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Hypoxia increases BK channel activity in the inner mitochondrial membrane

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Abstract

To explore the potential function of the BK channel in the inner mitochondrial membrane under physiological and hypoxic conditions, we used on-mitoplast and whole-mitoplast patches. Single BK channels had a conductance of 276 ± 9 pS under symmetrical K^+ solutions, were Ca^{2+}- and voltage-dependent and were inhibited by 0.1 μM charybdotoxin. In response to hypoxia, BK increased open probability, shifted its reversal potential (9.3 ± 2.4 mV) in the positive direction and did not change its conductance. We conclude that (1) the properties at rest of this mitoplast K^+ channel are similar to those of BK channels in the plasma membrane; (2) hypoxia induces an increase, rather than a decrease (as in the plasmalemma), in the open probability of this K^+ channel, leading to K^+ efflux from the mitochondrial matrix to the outside. We speculate that this increase in K^+ efflux from mitochondria into the cytosol is important during hypoxia in maintaining cytosolic K^+.

Keywords: BK channels; Hypoxia; Mitoplast

Living organisms maintain their homeostatic mechanisms for cell function and growth and an imbalance in such mechanisms leads to cell injury and potentially cell death. Such an imbalance occurs in many pathological conditions, such as cancer and hypoxia. In the past two decades, mitochondria have been shown to be actively involved in the process of cell fate and programmed cell death.

It has been known for more than a decade that an imbalance in K^+ flux across the cell membrane is associated with the onset of cell death [1]. Similarly, this has been closely associated with K^+ flux across the inner membrane of heart mitochondria [2]. How the properties of K^+ channels influence the permeability of the inner mitochondrial membrane and thereby control the release of cytochrome *c* may be important since this relation will influence cell fate. In order to understand further the relationship of BK in the mitochondrial inner membrane to other mitochondrial events, we needed to study first the effect of hypoxia on BK properties. We therefore recorded single-channel activity of the BK from the inner mitochondrial membrane to characterize and study its potential function. We then studied the behavior of BK under hypoxic conditions since hypoxia can modify mitochondrial function and lead to programmed cell death.

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Methods

Solutions. The storage solution used contained (mM): 150 KCl, 20 K–Hepes, and 1 K–EGTA. The hypotonic solution contained (mM): 5 K–Hepes and 1 $CaCl_2$. The hypertonic solution contained (mM): 750 KCl, 80 K–Hepes, and 1 $CaCl_2$. Bath solutions contained (mM): 150 KCl, 20 K–Hepes with different Ca^{2+} concentrations. The pipette solution contained (mM): 150 KCl, 20 K–Hepes 0 Ca^{2+}, and 1 EGTA. pH was always adjusted to 7.2. Experiments were performed at room temperature (22–25 °C). All chemicals were purchased from Sigma (St. Louis, MO).

Mitoplast preparation. Mitoplasts were prepared from human glioma cell line LN 229 (ATCC, Manassas, VA) or LN 405 (DSMZ, Braunschweig, Germany). We used these cells since they were shown to have BK in their mitoplasts [3]. The cell line cells were incubated in DMEM with 10% fetal calf serum (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 50 IU/ml Penicillin, 50 μg/ml streptomycin (Sigma, St. Louis, MO) in an incubator with 5% CO_2 in air at 37 °C in a H_2O saturated atmosphere. The cultured cells were reseeded into flasks at density of 5×10^4 cells/ml DMEM twice a week. Mitochondria were separated and harvested after several steps of centrifugations as previously described [4] and stored in a refrigerator for up to 36 h. Samples of 10 μl of suspension were added to 2 ml of a hypotonic solution in the bath to induce swelling following which the outer membrane was broken. Hypertonic solution (0.5 ml) was added later in order to restore isotonicity.

Identification of the inner mitochondrial membrane. To prove the mitochondrial origin of the mitoplast preparation, the mitoplasts were incubated with a mouse anti-inner mitochondrial membrane antibody (H6/C12, diluted 1:100, Serotec, Raleigh, NC) for 45 min in an isotonic solution [a 4:1 mixture of hypotonic and hypertonic solutions (see solutions section above)] with 0.1% BSA on ice. After washing with the isotonic solution, a FITC labeled rat anti-mouse antibody F(ab)2-fragment (diluted 1:100, Dianova Hamburg, Germany) was added. Forty-five minutes later, after washing, the mitoplasts were seeded into a multi-well dish, viewed and detected in fluorescence optics (Zeiss, Thornwood, NY). Negative controls excluding primary antibody were performed to demonstrate the specificity of the immunostaining.

Electrophysiology. Patch–clamp experiments were performed using an Axopatch 1C amplifier (Axon instruments, Foster City, CA, USA) or an EPC-7 amplifier (HEKA, Darmadt, Germany). Recordings were made in mitoplast-attached or whole-mitoplast mode with a pipette from borosilicate glass (WPI, Serasota, FL) pulled by means of a Sutter puller (P-87, Sutter instruments, Navato, FL), with a resistance of 10–25 MΩ measured in our pipette and bath solutions. The signals were low-pass filtered at a corner frequency of 5 kHz, sampled at a frequency of 20 kHz with NeuroData (DR-484, NeuroData instruments, NY) and recorded on VHS tapes on a Sony video-cassette recorder. Some other signals were low-pass filtered at a corner frequency of 0.5 kHz, sampled at a frequency of 1 kHz and recorded on hard disk. Data were then analyzed using the pClamp 6 software. Voltages in the text and figures are all given as at the inner side of the mitoplasts versus outside. Inward currents always deflect downward. The open probability was determined from the recorded data and defined as the time channels spent in the open state over the total time of the recorded data.

Results

BK channels are present on mitochondrial inner membrane

Most mitoplasts were under 1 μm in size and had one or multiple black caps on the surface, which are believed to be identical with the contact points between inner and outer membranes of mitochondria [5]. To determine whether the observed membrane vesicles were of mitochondrial origin, we used anti-inner mitochondrial membrane antibody (H6/C12) and detected staining under fluorescence microscopy (data not shown). Negative control without primary antibody showed no fluorescent labeling, verifying the specificity of the immunostaining (data not shown). Channel activity was found in patches of mitoplasts derived from either LN229 or LN405, when holding at a V_m ranging from −60 to 80 mV, in an external bath containing a 0.1–0.4 mM Ca^{2+}.

Voltage-dependence

The current traces from the majority of the mitoplast patches from LN229 or LN405 had only one or two channel openings and a maximum of 4 channels in a mitoplast-attached mode in the presence of an external Ca^{2+} concentration of 0.4 mM (Figs. 3A and 4A). An-all point histogram showed a typical distribution of open and closed states at different V_m, ranging from −60 to 60 mV (Fig. 1A). At negative V_m, such as −40 and −60 mV (Fig. 1A), channels were more likely to be in the closed than the open states. When V_m was held at more positive voltages (e.g., −20 mV), channels were more likely to stay open than close. Occasionally, substates could be seen. In symmetrical solutions of 150 mM K^+, the *I–V* curve could be fitted with a straight line by linear regression, with a reversal potential near 0 mV (Fig. 1B). The average single-channel slope conductance was 276 ± 9 pS ($n = 9$) (Fig. 1C), which is suggestive of a BK channel conductance.

Ca^{2+} dependence

To further ascertain that this channel is likely to be a BK channel, we performed additional experiments on on-mitoplast patches. We first used three different concentrations of Ca^{2+} in the external solution and measured P_o under these conditions. Fig. 2 shows the relationship between P_o and V_m at different bath concentrations of Ca^{2+}. P_o could be fitted with the Boltzmann equation and our analysis yielded a shift to the left as the concentration of Ca^{2+} in the bath was increased. As can be seen from Fig. 2, even at a single calcium concentration, P_o is voltage-dependent (see also Fig. 3D), with a lower P_o at negative V_m and higher P_o at positive V_m for all three concentrations of Ca^{2+}.

Effect of charybdotoxin

To further characterize the properties of the channel, we used an inhibitor of BK channels, charybdotoxin, in the bath in the whole-mitoplast configuration. At a concentration of 0.1 μM of charybdotoxin, P_o was reduced by 10–130% at a V_m that ranged from −60 to 40 mV (Fig. 3D) without affecting the single-channel amplitude (Fig. 3C), strongly suggesting that this channel is charybdotoxin-sensitive, as are other known BK channels [3].

Effects of low O_2 on BK channel

In order to test for the sensitivity of the BK for hypoxia, we perfused the mitoplasts with an external solution

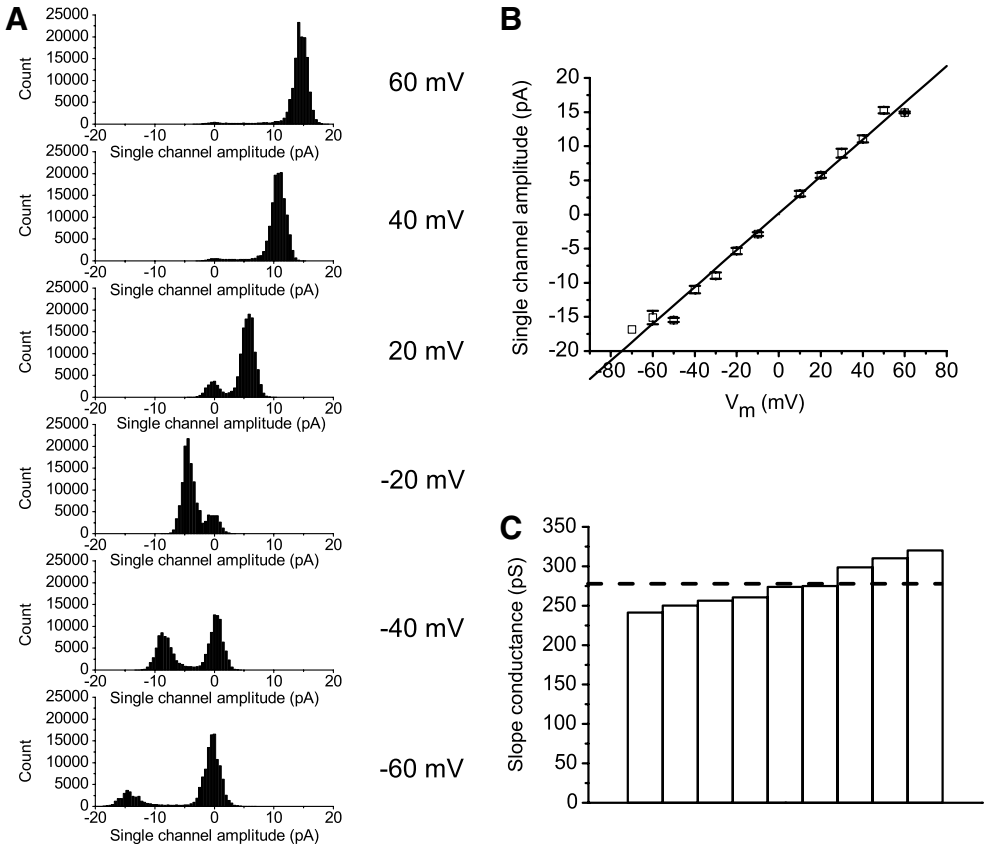

Fig. 1. Voltage dependence of BK channel and single-channel conductance. (A) Example of an all-point histogram of single BK-channel currents (from Fig. 3A) plotted as amplitudes (pA) of single-channel currents against count of current amplitudes at different V_m. (B) I–V curve of single BK channel plotted as current amplitudes of single channel against V_m ($n = 9$). The straight line was fitted by linear regression. Notice that the fitted line intercepted almost at 0pA with V_m of 0 mV. (C) Average single-channel conductance of 276 ± 9 pS was derived from nine individual patches.

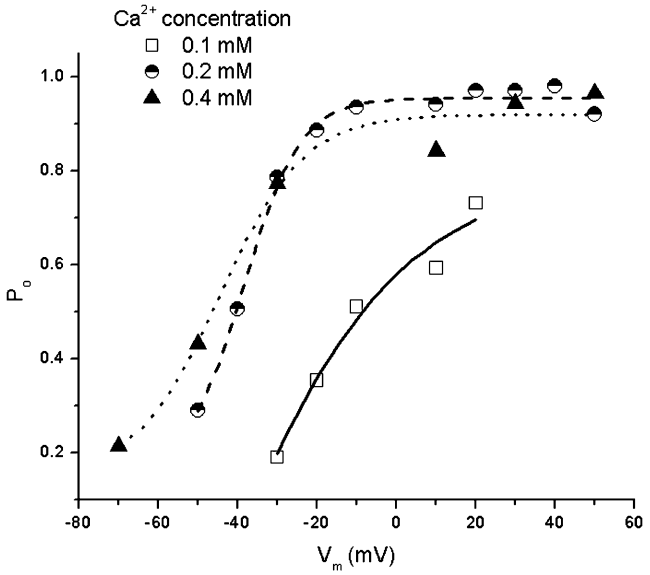

Fig. 2. Relationship between P_o and V_m at different Ca^{2+} concentrations for the BK channel. P_o was obtained from each concentration (0.1–0.4 mM) of Ca^{2+} at different V_m. Curves were fitted by Boltzmann's equation.

bubbled with 100% nitrogen for at least 2 h before carrying out the recording. Under normoxic conditions with 0.5 mM Ca^{2+} in the external solution, the channel showed a reversal potential at about 0 mV (Fig. 4C). Hypoxia shifted the reversal potential by 9.3 ± 2.4 mV ($n = 3$) to positive potentials (Fig. 4C). The reversal potentials were derived from linear fitting of the single-channel amplitudes. In another example, the reversal potential was extrapolated to be 100 mV. Hypoxia did not change the single-channel slope conductance (Fig. 4C).

In normoxic conditions with symmetrical K^+ solutions, NP_o plotted as a function of V_m had a peak at 20 mV (Fig. 4D). Hypoxia increased NP_o remarkably at all V_m. There was no peak at 20 mV and even at 40 mV the maximum total current had not been reached (Fig. 4D) indicating that hypoxia affects voltage dependence of the channels gating mechanism.

Discussion

Single-channel events were recorded from mitoplasts harvested from glioma cells. We have found that these events are due to mitochondrial BK channel activity for several reasons: (a) the single-channel slope conductance in symmetrical K^+ solutions (Fig. 1C) was in the range of conductance of BK channels previously studied [3]; (b) the current was voltage-dependent (Fig. 1A and B) and Ca^{2+}-sensitive (Fig. 2), as would be expected from a BK

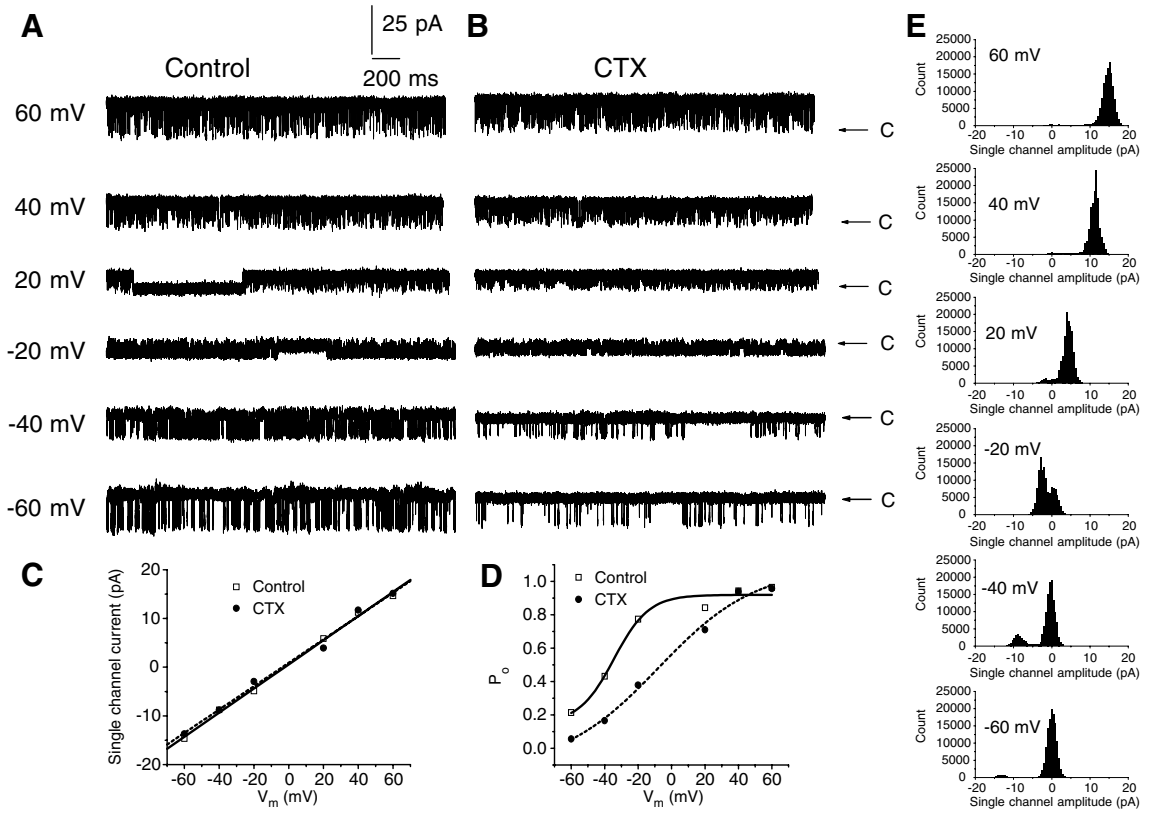

Fig. 3. Effects of CTX on single-channel activity of BK. Current traces of single BK channel recording at different V_m are plotted without CTX (A) or with 0.1 μM CTX (B). The letter "C"s and arrows in A and B indicate the close levels. (C) *I–V* curves of single BK channel plotted as current amplitudes of single channel against V_m in the absence and presence of CTX. The straight lines were fitted by linear regression. (D) Open probability of BK channels at different V_m in the absence and presence of CTX. (E) In the presence of CTX, an all-point histogram of single BK-channel currents plotted as amplitudes (pA) of single-channel currents against count of current amplitudes at different V_m.

channel (although Ca^{2+} sensitivity of the BK channels in our experiments is less than that of Siemen et al. [3]); (c) the current was inhibited by the BK channel inhibitor charybdotoxin (Fig. 3). Furthermore, we have been able to demonstrate that BK channels are expressed in the inner membrane of mitochondria in rat brain [6] using immunocytochemical and electromicroscopic techniques.

The discovery of BK channels in the inner mitochondrial membranes [3] has paved the way to explore the function of BK in the inner mitochondrial membrane under normal and abnormal conditions, such as in hypoxia. However, due to the size of the mitoplasts and technical difficulties, only a few reports have emerged. For example, pretreatment of the BK channel in the inner mitochondrial membrane of guinea pig ventricular cells with BK channel openers protected the heart from infarction [2]. Although it has been suggested that the complex modulation of the inner mitochondrial membrane potential upon the increase of mitochondrial Ca^{2+} concentration is important [3], the mechanisms by which the protection occurs are still unknown. We have now shown that BK channels are calcium-sensitive, and, most importantly, they respond to hypoxia by increasing open probability and shifting the reversal potential to induce an efflux of K^+ from the mitoplast.

Major advances have occurred in the past few decades regarding the biology of ionic fluxes in various conditions [7,8]. For instance, pH_i has been measured not only in the cytosol of cells but also in organellar pools. Ionic intracellular and intraorganellar concentrations, such H^+ concentrations in the ER, have already been studied by a number of investigators [8–10]. Kauppinen [11] has reported that the mitochondrial ΔpH is 0.53 (alkaline inside) in hearts beating at 1.5 Hz. Kim et al. [9] have found that the pH of the Golgi complex is close to 6.56, which is different from the cytosolic concentration. At present, there has been no data on intramitochondrial K^+ concentration in spite of the fact that the intracellular concentration of K^+ and therefore the exchange between intracellular and intraorganellar K^+ ions has become very important for determining cell fate.

We have been able to deduce from our current experiments the approximate K^+ concentration within the matrix. Although it has been proposed that the matrix K^+ concentration (180 mM) is greater than the cytosolic K^+ concentration [12] and this work is supported by Liu et al. [13], much lower matrix K^+ concentrations have also been reported in the matrix (67 mM) [14,15]. Our current work demonstrates that the K^+ concentration in the matrix is about that of the cytosol (150 mM) since, using

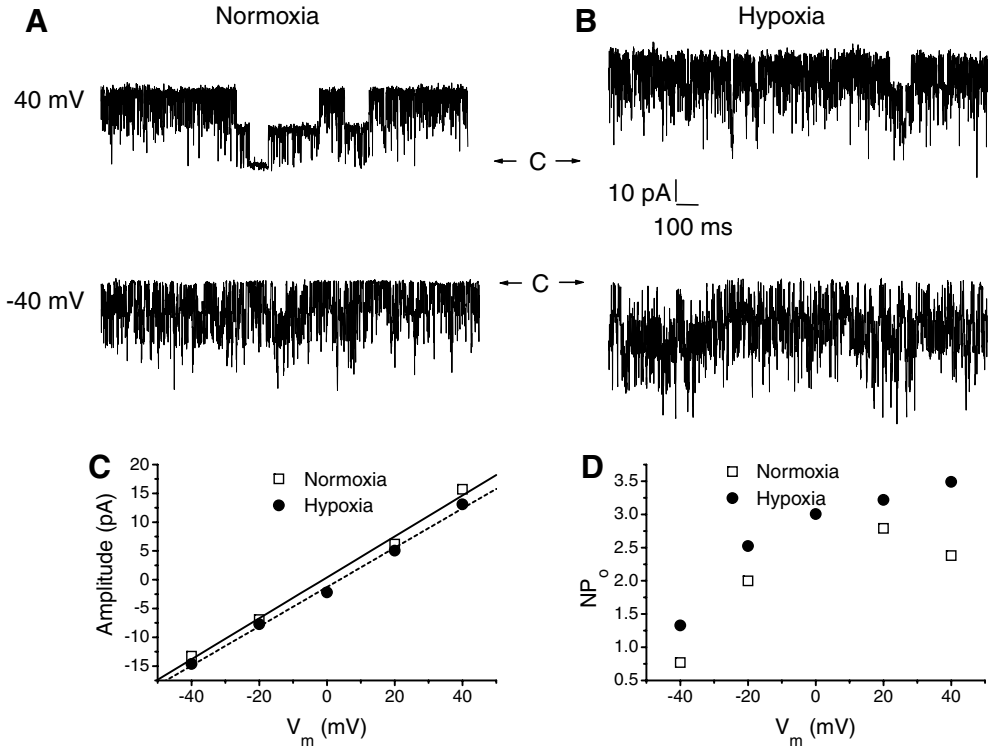

Fig. 4. Effect of hypoxia on single BK channel. Single-channel current traces at different V_m were plotted under normoxia (A) and hypoxia (B). (C) *I–V* curves of the single-channel amplitude at different V_m under normoxia and hypoxia. Curves were fitted by linear regressions. (D) NP_o of BK channel at different V_m under normoxia and hypoxia.

symmetrical K^+ solutions in our experiments, we found that conductance measurements yielded a straight line that passed by 0 mV.

We believe that mitochondrial BK channels open and promote K^+-ions efflux from the matrix to the outside during hypoxia. This conclusion is drawn from our experiments in which the reversal potential of mitochondrial BK shifted to a positive direction by hypoxia. Although the reversal potential was quite different among different patches, the trend during hypoxia was in the positive direction, indicating a K^+ efflux from the matrix. We therefore hypothesized that, during hypoxia, K^+ ions move out from the matrix to the intermembranous space. This K^+ efflux could be caused by two possible events: (1) an increase in mitochondrial Ca^{2+} concentration in the matrix [16,17] which can be the result of an increase in Ca^{2+} influx to the matrix via Ca^{2+} uniporter or a Ca^{2+} channel [18]. Such increase in Ca^{2+} concentration would likely increase the P_o of BK channels (Fig. 2). (2) An increase in K^+ efflux is due to mitochondrial depolarization induced by hypoxia [19], which, in turn, causes an increase in BK channel NP_o (Fig. 4D).

This response in open probability of the mitochondrial BK is totally different from that of the BK in the plasma membrane during hypoxia. Hypoxia has been reported to inhibit the BK channel activity in the plasma membranes of neurons [20,21], chemoreceptor cells [22], recombinant HEK-cells [23], alveolar epithelial cells [24], and myocytes [25]. We and others have also shown that hypoxia decreases the open probability in the cell-attached mode [20] and that hypoxia is without any effect on single-channel slope conductance [21–26]. Contrary to the hypoxic responses of BK in the plasma membrane, we observe here that the BK channel from the inner membrane of glioma mitochondria increases its open probability during hypoxia, while leaving its single-channel conductance unchanged. The reasons for this difference between plasma BK and mitochondrial BK in response to hypoxia might be related to (1) voltage differences between the two membranes [11]; (2) direct effect of hypoxia on membrane proteins [27]; or (3) Ca^{2+} levels inside the mitoplast as compared to regional Ca^{2+} levels in the cytosol [28,29].

In summary, we have found that (1) the K^+ concentration in the matrix was close to that of the cytosol; (2) BK channels in the inner mitochondrial membrane had similar single-channel properties as the BK in the plasma membrane; (3) BK channels in the inner mitochondrial membrane had a different response to hypoxia than BK in the plasma membrane.

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