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A novel potassium channel in skeletal muscle mitochondria

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ABSTRACT

In this work we provide evidence for the potential presence of a potassium channel in skeletal muscle mitochondria. In isolated rat skeletal muscle mitochondria, Ca^{2+} was able to depolarize the mitochondrial inner membrane and stimulate respiration in a strictly potassium-dependent manner. These potassium-specific effects of Ca^{2+} were completely abolished by 200 nM charybdotoxin or 50 nM iberiotoxin, which are well-known inhibitors of large conductance, calcium-activated potassium channels (BK_{Ca} channel). Furthermore, NS1619, a BK_{Ca}-channel opener, mimicked the potassium-specific effects of calcium on respiration and mitochondrial membrane potential. In agreement with these functional data, light and electron microscopy, planar lipid bilayer reconstruction and immunological studies identified the BK_{Ca} channel to be preferentially located in the inner mitochondrial membrane of rat skeletal muscle fibers. We propose that activation of mitochondrial K^+ transport by opening of the BK_{Ca} channel may be important for myoprotection since the channel opener NS1619 protected the myoblast cell line C2C12 against oxidative injury.

1. Introduction

Mitochondria play an important role in energy metabolism within the cell. Apart from this canonical function, mitochondria are also involved in regulation of cell death or survival, which is important for apoptosis or cell senescence. Within this context, potassium transport through inner mitochondrial membranes was found to play a central role in triggering cardio- and neuroprotection [1–3]. The transport of potassium ions is strictly ion channel-dependent and, accordingly, resembles plasma membrane ion channel activity [4–6]. The basic pharmacological properties of mitochondrial inner membrane potassium channels, such as ATP-regulated potassium ($mitoK_{ATP}$) channels [7], large conductance Ca^{2+}-activated potassium ($mitoBK_{Ca}$) channels [8], and voltage-dependent potassium (mitoKv1.3) channels [9], were found to be similar to some types of potassium channels present in the plasma membrane of various cell types [10]. Potassium channels in the inner mitochondrial membrane, which are of particular importance, must be regulated in subtle ways to prevent membrane potential collapse, but the precise molecular identity of these channels is still poorly understood.

The presence of the $mitoBK_{Ca}$ channel was originally described using the patch-clamp technique in the human glioma cell line LN229 [8]. This channel, with a conductance of 295 pS in LN229 cell mitochondria, 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 (stimulated by the potassium channel opener NS1619 and blocked by ChTx, iberiotoxin (IbTx) and paxilline) was observed in patch-clamp recordings from the mitoplasts of guinea pig ventricular cells [11]. Electrophysiological and pharmacological data have indicated that $mitoBK_{Ca}$ channels may protect guinea pig hearts from infarction. More recently, immunoblots of cardiac mitochondria using antibodies against the C-terminal part of the BK_{Ca} channel identified a 55 kDa protein. This putative channel was suggested to contribute to the cardioprotective effect of K^+ influx into mitochondria. It was also reported that cardiac $mitoBK_{Ca}$-channel activation by NS1619 (measured by flavoproteins oxidation) is amplified by 8-bromoadenosine-3′, 5′-cyclic monophosphate and forskolin [12]. This suggests that opening of $mitoBK_{Ca}$ is modulated by cAMP-dependent protein kinase.

The $mitoBK_{Ca}$ channel may offer a novel link between cellular/mitochondrial calcium signaling and mitochondrial membrane potential-dependent reactions. Altered intramitochondrial calcium levels directly affect the potassium permeability of the inner mitochondrial

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membrane, resulting in modulation of the membrane potential. This type of interaction can alter efficiency of oxidative phosphorylation in a calcium-dependent manner. In addition, the pharmacological, biophysical and molecular characteristics of this channel appear to be similar to the properties of the well-known plasma membrane large conductance potassium channel (BK_{Ca} channel) [13]. Until now, the mitoK_{ATP} channel has been described only in rat skeletal muscle [14]. In isolated rat skeletal muscle, mitochondria potassium channel openers stimulated respiration, depolarized the mitochondrial inner membrane and led to oxidation of the mitochondrial NAD-system in a strictly potassium-dependent manner [14].

The aim of this study was to search for large conductance, calcium-activated potassium channels in isolated rat skeletal muscle mitochondria and determine the bioenergetic consequences of their activation. For this purpose, we studied the effects of Ca^{2+}, BK_{Ca}-channel inhibitors (such as ChTx and IbTx) as well as activators (such as NS1619) on mitochondrial membrane potential and respiration of mitochondria isolated from rat skeletal muscle. To determine whether BK_{Ca} proteins are expressed in the inner mitochondrial membrane of rat skeletal muscle mitochondria, we utilized the techniques of Western blotting, immunocytochemistry and electron microscopy. Our data suggest the potential presence of BK_{Ca} channels in the inner mitochondrial membrane of rat skeletal muscle. Our observations were confirmed with results obtained from reconstitution of submitochondrial particles into black lipid membrane, in which single-channel activity of the mitoBK_{Ca} channel was recorded. We also observed a protective effect of the BK_{Ca}-channel opener NS1619 on C2C12 cells subjected to oxidative stress induced by hydrogen peroxide. As a result, we propose that activation of mitochondrial K^+ transport by BK_{Ca}-channel opening may be important for myoprotection.

2. Materials and methods

2.1. Materials

All reagents for cell culture were purchased from Life Technologies, Inc. (GibcoBRL, UK) and Sigma-Aldrich Co Ltd (UK). L-α-phosphatidyl-choline (asolectin) and *n*-decane for the black lipid membrane method were obtained from Sigma-Aldrich (Germany). All other chemicals were of the highest purity available commercially and, unless otherwise indicated, were obtained from Sigma-Aldrich Co Ltd (UK).

2.2. Isolation of rat skeletal muscle mitochondria and submitochondrial particles

Rat skeletal muscle mitochondria were prepared as previously described [14]. Briefly, albino Wistar rats weighing 250–350 g were sacrificed by decapitation, and the quadriceps and soleus muscles (4–5 g of tissue) were rapidly removed and transferred into ice-cold isolation medium (180 mM KCl, 10 mM EDTA-Na_2, pH 7.4). Muscles were minced with scissors, trimmed clean of visible fat and connective tissue, and placed in 30 ml of isolation medium supplemented with trypsin (1 mg per 1 mg of tissue). After 30 min, the tissue was homogenized using a motor-driven Teflon-glass Potter homogenizer. One ml of the homogenate was frozen at −80 °C for use in immunological studies (homogenate — H), and the remaining homogenate was centrifuged at 800 ×*g* for 6 min. The supernatant was decanted and centrifuged at 9000 ×*g* for 10 min at 4 °C. The final mitochondrial pellet (M fraction) was resuspended in medium containing 0.2 M sucrose and 20 mM HEPES, pH 7.2.

To obtain a purified mitochondrial fraction for Western blotting, the mitochondrial pellet was resuspended in EDTA-free isolation buffer (250 mM sucrose, 10 mM Tris), homogenized and centrifuged at 12,000 ×*g* for 20 min. The pellet was then resuspended in 1 ml of EDTA-isolation buffer, layered on the top of Percoll solution (30% Percoll, 0.25 M sucrose, 1 mM EDTA and 10 mM HEPES, pH 7.4) and spun at 35,000 ×*g* for 30 min. The mitochondrial fraction was collected, washed with EDTA-free isolation buffer, centrifuged twice at 16,000 ×*g* for 11 min and finally resuspended in the same buffer.

For the preparation of purified submitochondrial particles (SMP), the final mitochondrial suspension was loaded on Percoll solution (30% Percoll, 0.25 M sucrose, 1 mM EDTA and 10 mM HEPES, pH 7.4) and centrifuged at 35,000 ×*g* for 30 min. The mitochondrial fraction was collected and washed twice with EDTA-free isolation buffer, resuspended at 10–20 mg/ml, sonicated 8×15 s (Branson Ultrasonics S-250, sonication parameters: 38 duty cycle/amplitude and 1.5 input/out) and centrifuged at 16,000 ×*g* for 15 min to pellet unbroken mitochondria. The supernatant was centrifuged at 140,000 ×*g* for 35 min and the SMP were resuspended in isolation buffer without EDTA at 5 mg protein/ml. All procedures were carried out at 4 °C.

2.3. Mitochondrial membrane potential measurements

Measurements were performed at room temperature in a 1-ml cuvette and a Shimadzu RF-5001 spectrofluorometer (Tokyo, Japan) using 2.5 μM rhodamine 123, a membrane potential-sensitive fluorescent dye. Measurements were performed in medium containing 10 mM KH_2PO_4 (or NaH_2PO_4), 60 mM KCl (or NaCl), 60 mM Tris, 5 mM $MgCl_2$, 110 mM mannitol and 0.5 mM EDTA-Na_2, pH 7.4. To determine calcium effects on mitochondrial potential, the medium contained 10 mM KH_2PO_4 (or NaH_2PO_4), 60 mM KCl (or NaCl), 60 mM Tris, 110 mM mannitol and 0.5 μM EGTA, pH 7.4. As respiratory substrates, 10 mM glutamate and 5 mM malate were used. The protein concentration of skeletal muscle mitochondria during membrane potential measurements was 0.5 mg protein/ml. Samples were excited at 450 nm, and the fluorescence was recorded at 550 nm. To calculate the mitochondrial membrane potential, the change in fluorescence was calibrated using potassium diffusion potentials [15]. This was performed by adding 2 μg/ml valinomycin to the rotenone de-energized mitochondrial preparation, incubated in medium in which K^+ was replaced by Na^+. Thereafter, defined additions of KCl were made until no changes in rhodamine 123 fluorescence were recorded (equilibrium point). The corresponding potential was calculated using the Nernst equation.

2.4. Mitochondrial respiration

Mitochondrial oxygen consumption was measured at 25 °C using an Oroboros oxygraph (Anton Paar, Austria) in a medium containing 10 mM KH_2PO_4 (or NaH_2PO_4), 60 mM KCl (or NaCl), 60 mM Tris, 110 mM mannitol, 5 mM $MgCl_2$, 0.5 mM EDTA-Na_2, pH 7.4 (or with 0.5 μM EGTA for measurements in the presence of calcium chloride) and 10 mM glutamate and 5 mM malate as respiratory substrates. The concentration of mitochondria was 0.3 mg protein/ml. Mitochondria with respiratory control ratio of 4–6 during glutamate-malate-induced phosphorylating respiration were used.

2.5. Cell culture

C2C12 cells were routinely cultured in high glucose (4.5 g/l) Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 4 mmol/l L-glutamine. Cells were incubated in an atmosphere containing 5% CO_2/95% air at 37 °C. Under these conditions, cells remain myoblastic and do not differentiate.

2.6. Western blotting

The samples resuspended in Laemmli loading buffer were incubated at 95 °C for 10 min. In case of Na^+/K^+ ATPase labeling, the procedure was modified according to the protocol recommended by the manufacturer and the samples were heated at 60 °C for 5 min. Then, equal amounts of 30 μg of protein were resolved by 15% SDS-PAGE and electroblotted onto a nitrocellulose membrane (BIO-RAD). Membranes were blocked for 2 h at room temperature with 10% non-fat milk in TBS-T buffer (20 mmol/l Tris, 137 mmol/l NaCl, 0.1% Tween 20, pH 7.6) and incubated overnight at 4 °C with a specific primary antibody. Mitochondrial anti-adenine nucleotide translocase (anti-ANT, an affinity purified goat polyclonal antibody raised against a peptide mapping near the amino terminus of adenine nucleotide translocase 1) at a dilution of 1:5000 (Santa Cruz) was used to estimate the presence of mitochondria in studied fractions. To estimate the purity of the mitochondria preparation, an antibody recognizing SERCA1 (1:100 dilution, Novocasta Laboratories Ltd) and ATPase mouse monoclonal antibody (Na^+/K^+-ATPase α 1 subunit, 1:2000 dilution, Abcam, Cambridge, UK) was used as a marker of plasma membrane. A rabbit polyclonal anti-BK_{Ca} β4 subunit (*KCNMB4*) antibody (1: 200 dilution, Alamone Labs) was used to detect the presence of the BK_{Ca} BK_{Ca}-channel subunit in the inner mitochondrial membrane. After washing, blots were incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, UK) and viewed using an ECL detection system (Amersham Pharmacia Biotech, UK).

2.7. Immunocytochemistry

Adult Wistar rats were sacrificed by decapitation and the soleus muscles were removed and immediately frozen in isopentane pre-cooled with dry ice. Serial cross-sections were cut using a cryostat, mounted on poly-L-lysine coated slides and stored at −20 °C. Before staining, slides were warmed to room temperature for 30 min and then treated with cold methanol for 10 min and air dried for 15 min. Nonspecific antibody binding sites were blocked by pre-incubating sections with 5% normal donkey serum (NDS) diluted in PBS for 30 min. Sections were then incubated overnight at 4 °C in 5% NDS containing a mixture of primary antibodies: rabbit polyclonal anti-BK_{Ca} 4 subunit (*KCNMB4*) antibody (1:50 dilution, Alamone Labs) and monoclonal mouse anti-OxPhos Complex IV subunit I (1:200 dilution, Molecular Probes). Immunoreactions were visualized using Alexa-Fluor 488 conjugated donkey anti-mouse antibody (Molecular Probes) and Alexa-Fluor 555 conjugated donkey anti-rabbit antibody (Molecular Probes). Sections were then mounted in VECTASHIELD HardSet Mounting Medium with DAPI (Vector). Confocal images were acquired using a Leica microscope, model TCS SP2 using Ar (488 nm) and GeNe (543 nm) laser lines for the excitation of Alexa-Fluor 488 and Alexa-Fluor 555, respectively. Quantitative colocalization analysis was performed using ImageJ (rsb.info.nih.gov/ij/) pluging JaCOP [16].

2.8. Electron microscopy

Muscles from adult rats were removed immediately after decapitation, cut into 0.5–1 mm slices and fixed for 4 h at 4 °C in a fresh solution of 4% paraformaldehyde (Sigma) and 0.4% glutaraldehyde (Sigma) in phosphate buffer saline. Blocks of tissues were rinsed in PBS four times for 5 min, dehydrated in ethanol gradients, embedded in LR White Resin (Polysciences) and polymerized for 72 h at 56 °C.

After thin sectioning, samples were collected on carbon-formavar-coated nickel grids, blocked in PBS containing 1% bovine serum albumin and 0.1% Tween 20 for 30 min and labeled with rabbit antibodies directed against two subunits of Ca^{2+}-activated K^+ channel: anti-BK_{Ca} α subunit or anti-BK_{Ca} β4 subunit (Alamone Labs Ltd.) at 1:100 overnight at room temperature. After washing with 1% BSA/0.1% Tween/PBS, the samples were incubated in the anti-rabbit IgG (1:20) conjugated with 5 nm colloidal gold particles (Sigma) for 2 h at room temperature. After extensive washing (1%BSA/0.1%Tween/PBS 6×5 min, PBS 4×5 min, H_2O 2×5 min), sections were stained at room temperature with uranyl acetate for 1 h and lead citrate for 10 min. In control experiments, the primary antibodies were omitted. The sections were observed in a JEOL 1200 EX electron microscope.

2.9. Black lipid membrane (BLM) measurements

BLM technique was applied as previously described [17–19]. Briefly, the submitochondrial particles from skeletal muscle (4 mg of protein/ml, 1–3 μl) were added to the *trans* compartment. Incorporation of the ion channel into the BLM was usually observed within a few minutes. The studied compounds were added to both the *cis* and *trans* compartments. The chambers contained 50–450 mM KCl (*cis*/*trans*), 20 mM TRIS–HCl, pH 7.2 solution. BLMs were painted using asolectin in *n*-decane at a final concentration of 25 mg lipid/ml. A final accepted capacitance value of planar membrane ranged from 110 to 180 pF. Electrical connections were made by Ag/AgCl electrodes and agar salt bridges (3 M KCl) to minimize liquid junction potentials. Voltage was applied to the *cis* side, and the *trans* side was grounded. The current was measured using a Bilayer Membrane Amplifier (BLM-120, BioLogic). Signals were filtered at 500 Hz. The signal was digitized at a sampling rate of 100 kHz (A/D converter PowerLab 2/20, ADInstruments) and transferred to PC for off-line analysis by Chart v5.2 (PowerLab ADInstruments) and pCLAMP 9.0 (Axon Laboratory). The pCLAMP 9.0 software package was used for data processing. The single-channel recordings illustrated are representative of the most frequently observed amplitudes of the channel under the given conditions.

2.10. Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) enzyme activity was assayed using a commercial kit (Roche Molecular Biochemicals, Germany).

3. Results

3.1. Ca^{2+} induces ChTx-sensitive potassium transport in isolated skeletal muscle mitochondria

To investigate possible effects of Ca^{2+} on the potassium permeability of isolated rat skeletal muscle mitochondria, we determined mitochondrial membrane potential in sodium- and potassium-containing media. Measurements of mitochondrial membrane potential were performed using the membrane potential-sensitive fluorescence dye rhodamine 123 as it is shown in Fig. 1A. After addition of mitochondria to the medium, the fluorescence of rhodamine 123 decreased due to accumulation of the dye in the mitochondrial matrix. Additions of Ca^{2+} into potassium-containing medium below 10 μM caused mitochondrial depolarization, registered as a pronounced increase in rhodamine 123 fluorescence (Fig. 1A). In sodium-containing medium, however, depolarization was detectable only above 15 μM Ca^{2+} (i.e. depolarization around 3 mV was registered for 5 μM Ca^{2+} in potassium medium, for the same concentration of Ca^{2+} in sodium medium depolarization was around 0.4 mV). The depolarization of mitochondrial membrane evoked by Ca^{2+} in potassium-containing medium is not a consequence of its uptake due to the lack of membrane depolarization in sodium-containing medium. Furthermore, 50 nM IbTx (filled triangle in Fig. 1B) and 200 nM ChTx (filled squares in Fig. 1B) were able to abolish Ca^{2+}-induced mitochondrial inner membrane depolarization in potassium-containing medium (filled circles in Fig. 1B). In sodium-containing medium (open circles in Fig. 1B), however, both inhibitors were ineffective (data not shown). ChTx and IbTx are well-known inhibitors of the BK_{Ca} channel. Thus, the observed effect of Ca^{2+} on mitochondrial depolarization in potassium-containing medium could be a consequence of K^+ entry via mitoBK_{Ca} channels into the mitochondrial matrix. An increase of potassium transport across the mitochondrial inner membrane is usually accompanied by an increase in oxygen consumption [14]. Hence, further experiments were focused on the effects of Ca^{2+} on mitochondrial respiratory rate in potassium- and sodium-containing media. The results of these experiments are shown in Fig. 2. As expected, in potassium-containing medium, a Ca^{2+}-induced increase of mitochondrial oxygen consumption was observed, which was sensitive to IbTx (Fig. 2). IbTx did not affect respiration stimulated by ADP (data not shown). These findings strongly suggest the putative presence of a large conductance Ca^{2+} activated potassium channel in the inner membrane of rat skeletal muscle mitochondria. Therefore, the effects of the BK_{Ca}-channel opener NS1619 on mitochondrial membrane potential (Fig. 3A and B) and oxygen consumption were also tested (data not shown). Very similar to Ca^{2+}, NS1619 was observed to cause a potassium-specific mitochondrial depolarization as well as stimulation of respiration, both of which were sensitive to 200 nM ChTx and 50 nM IbTx. The IC_{50} value of NS1619 stimulation of respiration in skeletal muscle mitochondria was determined to be about 1 μM (data not shown).

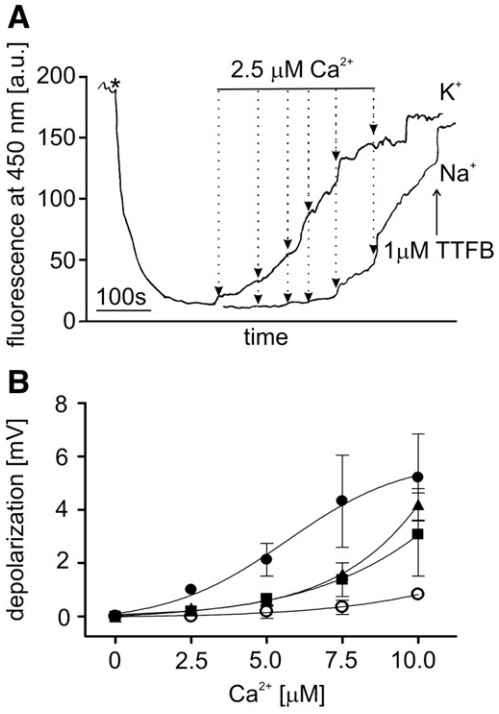

Fig. 1. Effect of Ca^{2+} on the membrane potential of isolated skeletal muscle mitochondria. (A) Original traces of mitochondrial membrane depolarization induced by Ca^{2+} in a potassium- and sodium-containing medium. The mitochondrial membrane potential was measured by rhodamine 123 fluorescence as described in the Materials and methods section. The suspension of rat skeletal muscle mitochondria (*) was added into 1 ml of potassium (K^+) or sodium (Na^+) medium, containing an additional 5 mM malate, 10 mM glutamate and 2.5 μM rhodamine 123. Complete membrane depolarization was achieved by addition of 1.5 μM TTFB. (B) Quantitative alterations of mitochondrial membrane potential after subsequent additions of calcium ions into potassium medium (filled circles) and sodium medium (open circles). Experiments were also performed in potassium medium in the presence of 200 nM ChTx (filled squares) or 50 nM IbTx (filled triangles). The experimental points are averages ± S.E.M of three independent experiments. The mitochondrial membrane potential was measured as described in the Materials and methods section.

3.2. Identification of large conductance Ca^{2+}-activated potassium channel in black lipid membrane (BLM)

Purified inner mitochondrial membranes (SMP) of rat skeletal muscle were reconstituted in black lipid membranes. The fusion of SMP

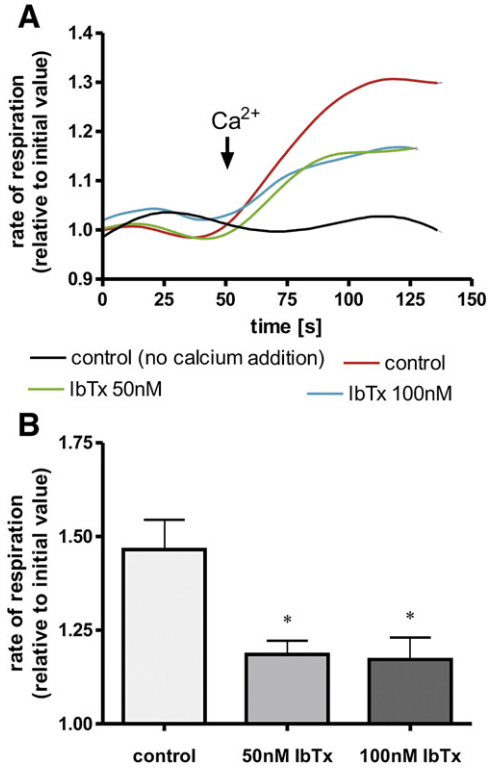

Fig. 2. Ca^{2+} induces an increase of respiration of isolated skeletal muscle mitochondria, sensitive to iberiotoxin. (A) Stimulation of mitochondrial respiration by $CaCl_2$. Rat skeletal muscle mitochondria (0.5 mg per 1 ml) were added to 1.5 ml of the mitochondrial respiration measurements medium (see Materials and methods), which contained 10 mM glutamate and 5 mM malate, plus 5 μM $CaCl_2$ and 50 or 100 nM IbTx. Traces represent derivatives of oxygen concentration changes in time (rate of respiration). The arrow indicates the addition of $CaCl_2$. The control respiration rate was from 1 to 3 nmol O_2/min/mg protein depending on mitochondrial preparation. (B) Inhibition of state 2 respiration stimulation of mitochondria by subsequent additions of $CaCl_2$ into the potassium-containing medium, in the absence of BK channel inhibitors (control), in the presence of 50 or 100 nM iberiotoxin. The experimental points are average ± S.E.M of three independent experiments. *$p<0.05$ *vs.* control.

to the bilayer was usually observed within 10–20 min after addition to the *trans* compartment. Three types of ion channels with different single-channel conductances and selectivities were observed ($n=55$).

First, an anion selective channel with conductance of ~125 pS in a gradient of 50/450 mM KCl (*cis*/*trans*) solution was observed ($n=42$) (Fig. 4A).

Second, single-channel recordings revealed a cation selective channel with conductance of ~150 pS in a gradient of 50/450 mM KCl (*cis*/*trans*) solution ($n=5$) (Fig. 4B).

Third, Fig. 4C shows the single-channel recordings of a large conductance potassium channel in a gradient of 50/450 mM KCl (*cis*/*trans*) solution at 0 mV ($n=8$) under control conditions. After addition of 300 μM Ca^{2+} (*cis*/*trans*), however, we observed an increase in channel opening probability ($n=6$), which was inhibited by 200 nM IbTx (*cis*/*trans*) ($n=2$). The channel conductance was equal to ~300 pS under gradient conditions. The reversal potential measured in the gradient 50/450 mM KCl (*cis*/*trans*) solution was ~35 mV. This result proves that the examined channel is cation selective. Taken together, our data indicate that the observed single-channel activity is similar to the mitoBK$_{Ca}$ channel previously reported in glioma and cardiac mitochondria [8,11].

3.3. Antibody directed against β4 subunit of the BK$_{Ca}$ channel shows immunoreactivity with skeletal muscle mitochondria

The presence of the β4 subunit of the BK$_{Ca}$ channel in purified skeletal muscle mitochondria was analyzed by Western blotting (Fig. 5). Immunoblot analysis was performed for the mitochondrial fraction (M) and total cell homogenate (H) prepared from rat skeletal muscle. The quality of purified mitochondrial fractions (M) was confirmed using antibodies against ATP/ADP translocase (ANT, molecular weight of 33 kDa), sarcoplasmic reticulum Ca^{2+}-ATPase 1 (SERCA-1, molecular weight of 110 kDa) and plasma membrane Na^+/K^+-ATPase α1 subunit. An increase of ANT labeling was observed in the M fraction in comparison to homogenate (H fraction). In contrast, a decrease of sarcoplasmic reticulum and plasma membrane was observed in the mitochondrial fraction compared to the homogenate. Using an antibody raised against the BK$_{Ca}$ β4 subunit, a single band at about 26 kDa was detected in the mitochondrial (M) as well as in the homogenate of rat skeletal muscle fraction (H) (Fig. 5). Competition studies performed with peptides specific to the BK$_{Ca}$ β4 subunit demonstrated elimination of signal in both fractions, as shown in Fig. 5. Together with the results of immunoblotting against ATP/ADP translocase, an enzyme specific for the inner mitochondrial membrane, and SERCA1, a marker of sarcoplasmic reticulum, and Na^+/K^+-ATPase α1 subunit, a marker of plasma membrane, these results suggest that the BK$_{Ca}$ channel might be present in skeletal muscle mitochondria. In addition, the results obtained using antibody against the pore-forming subunit of plasma membrane BK$_{Ca}$ channel suggest the presence of the α subunit of BK$_{Ca}$ with an apparent molecular weight of ~47 kDa in inner mitochondrial membranes (data not shown).

The intracellular localization of the mitoBK$_{Ca}$ channel in rat muscle was further confirmed by immunogold labeling for transmission electron microscopy using two antibodies directed against the BK$_{Ca}$ α

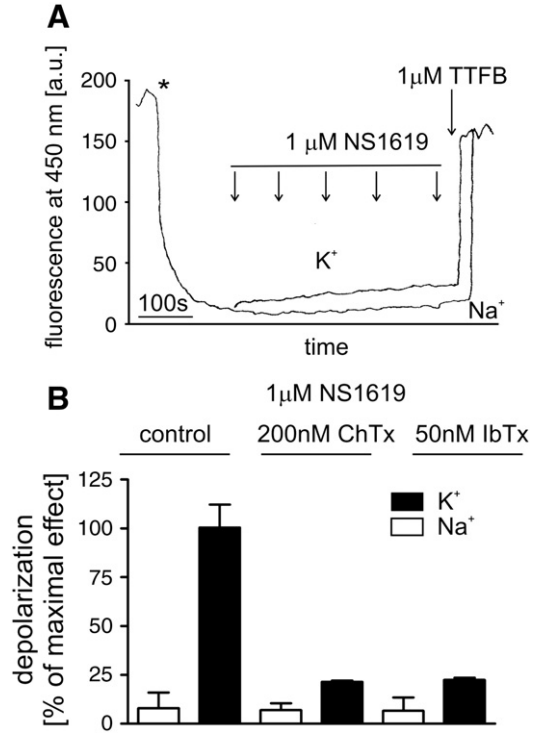

Fig. 3. BK channel opener NS1619 induces skeletal muscle depolarization sensitive to charybdotoxin and iberiotoxin. (A) Original trace of mitochondrial membrane depolarization induced by NS1619 in a potassium- and sodium-containing medium. The mitochondrial membrane potential was measured by rhodamine 123 fluorescence as described in Materials and methods. The suspension of rat skeletal muscle mitochondria (*) was added to 1 ml of potassium (K^+) or sodium (Na^+) medium, which contained 5 mM malate, 10 mM glutamate and 2.5 μM rhodamine 123. Complete membrane depolarization was reached with the addition of 1.5 μM TTFB. (B) The ion specificity of NS1619 induced mitochondrial depolarization and sensitivity to BK$_{Ca}$ blockers (200 nM charybdotoxin (ChTx) and 50 nM iberiotoxin (IbTx)). Mitochondrial membrane depolarization induced by 1 μM NS1619 was measured as described in Materials and methods in sodium (opened bars) or potassium (filled bars) medium. The columns represent the average ± S.E.M of three independent experiments. 100% depolarization was induced by 1 μM NS1619 (depending on mitochondrial preparation ~3 mV).

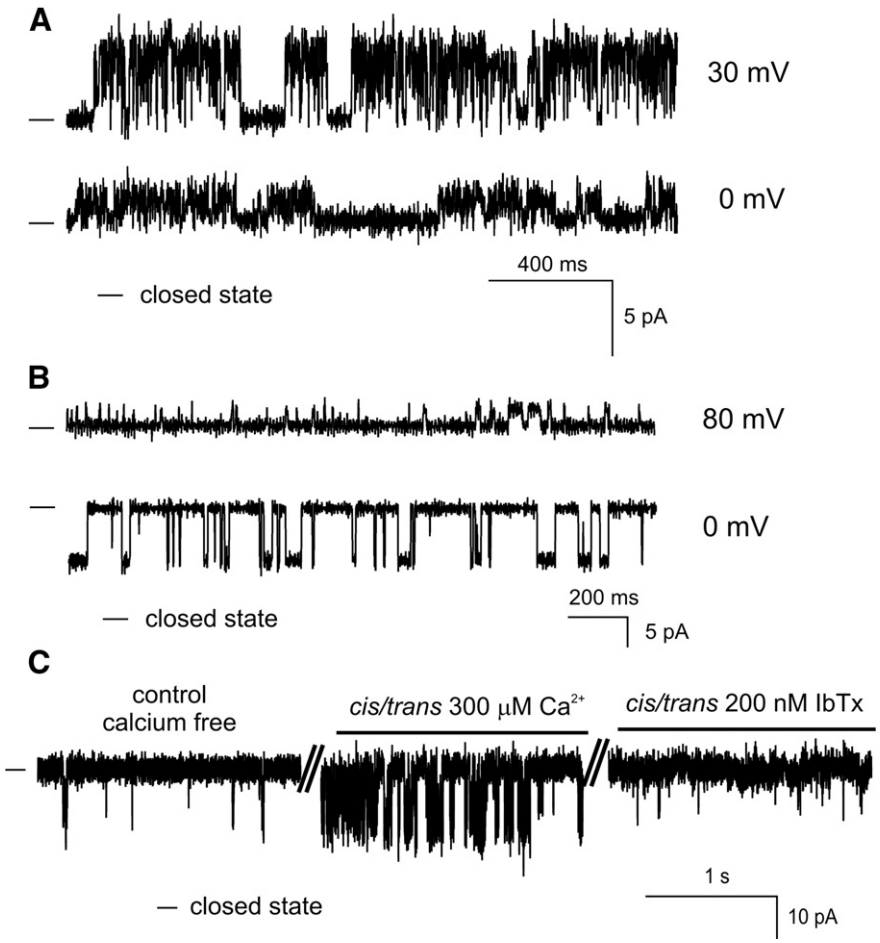

Fig. 4. Single-channel recordings of ion channel from skeletal muscle mitochondria in planar lipid bilayers. (A) Single-channel recordings of chloride channel in 50/450 mM KCl (*cis/trans*) gradient conditions at 0 and 30 mV. (B) Single-channel recordings of a small conductance potassium channel in 50/450 mM KCl (*cis/trans*) gradient conditions at 0 and 80 mV. (C) Single-channel recordings of a large conductance potassium channel in 50/450 mM KCl (*cis/trans*) gradient conditions at 0 mV under calcium free control conditions, after addition of 300 μM Ca^{2+} (*cis/trans*) and after addition of 200 nM IbTx (*cis/trans*). Dash (−) indicates the closed state of the channel. Recordings were low pass filtered at 500 Hz. Reconstitution of the inner mitochondrial membrane into planar lipid bilayer was performed as described in the Materials and methods section.

(data not shown) and BK_{Ca} β4 subunit (Fig. 6A). Ultrastructural analysis revealed that the β4 subunit of the mitoBK_{Ca} channel is localized to the plasma membrane and in the inner mitochondrial membrane. No immunostaining was observed when primary antibodies were omitted (data not shown).

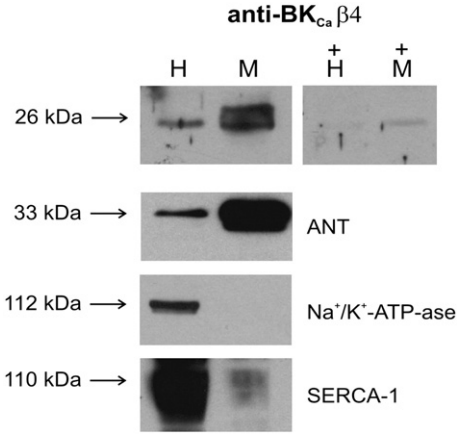

Fig. 5. Identification of subunit localization of the BK_{Ca} channel in isolated skeletal muscle mitochondria. Immunoblot of rat skeletal muscle homogenate (H) and mitochondria isolated from rat skeletal muscle (M) (see Materials and methods). Immunoreactive band of anti-BK_{Ca} β4 subunit (~26 kDa) was observed in the homogenate (H) as well as in the mitochondrial skeletal muscle protein fraction (M), which was displaced with antigenic peptide (H+), (M+). ANT represents the ATP/ADP translocase. The enrichment of the ANT signal and decrease in Na^+/K^+-ATPase immunoreactivity as well as SERCA-1 staining in mitochondria versus homogenate is apparent.

To determine the distribution of the BK_{Ca}-channel β subunit in skeletal muscle fibers, we performed immunohistochemistry with antibody directed against the BK_{Ca} β4 subunit (Fig. 6B). The distribution of immunoreactivity was remarkably similar to that of an antibody directed against subunit 1 of cytochrome *c* oxidase (Fig. 6C). As seen in the overlay (Fig. 6D), most of the BK_{Ca} β4 subunit immunostaining was found to co-localize with mitochondria (yielding a yellow staining), and only a minor fraction appeared to be exclusively plasma membrane associated (Fig. 6E, F, G). Quantitative colocalization analysis indicated very high degree of overlap between the two immunoreactivities (Pearson's coefficient $r = 0.862$, Mander's coefficients M1 = 0.915, M2 = 0.999) [16]. These results strongly indicate the presence of the BK_{Ca} channel in the inner mitochondrial membrane of rat skeletal muscle mitochondria.

3.4. Consequences of potassium transport by the mitoBK_{Ca} channel in skeletal muscle

It has been shown that increased potassium ion flux into the mitochondrial matrix, with potassium channel opener regulating the activity of the mitoK_{ATP} channel (i.e. diazoxide), is cytoprotective [20]. It has also been demonstrated that activation of mitoBK_{Ca} with NS1619 can protect cardiac cells from injury [11,12]. Hence, the effect of the BK_{Ca}-channel activator, NS1619, on C2C12 cell damage was tested. C2C12 cells are a murine skeletal myoblast line, which is widely used in studies on skeletal muscle impairment [21]. In the present

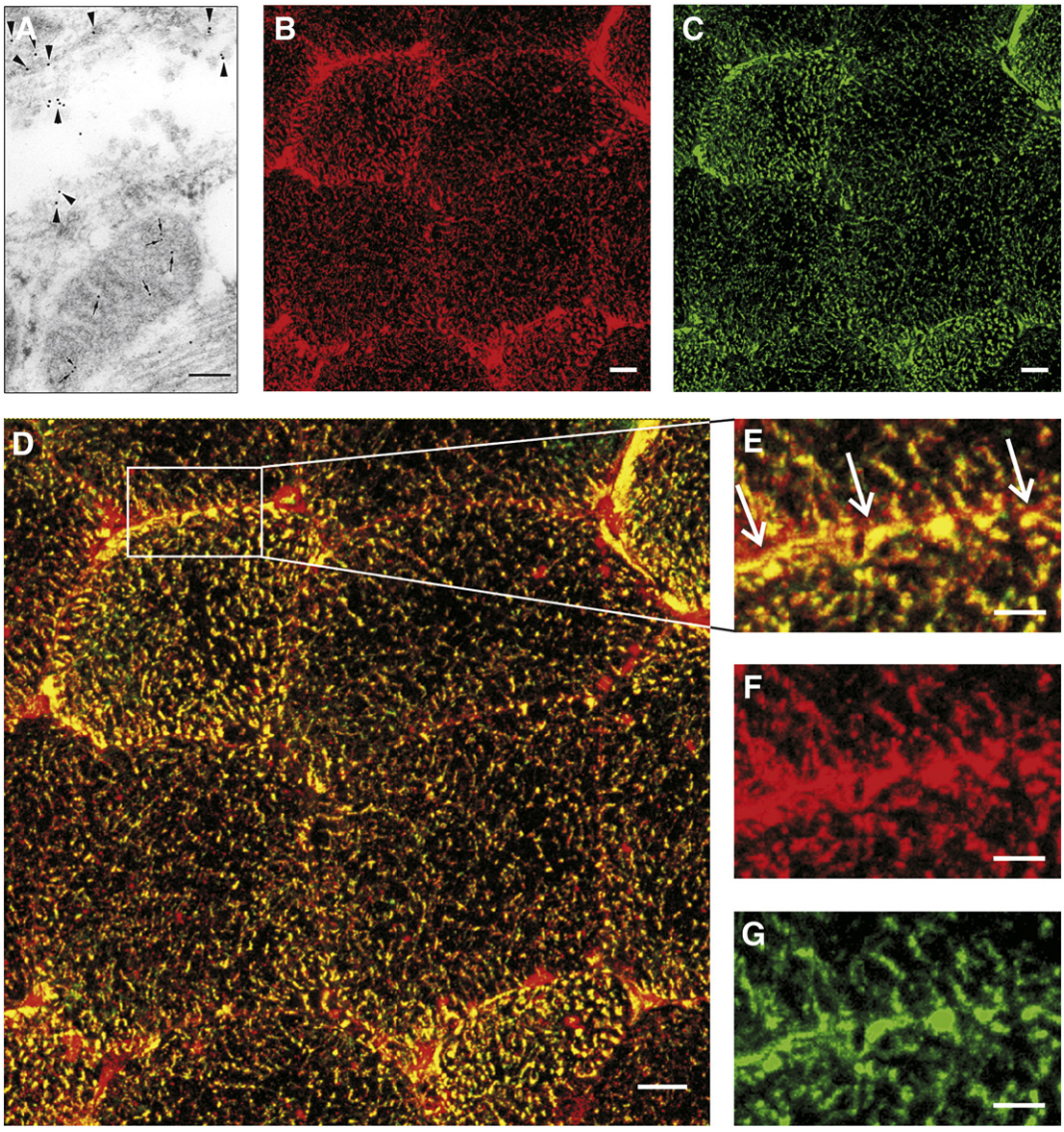

Fig. 6. Transmission electron and confocal microscopy images of rat skeletal muscle. (A) Ultrastructural analysis of freshly isolated rat muscles by transmission electron microscopy was performed with antibodies against the BK_{Ca} β4 subunit followed by immunogold labeling. Arrows indicate gold particles at the inner mitochondrial membranes and arrowheads at the plasma membrane. The bar represents 200 nm. (B–G) Confocal images of fluorescently-labeled BK_{Ca} β_4 subunit in rat skeletal muscle. The majority of BK_{Ca} β_4 immunoreactivity (red) strictly colocalizes with mitochondrial cytochrome c oxidase (green), while the yellow color in the overlay indicates colocalization. The arrow (E) indicates labeling of the plasma membrane.

experiments, C2C12 cells were subjected to oxidative stress induced by hydrogen peroxide. As shown in Fig. 7, the channel opener NS1619 protected approximately 50% of the cells from oxidative stress injury.

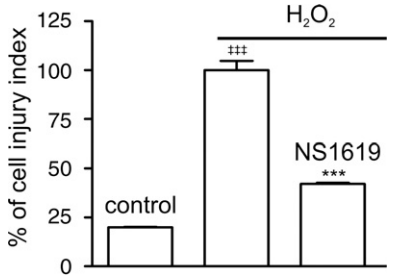

Fig. 7. Cytoprotective effect of the BK_{Ca}-channel opener NS1619 in the skeletal muscle cell line C2C12 in response to oxidative stress. C2C12 cells were incubated with 1 mM H_2O_2 for 6 h. Where indicated, 30 μM NS1619 was present for 15 min before exposure to oxidative stress and during the experiment. Results are expressed as a percentage of cell injury index, which was estimated by measurements of LDH activity in the medium and cell lysate. Experiments were performed in triplicate and results are shown as mean ± S.D. ‡‡‡$p<0.0001$ *vs.* control, ***$p<0.0001$ *vs.* H_2O_2.

Although a detailed mechanism of the specific myoprotective action of NS1619 remains to be elucidated, these preliminary experiments strongly suggest that the mitoBK_{Ca} channel has to be considered as a potentially myoprotective target.

4. Discussion

4.1. A BK_{Ca} channel is present in skeletal muscle mitochondrial inner membrane

In the present study, we provide evidence for the potential presence of a novel potassium channel in the inner membrane of skeletal muscle mitochondria. The pharmacological, biophysical and molecular properties of this channel appear to be similar to that of the well-known plasma membrane large conductance potassium channel (BK_{Ca} channel, *slo* 1) [13]:

(i) The mitochondrial channel is potassium selective and activated by calcium ions.
(ii) The channel is inhibited by IbTx and ChTx at nanomolar concentrations.

(iii) The channel is activated by the potassium channel opener NS1619 at low micromolar concentrations.
(iv) Reconstitution of skeletal muscle mitochondrial inner membrane preparations into planar lipid bilayer shows large conductance, calcium-activated (inhibited by IbTx) single-channel activity.
(v) Mitochondrial inner membranes from rat skeletal muscle show pronounced immunoreactivity with antibodies raised against the BK_{Ca} β4 subunits of the plasma membrane channel.

The latter finding (v) implies a close molecular similarity of the mitoBK$_{Ca}$ channel and the plasma membrane BK$_{Ca}$ channel. It is therefore reasonable to speculate that both channels might be different splice variants of the same gene product. The pore-forming α-subunits of BK channels are encoded by a single gene, *KCNMA1*, which undergoes extensive alternative pre-mRNA splicing. Distinct splice-variant mRNAs of α-subunits may be expressed in the same cell and differentially expressed between tissues or neighboring cells or compartments within cells [22]. Moreover, alternative pre-mRNA splicing from a single site of splicing generates physiologically diverse α-subunits that differ in their trafficking and regulation [23]. Furthermore, these properties can be modified by a physiological challenge such as oxidative stress or hormone treatment [24]. Further diversity is generated by co-assembly of α-subunits with a family of regulatory transmembrane β-subunits encoded by four distinct genes (*KCNMB1-4*). It is likely that at least one product of these processes locates in the inner mitochondrial membrane of skeletal muscle. These suggestions have to be confirmed by the molecular identification of mitoBK$_{Ca}$ composition in the future. Recently, a yeast two-hybrid assay showed that the β1 subunit can interact with a cardiac mitochondria cytochrome c oxidase subunit I [25]. Additionally, the data suggesting that the α subunit is also present in brain mitochondria have been reported [26]. Recently, we have shown that β4 subunit is a regulatory component of BK$_{Ca}$ channel in neuronal mitochondria [27].

Very likely, the mitochondrial channel has its charybdotoxin/iberiotoxin binding site close to the cytosolic compartment (probably on outer side of inner mitochondrial membrane) since, as peptides, these compounds cannot easily enter the mitochondrial matrix space. Consequently, the calcium binding site of the mitoBK$_{Ca}$ channel is close to the matrix compartment (cf. orientation of the plasma membrane BK$_{Ca}$ channel [13]). The tentative orientation of the channel in the mitochondrial membrane is depicted in Fig. 8. This

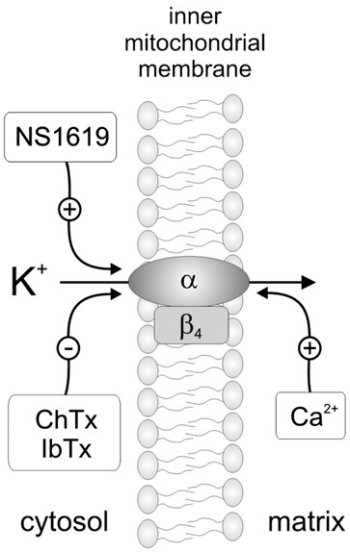

Fig. 8. Putative interactions of the mitochondrial BK$_{Ca}$ channel with channel activators (Ca^{2+}, potassium channel opener NS1619) and inhibitors (ChTx, IbTx).

type of potassium channel may contribute to complex and multi-targeted calcium ions effect on mitochondrial function.

Despite described studies in potassium-free medium further experiments on channel selectivity are needed. Due to complex ion transporting systems present in inner mitochondrial membrane this should be performed not with use of isolated mitochondria but rather with electrophysiological technique such as patch-clamp.

4.2. The mitoBK$_{Ca}$ channel — a potentially novel myoprotective target?

Similar to the mitoK$_{ATP}$ channel described in various tissues [18,28–32], this novel potassium channel is expected to affect mitochondrial metabolism due to regulation of matrix volume [33]. In addition to this classical physiological effect of mitochondrial potassium transport, a pivotal role of mitochondrial potassium channels in cardio- and neuroprotection has been implicated. Mechanism of cytoprotective action of an increased potassium flux into mitochondria has not yet been well-established (see discussion below).

It appears, therefore, also reasonable to suggest a possible cytoprotective effect of mitoBK$_{Ca}$-channel activation. To this end, we have tested a possible myoprotective action of the BK$_{Ca}$-channel opener NS1619 in response to hydrogen peroxide injury in C2C12 myoblast cells. Although the detailed mechanism of specific myoprotective action of NS1619 remains to be elucidated, these preliminary experiments strongly suggest that the mitoBK$_{Ca}$ channel should be considered as a potential novel myoprotective target. Other targets for the potassium channel opener NS1619 described in cardiac mitochondria may contribute to cytoprotective action of this compound [34,35]. Recently it was shown that an age of the rats used for cardiac mitochondria isolation reduced the opening effects of NS1619 [36].

The main question remains how the opening of the potassium channel in the inner mitochondrial membrane could protect cells against injury, such as injury resulting from oxidative stress and ischemic injury. Some proposals for explaining the mechanism of this phenomenon are based on experiments with mitoK$_{ATP}$ channel and may be applied to other electrogenic pathways of potassium influx into mitochondria such as mitoBK$_{Ca}$ channel.

Opening of the mitoK$_{ATP}$ channel could improve mitochondrial ATP production and/or handling [37,38]. It has been shown that hypoxia induces a decrease in mitochondrial oxygen consumption rate to approximately 40% of the prehypoxic value and that treatment with diazoxide preserves normal mitochondrial oxygen consumption rate during hypoxia [39]. Moreover, ATP concentration was significantly increased in potassium channel opener diazoxide-treated hearts [40]. Additionally, the protective effect of mitoK$_{ATP}$ activation could be mediated by decreasing Ca^{2+} overloading of mitochondria [41,42]. This is based on the observation that channel activation causes significant mitochondrial depolarization and that this may decrease calcium uptake by mitochondria. In fact, it was shown that mitoBK$_{Ca}$-channel activation reduce mitochondrial Ca^{2+} overload in rat ventricular myocytes [43]. It has also been demonstrated that opening of the mitoK$_{ATP}$ channel may modulate reactive oxygen species (ROS) level [44,45]. Potassium channel activity, or ROS crosstalk, may be further complicated by the fact that both the potassium channel [46] and mitochondrial suphonylurea receptor [47] are regulated by redox reactions. Recently, it was shown that hypoxia increases BK$_{Ca}$-channel activity in inner mitochondrial membrane [48].

Furthermore, it must be mentioned that the mitoBK$_{Ca}$ channel may offer a novel link between cellular/mitochondrial calcium signaling and mitochondrial membrane potential-dependent reactions, as altered intramitochondrial calcium levels directly affect the potassium permeability of the mitochondrial inner membrane, thus modulating the membrane potential. This type of interaction can modulate the efficiency of oxidative phosphorylation in a calcium-

dependent manner and may contribute to the dramatic increase of skeletal muscle oxygen uptake observed during the transition from rest to work at nearly unaltered concentrations of adenine nucleotides [49].

The consequences of the presence of BK_{Ca} channel in skeletal muscle mitochondria have to be further investigated. Especially with focus on other calcium transporting system such as calcium uniporter and calcium-activated permeability transition pore [50,51]. Additionally, Na^+/Ca^{2+} exchanger may contribute to matrix calcium level especially in conditions of elevated intracellular Na^+ level [52]. Probably, all these proteins may contribute to overall response of skeletal muscle mitochondria to changes in calcium ions concentration.

In summary, our results provide evidence for the potential presence of a calcium-activated potassium channel in skeletal muscle mitochondria. This channel is activated by the potassium channel opener NS1619 and is blocked by ChTx and IbTx. Furthermore, this channel is suggested to be myoprotective target and a possible signaling link between cytosolic/intramitochondrial calcium levels and the membrane potential of the mitochondrial inner membrane.

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Glossary

ANT: mitochondrial adenine nucleotide translocase
BK_{Ca} channel: large conductance Ca^{2+}-activated potassium channel
BLM: black lipid membrane
ChTx: charybdotoxin
IbTx: iberiotoxin
NS1619: 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazole-2-one
mitoBK_{Ca} channel: mitochondrial large conductance Ca^{2+}-activated potassium channel
mitoK_{ATP} channel: mitochondrial ATP-regulated potassium channel
ROS: reactive oxygen species
SERCA-1: sarcoplasmic reticulum Ca^{2+}-ATPase 1
SMP: submitochondrial particles
TTFB: 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole