Large-conductance Ca\(^{2+}\)-activated potassium channel in mitochondria of endothelial EA.hy926 cells

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Because ATP supplies in endothelial cells are relatively independent of mitochondrial oxidative pathways, studies on the bioenergetics of endothelial mitochondria have not been intensive to date. However, several recent observations suggest that endothelial mitochondria not only contribute to ATP generation but are also centrally involved in maintaining the fine regulatory balance among mitochondrial calcium concentrations, reactive oxygen species (ROS) production, and nitric oxide production (9, 10, 16). Endothelial mitochondria may also function as sensors of alternations in the local environment and contribute to the survival of endothelial cells under oxidative stress, and the mitochondrial ROS of endothelial cells are important signaling molecules (43).

Endothelial cells are involved in many aspects of vascular biology, including smooth muscle relaxation, vasodilatation, and regulation of blood flow. Actions of endothelium-derived relaxing substances commonly involve membrane hyperpolarization, and plasma membrane potassium channels serve as key molecules to produce the electrical events of the membrane (36). The three subtypes of plasma membrane calcium-activated potassium channels, i.e., large (BK\(_{Ca}\)), intermediate (IK\(_{Ca}\)), and small (SK\(_{Ca}\)) conductance, are present in the vascular wall (15). BK\(_{Ca}\) channels are preferentially expressed in vascular smooth muscle cells, whereas intermediate- and small-conductance channels are preferentially expressed in endothelial cells.

Potassium-selective ion channels that are similar to plasma membrane channels are found in the inner mitochondrial membrane (25, 34, 35): mitochondrial ATP-regulated potassium channels (mitoK\(_{ATP}\) channels), mitochondrial BK\(_{Ca}\) channels (mitoBK\(_{Ca}\) channels), voltage-dependent potassium Kv1.3 channels, and not yet functionally characterized twin-pore domain weakly inward rectifying K\(^+\) channel (TWIK)-related acid-sensitive K\(^+\) (TASK) subtype-3 channel. Potassium ions control mitochondrial metabolism, primarily via the regulation of matrix volume but also through the mitochondrial membrane potential (\(\Delta\Psi\)), respiration, and calcium transport (18). Potassium transport through the mitochondrial inner membrane was found to play a central role in the cytoprotection of various mammalian cells (24, 35). Mitochondrial potassium channels have been suggested to participate in neurodegenerative disorders and ischemic preconditioning. For example, the activation of a mitoK\(_{ATP}\) channel induces ischemic preconditioning of the endothelium in humans in vivo (7). The role of mitochondrial potassium channels in the regulation of the mitochondrial ROS level remains unclear. Studies on heart mitochondria have provided conflicting results; ROS production has been observed to increase (1, 20) or decrease (13, 19) upon the activation of potassium transport by the application of various potassium channel openers.

BK\(_{Ca}\) channels (MaxiK, Slo1) are present in the plasma membranes of different mammalian cell types. They are activated by changes in the concentration of free calcium and membrane depolarization. The channels assemble as tetramers.
of the pore-forming α-subunit, which may be associated with distinct β-subunits (β₁–β₄), depending on the tissue. Together, these subunits determine the electrophysiological and pharmacological properties of the channel. The properties of mitoBKCa channels are similar to those of surface BKCa channels (35). MitoBKCa channel activity is regulated by BKCa channel modulators, including the opener NS1619 and the inhibitors charybdotoxin, iberiotoxin, and paxilline. It is stimulated by calcium at a micromolar concentration and ΔΨ. In general, the activation of the mitoBKCa channel increases the mitochondrial respiratory rate and decreases ΔΨ. The presence of the mitoBKCa channel of 295-pS conductance (in 150 mM KCl) was originally described in the human glial cell line LN229 using the patch-clamp technique (31). Electrophysiological and pharmacological data from patch-clamp recordings of the mitoplasts of guinea pig ventricular cells have indicated that the mitoBKCa channel may protect guinea pig hearts from infarction (40).

With the use of the patch-clamp technique, it has been shown that hypoxia increases the mitoBKCa channel activity of rat liver and astrocyte mitochondria (8). Moreover, mitochondrial BKCa channels have been shown to contribute to the protection of cardiomyocytes isolated from chronically hypoxic rats (5). Recently, with the use of planar lipid bilayers, two electrophysiologically different types of mitoBKCa channels were observed in brain mitochondria (14). To determine the subcellular localization and distribution of the channel, rat brain fractions were examined by Western blot analysis, immunocytochemistry, and immunogold electron microscopy (11). These studies provide concrete morphological evidence for the existence of BKCa channels (α-subunit) in the inner mitochondrial membrane of rat brain cells. Moreover, rat skeletal muscle and brain mitochondria show immunoreactivity against antibodies targeting the BKCa β4-subunit (27, 32, 33). These findings indicate a close molecular similarity between the mitoBKCa channel and the plasma membrane BKCa channel, suggesting that both channels are splice variants of the same gene product. Studies of BKCa α-subunit in mitochondria revealed compartmentalization in sensory cells, whereas heterologous expression of a BK-DEC splice variant cloned from cochlea revealed a BK mitochondrial candidate (21).

So far, no information has been published about potassium channels in endothelial mitochondria, such as the mitoBKCa channel described in some other mammalian mitochondria. Therefore, the aim of our study was to search for a mitoBKCa channel in the mitochondria of endothelial EA.hy926 cells and to determine the electrophysiological and biochemical properties of this channel. With this purpose, we studied the effects of BKCa channel activators (Ca²⁺, NS1619, NS11021) and inhibitors (iberiotoxin, paxilline) on a single mitoBKCa channel conductance using the patch-clamp technique and on ΔΨ and respiration in isolated mitochondria. Moreover, we performed immunological experiments to detect the presence of mitoBKCa channel subunits in endothelial mitochondria. Our findings indicate that a mitoBKCa channel with properties similar to the surface membrane BKCa channel is present in endothelial mitochondria.

**MATERIALS AND METHODS**

*Cell culture.* The permanent human endothelial cell line EA.hy926 was originally derived from a human umbilical vein (12). In this study, cells were grown in DMEM (25 mM d-glucose) supplemented with 10% fetal bovine serum (FBS), 1% l-glutamine, 2% hypoxan-thine-aminopterin-thymidine, and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. During cell culture the medium was changed every 3 days. The EA.hy926 cells were cultured using 140-mm dishes (in vitro) or 250-mm dishes (in vivo) at 90–100% confluence. Cells that were between passages 5 and 12 were used in this study.

*Mitochondria and mitoplast preparation.* For the electrophysiological measurements, mitochondria were isolated before mitoplasts, i.e., mitochondria without their outer membranes. EA.hy926 cells were collected in PBS medium and harvested by centrifugation at 800 g for 10 min, resuspended in preparation solution containing 250 mM sucrose and 5 mM HEPES (pH 7.2), and homogenized (glass-glass homogenizer no. 19, Kontes Glass). The homogenate was next centrifuged at 9,200 g for 10 min to remove the small vesicles. The resulting pellet was resuspended in preparation solution and centrifuged at 790 g for 10 min. These two steps were performed to separate the fraction of purified mitochondria. The preparation solution was removed by two additional faster centrifugations (9,200 g, 10 min) in storage solution consisting of 150 mM KCl and 10 mM HEPES (pH 7.2). All procedures were performed at 4°C.

Mitoplasts were prepared from the mitochondrial fraction by a swelling procedure. The mitochondria were added to a hypertonic solution containing 5 mM HEPES (pH 7.2) and 100 μM CaCl₂ for ~1 min to induce swelling and breakage of the mitochondrial outer membrane. Afterward, the suspension was added to a hypertonic solution composed of 750 mM KCl, 100 μM CaCl₂, and 30 mM HEPES (pH 7.2) to restore the sample to an isotonic condition.

For the bioenergetic measurements, mitochondria were isolated from EA hy926 cells according to a very efficient isolation procedure, which produces highly active and well-coupled mitochondria (23). The final mitochondrial pellet was resuspended in medium containing 0.25 M sucrose and 15 mM Tris·HCl (pH 7.2) and centrifuged at 12,000 g for 10 min. The yield of the isolated mitochondria was equal to 3.4 ± 0.6 mg of mitochondrial protein per gram of cells (SD, n = 14).

**SDS-PAGE and immunoblotting.** Determination of protein levels was performed as previously described (23). The spectra Multicolor Broad Range Protein Ladder (Fermentas) was used as a molecular weight marker. Proteins were separated in 12 or 8% SDS-polyacrylamide gels and then electrotransferred to nitrocellulose membranes. The membranes were then incubated with anti-KCa1.1 and anti-sloβ₂ antibodies (APC-107 and APC-034, Alomone Laboratories), respectively, at dilutions of 1:200, in the presence or absence of blocking peptide. Cross-reactivity was also checked with antibodies raised against α-subunit of the plasma membrane Na/K-ATPase (MA3-929, Thermo Scientific) and calcinein, endoplasmic reticulum (ER) membrane marker (ab10286, Abcam), at dilutions of 1:200 or 2,000, respectively. Protein detection was achieved using an appropriate secondary antibody linked to horseradish peroxidase and an enhanced chemiluminescence system. Protein content was determined using the Bradford method (Bio-Rad).

**Fluorescence staining of mitoplasts.** To confirm the mitochondrial origin of the mitoplasts used in the patch-clamp experiments, the plasmid pcDNA3-green fluorescent protein (GFP) was used for the mitochondria-targeted expression of GFP (mGFP) in EA.hy926 cell transfection assays. Standard transfection assays were performed using Lipofectamine (Invitrogen) as described by the manufacturer. In brief, Lipofectamine-DNA complexes were formed by mixing 4 μL Lipofectamine with 1 μL (300 ng) pcDNA3-GFP. Cells were incubated for 5 h in transfection medium, the medium was then replaced with fresh DMEM medium, and the cells were further cultured. After 24 h, the cells were used for mitoplast isolation. The isolated mito-plasts were then labeled with antibodies raised against α-subunit of ATP synthase (anti-OxPhos Complex V subunit-α, Invitrogen), a marker protein of mitochondria, and the immunoreaction was visualized using the Alexa Fluor 546 Monoclonal Antibody Labeling Kit (Molecular Probes). Confocal images were acquired using a Leica microscope (TCS SP5, Leica Microsystems, Wetzlar, Germany). Mi-
toplasts with diameters of 2–5 µm were used for the patch-clamp experiments.

Patch-clamp experiments. Patch-clamp experiments on endothelial mitoplasts were performed as previously described for mitoplasts obtained from other mammalian mitochondria (3, 8). The experiments were carried out in mitoplast-attached single-channel mode using a pipette of borosilicate glass (Harvard, UK) with a mean resistance of ~15 MΩ, which was pulled using a Flaming/Brown Micropipette Puller. The patch-clamp glass pipette was filled with an isotonic solution containing 150 mM KCl, 100 µM CaCl₂, and 10 mM HEPES (pH 7.2). This isotonic solution was used as a control solution in all experiments. A low-calcium solution (1 mM Ca²⁺), which refers to calcium free solution, contained 150 mM KCl, 1 mM EGTA, 0.752 mM CaCl₂, and 10 mM HEPES (pH 7.2). All patch-clamp measurements were carried out in an air-conditioned room (24°C). The size of the pipettes and the formation of the gigaseal were monitored by measuring resistance. Electrical connections were made with Ag/AgCl electrodes and an agar salt bridge (3 M KCl) for the ground electrode. The current was recorded using a patch-clamp amplifier (Axopatch 200B, Molecular Devices). The currents were low-pass filtered at a corner frequency of 1 kHz and sampled at a frequency of 100 kHz with Clampex software (Molecular Devices). To apply substances, we used a perfusion system containing a holder with a glass pipe (made in our workshop), peristaltic pump, and teflon tubing. All channel modulators were added as dilutions in the isotonic solution containing 100 µM CaCl₂.

Data were processed using the Clampfit software. The presented single-channel recordings (Figs. 3A, 4, A–C, and 5, A–C) are representative of the most frequently observed conductance under the given conditions. The conductance was calculated from the current-voltage relationship. The probability of channel opening and the current amplitude were determined using the Single-Channel Search mode of the Clampfit software (update baseline, keep deltas and level contribution was equal to 10%). Calculations were made from segments of continuous recordings lasting 60 s and with N > 1,000 events.

Mitochondrial oxygen consumption and membrane potential measurements. The respiratory rates and ∆Ψ of the endothelial mitochondria were measured as previously described (23). Oxygen uptake was determined polarographically using a Rank Bros. oxygen electrode (Cambridge, UK) in 2.8 ml standard incubation medium, which consisted of 70 mM sucrose, 50 mM KCl, 2.5 mM KH₂PO₄, 2 mM MgCl₂, 10 mM Tris·HCl, 10 mM HEPES (pH 7.2), and 0.2% BSA at 37°C. ∆Ψ was measured simultaneously with oxygen uptake using a tetraphenylphosphonium-specific electrode. Changes in the composition of the incubation medium are described in Fig. 7 legend. All measurements were performed with 2 mg of mitochondrial protein in the presence of 0.15 mM ATP (to activate succinate dehydrogenase) and 10 µM glibenclamide (to inhibit the mitoK<sub>ATP</sub> channel). Succinate (5 mM) plus rotenone (2 µM) was used as a respiratory substrate. Resting, nonphosphorylating respiratory rate measurements were performed in the presence of 1.7 µM carboxyatractyloside (to inhibit ATP/ADP antipporter activity) and 0.4 µM/ml oligomycin (to inhibit ATP synthase). Phosphorylating respiration was measured using 120 µM ADP. Only high-quality mitochondria preparations, i.e., with an ADP-to-O ratio value of ~1.3 and a respiratory control ratio of ~2.5–3, were used in all experiments. O₂ uptake values (in nmol O₂/min µg protein⁻¹) and ∆Ψ (in mV) are presented.

1,3-Dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-[5-(trifluoromethyl)benzimidazole-2-one (NS1619) (Sigma, St. Louis, Mo) and 1-(3,5-bis-trifluoromethyl-phenyl)-3-[4-bromo-(1H-tetrazol-5-yl)-phenyl]-thiourea (NS11021) (NeuroSearch, Ballerup, Denmark), which were dissolved in methanol, were used to induce mitoBK<sub>Ca</sub> channel activity. Up to 2 µM iiberotoxin (Bachem, Bubendorf, Switzerland) (dissolved in water) or 10 µM psaxiline (Sigma) (dissolved in methanol) were used to inhibit the channel activity.

Statistical analysis. The results are presented as the means ± SD, obtained from at least six independent complete 60-s current traces (electrophysiological data) or three to four independent mitochondrial isolations (biochemical data), in which each determination was performed at least in duplicate. ANOVA test was used to identify significant differences; in particular, differences were considered to be statistically significant if P < 0.05, P < 0.01, or P < 0.001.

RESULTS

Immunological detection of mitoBK<sub>Ca</sub> channel proteins in mitochondria of endothelial EA.hy926 cells. Immunoblotting of total mitochondrial and mitoplast proteins allowed for the immunological detection of the human endothelial mitoBK<sub>Ca</sub> channel. Antibodies raised against the mammalian plasma membrane BK<sub>Ca</sub> channel pore (α-subunit BK<sub>Ca</sub>1.1) and auxiliary β-subunits were used. In the endothelial mitochondrial and mitoplast fractions, a protein band with a molecular mass of ~125 kDa was detected using the anti-BK<sub>Ca</sub>1.1 antibody (Fig. 1A). Moreover, the anti-slo<sub>β</sub>2 antibody cross-reacted with a single band of ~44 kDa (Fig. 1B). No reactivity was observed with the anti-slo<sub>β</sub>1 or anti-slo<sub>β</sub>4 antibodies (data not shown). With the use of the anti-BK<sub>Ca</sub>1.1 and anti-slo<sub>β</sub>2 antibodies (Fig. 1, A and B), much stronger signals were obtained for the mitoplast fraction than for the mitochondrial fraction (for lanes with...
equal total protein concentration loaded on the SDS-PAGE), proving that the detected proteins localized to the inner membrane of the endothelial mitochondria. Moreover, specific blocking peptides blocked the antibody-antigen interaction, demonstrating the specificity of the reaction in the immunoblotting assays. Conversely, mitochondrial (isolated mitochondria and mitoplasts) fractions probed with antibody against a plasma membrane marker (Na/K-ATPase) displayed no signal in contrast to a fraction containing total cell proteins, indicating the absence of surface membrane contamination in the mitochondrial fractions (Fig. 1C). Moreover, the immunodetection with antibody against ER marker (calnexin) revealed a presence of mitochondria-associated ER membrane only in the mitochondrial fraction but not in the mitoplast fraction (Fig. 1D). These results strengthen the identification of α- and β2-subunits of mitoBKCa channel in the EA.hy926 mitochondrial inner membrane. Therefore, it can be concluded that the endothelial mitoBKCa channel may contain subunits that are similar to the surface α-subunit KCa1.1 and the β-subunit sloβ2.

**Electrophysiological properties of the endothelial mitoBKCa channel.** Mitoplasts prepared from mitochondria isolated from EA.hy926 cells were used in electrophysiological experiments. To confirm that our patch-clamp experiments were carried out on mitoplasts, we transfected EA.hy926 cells with the plasmid pcDNA3-GFP, which encodes mGFP, the mitochondria-targeted GFP (Fig. 2A). To confirm the localization of mGFP in isolated mitoplasts (Fig. 2B), the cells were additionally fluorescently labeled with antibodies against ATP synthase subunit-α (Fig. 2C). Signals from mGFP and ATP synthase revealed their expected colocalization (Fig. 2D), confirming the presence of inner mitochondrial membrane vesicles (mitoplasts) in our preparations. Additionally, mitoplasts were easily recognizable because of their size, round shape, transparency, and the presence of their characteristic “cap” region that reflects remnants of the outer membrane (Fig. 2E).

Approximately 40% of the channels detected in the patch-clamp experiments showed potassium currents that are characteristic of the BKCa channel (31). Figure 3A shows representative single-channel recordings for the endothelial mitoBKCa channel at different voltages in a symmetrical isotonic solution containing 150 mM KCl, 10 mM HEPES, and 100 μM CaCl2, (pH 7.2). The current was measured as a function of applied potential from +60 mV to −60 mV. The channel showed a straight current-voltage relationship in the studied range with 150 mM KCl solutions on either side of the mitoplast. The channel conductance calculated from the current-voltage relationship (Fig. 3B) was equal to 270 ± 10 pS, thus indicating large-conductance channel activity. Rectification of the current was not observed (Fig. 3B). The probability of channel opening increased from −0.09 at −60 mV to −0.96 at positive voltage (+40 mV to +60 mV), as calculated from recordings in a symmetrical isotonic solution with 100 μM CaCl2 (Fig. 3C). The distribution of the mean time of channel closure and opening at different voltages was also analyzed (Fig. 3D). With increasing voltages (from −60 mV to +60 mV), the mean time of closure decreased from −100 to almost 0 ns, and, in contrast, the mean time of opening increased to −70 ms at +60 mV.

Substances known to modulate mitoBKCa channel activity were used to examine the potassium ion channel properties observed in our experiments. To test a possible dependence of the channel activity on Ca2+, we changed the calcium concentration in the measurement solution from a high (100 μM) to a low (1 μM) level. Figure 4A shows single-channel recordings at different voltages in the control isotonic solution with 100 μM or 1 μM calcium. In the presence of 1 μM Ca2+, a strong inactivation of the mitoBKCa channel was observed. Analysis of the probability of channel opening indicated a statistically significant inactivation effect at the low calcium concentration (data not shown). Figure 4, B–D, demonstrates the inhibitory effect of iberiotoxin and paxilline, known mitoBKCa channel...
inhibitors, on the mitoBKCa channel from EA.hy926 cells. As shown in representative single-channel recordings obtained in the isotonic control solution (with 100 μM Ca\(^{2+}\)) at +40 and −40 mV before and after the addition of 10 μM paxilline or 100 nM iberiotoxin, the inhibitory effect of paxilline was much stronger than that of iberiotoxin (Fig. 4, B and C). At +40 and −40 mV, paxilline caused a complete transition of the channel into a closed state (Fig. 4B), whereas the inhibitory effect of iberiotoxin was partial (Fig. 4C). At +40 mV, the probability of channel opening decreased from ~0.94 to 0.02 or 0.31 in the presence of paxilline or iberiotoxin, respectively (Fig. 4D, left). At −40 mV, the probability of channel opening decreased from ~0.17 to 0.01 or 0.07 in the presence of paxilline or iberiotoxin, respectively (Fig. 4D, right). Thus inhibition by iberiotoxin was more pronounced at the positive potential. Inhibitory effects of paxilline and iberiotoxin were also detected at other negative and positive voltages (data not shown). Moreover, 10 μM paxilline and 100 nM iberiotoxin irreversibly inhibited the mitoBKCa channel activity when 25-min washout of inhibitors was applied (data not shown). At 0.1 μM
Fig. 4. Effects of Ca\(^{2+}\), paxilline (Pax), and iberiotoxin (IbTx) on the activity of the endothelial mitoBKCa channel. A: single-channel recordings in 100 \(\mu\)M (control) or 1 \(\mu\)M (low) Ca\(^{2+}\) at different voltages. B and C: single-channel recordings at +40 and −40 mV under control conditions (100 \(\mu\)M Ca\(^{2+}\)) and after application of 10 \(\mu\)M Pax (B) or 100 nM IbTx (C). “−” indicates the closed state of the channel. Immediate effects of Ca\(^{2+}\) and Pax were observed, whereas IbTx inhibited with a 20-s delay. D: analysis of the probability of channel opening at +40 and −40 mV under control conditions (100 \(\mu\)M Ca\(^{2+}\)) and after application 10 \(\mu\)M Pax or 100 nM IbTx. Data are presented as means ± SD (\(n = 6\)). \(n\), Number of complete 60-s current traces. *** \(P < 0.001\); ** \(P < 0.01\) vs. control values. All presented data were acquired in a symmetric 150/150 mM KCl isotonic solution.
Ca^{2+}-regulated potassium channel in endothelial mitochondria

inhibitors of the mitoK_{ATP} channel, the ATP/Mg^{2+}
of NS1619, the inhibition by iberiotoxin was also more pro-
40 or H11002

initial values before Ca^{2+} leading to a decrease in the respiratory
rate and membrane potential in mitochondria isolated from
EA.hy926 cells.

mitoBK_{Ca} channel was observed in the presence of exoge-
nous Ca^{2+} (100 \mu M) and the lowest activity was observed
in the presence of 1 mM EGTA (a chelator of Ca^{2+}).

Similar to calcium ions, the potassium channel openers
NS1619 and NS11021 stimulated nonphosphorylating oxygen
uptake (Fig. 6, B and C, left) and decreased nonphosphorylating
\Delta \Psi (Fig. 6, B and C, right) in isolated EA.hy926 mito-
chondria. The effect of increasing concentrations of NS1619
up to 80 \mu M or NS11021 up to 4 \mu M on the respiratory rate
and \Delta \Psi is shown in Fig. 6B. The addition of NS1619 up to 50
\mu M or NS11021 up to 2.5 \mu M resulted in an increase in the
rate of respiration by \sim 100\% (by \sim 20–21 nmol O_{2}·min^{-1}·mg
protein^{-1}) and 64\% (by \sim 14–15 nmol O_{2}·min^{-1}·mg
protein^{-1}) in the absence or presence of 2 \mu M iberiotoxin,
respectively (Fig. 6B, left). At the same time, \Delta \Psi decreased
after the addition of up to 50 \mu M NS1619 or up to 2.5 \mu M
NS11021 by \sim 24 and 20 mV in the absence or presence of
iberiotoxin, respectively (Fig. 6B, right). Only at 10 \mu M
NS1619 or 2.5 \mu M NS11021, 2 \mu M iberiotoxin almost com-
pletely abolished the NS-induced respiration and \Delta \Psi depolar-
ization (Fig. 6B). In the presence of 20 to 50 \mu M NS1619 or 1
to 2.5 \mu M NS11021, iberiotoxin partially blocked NS-induced
respiration and \Delta \Psi depolarization, indicating a nonspecific
uncoupling effect of both activators on isolated endothelial
mitochondria. Moreover, a concentration of NS1619 above 50
\mu M and NS11021 above 2.5 \mu M caused a decrease in respi-
ration and a further decrease in \Delta \Psi, indicating an impairment
of the respiratory chain by higher concentrations of the potas-
sium channel openers. Nevertheless, we can conclude that in
endothelial mitochondria, NS1619 (at 10 \mu M) and NS11021
(at 0.5 \mu M) stimulates the iberiotoxin-sensitive K^{+} flux, de-
creases \Delta \Psi, and thus accelerates the mitochondrial respiration
rate.

Figure 6C illustrates the comparison of the efficiency of
iberiotoxin and saxilline in the inhibition of mitoBK_{Ca}
channel activity under NS-stimulated conditions. An example of the
effect of increasing the concentrations of the two inhibitors on
NS1619-induced respiration and \Delta \Psi depolarization is shown in
Fig. 6C, left. A further increase in the concentrations of
iberiotoxin (above 2 \mu M) and saxilline (above 20 \mu M) did not
cause any additional effects (data not shown). NS1619-induced
respiration was blocked by 2 \mu M iberiotoxin by 100% and by
20 \mu M saxilline by 72% (Fig. 6C, left). Similarly, iberiotoxin
almost completely restored NS1619-induced \Delta \Psi depolarization
(an increase by 4 mV), whereas saxilline only partially
restored this parameter (an increase by 1.5 mV). The difference
in the sensitivity of the endothelial mitoBK_{Ca} channel to
iberiotoxin and saxilline was also evident when the NS-
induced inhibitor-blocked changes in the respiratory rate and
\Delta \Psi were compared (Fig. 6C, right). A weaker inhibitory effect
of saxilline compared with iberiotoxin was observed with 10
\mu M NS1619, 0.5 \mu M NS11021 (Fig. 6C, right), and Ca^{2+}-
induced mitoBK_{Ca} channel activity (data not shown). These
results indicate a lower sensitivity of mitoBK_{Ca} channel activity
to saxilline in isolated endothelial mitochondria. A dif-
fERENCE in sensitivity to iberiotoxin and saxilline between patch-
clamp experiments (Figs. 4 and 5) and biochemical assays (Fig.
6C) could appear when the inhibitor is applied to a different
side of the inner mitochondrial membrane, i.e., from the outer
membrane or matricial space, respectively. However, our re-
sults indicate that Ca^{2+} (100 \mu M), NS1619 (10 \mu M), and
NS11021 (0.5 \mu M), when applied separately, can induce sim-
ilar iberiotoxin-sensitive mitoBK_{Ca} channel activities, i.e., an
\sim 7 nmol O_{2}·min^{-1}·mg protein^{-1} increase in respiratory rate
and an \sim 4 mV \Delta \Psi depolarization (Fig. 6A and C, right).
100 μM Ca\(^{2+}\) and 10 μM NS1619 (or 0.5 μM NS11021) were applied simultaneously, no further increase in the iberiotoxin-sensitive mitoBK\(_{\text{Ca}}\) channel activity was observed (data not shown).

Bioenergetic analysis of the effects of the known mitoBK\(_{\text{Ca}}\) channel modulators on the respiratory rate and ΔΨ in respiring endothelial mitochondria indicated the presence of Ca\(^{2+}\)- and NS-induced, iberiotoxin- and paxilline-inhibited K\(^{+}\) flux. To
Ca²⁺-Regulated Potassium Channel in Endothelial Mitochondria

Fig. 5. Effect of NS1619 on the mitoBKCa channel activity. A: single-channel current-time recordings at −40 mV (left) and the corresponding probability of channel opening (left, bottom) as well as currents recorded using a voltage ramp protocol from −70 to +70 mV (right) in a sequence of conditions: under control conditions (100 μM Ca²⁺), after decreasing the Ca²⁺ concentration to 1 μM, after reincreasing the Ca²⁺ concentration to 100 μM, and finally after the addition of 10 μM NS1619. B and C: single-channel recordings at −40 mV in the presence of 100 μM Ca²⁺ and 10 μM NS1619 and after application of 10 μM Pax-B or 100 nM IBTx. D: analysis of the probability of channel opening at +40 and −40 mV in the control solution in the presence of 10 μM NS1619 and after addition of 10 μM Pax or 100 nM IBTx. E and F: sequence of applied conditions as in A. Data on the probability of channel opening are presented as the means ± SD (n = 6). n, Number of complete 60-s current traces. ***P < 0.001, **P < 0.01, *P < 0.05 vs. values obtained in the absence of inhibitors.

Fig. 6. Mitochondrial respiratory control ratio was significantly lowered, nonphosphorylating activity of EA.hy926 mitochondria.

DISCUSSION

In the present study, we describe for the first time the functional properties of a mitoBKCa channel in endothelial mitochondria. The electrophysiological properties of this channel were studied in the inner mitochondrial membrane of the human endothelial cell line EA.hy926 using the patch-clamp technique in the mitoplast-attached mode. Large conductance (270 ± 10 pS), voltage dependence, a higher open-state probability at positive potentials, sensitivity to Ca²⁺, and NS1619 (a BKCa channel opener) and paxilline and iberiotoxin (BKCa channel inhibitors) indicate the similarity of this channel to the mammalian mitoBKCa channels previously reported in glioma (31), skeletal muscle (33), brain (14, 33), and cardiac (40) cells. Interestingly, a mitoBKCa channel with similar electrophysiological properties (although with a much higher conductance of 502–605 pS) has been recently described in nonmammalian mitochondria (potato tuber) mitochondria (22). In human endothelial EA.hy926 mitochondria, in addition to irreversible inhibition by paxilline and iberiotoxin, an immediate strong inactivating effect on mitoBKCa channel opening probability was observed in the presence of a low calcium level. Similar effects were reported in human glioma cells (31). In the case of the mitoBKCa channel in EA.hy926 mitochondria, after inactivation in the presence of low Ca²⁺, the activity of the channel was restored only when NS1619 was applied. It is not surprising as NS and Ca²⁺ have different binding sites. Thus, independent of Ca²⁺ presence, NS1619 can act on its own. The molecular mechanism of regulation of BKCa channels by Ca²⁺ is still unknown. Regulatory β subunits control the activity and sensitivity to Ca²⁺ of BKCa channels. The molecular mechanism of this regulation likely relies on the operation of two cytotoxic domains, regulator of K⁺ conductance (RCK1) and RCK2 (41). It has been demonstrated that the purified BKCa RCK1 domain adopts an α/β fold, binds Ca²⁺, and assembles into an octameric structure (41). Moreover, it seems that splice variation can regulate BKCa channel targeting to different subcellular compartments (42).

In our study, 10 μM paxilline caused a complete transition of the EA.hy926 mitoBKCa channel into a closed state, whereas the inhibitory effect of 100 nM iberiotoxin was partial. With the use of a single-channel patch-clamp technique, the partial inhibitory effect of iberiotoxin has also been observed in rat astrocyte mitoplasts (8). In our study, paxilline (10 μM) and iberiotoxin (100 nM) irreversibly inhibited the mitoBKCa channel activity of EA.hy926 mitoplasts. Paxilline (0.1 μM) still caused a irreversible inhibition when washout was applied. However, detailed kinetic studies with different doses of in-
hbitors and activator (calcium ions) and detailed studies on reversibility of the inhibitory effect would be important to understand regulation of the mitoBKCa channel. In the case of the BKCa channel, depending on calcium concentration, ~0.01–0.5 μM paxilline completely inhibits activity of α-subunit cloned from mouse brain and then expressed in Xenopus oocytes (29). It has also been shown that paxilline causes a reversible inhibition of BKCa channel activity with slow washout kinetics. The time course of relief of paxilline inhibition by elevated calcium has been more rapid that washout of inhibitor,
suggesting an allosteric interaction between calcium and paxilline (29).

In isolated EA.hy926 mitochondria, 10 μM NS1619 and 0.5 μM NS11021 induced a similar mitoBKCa channel activity, which was revealed as an ~7 nmol O2·min⁻¹·mg protein⁻¹ ibeiriotoxin-sensitive increase in nonphosphorylating respiration and an ~4 mV ibeiriotoxin-sensitive decrease in nonphosphorylating ΔΨ. These results indicate that in isolated endothelial mitochondria, NS11021 works at concentrations 20 times lower compared with NS1619. Thus NS11021 seems to be much more efficient activator of the mitoBKCa channel (this study) and the BKCa channel (4). However, 2 μM ibeiriotoxin completely abolished the NS-induced respiration and ΔΨ depolarization only at concentrations of openers not higher than 10 μM (with NS1619) or 0.5 μM (with NS11021). When compared with the full inhibitory effect of ibeiriotoxin on Ca2⁺-induced respiration and ΔΨ depolarization, this could indicate a nonspecific uncoupling effect of NS1619 and NS11021 within the concentration range of 20 to 50 μM and 1 to 2.5 μM, respectively, on isolated endothelial mitochondria (when 0.7 mg of mitochondrial protein is used) rather than the low sensitivity of the channel to ibeiriotoxin. Similarly, in some isolated mammalian mitochondria, the sensitivity of NS1619-

or NS11021-induced respiration and ΔΨ depolarization to the mitoBKCa channel blockers (ibeiriotoxin, charybdotoxin, and paxilline) is not complete (2, 20, 32, 33). Moreover, in mitochondria isolated from EA.hy926 cells, concentrations of NS1619 above 50 μM and NS11021 above 2.5 μM caused a decrease in respiration and a further decrease in ΔΨ, indicating an impairment of the respiratory chain by higher concentrations of the potassium channel openers. Thus our results indicate that the application of NS1619 and NS11021 to isolated mitochondria requires some caution because above a given concentration of these compounds, nonspecific mitochondrial uncoupling or even impairment of the respiratory chain may take place.

Plasma membrane IKCa and SKCa channels are preferentially expressed in endothelial cells (15). They have been characterized electrophysiologically in the endothelial cell line EA.hy926 (30). In EA.hy926 mitoplasts, we have rarely observed an activity of potassium channels other than mitoBKCa channels. Therefore, IKCa and SKCa were not characterized in this study. Thus it is difficult to confirm or exclude their existence in the inner mitochondrial membrane of endothelial EA.hy926 cells. Plasma membrane BKCa channels in endothelial cells have been characterized using electrophysiological, pharmacological, and molecular studies (15, 38). They play a role in the control of vascular tone, coupling local increases in intracellular Ca2⁺ to membrane hyperpolarization and vascular relaxation. In some freshly isolated endothelial cells, BKCa channels that are characterized by calcium and voltage dependence, ibeiriotoxin sensitivity, and a conductance between 190 and 300 pS are detected (6, 17, 26, 28). Interestingly, the properties of the BKCa channel in the human endothelial cell line EA.hy926 (26) are similar to those of the mitoBKCa channel described in this study. The BKCa channel of EA.hy926 cells has a single-channel conductance of 270 pS, which is the same as that of its mitoBKCa channel counterpart. Its activity also strongly depends on membrane potential, and Ca2⁺ concentration and is markedly increased by NS1619 (26).

In human EA.hy926 cells, the mRNA expression of the BKCa channel α-subunit has been previously detected, whereas the mRNA and protein expression of the BKCa channel β-subunit, corresponding to the β₄-subunit, has been undetectable (26). β-Subunit has not been searched in these cells until now.

**Fig. 6. Influence of mitoBKCa channel modulators on the nonphosphorylating respiratory rate and membrane potential (ΔΨ) in isolated EA.hy926 mitochondria.**

**A:** effect of Ca2⁺. **Left:** additions as indicated: 5 mM succinate, 100 mM CsCl, and 2 μM IbTx. The solid line trace shows the measurement obtained in the absence of activator (Ca2⁺). The numbers on the traces refer to the O2 consumption rates (in nmol O2·min⁻¹·mg protein⁻¹) or to the ΔΨ values (in mV). **Right:** IbTx-induced changes in respiratory rate and ΔΨ were calculated as the difference between the respective values measured in the absence and presence of 2 μM IbTx. ***P < 0.001, **P < 0.01, and *P < 0.05 vs. control value (no additions).** **B:** effect of increasing concentrations of NS1619 or NS11021 on nonphosphorylating respiratory rate (left) and ΔΨ (right) in the absence or presence of 2 μM IbTx. IbTx was added before activator addition. **C:** effect of IbTx and Pax. **Left:** additions as indicated: 5 mM succinate, 10 μM NS1619, and 2 doses of 1 μM IbTx (solid line) or 10 μM Pax (dashed line). Numbers on the traces as in A, left. **Right:** inhibitor-sensitive changes in respiratory rate and ΔΨ were calculated as the difference between respective values measured in the absence and presence of 2 μM IbTx or 20 μM Pax. MitoBKCa channel activity was induced by 10 μM NS1619 or 0.5 μM NS11021, ***P < 0.001 and *P < 0.05 vs. a value obtained with NS1619 and IbTx. The data address 3 different mitochondrial preparations, and each determination was performed at least in duplicate (means ± SD, 3 experiments, n = 8).
In the present study, the immunodetection of mitoBKCa channel proteins in the mitochondria of EA.hy926 cells indicates cross-reactivity with antibodies raised against the KCa1.1 α-subunit of the BKCa channel and the β-subunit of the sloβ2 BKCa channel. The existence of β2-subunit of the plasma membrane BKCa channel in EA.hy926 cells cannot be excluded. The mRNA transcript for β2-subunit has been shown by RT-PCR in porcine basilar and middle cerebral arteries (39). Our results suggest that the mitoBKCa channel present in the endothelial inner mitochondrial membrane may be structurally similar to the plasma BKCa channel. Specifically, it may be formed by the principal pore-forming α-subunit that interacts with an auxiliary β2-subunit. The predominant β2-subunit may determine the channel’s activity, including Ca2+ and membrane potential sensitivity, as in the case of the plasma BKCa channel. The relative molecular mass of the detected endothelial proteins (~125 and 44 kDa for the α- and β2-subunits, respectively) are the same as those of the mammalian proteins from the plasma membrane (11, 39) and mitochondria (27). The β2-subunit of mitoBKCa has been detected in astrocyte mitochondria (27) and potato tuber mitochondria (22). However, the molecular identity (gene and protein sequences) of the mitoBKCa channel still awaits elucidation. It has been recently shown that SLO2 coded for by a member of the BKCa family underlies mitoBKCa activity and anesthetic preconditioning-induced protection in both Caenorhabditis elegans and perhaps in mouse hearts as well (Slo2) (37).

In addition to our electrophysiological and immunological studies, the presence of a mitoBKCa channel in the mitochondria of human EA.hy926 cells was indicated in bioenergetic studies with isolated mitochondria. Under nonphosphorylating conditions, potassium channel activators Ca2+ (100 μM), NS1619 (10 μM), and NS11021 (0.5 μM) were able to modulate the respiratory rate (stimulation) and ΔΨ (depolarization) in isolated endothelial mitochondria. Conversely, mitochondrial respiratory rate inhibition and ΔΨ repolarization were observed when mitoBKCa channel inhibitors iberiotoxin and paxilline were applied. These effects were markedly dependent on the presence of K+ in the incubation medium. Moreover, we show for the first time (to our knowledge) that the activation of this mitoBKCa channel leads to a decrease in the yield of oxidative phosphorylation. In the phosphorylating mitochondria of EA.hy926 cells, NS1619-induced iberiotoxin-inhibited uncoupling decreased ATP synthesis. Thus the endothelial mitoBKCa channel can potentially modulate the tightness of coupling between respiration and ATP synthesis in mitochondria, thereby contributing to the maintenance of a balance between energy supply and demand in the cell. The observed effects of the mitoBKCa channel modulators indicate the activation of electrogenic potassium transport through the inner mitochondrial membrane of human endothelial EA.hy926 cells, as K+ influx into the matrix led to a decrease in ΔΨ and a stimulation of the respiratory rate. Thus the endothelial mitoBKCa channel may function as a possible signaling link between intramitochondrial calcium levels and mitochondrial ΔΨ and ROS. It has been previously observed that potassium channels affect mitochondrial matrix swelling, regulate the concentration of ROS, and change the mitochondrial ΔΨ and the transport of Ca2+ into mitochondria (25, 34, 35). However, the physiological role of the mitoBKCa channel in endothelial mitochondria, which is described for the first time in the present work and seems to significantly modulate mitochondrial metabolism, awaits exploration.

In conclusion, in the present study we identified and characterized a mitoBKCa channel in the inner mitochondrial membrane of endothelial EA.hy926 cells using the patch-clamp technique, immunoblotting, and functional measurements of oxygen uptake and ΔΨ with isolated mitochondria. The pharmacological, biophysical, functional, and molecular properties of this endothelial mitoBKCa channel are similar to those of the plasma membrane BKCa channels and mitoBKCa channels of other mammalian cells.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

P.B., W.J., and A.S. conception and design of research; P.B. and A.K. performed experiments; P.B. and A.K. analyzed data; P.B., W.J., and A.S. interpreted results of electrophysiological and biochemical experiments, respectively; P.B. and A.K. prepared figures; P.B. drafted manuscript, W.J. edited and revised manuscript; W.J. and A.S. approved final version of manuscript.
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