EXPERIMENTAL WORKS

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IDENTIFICATION OF DIFFERENT SUBTYPES OF K^+ CHANNELS IN THE MITOCHONDRIA OF RAT MYOMETRIUM USING K^+ CHANNELS MODULATORS

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Potassium ions affect Ca^{2+} transport in mitochondria, the magnitude of the electric potential on the inner mitochondrial membrane, metabolic processes in the matrix, and osmoregulation. The aim of this study was to identify different subtypes of K^+ channels in the mitochondria of rat myometrium. Isolated mitochondria were obtained from the myometrium of non-pregnant Wistar rats by differential centrifugation. Potassium ion accumulation was studied by spectrofluorimetry using the K^+-sensitive fluorescent probe PBFI-AM. Myometrial mitochondria effectively accumulate potassium ions within the concentration range of 25–150 mM. No increase in PBFI fluorescence was observed when K^+ ions were replaced by choline in equimolar concentrations. In the presence of voltage-operated K^+ channels inhibitor 4-aminopyridine, Ca^{2+}-dependent K^+ channels blockers charybdotoxin or paxilline, mitoK_{ATP} channels inhibitors glibenclamide, 5-hydroxydecanoic acid, or 200 μM ATP, a significant decrease in the PBFI fluorescence signal was observed. Conversely, application of Ca^{2+}-dependent K^+ channels specific activators NS11021 and NS1619, as well as of mitoK_{ATP}-specific activator cromakalim, resulted in increased mitochondrial K^+ accumulation. The efficiency of K^+ uptake increased further with the addition of 25–100 μM Ca^{2+} in the presence of 4-aminopyridine and ATP. The results obtained indicate the presence of voltage-operated and Ca^{2+}-dependent subtypes of K^+ channels, as well as of H^+/K^+ exchange system in myometrial mitochondria in addition to mitoK_{ATP} channels.

K e y w o r d s: mitochondria, potassium ions accumulation, K^+ channels modulators, calcium, rat myometrium.

Potassium ions play an essential role in the molecular physiology of mitochondria ensuring its physiological swelling that stimulates mitochondrial function and metabolism [1-2]. The regulation of mitochondrial matrix volume is a complex and dynamic process that affects various cellular activities, such as bioenergetics, Ca^{2+} regulation, and reactive oxygen species (ROS) formation [3-5]. Mitochondria significantly contribute to Ca^{2+}-dependent smooth muscle contraction [6-7]. Disruption of ion transport across the inner mitochondrial membrane is associated with the development of oxidative and nitrosative stress, which – if protective systems are insufficiently active – can lead to mitochondrial dysfunction [8-11]. These pathological processes are accompanied by disturbances in smooth muscle contractility and are implicated in a range of clinical conditions [12-13]. In particular, uterine atony or hypertonicity can result in weak labor or, conversely, in preterm contractions and deliveries [14-15]. This represents a significant medical and social issue in developed countries. The established link between smooth muscle contractile dysfunction and impaired mitochondrial activity has led to the recognition of mitochondrial disorders as a potential underlying cause of smooth muscle-related pathologies in contemporary biomedical literature.

It has been demonstrated that modulation of K^+ exchange across the inner mitochondrial membrane influences mitochondrial energetics, ROS production, organelle volume, and apoptotic signaling pathways [3, 5, 16-18]. The system responsible for

potassium ion movement across the inner membrane – including the H^+/K^+ exchanger and various K^+ channels – helps maintain osmotic balance between the mitochondrial matrix and the extramitochondrial environment [1-3, 19-21]. Activation of ATP-sensitive (mitoK_{ATP}) and Ca^{2+}-activated (particularly high-conductance, mitoBK_{Ca}) potassium channels has been shown to exert protective effects in cardiomyocytes and neurons [5, 22-25]. Conversely, inhibition of voltage-operated K^+ channels (mitoKv1.3) is associated with increased cell death and malignant transformation [26]. Mitochondrial K^+ channels have been implicated in various physiological and pathological processes, including ischemic heart disease, aging, apoptosis, and tumorigenesis [5, 27].

Potassium channels in mitochondria were first described as ATP- and glibenclamide-sensitive, i.e., ATP-sensitive K^+ channels [28]. Subsequently, various subtypes of mitochondrial potassium channels were identified in the liver, heart, endothelial cells, neurons, and fibroblasts [5, 23, 27, 29]. These channels were found to share biophysical and pharmacological properties with their counterparts in the plasma membrane [19, 22, 30-32]. The driving force for K^+ transport across the inner mitochondrial membrane into the matrix is the negative electrical potential on the matrix side and the concentration gradient of this ion [1-3].

While the presence of ATP-sensitive K^+ channels in myometrial mitochondria has been demonstrated in previous studies by the Department of Muscle Biochemistry at the Institute of Biochemistry of the National Academy of Sciences of Ukraine [33], the potential involvement of other K^+ channel subtypes in these subcellular structures of uterine smooth muscle remains unclarified.

Therefore, the aim of this study was to identify different subtypes of K^+ channels in rat myometrial mitochondria using specific inhibitors and activators.

Materials and Methods

Experiments were conducted on Wistar rats weighing 150–180 g. All procedures involving animals were carried out in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986) and the Law of Ukraine 'On the Protection of Animals from Cruelty'. Rats were anesthetized by chloroform inhalation and subsequently decapitated.

Isolation of myometrial mitochondria. Mitochondria were isolated from rat myometrium using a standard differential centrifugation protocol. Uterine tissue, cleared of blood and adipose tissue, was homogenized on ice in isolation buffer containing 10 mM HEPES (pH 7.2 at 4°C), 1 mM EGTA, 250 mM sucrose, and 0.1% bovine serum albumin (BSA). The homogenate was centrifuged at 1,000 × g for 15 min at 4°C. The resulting supernatant was further centrifuged at 12,000 × g for 15 min at 4°C. The final mitochondrial pellet was resuspended in 500 µl of buffer consisting of 10 mM HEPES (pH 7.2 at 4°C), 1 mM EGTA, 250 mM sucrose, 5 mM succinate, and 0.1% BSA.

Examination of K^+ accumulation in mitochondria. Mitochondrial suspensions were incubated with 10 µM PBFI-AM (a K^+-sensitive fluorescent probe, Potassium-Binding Fluorescent Indicator Acetoxymethyl Ester) and 0.02% Pluronic F-127 for 25 min at 25°C. After incubation, 50 mM tetraethylammonium (TEA^+) was added, followed by an additional 5-minute incubation. The mitochondrial suspension was then diluted with 5 ml of buffer containing 10 mM HEPES (pH 7.2 at 4°C), 250 mM sucrose, and 0.1% BSA, and centrifuged at 12,000 × g for 15 min at 4°C. The resulting pellet was resuspended in the same buffer and kept on ice. Protein concentration in the mitochondrial fraction was determined using the Bradford method [34], and ranged from 20 to 25 µg per sample.

Changes in K^+ content within the mitochondrial matrix were measured using a Quanta Master 40 spectrofluorimeter (PTI, Canada) equipped with FelixGX 4.1.0.3096 software. Probe fluorescence was recorded at excitation wavelengths of 340 nm and 380 nm, and an emission wavelength of 480 nm.

K^+ accumulation was assessed by changes in PBFI fluorescence intensity, expressed as the ratio of excitation at 340/380 nm. The signal at 340 nm corresponds to the maximum sensitivity of the probe to K^+, while the signal at 380 nm represents the isosbestic point of the probe. Ratiometric measurements reduce the influence of interfering factors [23, 35].

K^+ accumulation was studied in a medium with the following composition (in mM): 20 Hepes (pH 7.2 at 37°C), 2 K^+-phosphate buffer (pH 7.2 at 37°C), 1 $MgCl_2$, 130 KCl, 5 sodium succinate, 5 sodium pyruvate.

The ΔpH-induced release of K^+ was performed in a medium containing (in mM): 20 Hepes (pH 6.0-8.0 at 37°C), 250 sucrose, 5 Na_2HPO_4, 5 sodium suc-

cinate, 5 sodium pyruvate. In this case, the fluorescent response was expressed in relative units as F/F_o, where F_o is the initial level of fluorescence, F is the fluorescence intensity recorded as the experiment runs.

Statistical analysis. Data are presented as means ± SE, based on the indicated number of replicates. Differences between groups in fluorometric experiments were analyzed using unpaired Student's *t*-tests in Microsoft Excel and two-factor analysis ANOVA.

Chemicals and reagents. The following reagents were used in this study: PBFI-AM (Molecular Probes, USA); HEPES, sucrose, sodium succinate, sodium pyruvate, BSA, Pluronic F-127, ATP, $CaCl_2$, tetraethylammonium chloride, glibenclamide, 5-hydroxydecanoic acid, paxilline, charybdotoxin, cromakalim, NS1619, NS11021 (all from Sigma, USA); 4-aminopyridine (Sigma-Aldrich, USA). All other reagents were produced in Ukraine.

Solutions were prepared using bidistilled water with a specific electrical conductivity not exceeding 2.0 µcm/cm. Conductivity was measured using an OK-102/1 conductometer (Hungary).

Results and Discussion

The fluorescence signal of PBFI in isolated mitochondria increases significantly in the presence of potassium ions, accompanied by a shift of the excitation maximum toward the short-wavelength region (Fig. 1).

According to the literature, the concentration of potassium ions in the cytosol ranges from 100 to 150 mM, and in the mitochondrial matrix, it can reach up to 140-180 mM [2, 23]. This is primarily due to the strong negative membrane potential on the matrix side of the inner mitochondrial membrane, which in energized mitochondria can reach –180 to –190 mV [1, 3, 19].

To study K^+ uptake into the matrix of isolated mitochondria, experimental protocols often involve preincubation with 50 mM TEA^+ to deplete intramitochondrial K^+ [36], thereby shifting the K^+ concentration into the sensitivity range of PBFI (Fig. 2, *A*), dissociation constant $K_d \approx 8$ mM [21, 23]. This preliminary substitution of K^+ with TEA^+ facilitates more efficient K^+ accumulation in the matrix upon subsequent exposure (Fig. 2, *B*).

It was demonstrated that myometrial mitochondria efficiently accumulate potassium ions within the concentration range of 25–150 mM (Fig. 3, *A*). No increase in PBFI fluorescence was observed when K^+ ions were replaced with equimolar concentrations of choline (Fig. 3, *B*).

In both human and animal myometrium, several subtypes of K^+ channels have been identified at the plasma membrane level, including high- and low-conductance Ca^{2+}-activated, ATP-sensitive, and

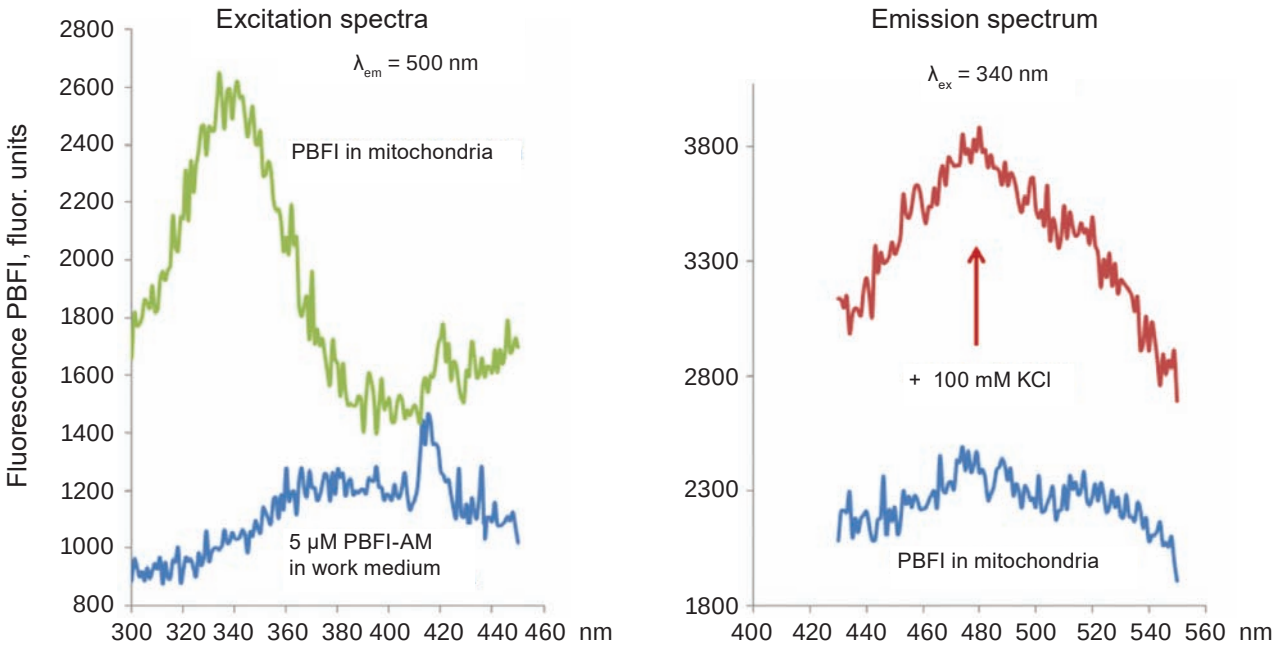

Fig. 1. Spectral characteristics of the K^+-sensitive fluorescent probe PBFI-AM

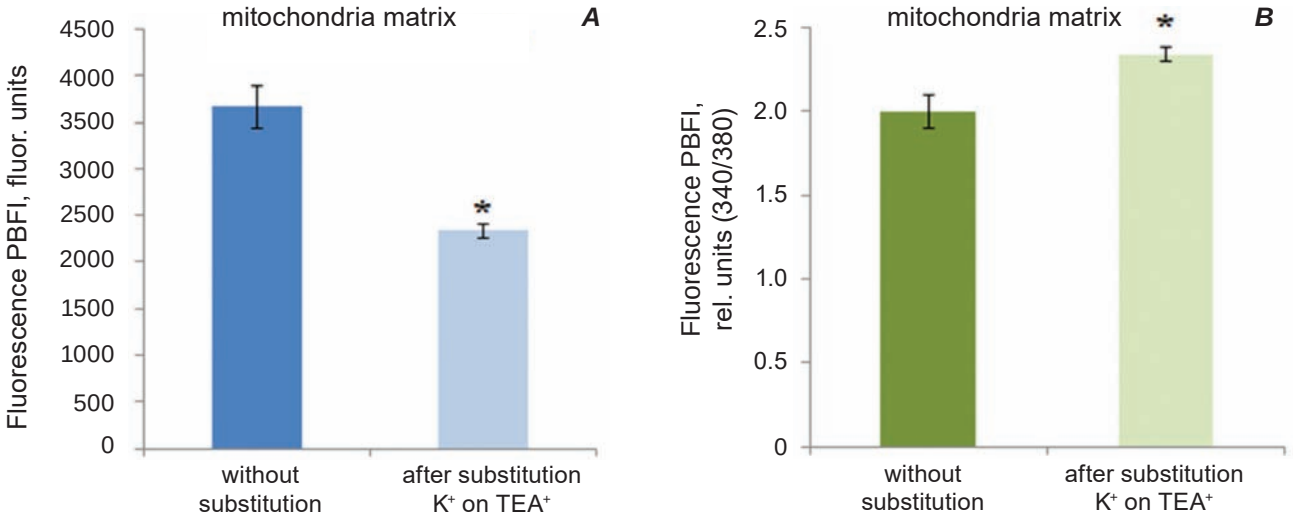

*Fig. 2. PBFI fluorescence intensity in the mitochondrial matrix (**A**) and K^+ accumulation efficiency (**B**) without and with mitochondria treatment with 50 mM TEA. *P < 0.05 vs without substitution, M ± m, n = 4*

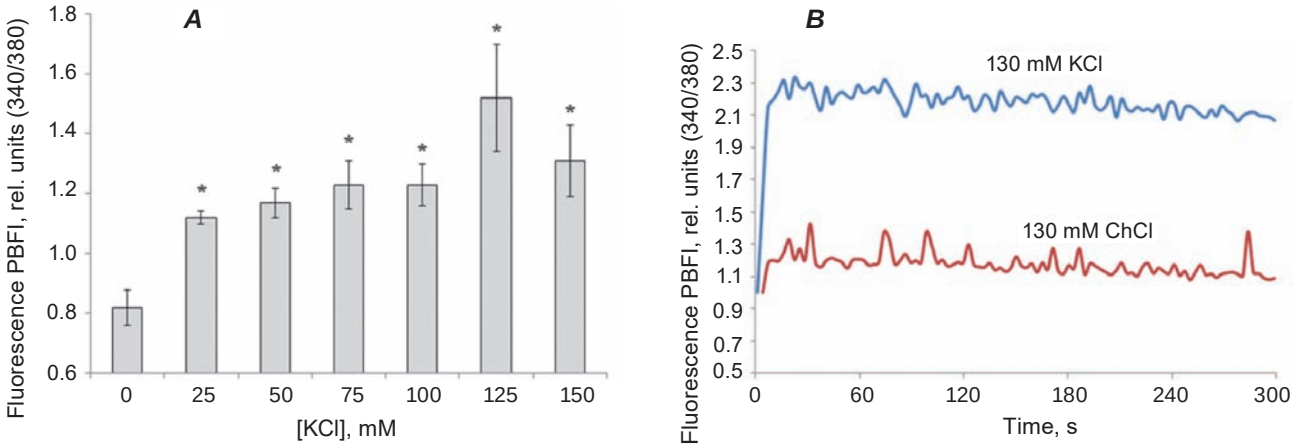

*Fig. 3. Accumulation of K^+ by isolated mitochondria in increasing concentrations (0 - 150 mM), * P < 0.01 vs 0 mM external KCl, M ± m, n = 4 (**A**). Changes in PBFI fluorescence in a medium containing 130 mM K^+ or choline in equimolar concentrations, results of a typical experiment (**B**)*

voltage-operated channels, among others [13, 37]. However, literature data suggest that the mitochondrial presence of the same channel subtypes is not obligatory. For instance, in cardiomyocytes, mitoBK$_{Ca}$ channels are found exclusively in the inner mitochondrial membrane, but not in the plasma membrane [16]. Several K^+ channel subtypes have been identified in mitochondria across different tissues, with the most common in mammals being mitoK$_{ATP}$, mitoBK$_{Ca}$, and mitoKv1.3 [5, 30-31].

In our study, application of the voltage-operated K^+ channel inhibitor 4-aminopyridine [37] resulted in a significant decrease in PBFI fluorescence, indicating reduced efficiency of mitochondrial potassium ion transport (Fig. 4).

In the presence of Ca^{2+}-activated K^+ channel blockers, such as charybdotoxin or paxilline [4-5, 30], potassium ion transport from the external medium into the mitochondrial matrix was also inhibited (Fig. 4). A similar inhibitory effect was observed with ATP-sensitive K^+ channel blockers, glibenclamide and 5-hydroxydecanoic acid [4, 23, 30], as well as with exogenous ATP, as evidenced by a significant decrease in PBFI fluorescence (Fig. 4). The inhibitory effect of ATP was further enhanced in the presence of 4-aminopyridine or paxilline.

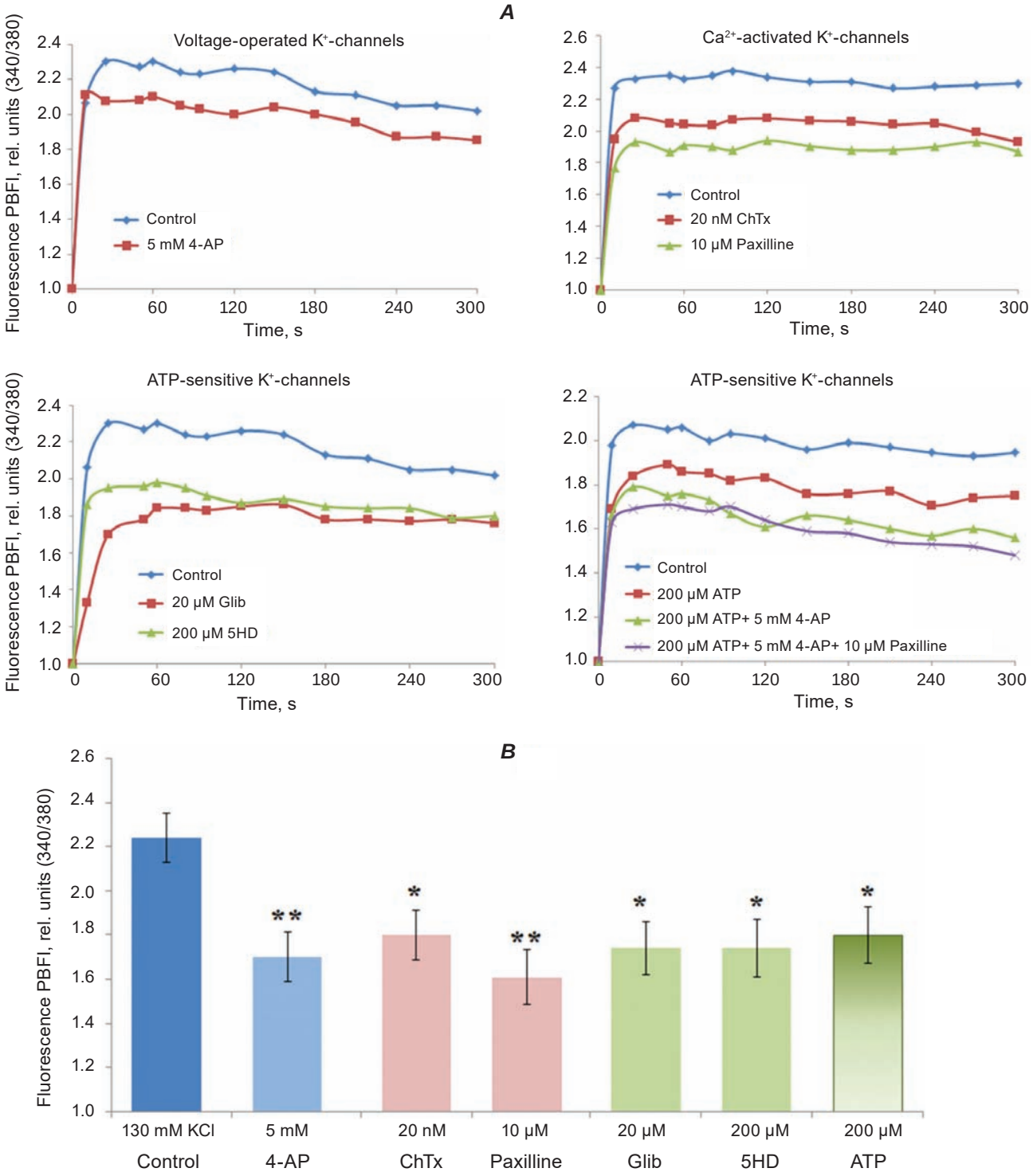

*Fig. 4. Dynamics of potassium ion accumulation by mitochondria in the presence of K^+ channel different subtypes inhibitors (**A**). Comparative effect of K^+ channel inhibitors on the efficiency of K^+ accumulation by mitochondria (**B**). * $P < 0.05$; ** $P < 0.01$ vs control, $M \pm m$, $n = 5$. 4-AP – 4-aminopyridine, ChTx – charybdotoxin, Glib – glibenclamide, 5HD – 5-hydroxydecanoic acid*

It is important to note that the concentrations of all inhibitors used in this study were consistent with those shown to be effective in blocking K^+ channels both in mitochondria of other tissues and at the plasma membrane level.

To further characterize potassium transport systems in the mitochondrial matrix, we employed specific activators of K^+ channel subtypes at concentrations previously validated in studies on other model systems. An increase in K^+ accumulation in mitochondria was observed in the presence of Ca^{2+}-activated K^+ channel activators NS11021 and NS1619 [5, 21, 27], as well as the ATP-sensitive K^+ channel opener cromakalim [19, 22] (Fig. 5). However, the stimulatory effect of cromakalim did not reach statistical significance.

Additionally, the mitoK_{ATP}-specific activator diazoxide (50 μM) [4, 22-23] did not elicit a detectable effect under the conditions of our experiments (data not shown).

These findings provide further evidence supporting the presence of functional K^+ channel subtypes in myometrial mitochondria.

The functional activity of Ca^{2+}-activated K^+ channels was confirmed by experiments evaluating the effect of exogenous Ca^{2+} on PBFI fluorescence intensity (Fig. 6). These studies were conducted in the presence of inhibitors of other K^+ channel subtypes – 4-aminopyridine and ATP. It was demonstrated that the efficiency of K^+ accumulation increased with the addition of Ca^{2+} in the concentration range of 25–100 μM, which approximates physiological levels near mitochondria located in the subplasmalemmal region and those in contact with the sarco(endo)plasmic reticulum (Fig. 6, *A*). This stimulatory effect of Ca^{2+} was not observed in the presence of the Ca^{2+}-activated K^+ channel inhibitor paxilline (Fig. 6, *B*).

Ca^{2+}-activated K^+ channels in mitochondria are widely expressed in cardiac and skeletal muscle tissues, the brain, fibroblasts, and endothelial cells [5, 30]. These channels are regulated by both the mitochondrial membrane potential and Ca^{2+} ions, with the effects of both activators being synergistic. Interaction between the four pore-forming subunits of these channels and cytochrome c oxidase has been demonstrated, suggesting a potential mechanism for direct regulation of the electron transport chain and oxidative phosphorylation [2, 27].

ATP-sensitive K^+ channels are widely expressed in cardiac and skeletal muscle, brain, and liver tissues [5, 19]. Overexpression of mitoK_{ATP} channels leads to mitochondrial swelling, whereas reduced expression is associated with membrane potential instability and decreased efficiency of oxidative phosphorylation [38]. Under physiological conditions (normoxia), stimulation of these channels promotes the generation of reactive oxygen species (ROS) as signaling molecules [19, 30]. This is accompanied by a decrease in the mitochondrial membrane potential, moderate uncoupling of oxidation and phosphorylation, and a subsequent reduction in ROS production.

Partial depolarization of the mitochondrial membrane protects against Ca^{2+} overload and prevents the opening of the mitochondrial permeability

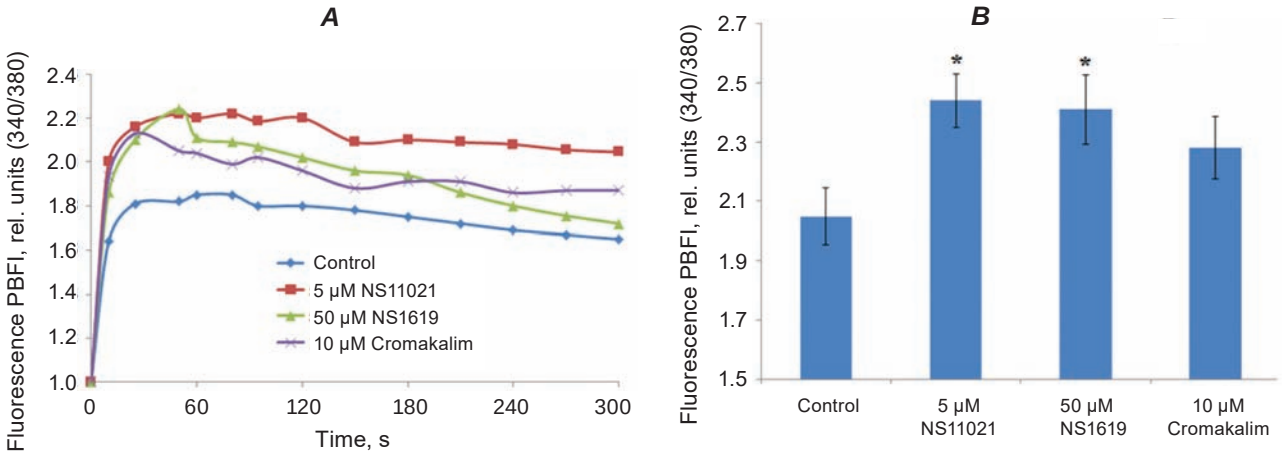

*Fig. 5. The effect of different subtypes K^+ channels specific activators on the accumulation of potassium ions by mitochondria. The effect of K^+-channel activators on the efficiency of K^+ ion accumulation by isolated mitochondria over time (**A**). Statistical processing of these results at the 1st minute of the transport process (**B**), *P < 0.05 vs control, M ± m, n = 6*

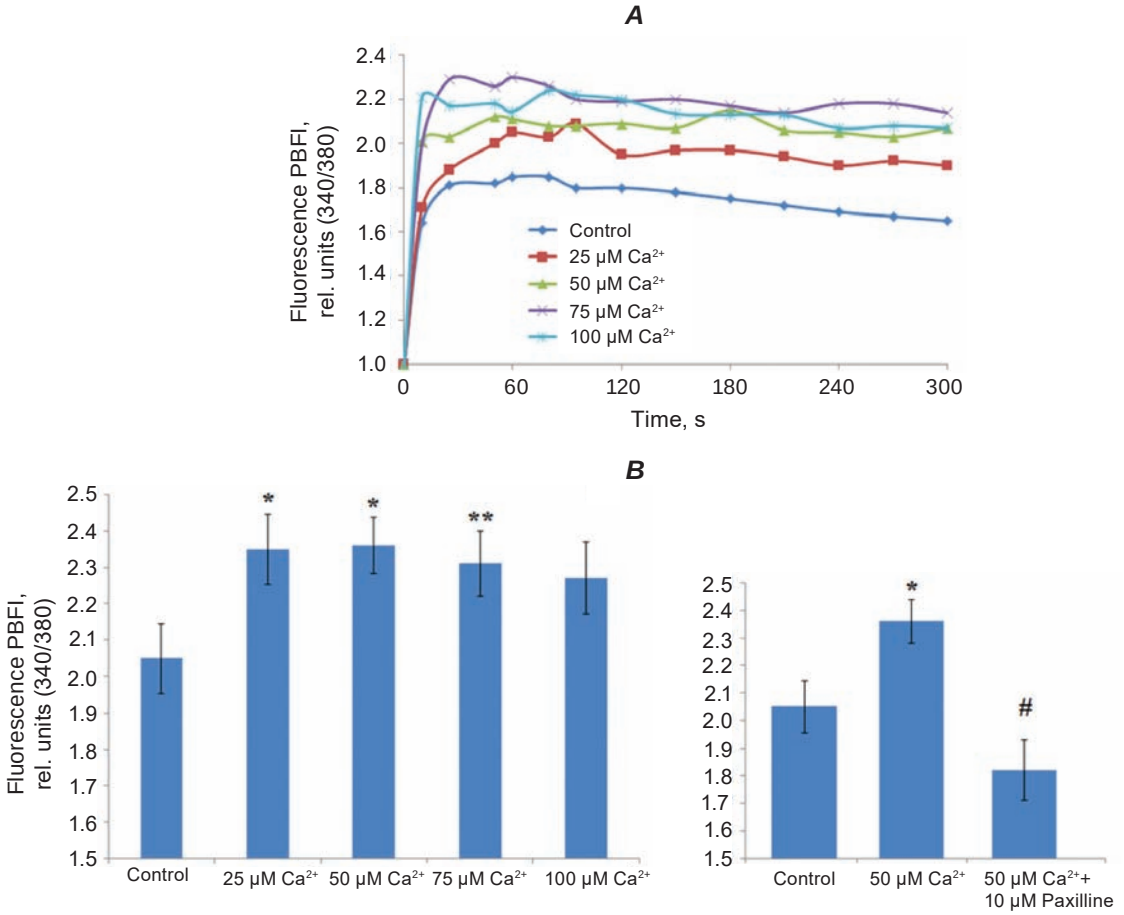

*Fig. 6. The dependence of potassium ion transport in mitochondria on the concentration of extramitochondrial Ca^{2+} in time (A). Statistical processing of preliminary data at the 1st minute of the transport process and its inhibition by the specific inhibitor of Ca^{2+}-dependent K^+ channels paxilline (the transport process was studied in the presence of 4 mM 4-aminopyridine and 200 μM ATP); * $P < 0.05$, ** $P = 0.05$ vs control, # $P < 0.01$ vs activated action of 50 μM Ca^{2+}, $M \pm m$, $n = 6$*

transition pore (mPTP) [1, 20, 30]. This mechanism may be critical for preventing mitochondrial dysfunction under ischemia–reperfusion conditions and the associated oxidative stress [23]. In particular, the mitoK$_{ATP}$ channel opener diazoxide has been shown to protect mitochondria under hypoxic or ischemic conditions and exhibits cardioprotective effects [5]. It is assumed that endogenously generated reactive oxygen and nitrogen species may play an important role in mediating these effects. Notably, myometrial ischemia poses a serious risk to the fetus and significantly complicates labor, emphasizing the potential relevance of mitoK$_{ATP}$ function in uterine physiology under stress conditions [39-40]. Evidence suggests that voltage-operated K^+ channels are involved in the initiation of apoptosis [30-31].

Our experimental results confirm the presence of voltage-operated and Ca^{2+}-activated K^+ channel subtypes in myometrial mitochondria, in addition to ATP-sensitive channels.

Activation of these K^+ channels promotes potassium influx into the mitochondrial matrix, enhances anion channel activity, and facilitates Cl^- accumulation in the organelle [3]. These ionic changes disrupt osmotic balance and drive water influx into mitochondria, leading to organelle swelling [20]. The activity of the H^+/K^+ exchanger serves as a compensatory mechanism to counteract these effects [19, 23].

In cases of mitochondrial dysfunction, irreversible depolarization of the inner mitochondrial membrane may occur, accompanied by opening of the mPTP and uncontrolled swelling [5, 20]. To date, no published data confirm the existence of an H^+/K^+ exchange system specifically in the inner membrane of myometrial mitochondria.

Our findings provide evidence for the presence of H^+/K^+ exchange in mitochondria, as demonstrated by the stimulation of K^+ release in response to acidification of the incubation medium (Fig. 7). In this experimental setup, mitochondria were not pretreated with tetraethylammonium, allowing the preservation of a physiological K^+ concentration within the matrix. The ΔpH-dependent efflux of potassium ions occurred in response to changes in extramitochondrial pH within a range close to physiological values.

Thus, the results presented in this study indicate the existence of a so-called "K^+ cycle" in myometrial mitochondria, which contributes to the maintenance of potassium homeostasis in these subcellular structures. A similar cycle has been well described in the mitochondria of cardiomyocytes [19]. Its core components include K^+ channels of various subtypes and the H^+/K^+ exchanger, all localized in the inner mitochondrial membrane.

It is proposed that the activity of the electron transport chain generates an electrochemical proton gradient across the mitochondrial membrane. The resulting negative membrane potential on the matrix side serves as the driving force for K^+ influx into the matrix. At the same time, excessive accumulation of K^+ is prevented by the coordinated action of ΔpH-dependent K^+ efflux mechanisms. In vivo, this system is further supported by the transport of inorganic phosphate (P_i) across the inner mitochondrial membrane, contributing to osmotic and ionic balance [19, 21].

Using a panel of selective inhibitors and activators, we demonstrated the presence of voltage-operated, Ca^{2+}-activated, and ATP-sensitive K^+ channels in mitochondria of uterine smooth muscle. Pinpointing these individual channel subtypes is crucial for both clarifying their contribution to myometrial physiology and guiding the development of subtype-specific pharmacological agents.

Conflct of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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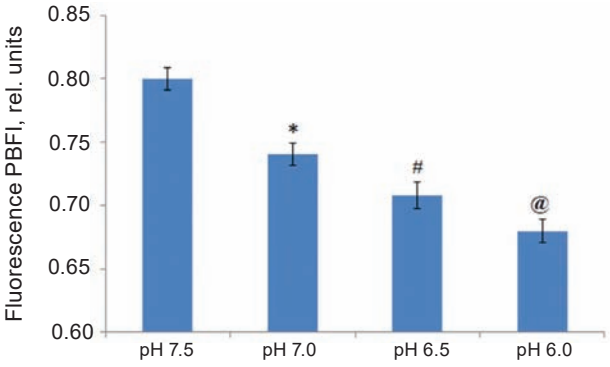

*Fig. 7. Dependence of K^+ release from the mitochondrial matrix on the pH of the medium. M ± m, n = 6. *P < 0.01 vs pH 7.5, #P < 0.05 vs pH 7.0, @P < 0.05 vs pH 6.5*

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ІДЕНТИФІКАЦІЯ K^+-КАНАЛІВ РІЗНИХ ПІДТИПІВ У МІТОХОНДРІЯХ МІОМЕТРІЯ ЩУРІВ ІЗ ВИКОРИСТАННЯМ МОДУЛЯТОРІВ K^+-КАНАЛІВ

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Іони калію регулюють транспорт Ca^{2+} в мітохондріях, величину електричного потенціалу внутрішньої мітохондрійної мембрани, перебіг метаболічних процесів у матриксі та осморегуляцію. Метою досліджень було ідентифікувати різні підтипи K^+-каналів у мітохондріях міометрія щурів. Ізольовані мітохондрії одержували з міометрія невагітних щурів лінії Вістар методом диференційного центрифугування. Акумуляцію іонів калію вивчали методом спектрофлуориметрії за допомогою K^+-чутливого флуоресцентного зонду PBFI-AM. Мітохондрії міометрія ефективно акумулювали іони калію в діапазоні концентрацій 25-150 мМ. У разі заміни іонів K на холін в еквімолярних концентраціях зростан-

ня флуоресценції зонда PBFI не спостерігали. У присутності інгібітора потенціал-керованих K^+-каналів 4-амінопіридину, блокаторів Ca^{2+}-залежних K^+-каналів харібдотоксину або паксиліну, інгібіторів мітоК$_{ATP}$ глібенкламіду, 5-гідроксидеканоєвої кислоти або АТР спостерігали суттєве зниження флуоресцентного сигналу від PBFI. Водночас використання специфічних активаторів Ca^{2+}-залежних K^+-каналів NS11021 та NS1619, а також специфічного щодо мітоК$_{ATP}$ кромакаліму супроводжувалося підвищенням акумуляції K^+ в мітохондрії. Ефективність акумуляції K^+ зростала за додавання 25-100 мкМ Ca^{2+} у присутності 4-амінопіридину та АТР. Одержані результати свідчать на користь наявності в мітохондріях міометрія крім мітоК$_{ATP}$, потенціал-керованих та Ca^{2+}-залежних підтипів K^+-каналів, а також системи H^+-K^+-обміну.

Ключові слова: мітохондрії, акумуляція іонів калію, модулятори K^+-каналів, кальцій, міометрій щурів.

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