

# ЕКСПЕРИМЕНТАЛЬНІ РОБОТИ

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## ACTIVATION OF GLYBENCLAMIDE-SENSITIVE MITOCHONDRIAL SWELLING UNDER INDUCTION OF CYCLOSPORIN OF A-SENSITIVE MITOCHONDRIAL PORE

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*Induction of mitochondrial swelling and increased generation of reactive oxygen forms by Ca ions have been shown in suspension of mitochondria from rat uterus. These effects were suppressed by the blocker of mitochondrial Ca<sup>2+</sup>-uniporter ruthenium red and MPTP inhibitor cyclosporin A, that evidences that the induction of mitochondrial permeability transition pore by Ca ions takes place. Ca<sup>2+</sup>-induced mitochondrial swelling was blocked by ATP-sensitive channel blocker glybenclamide but only if K<sup>+</sup> was present in the incubation medium. We also demonstrated that Ca<sup>2+</sup>-induced mitochondrial swelling can be eliminated in the presence of ROS scavengers N-acetyl cysteine and ascorbate. This effect of scavengers was also sensitive to K<sup>+</sup> and was not revealed in the medium that contained equimolar NaCl instead of KCl. Thus, our data gave us grounds to assume that the induction of MPTP by Ca ions evokes the activation of mitochondrial ATP-sensitive K<sup>+</sup>-channels, which are mediated by ROS.*

*Key words: mitochondria, mitoK<sub>ATP</sub> mitochondrial permeability transition pore, Ca<sup>2+</sup>, myometrium.*

In smooth muscles ATP-sensitive K<sup>+</sup>-transporting channels (K<sub>ATP</sub>-channels) participate in regulation of processes which are important for myocytes, such as muscular contraction, combination of excitement with energetic condition of myocyte and others. [1, 2]. The role of these channels in regulation of mitochondrial matrix volume, intensity of electron flow in the electron-transport chain and its coupled production of ATP in different tissues has been determined. [3–5]. Although there is a lot of available data on plasma membrane K<sub>ATP</sub>-channels, the evidence of similar channels in mitochondria membrane and, in particular, in smooth muscles is rather scarce and disputable. The presence of K<sub>ATP</sub>-channel subunits in sarcolemma of myometrium has been demonstrated in many studies [6, 7], and its role in regulation of myometrium contraction has been established. Nevertheless, presently the functioning of this structure in mitochondria of myometrium has been studied insufficiently. As activation of mitochondrial K<sub>ATP</sub>-channels (mitoK<sub>ATP</sub>-channels) has considerable physiological effects on general functioning of mitochondria in many tissues [8–11],

we have presumed that this structure may play an important role in myocytes of myometrium.

We have previously demonstrated activation of ATP- and diazoxide-sensitive K-transporter in mitochondria of rat uterine smooth muscle by photon-correlation spectroscopy and detection of side scattering in suspension of myometrium mitochondria. [12, 13]. Swelling of mitochondria in the environment containing K ions was inhibited to an extent under presence of ATP [12]. ATP inhibition was removed by the activator of K<sub>ATP</sub>-channels diazoxide and was not observed when NaCl or choline chloride were substituted equimolarly for KCl. Registrations of side scattering correlate with data of measurement of hydrodynamic diameter (HD) of mitochondria obtained using the method of photon-correlation spectroscopy of isolated mitochondria of the rat myometrium [13]. Thus, in a standard incubation medium with addition of oligomycin, the average HD of mitochondria of myometrium is 459 nm, which correlates with the real size of mitochondria *in vivo* [13]. The decrease of HD in the presence of ATP to 411 nm and its increase in the presence of ATP together

with diazoxide up to 451 nm have been observed only in the medium containing K ions [13]. So our previous data gives us a possibility to presume existence of a transmitter of K ions that possesses the characteristics of  $K_{ATP}$ -channels in the mitochondria of smooth muscle of the uterus.

It is well-known that Ca ions in muscles, including smooth ones, control the process of contraction-relaxation of muscle tissue, activity of  $Ca^{2+}$ -dependent enzymes, and act as secondary messengers in signal transduction from sarcolemma receptors to the inter-cell membrane structures [14]. Thus, the regulation of homeostasis of such ions in myocytes plays a crucial role in the normal functioning of tissue and organ in general. The evidence has been presented of the sensitivity of accumulation of Ca ions in mitochondria to presence of activators of  $mitoK_{ATP}$  [8, 11]. It has been demonstrated [8, 11] that activation of the channel may regulate  $Ca^{2+}$  transport into mitochondrial matrix without damage to the vital capacity of organelle itself. According to these results,  $mitoK_{ATP}$  may be an additional factor in the regulation of homeostasis of  $Ca^{2+}$  in a cell. Pathological conditions, which lead to the increased contractile activity of the myometrium, are accompanied by considerable concentration of Ca ions in cytoplasm [14]. If the mechanisms of regulation of  $Ca^{2+}$  homeostasis in the uterus were clarified, this would give ground for the development of new therapy for correction of such disorders. Therefore, the research of  $mitoK_{ATP}$  in myometrium may be promising both for basic and applied research.

The aim of the work was to study the effect of  $Ca^{2+}$  in high concentrations on glybenclamide-sensitive transport of  $K^+$  in mitochondria of smooth muscle of the uterus.

### Materials and Methods

**Mitochondrial isolation.** Sexually mature female white rats with body mass of 150 to 200 g, were anesthetized with diethyl ether or chloroform and decapitated. For extraction of mitochondria the uterus tissue, that had been cleaned up from blood and fat, was minced and homogenized on ice in 8 ml of isolation buffer containing 250 mM of sucrose, 1 mM of EDTA, 10 mM of HEPES, (pH 7.2 buffered with TRIS). The homogenate was centrifuged for 7 min at 1000 g at a temperature of 4 °C. The supernatant was separated and centrifuged for 7 min at 12 000 g at a temperature of 4 °C. The sediment was resuspended in extraction buffer (containing no EDTA) and kept

on ice. Protein content was determined by Bradford method [15].

Stock solutions of glybenclamide and cyclosporine A had been prepared in DMSO and added into cuvette immediately prior to measurement in the amount of 1  $\mu$ l per 1.8 ml of working volume (final concentrations of glybenclamide and cyclosporine A were 10  $\mu$ M); 1  $\mu$ l DMSO was added to the control sample. Concentrated solution of ruthenium red had been prepared in deionized water and added in the amount of 5  $\mu$ l per 1.8 ml of working volume (final concentration of ruthenium red was 10  $\mu$ M). Mitochondrial extraction buffer and working solutions were prepared on deionized water. Solution of  $CaCl_2$  had been added to the cuvette prior to measurement (up to a final concentration 100  $\mu$ M of  $CaCl_2$ ).

Measurement of mitochondrial swelling has been conducted by side scattering technique at 520 nm on the spectrofluorometer PTI Quanta Master 40 (Canada) in the thermostated fluorimetric cuvette with magnetic stirrer at 28 °C. Standard incubation medium contained: 10 mM HEPES (pH 7.2 at 28 °C, buffered with TRIS), 125 mM KCl, 5 mM  $Na_2HPO_4$ , 1 mM  $MgCl_2$ , 5 mM succinate, 5  $\mu$ M rotenon, oligomycin 2  $\mu$ g/ml. In case of usage of  $K^+$ -free medium, the medium contained all the above-mentioned components, excluding KCl, which isotonicity had been exchanged with 125 mM NaCl. Solutions of ascorbic acid and N-acetylcysteine were added before measurement up to final concentration of 200 and 100  $\mu$ M, respectively. Final protein concentration in the cuvette was 55-60  $\mu$ g/ml.

Measurement of reactive oxygen species (ROS) generation was conducted using ROS-sensitive probe 2,7-dichlorofluorescein-diacetate (DCF-DA) in spectrofluorometer PTI Quanta Master 40 (Canada) in a thermostated fluorimetric cuvette with magnetic stirrer at 28 °C in the incubation medium which contained: 10 mM HEPES (pH 7.2 at 28 °C, added 2M TRIS), 125 mM NaCl, 5 mM  $Na_2HPO_4$ , 1 mM  $MgCl_2$ , 5 mM succinate, 5  $\mu$ M rotenone, oligomycin 2  $\mu$ g/ml. Final protein concentration in cuvette was 50  $\mu$ g/ml. Nonfluorescent and membrane permeable form of dichlorofluorescein – DCF-DA – easily penetrates membrane. Once inside the mitochondrial matrix the acetate group of DCF-DA was cleaved by esterases yielding nonfluorescent product 2,7-dichlorofluorescein, which accumulated inside. Oxidation by ROS yields the fluorescent product dichlorofluorescein (DCF) [16, 17]. Small aliquote of stock solution of DCF-DA was added to the incubation

medium right before measurement to the final concentration of 4  $\mu\text{M}$ . Wavelengths of the excitation and emission of the fluorescence was 504 nm and 520 nm, respectively. Registration of fluorescence started 1 s after addition of aliquote of the suspension of mitochondria.

An analysis of the results and graph generation was conducted with Microcal Origin, version 5.0 (Microcal Software Inc., USA). Data were analyzed using Student's *t*-test. A value of  $P < 0.05$  was considered statistically significant.

Chemicals: sucrose, HEPES, KCl,  $\text{CaCl}_2$  (1 M solution), rotenone, oligomycin, succinate, N-acetylcysteine, cyclosporine A, ruthenium red, glybenclamide, 2,7-DCF-DA were by Sigma – Aldrich (USA); TRIS, EDTA, NaCl were by Fluka (Switzerland); other chemicals were by domestic manufacturer (Ukraine).

## Results and Discussion

Fig. 1 contains typical light scattering traces from uterus mitochondria respiring in  $\text{K}^+$  medium. In the absence of  $\text{CaCl}_2$  (Fig. 1, curve 1), the matrix swelled to a lower steady-state volume. Addition of 100  $\mu\text{M}$   $\text{CaCl}_2$  resulted in a faster swelling rate and consequently in higher steady-state volume (Fig. 1, curves 2). This effect of Ca ions was reversed by known blocker of  $\text{Ca}^{2+}$ -uniporter in mitochondria ruthenium red (RuR) (10  $\mu\text{M}$ ) as well as by the blocker of the mitochondria permeability transition pore (MPTP) cyclosporine A (10  $\mu\text{M}$ ). The intensity of light scattering in case of curves 3 and 4 (Fig. 1) coincided with that of the control curve (Fig. 1, curves 1, 3, 4). Dependence of light scattering of mitochondria on RuR and cyclosporine raises the possibility of involvement of MPTP in the dramatic increase of mitochondria matrix swelling in the presence of  $\text{CaCl}_2$  in high concentration.

$\text{Ca}^{2+}$ -induced mitochondria swelling in  $\text{K}^+$  medium was by 30% higher than in the medium without  $\text{K}^+$ , with equimolar substitution of 125 mM KCl by NaCl (Fig. 2, columns 1, 3). This data gives a possibility to admit other process, besides activation of MPTP, involved in mitochondria swelling in KCl medium. We have presumed an activation of  $\text{K}^+$  conductance throughout mitochondria inner membrane in the presence of 100  $\mu\text{M}$   $\text{CaCl}_2$ . To check our presumption, blocker of the  $\text{K}_{\text{ATP}}$ -channels glybenclamide was used. It appeared that addition of glybenclamide (10  $\mu\text{M}$ ) to KCl medium with respiring mitochondria in the presence of 100  $\mu\text{M}$

$\text{CaCl}_2$  resulted in the lowering of steady-state volume swelling of the mitochondria matrix (Fig. 2, columns 1 and 2), while in the absence of K ions, when 125 mM KCl was substituted by 125 mM NaCl (Fig. 2, column 3, 4), glybenclamide failed to cause such an effect. Results from Fig. 2 indicate the activation of  $\text{K}^+$ -dependent glybenclamide-sensitive mitochondria swelling in the presence of  $\text{Ca}^{2+}$  in high concentration.

It has been shown in mitochondria extracted from different tissues that the opening of MPTP is accompanied by the increase of ROS production [17, 18]. To prove this fact, regarding the myometrium mitochondria, we have measured generation of ROS in the suspension of mitochondria extracted from smooth muscle of the uterus. Potentiality of usage of probe DCF-DA for ROS measurement in isolated mitochondria was demonstrated by O'Brien et al. [17]. It is known that ROS may activate  $\text{K}_{\text{ATP}}$ -channels in myocytes [10, 19]. Operation of  $\text{K}^+$ -channels on the inner mitochondrial membrane, which existence had been demonstrated in the works [3–5, 10, 18, 19], could cause considerable error in measurement of ROS production, because activation of these channels under conditions of stress leads to

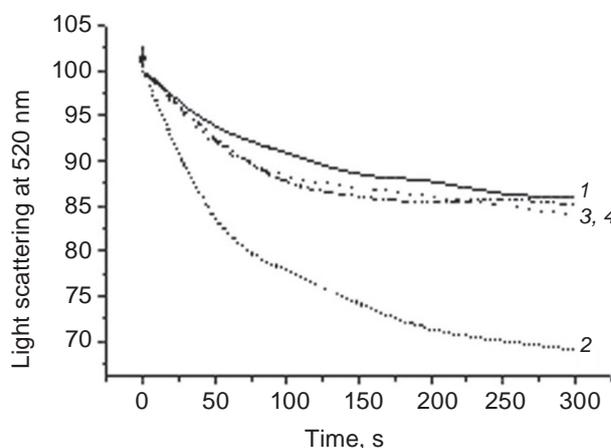


Fig. 1. Typical curves of the light scattering dynamics of uterus mitochondria under conditions of  $\text{Ca}^{2+}$  overloading. 1 – standard incubation medium with mitochondria (see “Materials and Methods” for details); 2 – 100  $\mu\text{M}$   $\text{CaCl}_2$  was added to the standard incubation medium with mitochondria just before the addition of mitochondria suspension; 3 and 4 – standard incubation medium with mitochondria, 100  $\mu\text{M}$   $\text{CaCl}_2$  and 10  $\mu\text{M}$  ruthenium red and 10  $\mu\text{M}$  cyclosporine A, respectively. Arrow signs the addition of the mitochondrial suspension ( $M \pm m$ ,  $n = 5$ )

a decrease of generation of free radicals in mitochondria. The last effect is thought to be a basis for cytoprotective action of these structures [18, 19]. Thus, the ROS production in uterus mitochondria with DCF-DA has been measured in the medium free from potassium ions with 125 mM NaCl, since there are reliable data that in the membrane of mitochondria of myometrium there are no structures providing the transportation of Na ions [20]. Addition of 100  $\mu$ M  $\text{CaCl}_2$  to the incubation medium led to a slight but reproducible increase of the ROS production in comparison with control curve (without Ca ions) (Fig. 3, curves 1, 2). At the same time in the presence of 10  $\mu$ M of cyclosporine A the level of DCF fluorescence corresponded to the control one, without Ca ions (Fig. 3, curves 2, 3). Thus, the results shown on Fig. 3, give us the basis to presume that the activation of cyclosporine-sensitive pore by Ca ions in the uterus mitochondria is accompanied by the increase of the level of ROS production.

Data of Queliconi et al. [10] indicate that ATP  $\text{mitoK}_{\text{ATP}}^+$ -channels may be activated under conditions of oxidative stress as a response to the increased level of ROS production. We have assumed such a mechanism of activation of  $\text{mitoK}_{\text{ATP}}$  in the myometrium mitochondria. To check our assumption,

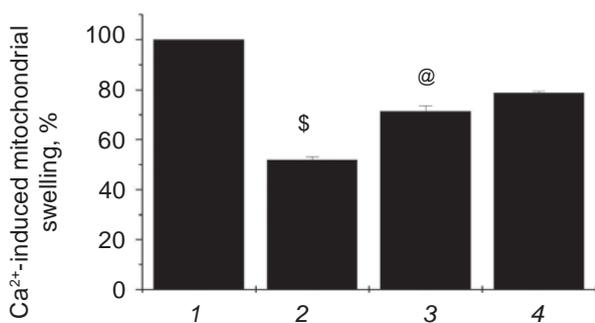


Fig. 2. Glibenclamide suppresses  $\text{Ca}^{2+}$ -induced mitochondrial swelling. 1, 2 –  $\text{Ca}^{2+}$ -induced mitochondrial swelling in a standard incubation medium without and with glibenclamide (10  $\mu$ M) respectively; 3, 4 –  $\text{Ca}^{2+}$ -induced mitochondrial swelling in  $\text{K}^+$ -free medium (with 125 mM NaCl instead of KCl) without and with glibenclamide (10  $\mu$ M) respectively; 100% is a difference between the plateau levels of light scattering of mitochondrial suspension in a standard incubation medium with and without 100  $\mu$ M  $\text{CaCl}_2$  (Fig., curves 1 and 2). \$ – difference is significant relative to 1 ( $P < 0.05$ ); @ – difference is significant relative to 1 ( $P < 0.05$ )

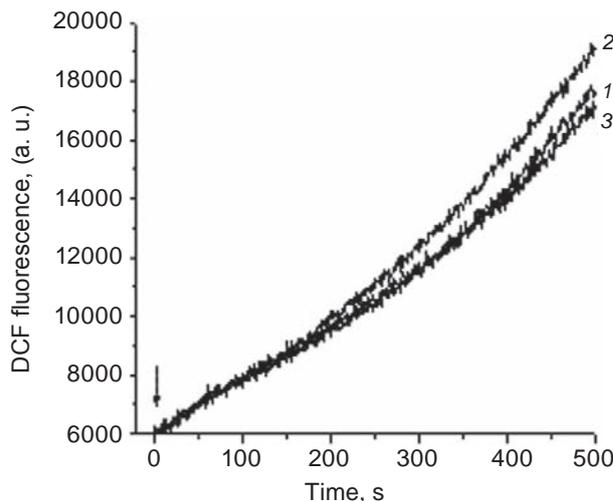


Fig. 3. Cyclosporin A suppresses  $\text{Ca}^{2+}$ -induced ROS generation in uterus mitochondria in  $\text{K}^+$ -free medium (with 125 mM NaCl instead of KCl). 1, 2 – ROS generation measured in mitochondria with DCF in medium without and with 100  $\mu$ M  $\text{CaCl}_2$  respectively (see “Materials and Methods” for details); 3 – ROS generation in mitochondrial suspension after addition of 100  $\mu$ M  $\text{CaCl}_2$  and 10  $\mu$ M cyclosporine

we have used scavengers of ROS N-acetyl cysteine and ascorbic acid in light scattering measurements of mitochondria in the presence of 100  $\mu$ M  $\text{CaCl}_2$  (Fig. 4, curves 1-4). It appeared that addition of 100  $\mu$ M of NAC, as well as 200  $\mu$ M of ascorbate together with 100  $\mu$ M of  $\text{CaCl}_2$ , led to the reliable decrease of mitochondria matrix swelling (Fig. 4, curves 3, 4). Yet the scavengers did not significantly influence  $\text{Ca}^{2+}$ -induced swelling of mitochondria after 125 mM KCl had been exchanged for equimolar NaCl. Thus, we have shown that the removal of ROS from the incubation medium leads to a considerable decrease of  $\text{Ca}^{2+}$ -induced uterus mitochondria swelling in  $\text{K}^+$  medium.

Concentration of  $\text{Ca}^{2+}$  in cytosol in myocyte under resting condition is about 100 nM [21]. Muscle contraction or metabolic stress causes the increase of  $\text{Ca}^{2+}$  concentration in cytosol by several orders of magnitude, reaching hundreds micromoles in particular microdomains of cytoplasm [22]. Homeostasis of  $\text{Ca}^{2+}$  in cells is strongly regulated by plasma membrane, as well as intracellular organelles – endo/sarcoplasmic reticulum and mitochondria. Thus, sarcoplasmic reticulum (SR) is one of the pools for  $\text{Ca}^{2+}$  inside the muscle cells [22]. Concentration of Ca ions in SR may reach 200  $\mu$ M and more.  $\text{Ca}^{2+}$  efflux from SR in response to some stimulus, after which con-

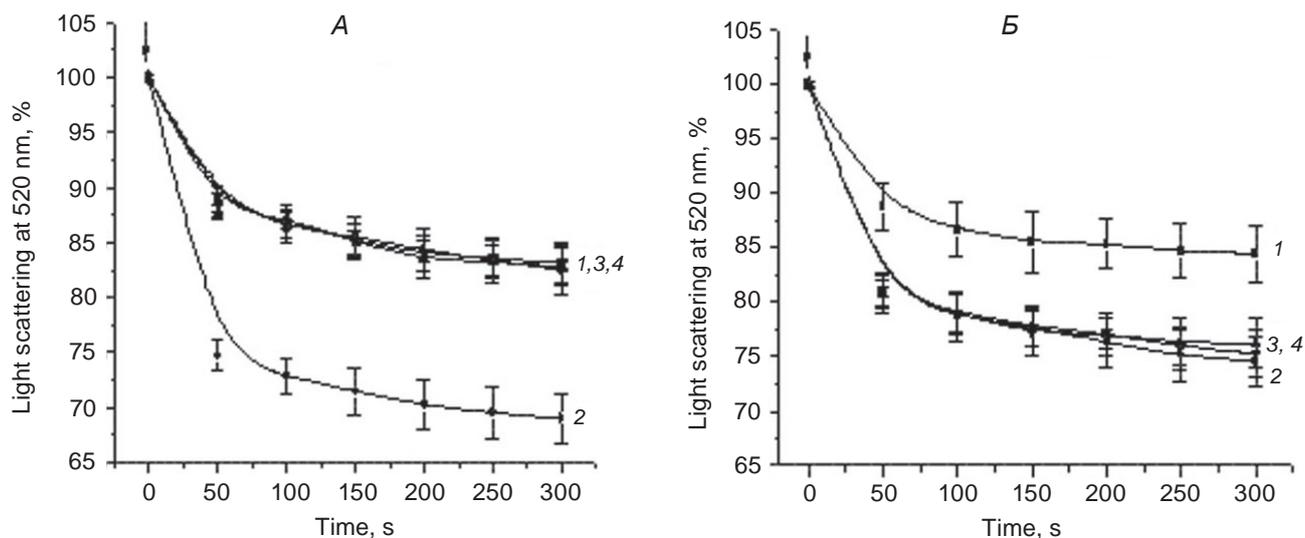


Fig. 4. ROS scavengers suppress  $\text{Ca}^{2+}$ -induced mitochondrial swelling in a standard incubation medium. A – mitochondrial swelling in a standard incubation medium; B – mitochondrial swelling in  $\text{K}^+$ -free medium with 125 mM NaCl instead of KCl (see “Materials and Methods” for details). 1, 2 – mitochondrial swelling in medium without and with 100  $\mu\text{M}$   $\text{CaCl}_2$  respectively (see “Materials and Methods” for details); 3, 4 – addition of 100  $\mu\text{M}$   $\text{CaCl}_2$  with 100  $\mu\text{M}$  NAC or 200  $\mu\text{M}$  ascorbate, respectively ( $M \pm m$ ;  $n = 5$ )

centration of Ca ions in this compartment decreases from 200 до 50  $\mu\text{M}$  [23] was shown. Concentration of  $\text{Ca}^{2+}$  in microdomains, which are located very close to SR, may reach 100  $\mu\text{M}$  [22, 24]. Close interaction between mitochondria and SR has been demonstrated [25]. Thus, mitochondria may be exposed to extremely high concentrations of Ca ions [18, 24].  $\text{Ca}^{2+}$  entry into mitochondrial matrix is provided by  $\text{Ca}^{2+}$ -uniporter and depends on membrane potential, which is close to -150 to -200 mV and is supported by the electron-transport chain [26, 22]. Low affinity of the uniporter for Ca ions with  $K_{0.5}$  value of 1-189  $\mu\text{M}$  is compensated by the high rate of  $\text{Ca}^{2+}$  accumulation [27]. It is suggested that due to high rate of ion accumulation of  $\text{Ca}^{2+}$ -uniporter, mitochondria are able to quickly withdraw the excess  $\text{Ca}^{2+}$  from cytosol, playing a role of highly capable intercellular  $\text{Ca}^{2+}$ -buffer, thus preventing from deregulation of metabolic processes by Ca ions and protecting the cell from death. But it is also well-known that high matrix concentrations of  $\text{Ca}^{2+}$  disrupt mitochondrial function [26, 27]. Thus, Chen et al. have demonstrated that in cardiomyocytes high local concentrations of  $\text{Ca}^{2+}$  in cytosol, as a result of spontaneous efflux of  $\text{Ca}^{2+}$  from SR, evoke an overloading of mitochondrial matrix by Ca ions and opening of the MPTP, which was depressed by blockers of  $\text{Ca}^{2+}$ -uniporter ruthenium red and Ru 360 [27]. Induction of MPTP is accompanied by swelling of mitochondrial ma-

trix as a result of osmotic arrival of water. This may be measured by light scattering at 520 nm [11]. We observed a considerable decrease of the light scattering of suspension of mitochondria after addition of 100  $\mu\text{M}$   $\text{CaCl}_2$  (Fig. 1, curve 2) in comparison with control (Fig. 1, curve 1), which prove the induction of MPTP by Ca ions in myometrium, because this process was fully depressed in the presence of MPTP inhibitor cyclosporine A (Fig. 1, curve 4). Sensitivity of mitochondria swelling to blocker of  $\text{Ca}^{2+}$ -uniporter RuR is a proof that  $\text{Ca}^{2+}$ -induced mitochondria swelling is accompanied by transport of Ca ions through the mitochondrial  $\text{Ca}^{2+}$ -uniporter (Fig. 1, curve 3), which is also confirmed by the data of Chen et al. [27].

Opening of  $\text{Ca}^{2+}$ -induced MPTP leads to dissipation of mitochondrial membrane potential, decrease of ATP synthesis, escape of cytochrome c from inter-membrane space and induction of necrosis or apoptosis [28]. It is also known that mitochondria possess functional plasticity in response to metabolic stimuli, which help them survive under stress conditions [29]. Activation of  $\text{K}^+$ -channels located on the inner mitochondrial membrane could be one of the factors providing the preservation of the physiological parameters of mitochondria functioning under stress conditions [3–5, 10, 19, 30]. The existence of  $\text{Ca}^{2+}$ -dependent and ATP-sensitive  $\text{K}^+$ -channels in mitochondria was shown [3–5, 31, 32].

Also pharmacological activation by selective activators has been demonstrated for both types of channels [20, 31]. Sato et al. have shown on cardiomyocytes that the influx of K ions via the Ca<sup>2+</sup>-dependent and ATP-sensitive channels, induced by NS-1619 and diazoxide, respectively, happens independent of each other, leading to additive effect [33]. Also, Ljubkovic et al. have demonstrated that the expression of pore forming mitoK<sub>ATP</sub> subunit Kir6.2 in mitochondria of cardiomyocytes exerts cytoprotective effect in conditions of Ca<sup>2+</sup> overloading in the absence of pharmacological activators of mitoK<sub>ATP</sub> which may also prove an activation of mitoK<sub>ATP</sub> in conditions of stress [9]. Besides this, it is known that Ca<sup>2+</sup>-sensitive K<sup>+</sup>-channels are activated under depolarization and increased Ca<sup>2+</sup> concentration [34]. Thus, under conditions we used, we may assume functioning of both structures. But, taking into the account that the activation of mitoK<sub>ATP</sub> leads to partial depolarization of mitochondrial membrane of myometrium, that was demonstrated by us earlier [13], and to a decrease of Ca accumulation in the matrix [8, 11], as well as that regulatory site for Ca<sup>2+</sup> on the mitochondrial Ca<sup>2+</sup>-sensitive K<sup>+</sup>-channel (mitoK<sub>Ca2+</sub>) is located on the matrix side of the inner membrane, we assume that activation of mitoK<sub>ATP</sub> may decrease a probability of opening of mitoK<sub>Ca2+</sub> [33, 35].

Our data proves the activation of mitoK<sub>ATP</sub> under conditions of overloading of mitochondria by Ca ions because K<sub>ATP</sub>-channel blocker glibenclamide considerably decreased Ca<sup>2+</sup>-induced decrease of mitochondria swelling in standard incubation medium containing K ions. Thus, swelling of mitochondria in the standard incubation medium with glibenclamide was substantially decreased in comparison with standard conditions. The blocker did not influence this process in K<sup>+</sup> free medium (Fig. 2, columns 1, 2). Thus, our data allow us to assume the participation of both MPTP and mitoK<sub>ATP</sub> in mitochondria swelling under standard conditions. In K<sup>+</sup>-free medium containing 125 mM NaCl instead of KCl, the plateau level of swelling caused by 100 mM CaCl<sub>2</sub> was 70% of control (standard conditions with KCl), but not 50%, as that was in the case with glibenclamide in KCl (Fig. 2, columns 2, 3). It is likely that contribution of both processes to Ca<sup>2+</sup>-induced mitochondrial swelling is not additive. According to data presented in Fig. 2, the contribution of Ca<sup>2+</sup>-induced MPTP to mitochondrial swelling in the standard incubation medium with glibenclamide (Fig. 2, column 2) is by 20% less than in the K<sup>+</sup>-

free medium with NaCl (Fig. 2, column 3). Thus, it may be assumed that the activation of glibenclamide-sensitive component of Ca<sup>2+</sup>-induced mitochondrial swelling leads to partial decrease of contribution of Ca<sup>2+</sup>-induced MPTP to mitochondrial swelling.

One of possible factors that activate mitoK<sub>ATP</sub> under conditions of Ca<sup>2+</sup> overload may be increased ROS generation as a result of opening MPTP [17]. In particular, such an effect of ROS has been shown for mitoK<sub>ATP</sub> of myocytes [19] and we assumed a possibility of such mechanism of activation of mitoK<sub>ATP</sub> of smooth muscle of the uterus. That is why the next logical step of our research was measurement of ROS generation in myometrium mitochondria. Using ROS-sensitive fluorescent probe DCF-DA, we have shown that the activation of Ca<sup>2+</sup>-induced MPTP is accompanied by the increase of ROS production (Fig. 3). ROS generation measurements in myometrium mitochondria have been conducted in the incubation medium that does not contain K ions in order to avoid possible influence of activation of K<sup>+</sup>-channels on the ROS production that was shown for mitoK<sub>ATP</sub> of different tissues [19, 36]. MPTP inhibitor cyclosporine A suppressed the increasing of ROS production. This may confirm our assumption that the increase of ROS generation under conditions of Ca<sup>2+</sup> overload is due to the induction of MPTP (Fig. 3, curve 3).

Thus, we have demonstrated that the induction of cyclosporine-sensitive MPTP by Ca ions causes intensive swelling of mitochondria of myometrium and is accompanied by the increase of ROS production. To check a possibility of activation of mitoK<sub>ATP</sub> of myometrium by ROS we have studied the influence of ROS scavengers in the measurements of mitochondrial swelling. To our surprise, it appeared that the exclusion of ROS from the incubation medium by 100 μM N-acetylcysteine (or 200 μM ascorbic acid as well) had not considerable influence on the steady-state level of Ca<sup>2+</sup>-induced mitochondrial swelling in K<sup>+</sup> free medium containing 125 mM NaCl (Fig. 4, B, curves 3, 4), but it might be admitted that elimination of ROS could decrease the negative effect of MPTP induction on mitochondria. Nevertheless, under control conditions (namely, medium containing 125 mM of KCl with no additions), we have observed full suppression of Ca<sup>2+</sup>-induced mitochondrial swelling by ROS scavengers (Fig. 4, A, curves 3, 4). If we assume that mitoK<sub>ATP</sub> of myometrium is activated by ROS, then the results shown in Fig. 4, A somewhat contradict

the results presented in Fig. 2. It might be expected that if scavengers are present (Fig. 4, A, curves 3, 4), only the contribution of activation of  $\text{mitoK}_{\text{ATP}}$  to the swelling process will be suppressed, and the component responsible for MPTP-induced swelling would not change. Moreover, if we take into account that in the medium containing NaCl the ROS scavengers did in no way influence the steady-state level of  $\text{Ca}^{2+}$ -induced mitochondrial swelling (Fig. 4 B, curves 3, 4). Nevertheless, persistence of the effect of ROS scavengers in the standard  $\text{K}^+$ -medium and its absence in the  $\text{K}^+$ -free medium, in our opinion, may prove the above-mentioned assumption that the activation of glybenclamide-sensitive component of swelling in the presence of  $100 \mu\text{M CaCl}_2$  is mediated by ROS and connected to the transport of K ions through the  $\text{mitoK}_{\text{ATP}}$ .

Thus, the results of our study allow us to make the assumption that the activation of  $\text{mitoK}_{\text{ATP}}$  in myometrium is mediated by ROS under conditions of the  $\text{Ca}^{2+}$ -induced cyclosporine-sensitive MPTP. It is necessary to take into consideration that mitochondria not only provide a cell with ATP, but also serve as a high capacity inner cell  $\text{Ca}^{2+}$  pool, which participates in  $\text{Ca}^{2+}$  homeostasis in cytosol [18, 26]. Ca ions in muscle cells couple processes of excitation and contraction. That is why the disorder in normal functioning of these subcellular structures in myocytes would lead to disruption of electro-mechanical coupling in muscles [37]. Because transport of  $\text{K}^+$  into mitochondria regulates accumulation of Ca in matrix [9, 11, 19, 20], activation of  $\text{mitoK}_{\text{ATP}}$  may be considered as a defensive mechanism, encouraging not only normal mitochondria functioning and viability of myocyte in general, but also participating in regulation of  $\text{Ca}^{2+}$  homeostasis in cells.

The contribution of  $\text{mitoK}_{\text{Ca}^{2+}}$  to  $\text{Ca}^{2+}$ -induced change of uterus mitochondria swelling remains unclear. Despite its activation has been shown by pharmacologically and physiologically active substances under conditions of metabolic stress, the mechanisms of its activation and ways of its regulation in smooth muscles are not known for sure. For example, the inhibition of this structure in smooth muscles of vessels by active forms of oxygen has been shown in some works [38, 39], that might prove impossibility of activation of  $\text{mitoK}_{\text{Ca}^{2+}}$  under conditions of  $\text{Ca}^{2+}$ -induced pore opening. Other works have demonstrated that mediated by protein kinase A activation of  $\text{mitoK}_{\text{Ca}^{2+}}$  exerts cytoprotective effect under stress conditions, caused by  $\text{Ca}^{2+}$  overload in cytosol [35]. The absence of reliable data about functioning of

this structure in smooth muscles, in particular, in myometrium determines a necessity to conduct studies in this direction. Thus, in future researches we are planning to study a possibility of  $\text{mitoK}_{\text{Ca}^{2+}}$  activation in the mitochondria of myometrium under conditions of  $\text{Ca}^{2+}$ -induced MPTP and distinguish a separate contribution of  $\text{mitoK}_{\text{Ca}}$  and  $\text{mitoK}_{\text{ATP}}$  to countering the negative effects of induction of cyclosporine-sensitive MPTP.

### АКТИВАЦІЯ ЧУТЛИВОГО ДО ГЛІБЕНКЛАМІДУ НАБУХАННЯ МІТОХОНДРІЙ МІОМЕТРІЯ ЩУРІВ В УМОВАХ ІНДУКЦІЇ ЦИКЛОСПОРИН А-ЧУТЛИВОЇ МІТОХОНДРІАЛЬНОЇ ПОРИ

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Показано, що іони Са індуюють набухання мітохондрій міометрія щурів та активують процеси окислення, які супроводжуються підвищеною генерацією активних форм кисню. Ці ефекти пригнічуються в присутності блокатора мітохондріального  $\text{Ca}^{2+}$ -уніпортера рутенієвого червоного та блокатора мітохондріальної пори циклоспорину А, що свідчить про активацію іонами Са мітохондріальної пори перехідної проникності в мітохондріях міометрія. Індуковане  $100 \mu\text{M CaCl}_2$  набухання мітохондрій було чутливим до присутності блокатора АТР-чутливих  $\text{K}^+$ -каналів глібенкламід (10  $\mu\text{M}$ ) в середовищі, яке містило іони К і нечутливим до глібенкламід у безкалієвому середовищі.  $\text{Ca}^{2+}$ -індуковане набухання мітохондрій усувалося скавенджером активних форм кисню N-ацетилцистеїном і аскорбатом у середовищі, яке містило  $\text{K}^+$  і було нечутливим до цих речовин у безкалієвому середовищі. Таким чином, можна зробити припущення, що в умовах індукції іонами Са мітохондріальної пори перехідної проникності відбувається активація АТР-чутливих  $\text{K}^+$ -каналів у мітохондріях міометрія, яка опосередковується активними формами кисню.

**Ключові слова:** мітохондрії,  $\text{mitoK}_{\text{ATP}}$ -канал, мітохондріальна пора перехідної проникності,  $\text{Ca}^{2+}$ , міометрій.

**АКТИВАЦІЯ ЧУВСТВИТЕЛЬНОГО  
К ГЛИБЕНКЛАМИДУ  
НАБУХАННЯ МИТОХОНДРИЙ  
МИОМЕТРИЯ КРЫС В УСЛОВИЯХ  
ИНДУКЦИИ ЦИКЛОСПОРИН  
А-ЧУВСТВИТЕЛЬНОЙ  
МИТОХОНДРИАЛЬНОЙ ПОРЫ**

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Показано, что ионы Са индуцируют набухание митохондрий миометрия крыс и активируют процессы окисления, сопровождающиеся повышенным образованием активных форм кислорода. Эти эффекты подавляются в присутствии блокатора митохондриального Са<sup>2+</sup>-унипортера рутениевого красного и блокатора митохондриальной поры циклоспорина А, что свидетельствует об активации ионами Са митохондриальной поры переходной проницаемости в митохондриях миометрия. Индуцированное 100 мкМ СаСl<sub>2</sub> набухание митохондрий уменьшалось в присутствии блокатора АТФ-чувствительных К<sup>+</sup>-каналов глибенкламида (10 мкМ) в среде, содержащей ионы К и не изменялось под действием глибенкламида в безкальевой среде. Са<sup>2+</sup>-индуцированное набухание митохондрий устранялось сквенджером активных форм кислорода N-ацетилцистеином и аскорбатом в среде, содержащей ионы К и не зависило от этих веществ в безкальевой среде. Таким образом, можно предположить, что при индукции ионами Са митохондриальной поры переходной проницаемости происходит активация АТФ-чувствительных К<sup>+</sup>-каналов в митохондриях миометрия, которая опосредуется активными формами кислорода.

**Ключевые слова:** митохондрии, митоK<sub>АТФ</sub>-канал, митохондриальная пора переходной проницаемости, Са<sup>2+</sup>, миометрий.

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