PROTECTIVE EFFECTS OF POTASSIUM TRANSPORT IN MITOCHONDRIA FROM RAT MYOMETRIUM UNDER ACTIVATION OF MITOCHONDRIAL PERMEABILITY TRANSITION PORE

O. B. VADZYUK

Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine, Kyiv; e-mail: olga_vadzyuk@hotmail.com

We demonstrated using PBFI K⁺-sensitive fluorescent probe an enhancement of both components of K⁺-cycle – the ATP-sensitive K⁺-uptake and quinine-sensitive K⁺/H⁺-exchange – under the Ca²⁺-induced opening of mitochondrial permeability transition pore (MPTP) in rat myometrium mitochondria. Addition of CaCl₂ (100 μM) to K⁺-free medium results in the enhancement of reactive oxygen species (ROS) production, which was eliminated by cyclosporine A. Addition of CaCl₂ to K⁺-rich medium did not increase the rate of ROS production, but blocking of mitoK⁺_ATP⁻channels with glybenclamide (10 mcM) increased production of ROS. We conclude that K⁺-cycle exerts a protective influence in mitochondria from rat myometrium by regulation of matrix volume and rate of ROS production under the condition of Ca²⁺-induced MPTP.

Key words: Ca ions, MPTP, glybenclamide, K⁺_ATP⁻channels, K⁺/H⁺-exchanger, mitochondria.

Mitochondria play an important role in regulation of cell life and death as they are involved in realization of the most important functions, such as providing of energy substrates and control over cell death [1]. The inner membrane of mitochondria is encrusted with respiratory chain complex and ATP-synthase, which produces ATP, using energy of Δμ_H⁺ in the process. The inner membrane of mitochondria is thus impermeable to ions, and their matrix homeostasis is regulated via functioning of ion transporting systems of channels and exchangers [1, 2]. The mitochondrial ion homeostasis is a finely tuned process, imbalances in it may adversely affect mitochondrial functions and may provoke cell death. Particularly, matrix overload with Ca²⁺ is known to induce cyclosporine A (CsA) – sensitive mitochondrial permeability transition pore (MPTP), that exists in either high or low conductance state. The high-conductance state causes inner membrane rupture followed by cell death. Imbalanced Ca²⁺ homeostasis and MPTP induction caused by it are associated with a number of pathologies such as neurodegenerative diseases and disorders of heart, smooth and skeletal muscles [3, 4].

Mitochondria are capable of maintaining normal functioning under adverse conditions [5]. K⁺ transport across the inner membrane may be one of the factors that help to sustain the mitochondrial function within physiological margins [6]. K ions homeostasis is maintained by potassium channels, which provide for accumulation of the ion in mitochondria, and by K⁺/H⁺-exchanger, which releases K⁺ in cytosol in exchange for H⁺. The latter’s function serves to maintain stable matrix volume and mitochondrial membranes’ integrity. Potassium channels, i.e. mitochondrial K⁺_ATP⁻channels (mitoK⁺_ATP⁻channels) regulate matrix volume, function of electron-transport chain, as well as indirectly affect F₀/F₁-ATPase and generation of reactive oxygen species (ROS) [2, 7]. For instance, Costa et al. [8] demonstrated, that cooperative activity of mitoK⁺_ATP⁻channel and K⁺/H⁺-exchanger in muscle mitochondria is responsible for creating and supporting a new equilibrium matrix volume. K⁺ influx through the mitoK⁺_ATP⁻channel causes alkalinization of matrix that mediates increased ROS production [9]. The last effect, in particular, is perceived as key to cytoprotective mitoK⁺_ATP⁻channel effects under MPTP activation. For example, Costa et al. [8] showed, that mitoK⁺_ATP⁻channel activation indirectly prevents MPTP opening through increased ROS production and consequent protein kinase C activation, which is one of the key enzymes protecting cells from death [10]. These effects are probably involved in the phenomenon of preconditioning – the protective influence of series of short ischemic periods prior to prolonged ischemia [7, 10]. Cell damage under adverse conditions, namely oxidative stress, has been proven
to include the disruption of Ca\textsuperscript{2+} homeostasis, which
in turn causes necrotic or apoptotic cell death [11].
There is also data supporting activation and cytoprotective
effects of mitoK\textsubscript{ATP}-channels under increased
cytosol Ca\textsuperscript{2+} concentration and induction of MPTP
opening [12].

Nevertheless, the mechanism underlying cell
protection by mitoK\textsubscript{ATP}-channel has not been
studied thoroughly. The mitoK\textsubscript{ATP}-channels’ priority
role over K\textsubscript{ATP}-channels of plasma membrane in
preconditioning remains a speculative subject as
well. The influence of activation of K’ transport in
smooth muscle mitochondria on ROS generation
has not been investigated. Also, there is no data as
to specifics has not been investigated. Also, there is no data as
to specifics has not been investigated. Also, there is no data as
pertaining to Ca\textsuperscript{2+} overload. Therefore, we pursued the aim to
investigate, using probes and selective blockers of
K’ transport, dynamics of changes in matrix volume
and ROS generation in isolated rat myometrium mito­
chondria.

**Materials and Methods**

**Isolation of mitochondrial fraction from myo­
metrium.** Mature female white rats (150-200 g
body mass) were anesthetized with ethyl ether and
decapitated. Uterus tissue was cleaned from blood
and fat, minced and homogenized on ice in 8 ml of
isolation buffer solution of the following compo­sition: 250 mM sucrose, 1 mM EDTA, 10 mM HEPES
(pH 7.2 titred by 2 M Tris). The homogenate was
centrifuged for 7 min at 1000 g and 4 °C. The
supernatant was centrifuged for 7 min. at 12 000 g
and 4 °C. The sediment of mitochondria was
resuspended in isolation buffer without EDTA and
stored on ice. Protein content was determined using
Bradford assay.

Concentrated glibenclamide and CsA solutions
in dimethyl sulfoxide were applied introduced
into incubation medium in cuvette at 1 μl per 1.8 ml
of working volume (final concentration of 10 μM).
CaCl\textsubscript{2} solution was introduced in the cuvette to final
concentration of 100 μM.

**Measurement of K’ influx in mitochondria
loaded with PBFI (potassium-binding benzofuran
isophthalate) probe.** The mitochondria were loaded
with PBFI probe as described [8]. The sediment after
the second centrifugation was resuspended in 500 μl
of buffer solution containing 250 mM sucrose,
10 mM HEPES (pH 7.2, 25 °C), 10 mM pyruvate.
The suspension was incubated with 40 μM ace­
toxymethyl ether of PBFI (PBFI-AM) and 0.5 μl of 20% solution of F-127 non-ionic surfactant for 10 min at
room temperature. The suspension was then mixed
for 2 min with 500 μl of buffer for K ions substitu­tion
in mitochondrial matrix, consisting of 175 mM
sucrose, 10 mM HEPES (pH 7.2 at 25 °C), 5 mM
succinate, 5 mM Na\textsubscript{2}HPO\textsubscript{4}, 1 mM MgCl\textsubscript{2}, and 50 mM
tetraethyl ammonium. 7 ml of mitochondria isolation
buffer was then added to the mix, followed by
centrifugation for 10 min at 12000 g and 4 °C. The
sediment was resuspended in buffer with 250 mM
sucrose and 10 mM HEPES (pH 7.2 at 4 °C and no
EDTA added) and kept on ice. Probe fluorescence
was registered at 340 nm and 380 nm wavelength of
excitation and 480 nm wavelength of emission. Final
protein concentration in a sample was 55-60 μg/ml.

The measurements were performed with PTI Quanta Master 40 spectrofluorometer (Canada) in a
standard fluorometric cuvette in thermostatic sam­
ples. Final concentration in a sample was 55-60 μg/ml.

Mitochondria swelling was measured by
registering side lateral light scattering at 520 nm
in standard incubation medium (see above) as de­
scribed [8]. The signal registration begun 1 s after
the added mitochondria suspension had been intro­
duced in the incubation medium.

ROS generation was assayed with ROS-sensi­tive
2,7’-dichlorofluorescein diacetate probe (DCF­
DA). Probe solution was introduced into incubation
medium, and the readings were taken immediately.
Final probe concentration was 4 μM. Fluorescence
excitation wavelength was 540 nm, and emission
was registered at 520 nm 1 s after mitochondria
suspension had been introduced into the incubation
medium.

The results were analyzed and plotted with
Microcal Origin v. 5.0 Software (Microcal Software,
USA). Data was evaluated with Student’s t-test,
the difference between groups was considered signifi­
cant if P < 0.05.

We used the following reagents: Na\textsubscript{2}HPO\textsubscript{4},
MgCl\textsubscript{2} – of local origin, of chemically pure grade;
ATP, diazoxide, sucrose, HEPES, KCl, rotenone, oli-
gomycin, succinate, CsA, ruthenium red, glibenclamide, DCF-DA – by Sigma–Aldrich (USA), CaCl₂ (1 M solution), Tris, EDTA, NaCl – by Fluka (Switzerland), PBFI-AM by Molecular Probes (USA).

Results and Discussion

Mitochondria isolation in sucrose-rich K⁺-free medium leads to a markedly decreased content of these cations in matrix. Afterwards, as the mitochondria are introduced into incubation medium containing KCl, P, anions, and respiratory chain substrates, they begin to uptake K⁺ intensively, which has been demonstrated by various methods, including fluorescent spectroscopy with potassium-sensitive PBFI probe [8]. In order to ensure the adequacy of the probe’s response to K⁺ in isolated mitochondria from the rat myometrium, we studied intensity of fluorescence PBFI loaded in mitochondria depending on KCl concentration in incubation medium. Introduction of mitochondria loaded with PBFI into the incubation medium with various KCl concentrations (see Materials and Methods) led to dose-dependent increase in PBFI fluorescence, which testifies to the adequacy of response of the probe to K⁺ accumulation in mitochondrial matrix (Fig. 1, A). The maximum probe’s response was registered at KCl concentrations close to physiologic (125 mM), and consequently this concentration was used in the standard incubation medium. K⁺ influx into mitochondrial matrix in these experimental conditions is by diffusion due to high membrane potential, and by K⁺-transport channels (ATP-sensitive, in particular) on the inner mitochondrial membrane [13]. As has been demonstrated in numerous publications, the functioning of mitoK⁺,ATP-channels is blocked by ATP [8]. ATP in concentration of 200 µM in the standard incubation medium inhibited K⁺ accumulation in myometrium mitochondria as well (Fig. 1, B, 8). The inhibiting effect of ATP was totally eliminated by mitoK⁺,ATP-channel activator diazoxide (Fig. 1, B, 9), which is also in accordance with data from experiments performed on mitochondria from other tissues [2, 8, 14]. Thus, our results corroborate the existence of K⁺,ATP-channels in the rat myometrium mitochondria.

As has been mentioned by various authors, the pathological conditions inhibiting normal muscle tissue and cell functioning are mediated by disruptions in Ca²⁺ homeostasis, increase in its cytosol concentrations, sometimes by orders of magnitude above the physiological values, and may lead to negative consequences for the myocyte and the muscle in general [11]. Hence, the understanding of possible mechanisms underlying myocytes protection under damaging conditions with increased cytosol Ca²⁺ is an important research task. The possibility of regulation of K⁺,ATP-channels by Ca²⁺ has been demonstrated [15]. Since the activation of these channels exhibits a cytoprotective effect in conditions associated with imbalanced Ca²⁺ homeostasis, it is interesting to investigate their functional properties in smooth muscle mitochondria under high Ca²⁺ concentration. K⁺ accumulation in matrix was found to decrease in incubation medium with 100 µM of CaCl₂ (Fig. 1, B, 2). Ca²⁺-induced decrease in K⁺ accumulation was eliminated by 0.5 mM EGTA, a Ca²⁺-chelator (Fig. 1, B, 4). The data presented on Fig. 1, B, support the assumption that this influence of Ca²⁺ on K⁺ accumulation in myometrium mitochondria is mediated via Ca²⁺-uniporter. The inhibiting effect of Ca²⁺ on K⁺ accumulation was eliminated by 10 µM of ruthenium red, an inhibitor of Ca²⁺-uniporter (Fig. 1, B, 3).

The blockade of total accumulation of K⁺ in mitochondria may be caused by either partial inhibition of K⁺ uptake into the matrix or by activation of K⁺ release. Diazoxide, an activator of K⁺,ATP-channels, in concentration of 50 µM did not exert any effect on K⁺ accumulation in the presence of 100 µM of CaCl₂ (Fig. 1, B, 6). Lack of activation of K⁺ accumulation by diazoxide in our conditions may have at least two explanations: a) K⁺,ATP-channels are already in active state in the presence of Ca²⁺, or b) K⁺,ATP-channels are inhibited, and the activator cannot affect their functional state. Since introduction of 10 µM glibenclamide, an inhibitor of K⁺,ATP-channels, in the presence of Ca²⁺ restores fluorescent signal to control levels, it evidences in favor of our first assumption (Fig. 1, B, 5). We have demonstrated the specificity of inhibiting effect of glibenclamide on K⁺ transport in myometrium mitochondria in particular in our previous work [16]. We have also demonstrated inhibition by glibenclamide of ATP-sensitive K⁺ transport in myometrium mitochondria with PBFI probe (Fig. 1, B, 10). This tendency of increase in PBFI signal in the presence of K⁺,ATP-channels inhibitors allowed us to formulate a hypothesis of simultaneous activation of mitoK⁺,ATP-channels and K⁺/H⁺-exchanger, which was confirmed in further experiments with quinine, an inhibitor of K⁺/H⁺-exchanger. Namely, quinine, when introduced in 0.5 mM concentration, restored K⁺ to values higher than that of the control

Fig. 1. K ions accumulation in isolated mitochondria from myometrium under various conditions. A, fluorescence of PBFI probe loaded in isolated mitochondria depending on KCl concentration in incubation medium; 1 – 125 mM KCl, 2 – 50 mM KCl, 3 – 10 mM KCl, 4 – 50 mM choline chloride (in cases 2, 3 and 4 the incubation medium contained also sucrose, the final osmolarity was 250 mOsm/l). B, effect of Ca²⁺, inhibitors of K⁺ transport and Ca²⁺ uniporter on total accumulation of potassium ions in isolated mitochondria; 1 – K⁺ accumulation in standard incubation medium, 2 – K⁺ accumulation in the presence of 100 µM CaCl₂, 3 – 100 µM CaCl₂ and 10 µM ruthenium red, 4 – 100 µM CaCl₂ and 0.5 EGTA, 5 – 100 µM CaCl₂ and 10 µM glibenclamide, 6 – 100 µM CaCl₂ and 50 µM diazoxide, 7 – 100 µM CaCl₂ and 0.5 mM quinine, 8 – K⁺ accumulation in standard incubation medium in the presence of ATP 200 µM, 9 – with addition of ATP 200 µM and diazoxide 50 µM, 10 – with addition of ATP 200 µM, diazoxide 50 µM and glibenclamide 10 µM. * Denotes significant differences from bar 2 (P < 0.05)

(Fig. 1, B, 7). We can thus assume that activation of mitoK⁺₁₅₅ channels is coupled with activation of K⁺/H⁺-exchanger. Therefore, we can assume from these results, that in our conditions we observe potassium cycle activation in the presence of calcium in mitochondria of myometrium, particularly K⁺ influx into matrix through the K⁺₁₅₅-channels and release the K⁺/H⁺-exchanger, which is eliminated by the specific inhibitors – glibenclamide, ATP and quinine. Garlid et al. argue that the regulation of mitochondrial ma-
matrix volume is the main function of potassium cycle under physiological conditions, which may also protect mitochondria under stress [2, 14]. It is known that increased mitochondrial volume is a condition for activation of the K+/H+-exchanger as a participant in potassium cycle. The activation of the potassium cycle helps to establish new matrix volume equilibrium, yet it is the K+/H+-exchanger that maintains integrity of mitochondrial membranes [2, 8]. Basing on our data we assume that potassium cycle activation, and the K+/H+-exchanger in particular, may be involved in maintenance of integrity of myometrium’s mitochondrial membranes under Ca2+ overload and induction of MPTP.

It has been demonstrated that opening of Ca2+-induced MPTP may result in uncontrolled increase in matrix volume followed by outer membrane rupture and induction of apoptosis or necrosis [11]. The change in mitochondrial volume resulting from MPTP opening may be observed by registering of side light scattering at 520 nm [17]. Introduction of mitochondrial suspension in the standard incubation medium with 100 µM CaCl2 (see Material and Methods section) caused a noticeable drop in lateral light scattering in comparison to control (Fig. 2, A). According to our results, ruthenium red (10 µM), CsA (10 µM) and EGTA inhibited Ca2+-induced swelling of mitochondria (Fig. 2, A). The Ca2+-sensitivity of changes in lateral light scattering of mitochondria suspension leads to the following conclusions: a. Ca2+-induced swelling of mitochondria is mediated by transport of Ca2+ into matrix through mitochondrial uniporter as it is inhibited by ruthenium red and EGTA, a Ca2+ chelator; b. this process is mediated by MPTP induction as it is inhibited by CsA, which is an MPTP inhibitor.

One of the functions of mitochondrial potassium cycle and the mitoK+ATP-channels has been presumed to be matrix volume regulation [2]. We hence investigated the possible involvement of the mitoK+ATP-channels in Ca2+-induced swelling of mitochondria from myometrium. We found that glibenclamide, an inhibitor of mitoK+ATP-channels, in concentrations of 10 µM partially inhibited the changes in lateral light scattering of mitochondrial suspension induced by Ca2+ (Fig. 2, B). It must be noted that the inhibitor itself did not affect the process in any way in the absence of Ca ions (data not shown). Our data are in accordance with the results of Jaburek et al. [18], who propose the existence of various functional states for mitoK+ATP-channels with differing sensitivity to inhibitors. Also, glibenclamide did not affect the change of mitochondrial volume in K+-free medium (replaced equimolarly with Na+) (Fig. 2, B). These results may indicate that changes in light scattering of mitochondria suspension in the presence of 100 µM CaCl2 are caused by two processes: the induction of CsA-sensitive MPTP and the swelling of mitochondria due to K+ influx through potassium channels, i.e. glibenclamide-sensitive mitoK+ATP-channels. Costa et al. suppose that one of the main physiological effects of mitoK+ATP-channels activation on mitochondrial function is the increase in stable matrix volume, and the protective effect against damage to the cell that is ascribed to these channels results directly from regulation of mitochondrial volume [8].

ROS are by-products of cellular metabolism and are produced as a result of functioning of mitochondrial electron-transport chain, among other processes. They participate in intracellular signal pathways, regulating numerous processes under physiological conditions, including gene expression and muscle contraction. Nevertheless, under pathological conditions associated with imbalance in intracellular Ca2+ homeostasis the increased ROS generation leads to MPTP opening followed by necrosis or apoptosis [11]. Taking into account the cytoprotective effect of mitoK+ATP-channels activation under conditions arising from increased concentration of Ca2+ [3, 17], we also aimed to investigate the effect of K+ transport in mitochondria of myometrium on the rate of ROS generation in the presence of Ca2+. Under standard conditions (Ca2+-free) ROS generation does not result in MPTP opening, as introduction of CsA, an inhibitor of MPTP, does not affect the process in any way (data not shown). It is worth mentioning that in the incubation medium containing K+ the rate of ROS generation was higher than in the potassium-free medium (replaced equimolar Na+) (Fig. 3), which is in accordance with data by others [19] that activation of K+ influx in mitochondria potentiates ROS generation in heart mitochondria. While addition of 100 µM CaCl2 did not cause further increase in ROS generation in the medium containing K+, it did result in higher ROS generation in the potassium-free medium, and these changes were eliminated by CsA (data not shown). Glibenclamide (10 µM) potentiates rate of ROS generation in the standard medium with KCl and did not affect the process in the potassium-free medium (Fig. 3), which proves yet again the specificity of its
Fig. 2. Mitochondria swelling induced by CaCl₂. Dynamics of changes in light scattering of mitochondria suspension in the presence of: A, blockers of Ca²⁺ transport and MPTP (result of a typical experiment are shown); B, K⁺<sub>ATP</sub>-channels inhibitors in standard medium; C, K⁺<sub>ATP</sub>-channels inhibitors in K⁺-free medium with KCl replaced by NaCl on equimolar basis. 1 – incubation with CaCl₂ (100 µM); 2 – incubation with CaCl₂ (100 µM) and ruthenium red (10 µM); 3 – incubation with CaCl₂ (100 µM) and CsA (10 µM); 4 – incubation with CaCl₂ (100 µM) and EGTA (0.5 mM); 5 – control (standard medium with no alterations); 6 – incubation with CaCl₂ (100 µM) and glibenclamide (10 µM)
effect on K⁺ transport in mitochondria isolated from the myometrium. Thus, MPTP induction under inhibited K⁺ transport results in the increased matrix ROS generation rate, while activation of K⁺ transmembrane transport may protect the mitochondria to an extent from the damage caused by overproduction of ROS under opening of MPTP, which corroborates the data by Facundo et al., who demonstrated that activation of mitoK⁺ATP-channels of cardiomyocytes decreases ROS generation in the mitochondria in response to increased local oxidant levels [14].

Therefore, the obtained results lead to conclusion that Ca²⁺ induces opening of CsA-sensitive MPTP in mitochondria from the myometrium, followed by matrix swelling and increased rate of ROS generation. We suppose that both components of potassium cycle in the myometrium mitochondria – accumulation in matrix, including influx through mitoK⁺ATP-channels, and release through K⁺/H⁺-exchanger – are activated under the induction of MPTP. Activation of K⁺ influx into the matrix intensifies ROS generation under normal conditions and inhibits it under induction of MPTP. While the induction of MPTP is associated with swelling of mitochondria and accumulation of cytotoxic ROS, the activation of potassium cycle, on the other hand, regulates the rate of ROS generation and matrix volume, thus preventing membrane rupture and overproduction of damaging ROS.
підвищення продукції активних форм кисню в мітохондріях міометрії. Зроблено висновок, що в умовах відкривання Ca^{2+}-індукуваної мітохондриальної пори калієвий цикл виявляє протекторний ефект у мітохондриях міометрії щурів, регулюючи об’єм матриксу та швидкість утворення АФК.

Ключові слова: іони Ca, циклоспорин-чувствительна мітохондриальна пора, глібенкламід, K\textsubscript{АТР}-канали, K+/H\textsuperscript{+}-обмін, мітохондриї.

ПРОТЕКТОРНИЙ ЕФФЕКТ ТРАНСПОРТА K\textsuperscript{+} В МІТОХОНДРИЯХ МІОМЕТРИЇ КРЯС В УСЛОВИЯХ ОТКРЫВАНИЯ МІТОХОНДРИАЛЬНОЇ ПОРЫ

О. Б. Вадзюк

Інститут біохімії ім. А. В. Палладіна НАН України, Київ;
e-mail: olga_vadzyuk@hotmail.com

Методом флуоресцентної спектроскопії з利用сям K-чувствительного зонда було показано, що в умовах індукції іонами Ca мітохондриональна пора активируется обе компоненти K-цикл в мітохондриях міометрії кріс – АТР- і глібенкламид-чувствит.

Ключові слова: іони Ca, циклоспорин-чувствительна мітохондриальна пора, глібенкламід, K\textsubscript{АТР}-канали, K+/H\textsuperscript{+}-обмін, мітохондриї.

References


Received 13.05.2015