changes in the volume of isolated mitochondria. It is generally accepted that changes in mitochondrial volume are determined by the transport of water across the membrane. Osmoregulation is controlled primarily by the coordinated functioning of the K^+ channels and the H^+-K^+-exchanger of the inner membrane [Nowikovsky et al., 2012; Natarajan et al., 2021]. Disruption of osmoregulation occurs due to the development of mitochondrial dysfunction, Ca^{2+}-overload and the opening of the permeability transition pore [Kaasik et al., 2007; Nowikovsky et al., 2012; Natarajan et al., 2021]. Changes in the activity of H^+-Ca^{2+}-exchanger may result in changes in volume of isolated mitochondria.

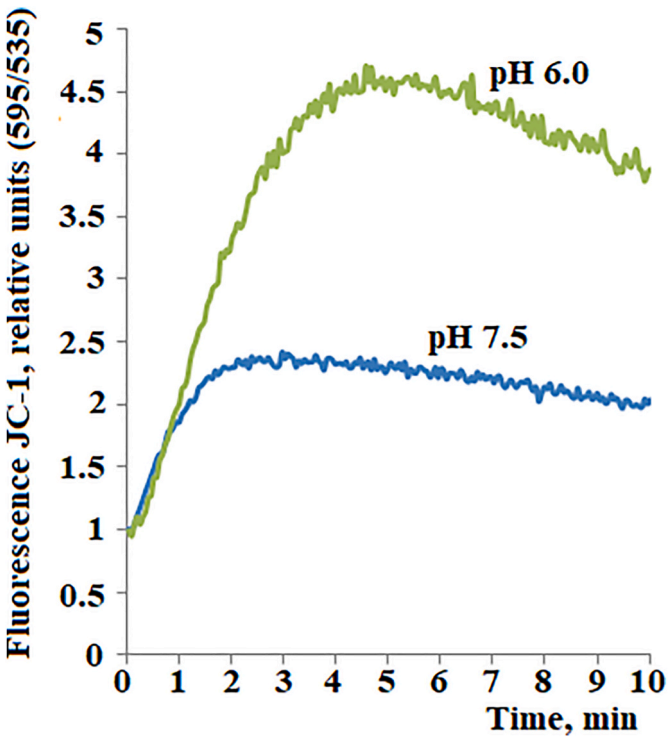

Fig. 6. Changes of the mitochondria inner membrane electrical potential under the conditions of H^+-Ca^{2+}-exchanger functioning determined by the fluorescence of JC-1. The result of a typical experiment.

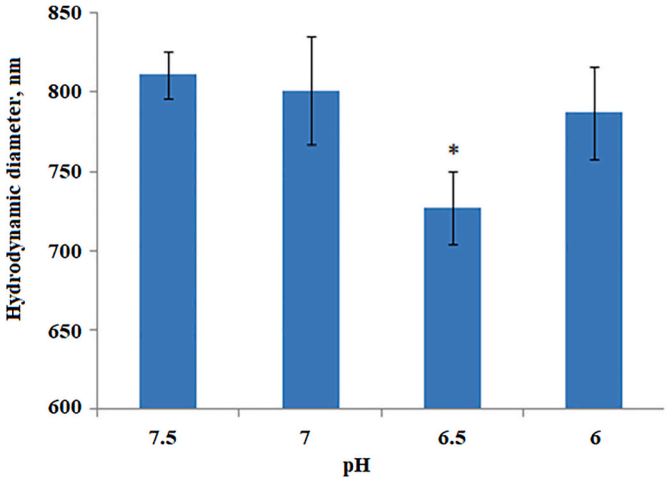

Fig. 7. Changes in the hydrodynamic diameter of isolated mitochondria under condition of ΔpH-induced release Ca^{2+} from the matrix. Data are means ± SE, n = 6, *- P < 0,05 *vs.* pH 7.5.

Photon correlation spectroscopy is a precise method for analyzing changes in the size of objects that are in Brownian motion. This method analyzes the intensity of scattering of monochromatic light by the surface of the objects under study, resulting in information on changes in their hydrodynamic diameter [Merkus, 2009].

We found that the H^+-Ca^{2+}-exchanger functioning (the acidification of the mitochondrial incubation medium from 7.5 to 6.5) was accompanied by a significant decrease in the value of the hydrodynamic diameter (Fig. 7). Further acidification of the medium did not change the volume. We assume that the stimulation of energy-dependent Ca^{2+} transport into mitochondria can lead to increased influx of K^+ and water molecules into the matrix, which will result in organelle swelling and a

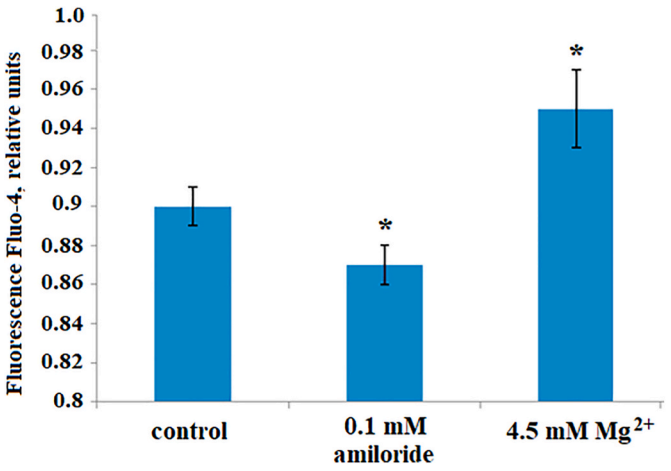

Fig. 8. The effects of modifiers of transmembrane cation exchange on ΔpH-induced Ca^{2+} transport from the mitochondria. Data are means ± SE, n = 5, * – P < 0.05 *vs.* control.

corresponding increase in hydrodynamic diameter. Initiation of ΔpH-dependent release of Ca^{2+} will reduce the studied parameter in accordance with these considerations. However, more significant acidification of the extramitochondrial environment can ionize the outer membrane of the organelle, which will raise the hydrodynamic diameter (increase the size of the water shell around the mitochondria), eliminating the previous effect. Thus, the obtained results may be a consequence of the H^+-Ca^{2+}-exchanger transport system functioning in the mitochondrial membrane.

It has been shown (Fig. 8) that magnesium ions inhibit and diuretic amiloride stimulates H^+-Ca^{2+}-exchanger. Another authors [Gunter and Pfeiffer, 1990; Pradhan et al., 2011] observed an inhibition of the Ca^{2+}-uniporter by Mg^{2+}. They associated that effect with either direct influence on the channel structure, or with decreasing of a negative charge, which provided the uniporter driving force. For H^+-Ca^{2+}-exchanger, we assume direct impact of Mg^{2+} on the transport system. Penetration of the blocking cation into the matrix and its relevant competition with Ca^{2+} for binding sites is unlikely because of the barrier function of the inner membrane. The studies using amiloride and its analogs have shown the inhibitory effect of diuretics on calcium uniporter [Gunter and Pfeiffer, 1990], while the corresponding effect on H^+-Ca^{2+}-exchanger was the opposite (Fig. 8).

In our experiments, preliminary Ca^{2+} accumulation in energy-dependent process can led to depolarization of the mitochondrial inner membrane, which opened the possibility of the cation release from the matrix upon concentration gradient through the structure of MCU. Since the effects of known modifiers of Ca^{2+} transport at the level of uniporter and H^+-Ca^{2+}-exchanger are opposite, the latter assumption is unlikely. On the other hand, according to our previous results [Danylovych et al., 2015], the permeability transition pore does not play a significant role in Ca^{2+} transport in isolated mitochondria in these conditions.

The functioning of H^+-Ca^{2+}-exchanger is ensured by protein LETM1 (~70 kDa). In the structure of LETM1 proteins family, N-terminal end, located in the intermembrane space of mitochondria, has been described. Also a transmembrane domain, which regulates the oligomerization to hexamer complex (~400 kDa), is distinguished, and C-terminal domain, located in the matrix. It includes, in particular, typical calcium-binding protein domain known as the "EF-hand" [Nowikovsky et al., 2012; Shao et al., 2016; Lin and Stathopulos, 2019]. The latter can ensure the functioning of LETM1 as H^+-Ca^{2+}-exchanger or act as a sensor, perceiving the changes in Ca^{2+} concentration in the matrix. It was used antibodies to the LETM1 (Anti-LETM1) to identify the molecular structure of the H^+-Ca^{2+}-exchanger in our experiments.

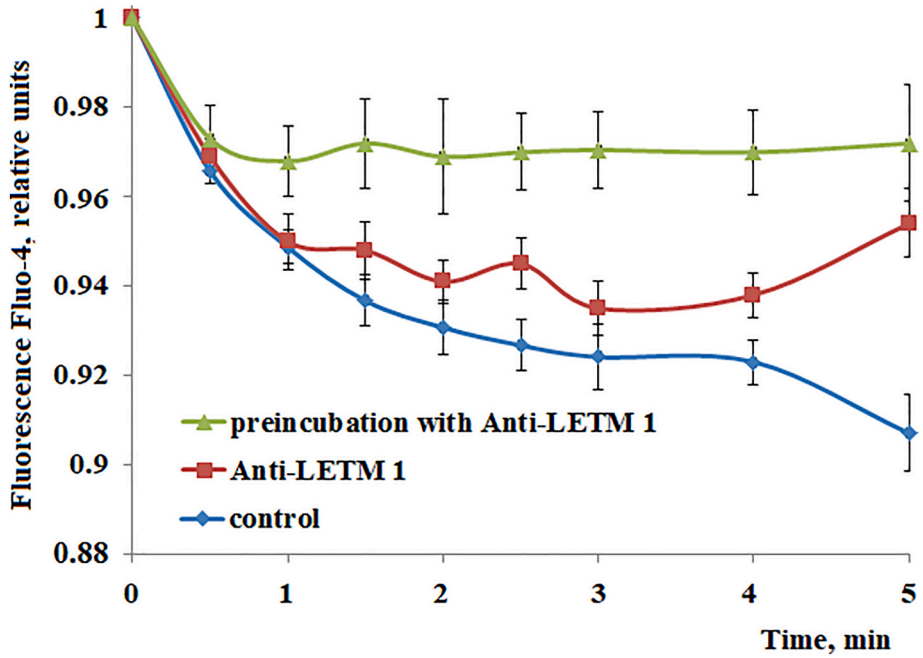

Fig. 9. Effect of the anti-LETM1 on ΔpH-induced Ca^{2+} transport from the matrix of the mitochondria (2.5 μg antibody to 100 μg mitochondrial protein). Data are means ± SE, n = 6.

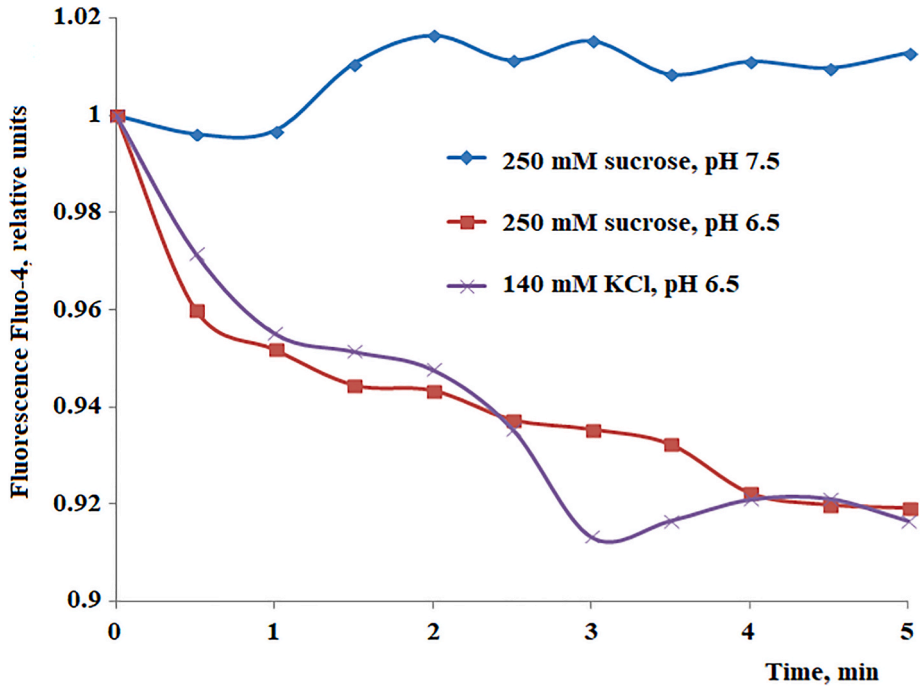

Fig. 10. ΔpH-induced Ca^{2+} transport from the mitochondrial matrix under condition of the replacement of sucrose medium by KCl. The result of a typical experiment.

Anti-LETM1 suppressed H^+-Ca^{2+}-exchanger in the myometrial mitochondria (Fig. 9). Thus, there is reason to assume that the rat myometrial mitochondria have the transport system of H^+-Ca^{2+}-exchanger which is represented by protein LETM1. However, these antibodies failed to inhibit the transport system completely even in high concentration. This result can be explained by species-specificity of the protein transporter. There is information on LETM1 interactions with the systems implementing the transport of substances into mitochondria [Nowikovsky et al., 2012], which possibly provides communication of the protein with external membrane of the organelles.

LETM1 is associated with a huge protein complex in the inner mitochondrial membrane. The absence of this protein leads to mitochondrial depolarization and swelling, and loss of cristae. Therefore, LETM1 is also considered as a part of the molecular structure that is involved in the maintenance of potassium homeostasis of cellular organelles. It is assumed that LETM1 can also act as the H^+-K^+-exchanger, regulating K^+-homeostasis and mitochondrial volume [Nowikovsky et al., 2012; Lin and Stathopulos, 2019; Natarajan et al., 2021]. The

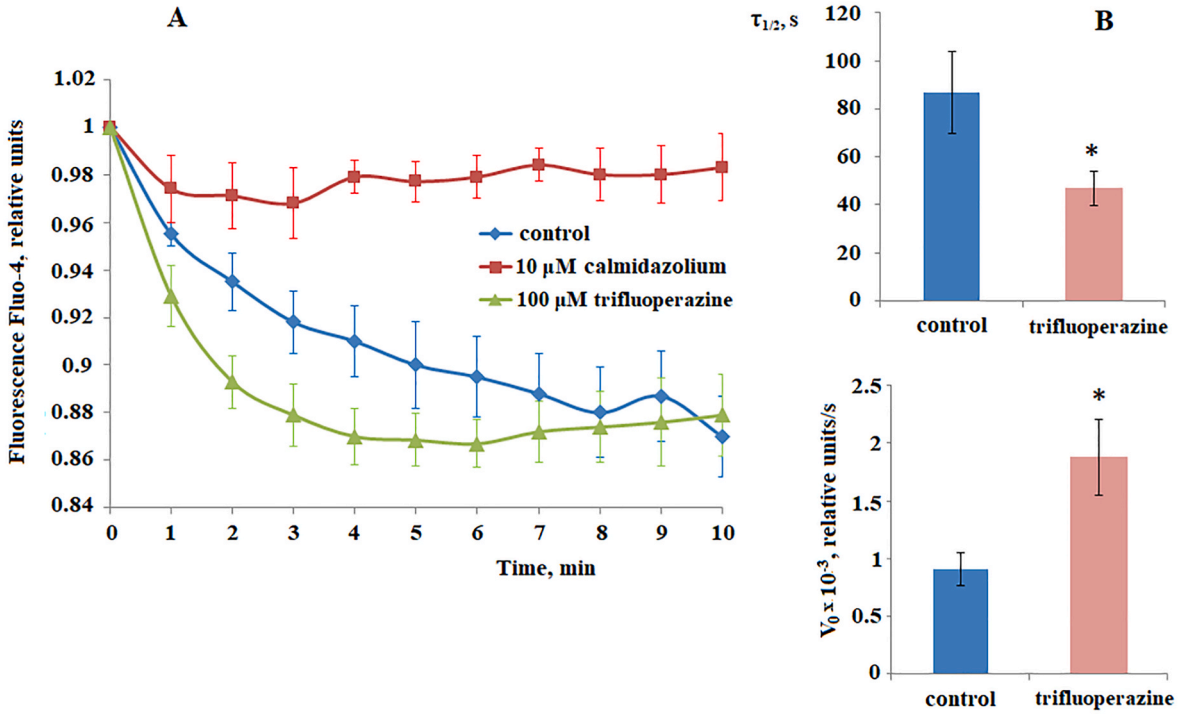

Fig. 11. Effect of the calmodulin antagonists on ΔpH-induced Ca^{2+} transport from the mitochondria (pH 6.5). (A) and changes of the main kinetic parameters of Ca^{2+} release (B). Data are means ± SE, n = 5, * - P < 0.05 *vs.* control.

effect replacement of sucrose medium by KCl on the pH-dependent release of Ca^{2+} from mitochondria was absent (Fig. 10). This result suggests that H^+-K^+-exchanger and H^+-Ca^{2+}-exchanger, probably, are different structures.

The presence of calmodulin in mitochondria is an established experimental fact [Odagiri et al., 2009]. To date, there is no information on the possibility of H^+-Ca^{2+}-exchanger regulation of the myometrial mitochondria by the Ca^{2+}-calmodulin complex. Therefore, the effects of calmodulin antagonists on the ΔpH-dependent release of Ca^{2+} from mitochondria isolated from the myometrium were elucidated. The almost complete blocking of the H^+-Ca^{2+} - exchanger by calmidazolium (Fig. 11, A) convincingly demonstrates the important role of calmodulin in the regulation of the H^+-Ca^{2+}-exchanger. The effect of trifluoperazine was less unambiguous. Analysis of the main kinetic parameters of the transport process - the initial velocity (V_0) and the value of the characteristic time ($\tau_{1/2}$) revealed that under the action of the studied antagonist the calcium release half-time decreased and the initial transport velocity increased (Fig. 11, B). That is, the mechanism of action of trifluoperazine (a milder antagonist of calmodulin) is different from that of calmidazolium. The first only reduces the effectiveness of the interaction between calmodulin and the transport system, while the second acts on the complex calmodulin-transport system, inhibiting the decay of the latter. It is a non-competitive inhibitor and also has a higher affinity for calmodulin [Roufogalis et al., 1983; Sobieszek, 1989]. These data indirectly suggest that calmodulin may act as a regulator of H^+-Ca^{2+}-exchanger by inhibiting it.

5. Conclusions

We have shown the possibility of the existence of H^+-Ca^{2+}-exchanger in the mitochondria of the myometrium and studied its properties (Fig. 12), namely:

- it does not depend on the gradient of sodium and potassium ions;
- is activated at physiological pH values;
- is carried out in stoichiometry 1: 1 and is electrogenic;

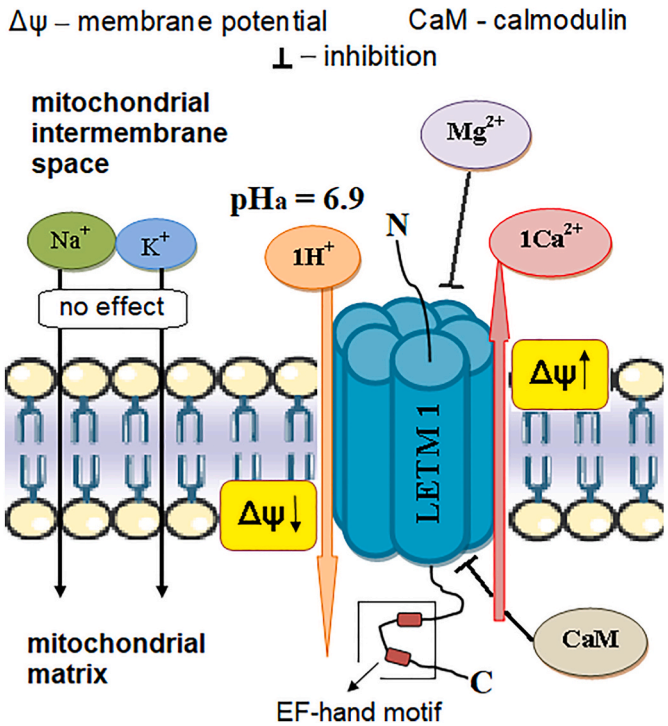

Fig. 12. Properties of H^+-Ca^{2+}-exchanger (system of secondary-active transport of Ca^{2+} from the matrix) in the mitochondria of the myometrium.

- inhibited by antibodies against LETM1 protein;
- modulated by the diuretic amiloride and magnesium ions;
- calmodulin may act as a regulator of H^+-Ca^{2+}-exchanger.

Thus, in addition to the energy-dependent accumulation of Ca^{2+} in the mitochondria of the myometrium, there is a system of secondary-

active transport of Ca^{2+} from the matrix of these organelles into the myoplasm. The basis of this phenomenon may be the functioning of H^+-Ca^{2+}-exchanger of the inner membrane of mitochondria.

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CRediT authorship contribution statement

Yurii V. Danylovych: Conceptualization, Investigation, Formal analysis, Data curation, Writing – review & editing. **Hanna V. Danylovych:** Conceptualization, Methodology, Investigation, Data curation, Writing – review & editing. **Oksana V. Kolomiets:** Investigation, Formal analysis, Writing – review & editing. **Marina D. Sviatnenko:** Investigation, Formal analysis, Writing – review & editing. **Sergiy O. Kosterin:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Danylovych Hanna reports financial support was provided by Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine.

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