ORIGINAL ARTICLE

Mitochondria from rat uterine smooth muscle possess ATP-sensitive potassium channel

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Abstract The objective of this study was to detect ATP-sensitive K⁺ uptake in rat uterine smooth muscle mitochondria and to determine possible effects of its activation on mitochondrial physiology. By means of fluorescent technique with usage of K⁺-sensitive fluorescent probe PBFI (potassium-binding benzofuran isophthalate) we showed that accumulation of K ions in isolated mitochondria from rat myometrium is sensitive to effectors of KATP-channel (ATP-sensitive K⁺-channel) – ATP, diazoxide, glibenclamide and 5HD (5-hydroxydecanoate). Our data demonstrates that K⁺ uptake in isolated myometrium mitochondria results in a slight decrease in membrane potential, enhancement of generation of ROS (reactive oxygen species) and mitochondrial swelling. Particularly, the addition of ATP into incubation medium led to a decrease in mitochondrial swelling and ROS production, and an increase in membrane potential. These effects were eliminated by diazoxide. If blockers of KATP-channel were added along with diazoxide, the effects of diazoxide were removed. So, we postulate the existence of KATP-channels in rat uterus mitochondria and assume that their functioning may regulate physiological conditions of mitochondria, such as matrix volume, ROS generation and polarization of mitochondrial membrane.

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1. Introduction

The mitochondria are involved in a variety of cellular metabolic functions, ranging from ATP supply to triggering of apoptosis (Galluzzi et al., 2012). Thus, the proper mitochondrial functioning is essential for cellular physiology. K⁺-transporting channels, which were discovered in mitochondrial inner membrane in last decades, exert influence not only on mitochondrial physiology, but also on cell functioning both under normal and stress conditions (Costa et al., 2006; Wojtovich and Brookes, 2008; Garg and Hu, 2007). The ATP-sensitive mitochondrial K⁺-channels (mitoKATP) are the most studied among the types of mitochondrial K⁺-channels. The mitoKATP channels are presumed to regulate different characteristics of mitochondria, such as matrix volume, respiration rate, etc., and mediate cell survival under stress conditions (Costa et al., 2006; Wojtovich and Brookes,
2. Material and methods

2.1. Isolation of mitochondria from rat uterus

All procedures performed on animals were approved by the Institutional Commission on Humane Treatment of Laboratory Animals of the Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine. The animals were housed in plastic cages and fed standard laboratory diet and water ad libitum. The study was carried out on sexually mature white female laboratory rats of no particular breed, average weight 150–200 g.

Animals were anesthetized with diethyl ether or chloroform and decapitated. For the extraction of mitochondria, the tissue of uterus was cleaned up from blood and fat, minced, and homogenized on ice in 8 ml of isolation buffer containing 250 mM of sucrose, 1 mM of EDTA, and 10 mM of HEPES (pH 7.2 buffered with TRIS). The homogenate has been centrifuged for 8 min, and the supernatant has been separated and centrifuged for 7 min at 12,000 g, and temperature of 4 °C. The pellet was resuspended in isolation buffer and kept on ice. PBFI fluorescence was measured with excitation ratio technique (excitation wavelength 340/380 nm, emission wavelength 480 nm), in which the signal at 340 nm corresponds to the maximal sensitivity of the probe to K⁺ and the signal at 380 nm corresponds to the isosbestic point of the probe. Ratios for all proteins were used.

2.2. Mitochondrial membrane potential assay

Mitochondrial membrane potential was assayed with voltage-sensitive fluorescent probe TMRM (tetramethylrhodamine acetoxymethyl ester, 50 nM). Fluorescence signal was excited at 540 nm and registered at the wavelength of emission of 580 nm. The measurements started with the addition of aliquot of mitochondrial suspension. Complete depolarization of mitochondria was achieved by the addition of protonophore CCCP (carbonyl cyanide m-chlorophenyl hydrazone) to a final concentration of 2.5 μM or 100 μM CaCl₂.

2.3. Measurements of K⁺ influx into mitochondria loaded with PBFI-AM

Mitochondria were loaded with PBFI as described in (Costa et al., 2006). Briefly, pellets after second centrifugation were resuspended in 0.5 ml of buffered medium (pH 7.2, 25 °C) containing sucrose (250 mM) and pyruvate (10 mM). This suspension was incubated with 40 μM PBFI under gentle stirring for 10 min. An aliquot of PBFI solution in DMSO was previously mixed in 2:1 ratio (vol/vol) with the non-ionic surfactant F-127 before adding to the mitochondrial suspension. The mitochondrial suspension was then diluted with TEA⁺ medium containing sucrose (250 mM), 5 sucinate, 5 Na₂HPO₄, 1 MgCl₂, 50 tetraethylammonium chloride (TEA-Cl) and incubated for 2 min under gentle stirring at 25 °C. This incubation was designed to substitute matrix K⁺ with TEA⁺. Then the suspension was diluted with 7 ml of ice-cold isolation buffer, centrifuged at 12,000g for 8 min, resuspended in isolation buffer and kept on ice. PBFI fluorescence was measured with excitation ratio technique (excitation wavelength 340/380 nm, emission wavelength 480 nm), in which the signal at 340 nm corresponds to the maximal sensitivity of the probe to K⁺ and the signal at 380 nm corresponds to the isosbestic point of the probe. Ratios for all proteins were used.

2.4. Mitochondrial swelling

Changes in mitochondrial matrix volume were followed using light-scattering technique. Measurements were conducted on
Mitochondria from rat uterine smooth muscle

2.5. Measurement of ROS generation

Measurements had been conducted using ROS-sensitive probe DCF-Da (2,7-dichlorofluorescein-diacetat) (Gomes et al., 2005). A small aliquot of stock solution of DCF-Da was added into the incubation medium immediately prior to the measurement of the final concentration of 4 M. Wavelengths of the excitation and emission of fluorescence were 498 nm and 523 nm respectively. Registration of fluorescence was started at the 1 s. after the addition of aliquot of the suspension of mitochondria. Measurements were done in standard and K+-free incubation media with the composition described above (see above for details).

2.6. Statistical analysis

Analysis of the results and graph generation has been conducted by Microcal Origin software, 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.

2.7. Chemicals

Sucrose, HEPES, KCl, CCCP, rotenone, oligomycin, succinate, glibenclamide, 5HD, 2,7-DCF-Da, TMRM were obtained from Sigma – Aldrich (USA); PBFI-AM – Molecular Probes (USA), TRIS, EDTA, NaCl CaCl2 (1 M solution) were obtained from Fluka (Switzerland); other chemicals were obtained from a domestic manufacturer (Ukraine).

3. Results

3.1. Measurements of the mitochondrial membrane potential

Experimental work has been performed on isolated fraction of rat uterus mitochondria prepared as described in the Methods section. Fluorescent probe TMRM is widely used for measurements of the inner mitochondrial membrane potential. Cationic molecule of TMRM easily moves through inner membrane toward its negative potential which can reach -200 mV in well coupled mitochondria (Suski et al., 2012). The mitochondria were coupled and maintained membrane potential as it can be seen in Fig. 1A. Accumulation of TMRM in the mitochondrial matrix after the addition of aliquot of mitochondrial suspension into the standard incubation medium was done with respiration substrate results in fluorescence quenching with subsequent setting of the steady-state level. Protonophore CCCP (2.5 M) or CaCl2 (100 M) caused rapid a dissipation of mitochondrial potential and restored the initial level of fluorescence signal. The same level of fluorescent signal was obtained in samples previously incubated with 2.5 M CCCP (Fig. 1A). These results confirm the well-coupled conditions of the mitochondrial suspension, we used for this study.

As it can be seen in Fig. 1B the ATP-sensitive K+ transport in uterus mitochondria leads to a decrease in Δψ. Addition of ATP (200 M) caused a small increase in mitochondrial potential in the standard incubation medium if compared to control conditions. This effect was eliminated by the addition of diazoxide (50 M) along with ATP. Neither ATP nor diazoxide influenced the membrane potential in K+-free medium (Fig. 1B). Thus K+ transport in mitochondria through the ATP-sensitive pathway results in slight depolarization of the inner mitochondrial membrane.

3.2. Measurement of K+ influx into PBFI-loaded mitochondria

K+-sensitive fluorescent indicator PBFI is effectively used for direct measurement of K+ transporting processes in living objects (Costa et al., 2006). Costa et al. demonstrated that PBFI can be used to detect K+ uptake directly in the mitochondrial matrix in isolated rat heart mitochondria (Costa et al., 2006). Using the methodology described in this paper (Costa et al., 2006) we studied the K+ uptake in isolated rat uterus mitochondria. Mitochondria loaded with PBFI accumulate K ions in the standard incubation medium as shown in Fig. 2. We had ensured that the intensity of fluorescence emission of PBFI loaded to mitochondria was directly proportional to the concentration of K ions absorbed from the incubation media with different concentration of KCl (data not shown). The K+ uptake into mitochondrial matrix was sensitive to ATP (200 M), diazoxide (50 M) and glibenclamide (1 M). Addition of ATP (200 M) slowed down the increase in PBFI fluorescence signal in isolated rat uterus mitochondria in the standard incubation medium. The blocking effect of ATP on K+ uptake was prevented by diazoxide, while glibenclamide eliminated the effect of mitoKATP activator (Fig. 2).

3.3. Light scattering studies of isolated mitochondria

The mitochondrial swelling could be detected by means of registration of light scattering signal on 520 nm. This technique is widely used for the studying of mitoKATP activation in isolated mitochondria (Costa et al., 2006; Garlid and Halestrap, 2012). Light scattering values are inversely dependent on the volume of mitochondria. This measurement is based on the fact that mitochondria isolated in K+ -free medium are depleted of K ions, easily accumulate them and swell when added to K+ -rich medium, resulting in a loss of light scattering. Fig. 3A contains typical light scattering traces from rat uterus mitochondria respiring in K+ -rich medium. In the absence of ATP, the matrix swelled to a higher steady-state volume. Addition of 200 M ATP resulted in a slower swelling rate and consequently in a lower steady-state volume, which means that ATP-sensitive K+ uptake is blocked under these conditions. This effect of ATP was reversed by diazoxide (50 M). Another KATP-channel blocker glibenclamide (10 M) added along with diazoxide and ATP lowered the rate of swelling and steady-state volume (Fig. 3A and B). But ATP and other effectors caused such effects only when K ions were present in the incubation medium. There was no dependency of light
scattering of mitochondria on ATP, diazoxide or glibenclamide in K+-free medium (Fig. 3BII).

3.4. ROS measurements in isolated mitochondria

Under basic conditions in the standard incubation medium supplied with a substrate of respiration the isolated mitochondria produce moderate level of ROS as normal by-products of electron-transporting chain (Fig. 4A). Application of ATP (200 μM) attenuated ROS production. The rate of ROS production was restored when diazoxide was added along with ATP. The stimulating effect of diazoxide on the generation of ROS was fully eliminated by 5-HD (200 μM) (Fig. 4A, BI).

The same results were obtained with 1 μM glibenclamide (data not shown). All the effectors mentioned above had no influence on the rate of ROS production in the absence of K ions (Fig. 4BII).

4. Discussion and conclusions

Although functioning and regulation of mitoK<sub>ATP</sub>-channel from other tissues is studied widely, the problem of existence of this structure in mitochondria from uterus has not been discussed yet. So, the major objective of this study was to reveal the ATP-sensitive K+ transporting pathway in mitochondria from rat uterus. To accomplish it we studied the K+-dependent mode of action of the effectors of KATP-channels on mitochondrial physiology and directly measured K+ uptake into the matrix of mitochondria from rat uterus.

The existence and physiological role of K<sub>ATP</sub>-channel in plasma membrane of myometrium cells is well defined (Brainard et al., 2007; Zhu et al., 2013). This type of potassium channels is one of the most abundant in myometrium plasma membrane. Its main function, as in other types of smooth muscles, is to regulate excitability and provide for relaxation of uterus smooth muscle, which is crucial for its proper functioning (Brainard et al., 2007 Zhu et al., 2013). It was shown that both the known subunits of the K<sub>ATP</sub>-channel are expressed in plasma membrane of human and rat myometrium (Brainard et al., 2007). Although the structure and mechanisms of regulation of uterus plasma membrane K<sub>ATP</sub>-channel are actively investigated and discussed, there are very few data about this
channel in the inner membrane from uterus mitochondria (Vadzyuk and Kosterin, 2015), though there are plenty of data regarding mitoK$_{\text{ATP}}$ in mitochondria from other tissues, such as heart, liver, brain, and vessels (Garlid and Halestrap, 2012; Brainard et al., 2007; Zhu et al., 2013).

Study of mitoK$_{\text{ATP}}$ in living cell is complicated by the fact that both mitoK$_{\text{ATP}}$ and plasma membrane K$_{\text{ATP}}$-channel are regulated by the same effectors with the exception of 5-HD (Garlid and Halestrap, 2012). Also there are some contradictory data about the specificity of diazoxide toward

Figure 3 Measurements of mitochondrial swelling in standard K$^+$-rich medium (A, BI) or in K$^+$-free medium with NaCl (equimolar substitution) (II). (A) Typical traces of light scattering changes in the standard incubation medium. (B) Averaged swelling rates relative to controls (with no additions, columns 1 and 2 respectively). Effectors of mitoK$_{\text{ATP}}$ were added as follows: (1)-none; (2)-ATP; (3)-ATP and diazoxide; (4)-ATP, diazoxide and glibenclamide. Concentrations of substances were: ATP 200 mcM, diazoxide 50 mcM, and glibenclamide 10 mcM. Additions of aliquots of mitochondrial suspension signed by arrow (mito). At the 0.05 level, the means are significantly different. @-column 2I is significantly different from 1I, #-column 3I is significantly different from 2I, &-column 4I is significantly different from 3I.

Figure 4 Mitochondrial reactive oxygen species production increases with K$^+$ uptake. (A) Relative fluorescence of 2,7-dichlorofluoresceine-diacetat expressed in arbitrary units (a.u.) is plotted against time (results of typical experiments are shown). ATP (200 mcM) was present in all traces except for the trace marked “none”. Diazoxide (diaz) and 5HD were added where mentioned in concentration 50 and 200 mcM respectively. (B) Rates of ROS production by isolated rat uterus mitochondria are plotted as percentage of control rate obtained in control conditions in the standard incubation medium with no additions (1I). I-The standard incubation medium, II-K$^+$ free medium, with KCl substituted with equimolar NaCl. (1)-No additions, (2)-ATP, (3)-ATP and diazoxide, (4)-ATP, diazoxide and 5HD, concentrations of effectors were: ATP 200 mcM, diazoxide 50 mcM, and 5HD, concentrations of effectors were mentioned above. Additions of aliquots of mitochondrial suspension signed by arrow (mito). At the 0.05 level, the means are significantly different. @-column 2I is significantly different from 1I, #-column 3I is significantly different from 2I, &-column 4I is significantly different from 3I.
the K\textsubscript{ATP}-channels (Garlid and Halestrap, 2012). We conducted the current study on the isolated mitochondrial fraction, so that data we obtained cannot be related to plasma membrane K\textsubscript{ATP}-channels. In order to assume the potassium specificity of the substances we used, we repeated the same experiments in K\textsuperscript{+}-free medium and made sure that all effects of ATP, diazoxide, glibenclamide and 5-HD on studied physiological processes in mitochondria appeared only if potassium ions were present. This is a strong indication that their influence on mitochondrial physiology is mediated by K\textsuperscript{+}-dependent processes. Also, we directly demonstrated that K\textsuperscript{+} uptake into the matrix of isolated mitochondria from rat uterus loaded with PBF-I is sensitive to diazoxide and blockers of K\textsubscript{ATP}-channel in the way that it is activated by diazoxide after blocking by ATP, and blocked if another blocker glibenclamide is added along with ATP and diazoxide (Fig. 2).

Thus, we studied K\textsuperscript{+}-influx pathway in rat uterus mitochondria by means of four different assays, mitochondrial swelling, measurements of mitochondrial membrane voltage and ROS production, and direct measurement of K\textsuperscript{+} uptake with K\textsuperscript{+}-sensitive fluorescent probe. Taking into account all of the above, we may assume that all effects of activator and inhibitors of K\textsubscript{ATP}-channel on studied physiological processes in mitochondria are mediated by the transporting of K ions through the ATP-sensitive pathway in rat uterus mitochondria, namely mitoK\textsubscript{ATP}, similar to those from other tissues (Costa et al., 2006; Garg and Hu, 2007).

Our data allow us to assume that consequences of the activation of ATP-sensitive K\textsuperscript{+} uptake in rat uterus mitochondria are (1) mild mitochondrial depolarization, (2) mitochondrial matrix swelling and (3) slight enhancement of ROS production. It should be said that our data regarding the influences of activators and blockers on functioning of mitochondria from myometrium are in concordance with those described in mitochondria isolated from other tissues (Costa et al., 2006; Garg and Hu, 2007).

The inner membrane potential is an important indicator of the mitochondrial energetic state (Suski et al., 2012). As it is a driving force for ATP synthesis and Ca\textsuperscript{2+} uptake, its value determines the proper functioning of the mitochondria and the cell as a whole. Thus, one of our aims was to determine whether the ATP-sensitive K\textsuperscript{+} transport in uterus mitochondria could influence the mitochondrial inner membrane potential. It should be said that small depolarizing effect of mitoK\textsubscript{ATP} activation on mitochondrial inner membrane was reported on isolated vascular and renal mitochondria (Katakam et al., 2014; Cancerini et al., 2003). The same results were obtained for uterus mitochondria (Fig. 1B). This could be explained in that way, that K\textsuperscript{+} uptake results in an increase in mitochondrial matrix volume, which in turn activates K\textsuperscript{+}/H\textsuperscript{+} exchanger, promotes H\textsuperscript{+} extrusion out of matrix, and provides a mild uncoupling effect (Costa et al., 2006). This uncoupling stimulates electron transporting chain. The result of it could be enhancement of ATP synthesis, as well as amplification of ROS production (Suski et al., 2012). It should be noticed that mitochondrial uncoupling could have protective effect by itself (Minners et al., 2000; Hausenloy et al., 2004). Minners et al. showed that mitochondrial uncoupling agents confer protection under stress conditions (Minners et al., 2000).

Another beneficial consequence of partial mitochondrial depolarization as a result of K\textsuperscript{+} transporting into mitochondria is attenuation of Ca\textsuperscript{2+} uptake. As Ca\textsuperscript{2+} influx into mitochondria is driven primarily by \(\Delta\psi\), a small change in \(\Delta\psi\) can induce a large change in matrix Ca\textsuperscript{2+} concentration (Ishida et al., 2001). Indeed, Holmuhamedov et al. showed that the pharmacological activation of cardiac mitoK\textsubscript{ATP} by diazoxide decreased the accumulation of Ca ions into the mitochondrial matrix (Holmuhamedov et al., 1999). It is known that excessive Ca\textsuperscript{2+} accumulation in mitochondrial matrix may trigger the induction of the mitochondrial permeability transition pore opening and cell death, and the prevention of Ca\textsuperscript{2+} overload may protect mitochondria from the induction of apoptosis (Holmuhamedov et al., 1999). Therefore, we assume that the mild uncoupling effect of mitoK\textsubscript{ATP} activation could have beneficial consequences on physiology of uterus mitochondria under normal as well as under stress conditions, and effectively prevent Ca\textsuperscript{2+} overload.

There is a general agreement that K\textsuperscript{+} uptake into mitochondria, accompanied by the movement of anions, evokes osmotic entry of water. This leads to an increase in matrix volume with further activation of K\textsuperscript{+}/H\textsuperscript{+}-exchange and settlement of new steady-state matrix volume without the disruption of the outer mitochondrial membrane. The primary physiological consequence of the mitochondrial matrix swelling is the enhancement of respiration and contraction of intermembrane space, resulting in an increased membrane permeability to ADP and, as a result, the facilitation of ATP synthesis (Costa et al., 2006). Thus, mitochondrial matrix swelling after activation of mitoK\textsubscript{ATP} could occur in response to an increased cellular need in ATP. These effects could be crucial for mitochondrial functioning under ischemic conditions, as well as under conditions of normoxia. As we showed earlier (Vadzyuk and Kosterin, 2015) the activation of mitoK\textsubscript{ATP} has a protective effect on rat uterus cell under oxidative stress conditions. The explanation of this phenomenon may consist, as well, in mild uncoupling and mitochondrial swelling as the consequences of the activation of mitoK\textsubscript{ATP} in rat uterus cells.

ROS are natural by-products of electron transporting chain, and intensity of their generation directly depends on the mitochondrial respiration rate (Suski et al., 2012). ROS are known signaling molecules, which trigger different pathways resulting in stress adaptation or cell death (Brown and Griendling, 2015). Thus, their production under conditions of normoxia should be tightly controlled. We assume that the activation of ATP-sensitive K\textsuperscript{+} uptake in mitochondria could be one of mode of regulation of the intensity of ROS production in uterus mitochondria. As data of Fig. 4 show, when mitoK\textsubscript{ATP} channel is closed by ATP or 5-HD, ROS production is substantially lower than in conditions where ATP-sensitive K\textsuperscript{+} uptake is allowed. It is known that ROS regulates homeostasis of Ca\textsuperscript{2+} in smooth muscle and mediates muscle relaxation (Brown and Griendling, 2015; Zhang et al., 2013), so we assume that the activation of mitoK\textsubscript{ATP} channel in rat uterus cells may contribute to the relaxation of myometrium by means of amplification of ROS generation. On the other hand, ROS induced by activation of mitoK\textsubscript{ATP} could promote cell adaptation to environmental stress (Brown and Griendling, 2015). It was showed that ROS scavengers blocked protection induced by diazoxide under conditions of ischemia (Semirabet et al., 2008). This clearly indicates that a relationship exists between mitoK\textsubscript{ATP}, ROS and cell protection. Therefore the impact of mitoK\textsubscript{ATP} activation on ROS production may have a protective effect under stress conditions and...
regulate contraction and relaxation under normal conditions. We have shown earlier that diazoxide mildly increased ROS production under normal conditions in rat uterus cells, but decreased severe peroxidation under chemically induced oxidative stress conditions if cells had been incubated with diazoxide prior to the induction of stress. This effect may be explained also by ROS-mediated cytoprotection and may involve other intermediates, such as protein kinases.

Our results allow us to assume that ATP-sensitive K⁺-channel in rat uterine smooth muscle mitochondria exists and exerts versatile effects on mitochondrial physiology – regulates matrix swelling, ROS production and change inner membrane potential, which may be a response of mitochondria on cell demands under different metabolic challenges, and may have an important role in physiology of the smooth muscle as a whole.

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