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Mechanosensitivity of mitochondrial large-conductance calcium-activated potassium channels☆

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ABSTRACT

Potassium channels have been discovered in the inner mitochondrial membrane of various cells. These channels can regulate the mitochondrial membrane potential, the matrix volume, respiration and reactive species generation. Therefore, it is believed that their activation is cytoprotective in various tissues. In our study, the single-channel activity of a large-conductance calcium-activated potassium channel ($mitoBK_{Ca}$) was measured by the patch-clamp technique on mitoplasts derived from mitochondria isolated from human glioma U-87 MG cells. Here, we show for the first time that mechanical stimulation of $mitoBK_{Ca}$ channels results in an increased probability of channel opening. However, the mechanosensitivity of $mitoBK_{Ca}$ channels was variable with some channels exhibiting no mechanosensitivity. We detected the expression of mechanosensitive BK_{Ca}-STREX exon in U-87 MG cells and hypotesize, based on previous studies demonstrating the presence of multiple BK_{Ca} splice variants that variable mechanosensitivity of $mitoBK_{Ca}$ could be the result of the presence of diverse BK_{Ca} isoforms in mitochondria of U-87 MG cells. Our findings indicate the possible involvement of the $mitoBK_{Ca}$ channel in mitochondria activities in which changes in membrane tension and shape play a crucial role, such as fusion/fission and cristae remodeling.

1. Introduction

Mitochondria are cellular organelles that play a central role in the production of ATP, which is then consumed for all types of cellular functions, from metabolism to cellular movement. Mitochondria are constantly changing shape through fusion, fission and cristae remodeling to best meet the energy requirements of the cell [1]. The mechanisms of this process are still not fully understood, even though it is known that altered mitochondrial dynamics are linked to various pathologies. The inner mitochondrial membrane is a complex structure composed of mitochondrial cristae. Mitochondrial cristae change during metabolism, and their opening is critical for the apoptosis progress [2]. The above processes, i.e., cristae remodeling, fusion and fission, must affect the mechanical tension in mitochondrial membranes. However, data on the mechanical sensitivity of mitochondria are very limited.

Several channel types have been discovered in mitochondria, among which potassium channels constitute the most abundant group. They include, for instance, $mitoK_{ATP}$ [3], $mitoBK_{Ca}$ [4,5], and mitoKv1.3 [6]. These channels are located in the inner mitochondrial membrane and can regulate mitochondrial physiology, including membrane potential, matrix volume, respiration rate and generation of reactive oxygen species. Accumulating experimental evidence suggests that their activation is cytoprotective in various tissues [7,8]. On the other hand, it was recently found that blocking the activity of mitoKv1.3 in glioma cells induces apoptosis and hence these channels are promising oncotherapy targets [9].

The $mitoBK_{Ca}$ channel was originally identified in the human glioma cell line LN229 using the patch-clamp technique [4]. This channel, with a conductance of 295 ± 18 pS measured in 150 mM KCl, was stimulated by Ca^{2+} and blocked by charybdotoxin (ChTx). In later studies, the presence of a channel with properties similar to the surface membrane calcium-activated K^+ channel was observed in patch-clamp recordings from the mitoplasts of guinea pig ventricular cells [10]. The guinea pig channels were stimulated by the potassium channel opener NS1619, blocked by ChTX, iberiotoxin (IbTX) and, another BK_{Ca} channel blocker of established mechanism of action, paxilline [11]. From our studies, we know that BK_{Ca} is expressed and active in mitochondria of several cell types including astrocytes, fibroblasts and endothelium [12–14]. Rat skeletal muscle mitochondria were found to show pronounced immunoreactivity against antibodies targeting the BK_{Ca} β4 subunits [15]. This finding implies a close molecular similarity

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between the mitoBK$_{Ca}$ channel and the plasma membrane BK$_{Ca}$ channel. It is therefore reasonable to speculate that both channels are splice variants of the same gene product. Indeed, the pore-forming α-subunits of BK$_{Ca}$ channels are encoded by a single gene, *KCNMA1*, which undergoes extensive alternative pre-mRNA splicing and a mitochondria-specific splice variant BK$_{Ca}$-DEC has been recently identified [5]. It has been suggested that the mechanosensitivity of the plasma membrane BK$_{Ca}$ is due to the presence of a STREX exon in specific BK$_{Ca}$ splice variants [16].

In this work, we have shown for the first time that a subpopulation of mitochondrial BK$_{Ca}$ channels is mechanosensitive. However, mechanosensitivity was not observed for all mitoBK$_{Ca}$ channels. We detected expression of different exons of various BK$_{Ca}$ splice variants and argue that this variable mechanosensitivity could be due to the presence of different BK$_{Ca}$ isoforms in mitochondria of U-87 MG cells. Our findings indicate a possible role of the mitoBK$_{Ca}$ channel in the mechanosensitivity of mitochondria, which could be important for processes such as fusion/fission and cristae remodeling.

2. Materials and methods

2.1. Cell culture

Astrocytoma U-87 MG cells were cultured in DMEM (Laboratory of General Chemistry, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences) supplemented with 10% FBS (Gibco, USA), 100 U/mL penicillin (Sigma-Aldrich), 100 μg/mL streptomycin (Sigma-Aldrich) and 2 mM L-glutamine (Gibco, USA) at 37 °C degrees in a humidified atmosphere with 5% CO_2. The cells were fed and reseeded every third day.

2.2. RNA isolation

Total RNA was isolated from human astrocytoma U-87 MG cells using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Briefly, cells were harvested and centrifuged at 800 × *g* for 10 min. Next, cells were lysed in RLT buffer and the lysate was centrifuged for 3 min at maximum speed. One volume of 70% ethanol was added to the supernatant from the previous step. The sample was transferred to an RNeasy Mini spin column placed in a collection tube and centrifuged for 15 s at ≥8000 × *g*. The flow-through was discarded. Buffer RW1 was added to the RNeasy column, which was centrifuged for 15 s at ≥8000 × *g*. DNase I stock solution was added to Buffer RDD, mixed and centrifuged briefly. DNase I incubation mix from the previous step was directly added to the RNeasy column and incubated at RT for 15 min. Next, Buffer RW1 was added to RNeasy the column and the column was centrifuged for 15 s at ≥8000 × *g*. Last, the RNA was cleaned up with RW1 and RPE buffers, and RNA was eluted from the RNeasy column with RNase-free water by centrifugation for 1 min at ≥8000 × *g*. The RNA concentration was measured using a Cary 60 UV–Vis spectrophotometer (Agilent Technologies, USA).

2.3. cDNA synthesis

RNA was reverse transcribed using a RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific). Then, 2 μg of total RNA (~4 μL – depending on the RNA preparation) were mixed with 1 μL of Random Hexamer primers, adjusted with DEPC-treated water to 12 μL, mixed gently, briefly centrifuged, incubated at 65 °C for 5 min and chilled on ice. Next, 4 μL 5× Reaction Buffer, 1 μL RiboLock RNase Inhibitor (20 U/μL), 2 μL dNTP Mix (10 mM) and 1 μL RevertAid M-MuLV RT (200 U/μL) were added to the above reverse transcription mixture. The total volume was 20 μL. cDNA synthesis reactions were also carried out with DEC specific reverse primer 5′-GGTACTCATGGGCTTGATTT-3′. The cDNA synthesis reaction was run for 5 min at 25 °C, followed by 60 min at 42 °C and terminated by heating for 5 min at 70 °C. The resulting cDNA was used as a template for PCR amplification.

2.4. Polymerase chain reactions

PCR reactions were carried out with the cDNA obtained in the reverse transcription reaction to detect the alternatively spliced exons STREX and DEC.

PCR was conducted with the following primers: BK$_{Ca}$-All, forward primer 5′CCCGCAGACACTGGCCAATAG 3′, reverse primer 5′-GAGCA TCTCTCAGCCGGTAA-3′; BK$_{Ca}$-STREX_L, forward primer 5′-GAGACGG GCATGTTGTTTTG-3′, reverse primer 5′-GAGCATCTCTCAGCCGG TAA-3′; BK$_{Ca}$-STREX_S, forward primer 5′-GAGACGGGCATGTTGTT TTG-3′, reverse primer 5′-ATCAGCTTAGGCGAGGTGTT-3′; BK$_{Ca}$-DEC, forward primer 5'-GGGACAAACAGAATGCAACA-3′, reverse primer 5′-GGTACTCATGGGCTTGATTT-3′, BK$_{Ca}$-STREX_DEC, forward primer 5′-GAGACGGGCATGTTGTTTTG-3′, reverse primer 5′-CTTGGCCCATTC TATTCATCC-3′.

The PCR was performed with REDTaq® Ready Mix™ PCR Reaction Mix (Sigma-Aldrich). Briefly, 2 μL of cDNA was mixed with 10 μL (for BK$_{Ca}$-All, BK$_{Ca}$-STREX_L, BK$_{Ca}$-STREX_S, BK$_{Ca}$-STREX_DEC) or 12.5 μL (for BK$_{Ca}$-DEC) REDTaq Ready Mix; 1 μL forward primer; 1 μL reverse primer; and 0.5 μL DMSO (for BK$_{Ca}$-DEC) and adjusted to 20 μL (for BK$_{Ca}$-All, BK$_{Ca}$-STREX_L, BK$_{Ca}$-STREX_S, BK$_{Ca}$-STREX_DEC) or 25 μL (for BK$_{Ca}$-DEC) with nuclease-free water.

PCR amplification was performed in a Thermal Cycler C1000™ (BioRad) as follows: initial denaturation at 95 °C for 5 min (for BK$_{Ca}$-DEC) or at 94 °C for 60 s (for BK$_{Ca}$-All, BK$_{Ca}$-STREX_L, BK$_{Ca}$-STREX_S, BK$_{Ca}$-STREX_DEC); 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 58.4 °C for 30 s and extension at 72 °C for 30 s for BK$_{Ca}$-DEC; 10 cycles of denaturation at 94 °C for 15 s, primer annealing by a touch-down protocol from 59 °C to 49 °C for 30 s, extension at 72 °C for 45 s (for BK$_{Ca}$-All, BK$_{Ca}$-STREX_S) or 120 s (for BK$_{Ca}$-STREX_L, BK$_{Ca}$-STREX_DEC) followed by 20 cycles of denaturation at 94 °C for 15 s, primer annealing at 49 °C for 30 s and extension at 72 °C for 45 s (for BK$_{Ca}$-All, BK$_{Ca}$-STREX_S) or 120 s (for BK$_{Ca}$-STREX_L, BK$_{Ca}$-STREX_DEC); a final extension step at 72 °C for 5 min (for BK$_{Ca}$-DEC) or for 30 s (for BK$_{Ca}$-All, BK$_{Ca}$-STREX_L, BK$_{Ca}$-STREX_S, BK$_{Ca}$-STREX_DEC). The predicted sizes of the PCR products were as follows: BK$_{Ca}$-All, 167 bp; BK$_{Ca}$-STREX_L, 1313 bp; BK$_{Ca}$-STREX_S, 274 bp BK$_{Ca}$-DEC, 113 bp; BK$_{Ca}$-STREX_DEC, 1598 bp. The PCR products were separated by electrophoresis in 1% agarose gel and were visualized with Midori Green (Nippon Genetics Europe GmbH).

2.5. Mitochondria isolation

Mitochondria were prepared from the human astrocytoma (glioblastoma) U-87 MG cell line as previously described with modifications [12]. Human astrocytoma cells from one to three culture flasks were collected in PBS and centrifuged at 400 × *g* for 10 min. The cell pellet was resuspended and homogenized in a preparation solution (250 mM sucrose, 5 mM HEPES, pH = 7.2). Next, the homogenate was centrifuged at 9200 × *g* for 10 min. The pellet was then suspended and centrifuged at 400 × *g* for 10 min. The supernatant was transferred to a new tube and centrifuged at 9200 × *g* for 10 min. The pelleted mitochondria were then resuspended in preparation solution and centrifuged at 9200 × *g* for 10 min. Finally, the mitochondria were resuspended in 0.1 mL of preparation solution. All procedures were performed at 4 °C.

2.6. Patch-clamp experiments

Patch-clamp experiments on mitoplasts were performed as described previously, with modifications [12,13,17]. Briefly, mitoplasts were prepared from a 1–3 μL of sample of human astrocytoma mitochondria placed in 400 μL of hypotonic solution (5 mM HEPES, 100 μM $CaCl_2$, pH = 7.2) for approximately 1–3 min to induce swelling