Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2008;22:127-136

Accepted: June 09, 2008

# Hypoxia Increases Activity of the BK-Channel in the Inner Mitochondrial Membrane and Reduces Activity of the Permeability Transition Pore

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**Key Words**

BK-channel • Permeability transition pore • Hypoxia • Inner mitochondrial membrane • Mitochondrial membrane potential

**Abstract**

Hypoxia can cause severe damage to cells by initiating signaling cascades that lead to cell death. A cellular oxygen sensor, other than the respiratory chain, might exist in sensitive components of these signaling cascades. Recently, we found evidence that mitochondrial ion channels are sensitive to low levels of oxygen. We therefore studied the effects of hypoxia on the mitochondrial BK-channel (mtBK), on the mitochondrial permeability transition pore (PTP), and on their possible interaction. Using single-channel patch-clamp techniques we found that hypoxia inhibited the PTP but substantially increased the mtBK activity of mitoplasts from rat liver and astrocytes. Experiments measuring the mitochondrial membrane potential of intact rat brain mitochondria (using the fluorescence dye safranine O) during hypoxia exhibited an increased $Ca^{2+}$-retention capacity implying an impaired opening of the PTP. We also found a reduced $Ca^{2+}$-retention capacity with 100 nM iberiotoxin, a selective inhibitor of BK-channels. We therefore conclude that there is interaction between the mtBK and the PTP in a way that an open mtBK keeps the PTP closed. Thus, the response of mitochondrial ion channels to hypoxia could be interpreted as anti-apoptotic.



## Introduction

Cerebrovascular diseases (including stroke) are the third leading cause of death in the United States (150 074 cases in 2004) and in Germany (67 117 cases in 2005) [1, 2]. About the same number of patients will be left with severe and permanent disability. As known from stroke, ischemic-brain injury related hypoxia has dramatic effects on neuronal energy metabolism and on cell survival. Around a core region of ischemia, in which cells decay by necrosis, it extends the penumbra region in which cells die by hypoxia-induced apoptosis. It is this region in which efforts of neuroprotection could help to improve the patient´s outcome after stroke. An unconditional prerequisite, however, is a better understanding of the cellular events during hypoxia.

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A shortage of oxygen may cause alterations of cell signaling in several respects. Among others hypoxia could *i)* induce a hypoxia-inducible factor (HIF) [3], *ii)* cause production of excess reactive oxygen species (ROS) by the enzymes of the mitochondrial respiratory chain [4], *iii)* cause alterations in pH homeostasis [5], or *iv)* directly affect other elements of the signaling chains leading to different forms of cell death or to cell survival. A cellular oxygen sensor other than the respiratory chain might exist in components of these signaling cascades [6].

Ion channels of the mitochondrial membranes play a role in cell survival. The mitochondrial permeability transition pore (PTP) in the inner mitochondrial membrane is considered to trigger the intrinsic pathway of apoptosis which includes release of pro-apoptotic factors from the mitochondria [7, 8]. Thus knowledge of the behaviour of the PTP under hypoxia seems important for understanding the pathophysiological process of hypoxic-ischemic brain injury caused by stroke.

It has been suggested that the PTP is kept closed by open K-channels thus protecting the cell from apoptosis [9-11]. The large conductance $Ca^{2+}$-activated K channel of the inner mitochondrial membrane (mtBK) was first identified in 1999 [12]. It shows characteristics which are similar to the plasma-membrane channel. It has a cytoprotective role against infarction in cardiac cells and, if activated, contributes to $K^+$ efflux thus inducing cell survival [11]. Initial experiments on mitochondria of human glioma cells (LN229) demonstrated that hypoxia induced an increased open probability of the mtBK [13].

The aims of this paper are *i)* testing if hypoxia has similar effects on mtBK from mitochondria of a non-human, non-neoplastic cell line, *ii)* identifying the kinetic component responsible for channel activity, *iii)* testing for effects of hypoxia on the PTP, *iv)* proving the observed hypoxia modulation in intact rat-brain mitochondria, and finally *v)* exploring the interaction of mtBK and PTP in intact brain mitochondria. We thus intend to contribute to a better understanding of the neuroprotective effect of the mtBK.

## Materials and Methods

*Preparation of mitochondria from rat liver (RLM)*

Wistar rats 300 g (7 weeks old) were sacrificed as approved by Animal Health and Care Committee of the State of Sachsen-Anhalt, Germany. The liver was rapidly removed and washed twice in ice-cold isolation medium containing 250 mM sucrose (pH=7.4). The liver was homogenized in small pieces in a solution of 250 mM sucrose and 1 mM EDTA (pH=7.4) and centrifuged for 5 min at 800 × g. The supernatant was centrifuged for 4 min at 5100 × g. The pellet was resuspended in isolation medium, homogenized by hand in a 2-ml tight glass homogenizer, and centrifuged for 2 min at 12 300 × g. Resuspension and centrifugation were repeated for 10 min at 12 300 × g. The final pellet was resuspended in 1.5 ml solution with 250 mM sucrose and 0.5 mM EDTA (pH=7.4). Mitochondrial protein concentration was determined by the biuret method. All procedures were performed at 4 °C.

*Preparation of mitochondria from rat brain (RBM)*

Brain mitochondria were prepared from Wistar rats for the measurement of mitochondrial membrane potential. The rats were killed by decapitation, and the brain was rapidly removed and washed in ice-cold MSE-medium containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA and 5 mM HEPES (pH=7.4). After removing meninges and major blood vessels, the brain was minced with scissors, homogenized in 10 ml MSE-medium with 10 mg protease type I and 0.1% BSA and then diluted by 20 ml MSE-medium with 0.1% BSA. The suspension with the brain was centrifuged for 3 min at 2000 × g and the supernatant was centrifuged for 8 min at 12 000 × g. The pellet was resuspended in 10 ml MSE-medium with 6 mg digitonin and homogenized. After 10-min incubation at 4 °C and the centrifugation for 10 min at 12 000 × g, the resulting pellet was resuspended in 1.5 ml MSE-medium. Mitochondrial protein concentration was determined by the biuret method.

*Cell culture and preparation of astrocyte mitochondria*

Mitoplasts for patch-clamp experiments were prepared from rat astrocytes [14]. The cells were cultured in DMEM medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10 µg/ml tylosin at 37 °C in a humidified atmosphere with 5% $CO_2$. The cells were fed and reseeded every third day. The cells were harvested by centrifugation at 800 × g for 10 min, resuspended in the preparation solution for the mitochondria isolation (250 mM sucrose, 5 mM HEPES, pH=7.2) and homogenized. One fast (9200 × g, 10 min) and one slow (800 × g, 10 min) centrifugation step were performed to separate the fraction of purified mitochondria. Sucrose was removed by two further fast centrifugation steps (9 200 × g, 10 min) in the storage solution containing 150 mM KCl and 10 mM HEPES (pH=7.2). All procedures were performed at 4 °C.

*Patch-clamp experiments*

Mitoplasts were prepared from a sample of the RLM suspension put into a hypotonic solution containing 5 mM HEPES and 0.2 mM $CaCl_2$ (pH 7.2) for 45 s. Thereafter, adding a hypertonic solution composed of 750 mM KCl, 30 mM HEPES and 0.2 mM $CaCl_2$ restored isotonicity. The pipette was filled by an isotonic solution containing 150 mM KCl, 10 mM HEPES and 0.2 mM $CaCl_2$. Astrocyte mitoplasts were prepared in the same way. Reliability of the method in yielding unambiguously mitoplasts has been tested earlier in several ways, i) mitoplasts can be recognized by their absolutely round shape together with the presence of one or several black spots, called caps, only in phase contrast, ii) staining with a mouse anti-inner

mitochondrial membrane antibody, iii) staining and mitochondrial localization with an anti-BK channel antibody, and iv) occasional co-activity of the mtBK-channel with the mitochondrial PTP [11, 12].

For applying substances (iberiotoxin, Ibtx) from the membrane outside (inside of the patch pipette) to the ion channels we used the method of back-filling. The first part of the pipette tip was filled with control solution. Afterwards the second part of the pipette solution containing the test substance was added from the back by a syringe. Test solutions from the membrane outside (outside of the patch pipette) were added by a peristaltic-pump driven capillary-pipe system.

The single-channel current was recorded using an EPC-7 amplifier (HEKA electronics, Lambrecht, Germany). The currents were low-pass filtered at a corner frequency of 0.5 kHz and sampled at a frequency of 2.5 kHz by means of the pClamp 9.2 software (Axon Instrument, Foster City, CA, USA). Recording was made in mitoplast-attached mode with a pipette of borosilicate glass (Harvard Apparatus, Kent, UK) with a resistance of 10-20 MΩ measured in the above mentioned solutions.

*Data analysis*

Data were analyzed using the pClamp 9.2 software. Voltages in text and figures are all given as at the inner side of the mitoplasts versus outside. Inward currents were defined downward. The open probability ($P_o$) of the mtBK was determined by means of the single-channel search mode of the pClamp software. The open probability of PTP was determined by means of all-points analysis. For this purpose currents were recorded for one minute and analyzed point by point for the amplitudes drawn as histograms. Occupation of the open or closed states was determined as the area below the corresponding Gauss-curve and weighed by their corresponding current amplitude [15]. Occupation of the weighed open-states area divided by the total area weighed by the maximum-current amplitude gave the $P_o$. SEM was used throughout the paper unless otherwise specified. Significance was tested by Student´s t-test.

*Measurements of mitochondrial membrane potential*

Mitochondrial electrical membrane potential ($\Delta\Psi$) was estimated by the safranine O method [16]. Safranine O was used at 5 μM in experiments performed in Hansson medium containing 125 mM KCl, 20 mM Trizma base, 1 mM $MgCl_2$, 1 μM EGTA and 1 mM phosphate ($KH_2PO_4/Na_2HPO_4$) [17]. Accumulation of safranine O in mitochondria was driven by $\Delta\Psi$ and results subsequently in decrease of fluorescence. Depolarization results in the release of safranine O from mitochondria and a subsequent increase in fluorescence. Changes of safranine O fluorescence were recorded using a CARY Eclipse (Varian Instruments, Walnut Creek, CA, USA) fluorescence spectrophotometer operating at excitation and emission wavelengths of 525 nm and 587 nm.

*Materials*

Ibtx (a toxin from the red Indian scorpion Buthus tamulus), safranine O, EDTA, EGTA, HEPES, Trizma base, and protease type I were purchased from Sigma (Deisenhofen, Germany). BSA and digitonin were purchased from SERVA (Heidelberg, Germany). The other chemicals were obtained from Merck KGaA (Darmstadt, Germany).

## Results

*Identification of the mitochondrial BK-channel*

In order to test for the oxygen sensitivity of mitochondrial ion channels, we started recording single-channel currents from mitoplasts (i.e. inner mitochondrial membrane) of rat astrocytes by means of the patch-clamp technique. In about 1 out of 10 patches, the mean current amplitudes of channels was 13.1±1.3 pA (n=7) at a holding potential ($E_H$) of 40 mV and 4.6±1.3 pA at -20 mV. The channel activity was considerably reduced when the membrane-patch was achieved with a patch pipette that had been filled from the back with 100 nM Ibtx, a specific inhibitor of the $K_{(Ca)}$-channel of the BK-type (Fig. 1A). States of smaller current amplitude appeared occasionally and were identified as substates of the channel by their Ibtx sensitivity. Fig. 1B demonstrates the decline of $P_o$ by Ibtx. This decline was obvious throughout the potentials used. Fig. 1B shows additionally the voltage dependence of the channel with larger $P_o$-values at depolarizing potentials. Finally, Fig. 1C demonstrates the time course of the effects of Ibtx which is rather slow, a time period caused by the diffusion of the toxin through the interior of the pipette to the patch containing the mtBK.

The characteristics of the described single-channel activity, namely (i) a single-channel conductance of 296 ± 18 pS (n=7, see also Fig. 2B), (ii) voltage dependence with a larger $P_o$ at depolarizing potentials, (iii) $Ca^{2+}$-dependence (see next paragraph), and (iv) sensitivity to Ibtx, makes it very likely that it is caused by a $K_{(Ca)}$-channel of the BK-type as was described earlier by Siemen et al. [12] and Gu et al. [13].

*Increased mtBK activity in hypoxic solutions bubbled with nitrogen*

In order to test the effect of hypoxia on the mtBK of rat astrocytes we filled the flow system with isotonic solution (containing 1 μM $Ca^{2+}$) that had been bubbled with 100% nitrogen for 2 hours. The $O_2$ content of this solution had been determined to be 21.1±1.2 nmol/ml (n=5) in the closed chamber of a Clark-electrode (OROBOROS, Innsbruck, Austria) as compared with 222.3±13.1 nmol/ml after stirring the same solution for 17 min in the open chamber. Single-channel recordings in Fig. 2A demonstrate a considerable increase of $P_o$

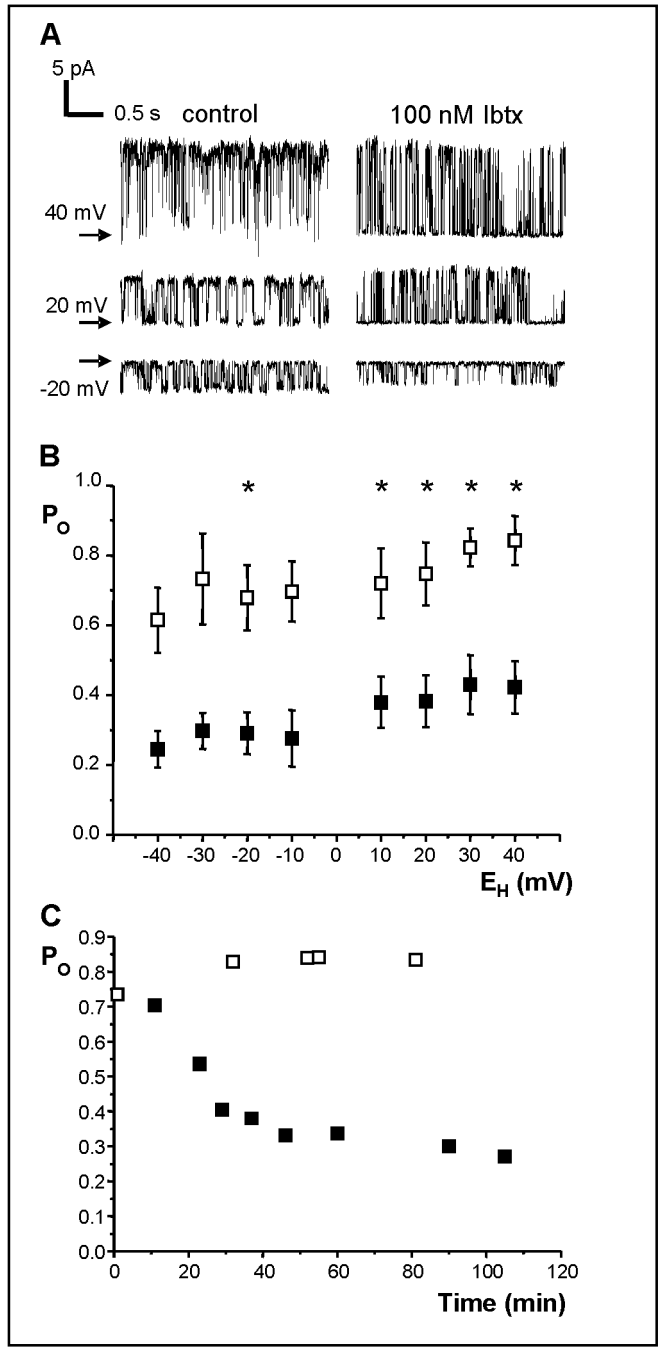


**Fig. 1.** Identification of the mtBK by blockade with iberiotoxin (Ibtx). A: Single-channel recordings of the mtBK of rat astrocytes in isotonic KCl-solution (control) and in the same solution 93 min later at a time at which (according to C) diffusion of 100 nM Ibtx to the tip of the patch pipette could be assumed to be complete (back-filling, right panel). Holding potentials as indicated. Arrows mark closed state of the channel. B: Ibtx-effect on the open probability ($P_o$) of the mtBK across the voltage range. $P_o$ before (open squares) and after (filled squares) Ibtx (100 nM) reached the channel. Under the influence of Ibtx the mean $P_o$ of the mtBK decreased by 54.2 ± 1.9% (3 independent experiments) at holding potentials between -40 mV and 40 mV (+40 mV, +30 mV, +20 mV, +10 mV, -20 mV: $P<0.05$). C: Decrease of $P_o$ (filled squares) reflecting the time course of diffusion of 100 nM Ibtx to the tip of the patch pipette ($E_H$ = +40 mV). Same experiment as in A, representative of 3 experiments with similar results. Control experiment without added Ibtx does not show any decrease of $P_o$ (open squares). For back-filling and determination of $P_o$ see methods.

measured in the hypoxic solution as compared with the control solution (both containing 1 µM $Ca^{2+}$). In comparison with Fig. 1A (left panel) where 200 µM $Ca^{2+}$ had been added to the isotonic solution, the left panel in Fig. 2A additionally demonstrates the earlier mentioned $Ca^{2+}$-sensitivity of the channel. At +20 mV the mean $P_o$ was reduced from 0.76±0.10 (±SD, n=3) to 0.06±0.02 (±SD; $P<0.01$, n=3) in solutions containing 200 µM $Ca^{2+}$ or 1 µM $Ca^{2+}$, respectively. Hypoxia does not modify the single-channel conductance (Fig. 2B) though the control after hypoxia in Fig. 2A shows an increased number of events of reduced amplitude. They represent an increased probability of the channel to occupy a subconductance state which appeared in some but not all experiments. Hypoxia does shift, however, the reversal potential by 5.0±0.4 mV (n=3) into the negative direction. This effect was partially reversible. We are not entirely sure if this is a true effect of hypoxia as earlier experiments in a different preparation and at 400 µM $Ca^{2+}$ had shown a shift by a few mV into the opposite direction [13].

The increase of mean $P_o$ (n=3) by hypoxia is quantified in Fig. 3. Additionally, panel A (with the absolute $P_o$-values) and panel B (with the normalized $P_o$-values) demonstrate an increased activity at depolarizing potentials. Even at very small $P_o$, the values measured in hypoxic solutions tended to be insignificantly larger than the controls. This becomes clearer if $P_o$ is drawn on a logarithmic scale (not shown, but compare Fig. 5B). $P_o$ was always insignificantly smaller in the control after hypoxia than in the control.

*Increased mtBK activity in dithionite-containing solutions*

Since we found that hypoxia induced by bubbling solutions with $N_2$ modified ion-channel activity we tested furthermore whether 1 mM sodium dithionite (DTN) containing solutions had the same effect. Again the activity of the mtBK increased reversibly (Fig. 4A). Fig. 4B shows the results of a dwell-time analysis for this experiment as histograms of a one-minute segment for each condition. The left panel shows the open-time distribution while the right panel demonstrates the closed-time distribution. A

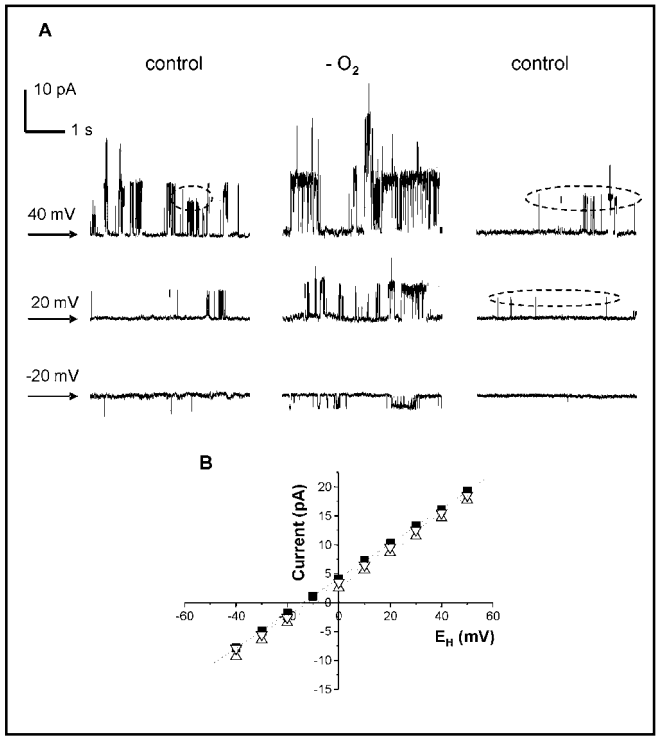


**Fig. 2.** Hypoxia increases $P_o$ of the astrocyte mtBK but does not change conductance. For testing the effect of hypoxia on the mtBK, we used a control solution (containing 1 µM $Ca^{2+}$ which may not be too far from the physiological $Ca^{2+}$ concentration) and a hypoxic solution (control solution bubbled with 100% nitrogen for 2 hours). A: Single-channel recordings at 3 different potentials demonstrate the increase of $P_o$. Arrows mark closed state of the channel. At least 2 channels were present in this patch. The channel occassionally switched to substates of smaller conductance (marked by circles) which were more frequent in the control after hypoxia in this patch. B: The slope of the current-voltage curves show that the conductance of the mtBK was not changed by hypoxia (one out of 3 experiments). The mean reversal potential is at -5.5 ± 2.2 mV in control solution (open triangle, tip up). Hypoxia shifted the reversal potential by 5.0 ± 0.4 mV (n=3) to the negative direction (filled squares). This shift was partially reversibly in control solution (open triangle, tip down).

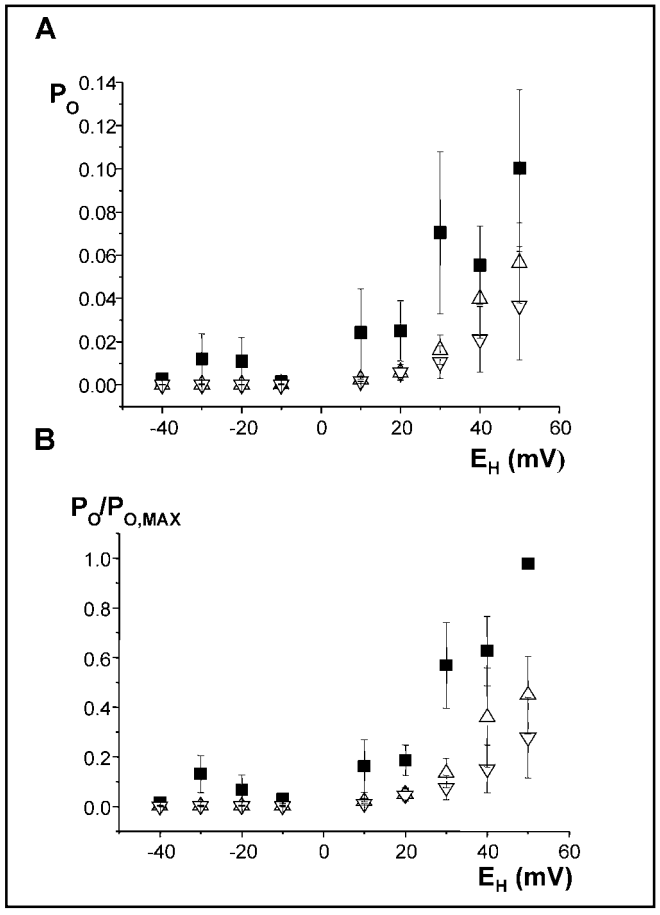


**Fig. 3.** The increase of $P_o$ of the mtBK by nitrogen-induced hypoxia becomes more obvious at larger $P_o$-values, i.e. at more positive voltages. A: Increased $P_o$ in hypoxic solution (filled squares) as compared with control solution (before hypoxia: open triangle, tip up, and after hypoxia: open triangle, tip down), particularly at positive $E_H$ (n=3). Control solution and hypoxic solution contained 1 µM $Ca^{2+}$ so that the $Ca^{2+}$-activated channel showed little activity only ($P_o$ smaller than 0.15 at all $E_H$ and almost 0 at negative $E_H$). (Note larger $P_o$ induced by 200 µM $Ca^{2+}$ in Fig 1B for comparison). B: $P_o$ normalized to largest $P_o$ of the experiment ($P_o/P_{o,max}$). Hypoxia values differed significantly to control values before at 30 mV and at 50 mV ($P<0.05$) and to control values after hypoxia at 20 mV and 30 mV ($P<0.05$), at 40 mV ($P<0.02$), and at 50 mV ($P<0.01$). Error bars give SD.

least-square fit of the histogram gave best results with two exponentials for the open times and three exponentials for the closed times. The resulting time constants (τ-values) are collected in Table I.

Hypoxia reversibly increases the second open-time constant and it irreversibly increases the slowest closed time constant. $P_o$ in 1 mM DTN resulted in larger values as compared with control solutions without DTN (n=4; absolute values in Fig. 5A). Again the $P_o$-values were larger in the depolarizing range and the controls after DTN were smaller than the controls before (demonstrated on logarithmic scale in Fig. 5B). Data can be fitted by a straight line the steepness of which did not change in DTN. An e-fold change of $P_o$ was caused by a change of $E_H$ of 13.2 mV (before), 13.6 mV (1 mM DTN), and 9.9 mV (control after).

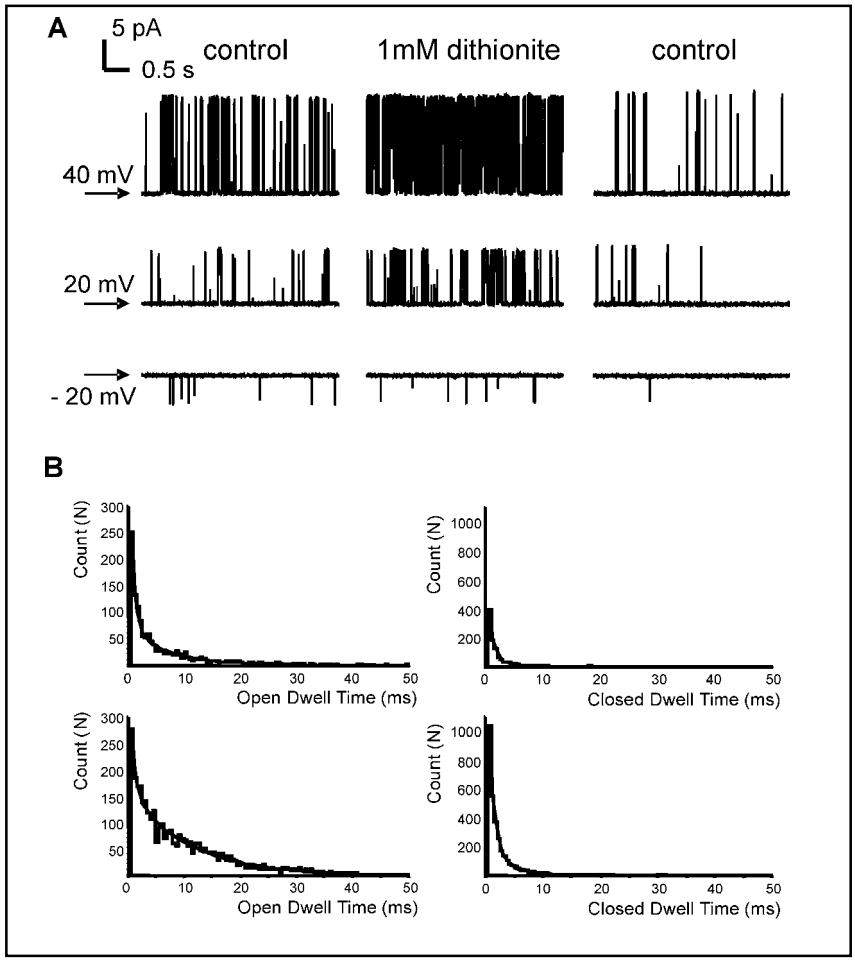


**Fig. 4.** DTN-induced hypoxia increases activity of the mtBK. A: Single-channel recordings of mtBK before, during, and after application of 1 mM DTN solution are shown at 3 different $E_H$. $P_o$ was remarkably increased in DTN solution. Arrows mark closed state of the channel. B: Corresponding open–time histograms (left) and closed-time histograms (right) of the same experiment as in A at 40 mV before (upper part) and during (lower part) application of DTN solution as determined by all-points analysis. Distributions of open-time histograms were fitted by two exponentials, distributions of closed-time histograms by three exponentials. Time constants given in Table I.

*Hypoxia reduces activity of the permeability transition pore*

It is our working hypothesis that mitochondrial K-channels are keeping the permeability transition pore (PTP) closed. Therefore, we tested if hypoxia has an effect on the PTP itself. We recorded single-channel current of the PTP from rat liver mitochondria in isotonic solutions with 1 mM DTN (n=4) or bubbled for 2 h by $N_2$ (n=4) and the respective controls before and after hypoxia. The PTP showed, as usual, very large conductance (>1 nS) with several sub-conductance states and could be inhibited by the specific inhibitor cyclosporine A (CSA; see [18] for the complete concentration-response curve) but was neither affected by 100 nM Ibtx (n=4, not shown), nor spontaneously on its own (tested for more than 24 min). Conductance and appearance of sub-states was unchanged in hypoxic solutions as compared with control solution (both containing 200 µM $Ca^{2+}$ for activating the PTP, Fig. 6A, C). However, the mean $P_o$ of the PTP was irreversibly and significantly decreased by 57% in DTN (0.45 ± 0.04, 0.19 ± 0.02, and 0.22 ± 0.03 before, during and after DTN solution), respectively (n=4)

| | control (before) | 1 mM DTN |
|---|---|---|
| open time constant (τ) | $\tau_1$ = 0.70 ms | $\tau_1$ = 0.82 ms |
| | $\tau_2$ = 7.63 ms | $\tau_2$ = 10.80 ms |
| closed time constant (τ) | $\tau_1$ = 0.06 ms | $\tau_1$ = 0.24 ms |
| | $\tau_2$ = 1.05 ms | $\tau_2$ = 1.27 ms |
| | $\tau_3$ = 6.68 ms | $\tau_3$ = 8.16 ms |

**Tab. 1.** The time constants of the mtBK in control and under DTN ($E_H$ = 40 mV).

or insignificantly by 44% in $N_2$-bubbled solution. 0.41± 0.08, 0.23 ± 0.07, and 0.16 ± 0.12 are the respective values (n=4, Fig. 6B).

*Effects of hypoxia can be reproduced on intact brain mitochondria*

Finally, we had to ensure that the effects of hypoxia measured in patch-clamp experiments on mitoplasts from RLM and from rat astrocytes, are representative for the situation in intact RBM. Therefore, the mitochondrial membrane potential was measured by application of

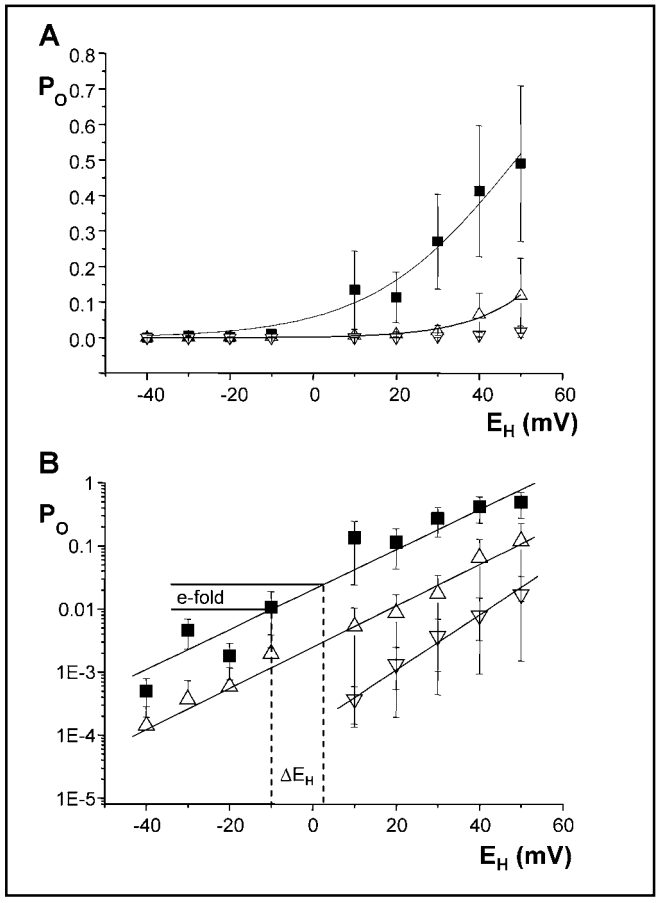


**Fig. 5.** $P_o$ of the mtBK was increased by DTN-induced hypoxia (n=4). A: $P_o$ in 1 mM-DTN solution (filled squares) was larger than in control solution (before hypoxia: open triangle, tip up, and after hypoxia: open triangle, tip down) at an $E_H$ ranging from -40 mV to 50 mV. Continuous curve fitted by Boltzmann equation. B: Same data on logarithmic scale demonstrate increase of $P_o$ also at negative potentials. Data can be fitted by a straight line. Increase of the holding potential ($\Delta E_H$) that induces an e-fold change of $P_o$ is a measure for the voltage dependence of the channel which is unchanged by DTN. Error bars give SD. Shift of the fits for control data (before) and for DTN data was -25 mV (determined at $P_o$ = 0.5; fits differed slightly in steepness) in A and -29 mV in B, respectively.

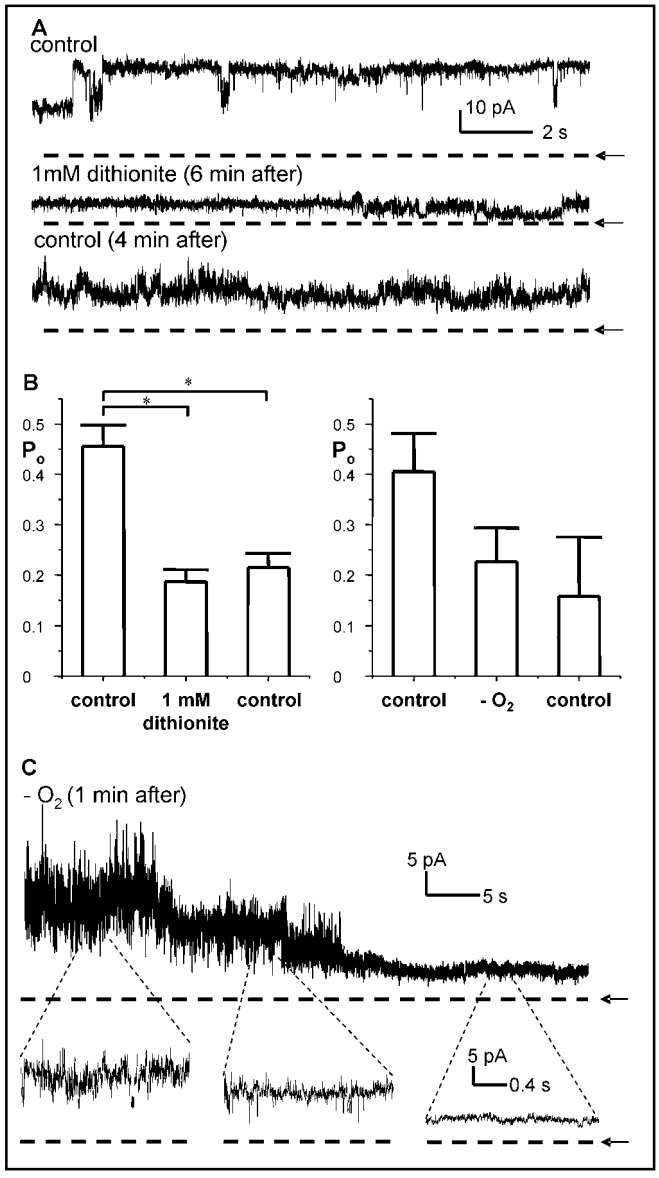


**Fig. 6.** Hypoxia inhibits the PTP of rat liver mitochondria irreversibly. A: Single-channel current through the PTP at $E_H$ = 20 mV before, during, and after application of 1 mM DTN by the flow system. Closed states marked by dotted lines. B: $P_o$ of the PTP at $E_H$ = 20 mV before, during and after hypoxia established by 1 mM DTN (left panel, *: P<0.05, n=4) or by switching to hypoxia (right panel, P=0.07, n=4). Error bars give SEM. C: Single-channel current trace recorded when hypoxia (by $N_2$-bubbling) became effective inhibiting the pore. Short segments of the trace are shown below at an enlarged time scale. Dashed line indicates fully closed state of the PTP.

safranine O to intact RBM. After addition of mitochondria to the cuvette, safranine O was accumulated in mitochondria by negative membrane potential decreasing the fluorescence signal (Fig. 7). Addition of $Ca^{2+}$ caused depolarization of the mitochondrial membrane. It turned out by titration with aliquots of 50 µM $Ca^{2+}$ in Hansson medium that 200 µM were able to induce depolarization of the mitochondrial membrane (Fig. 7A). In control experiments with 10 µM CSA, application of 400 µM $Ca^{2+}$ was necessary to induce a depolarization making it likely

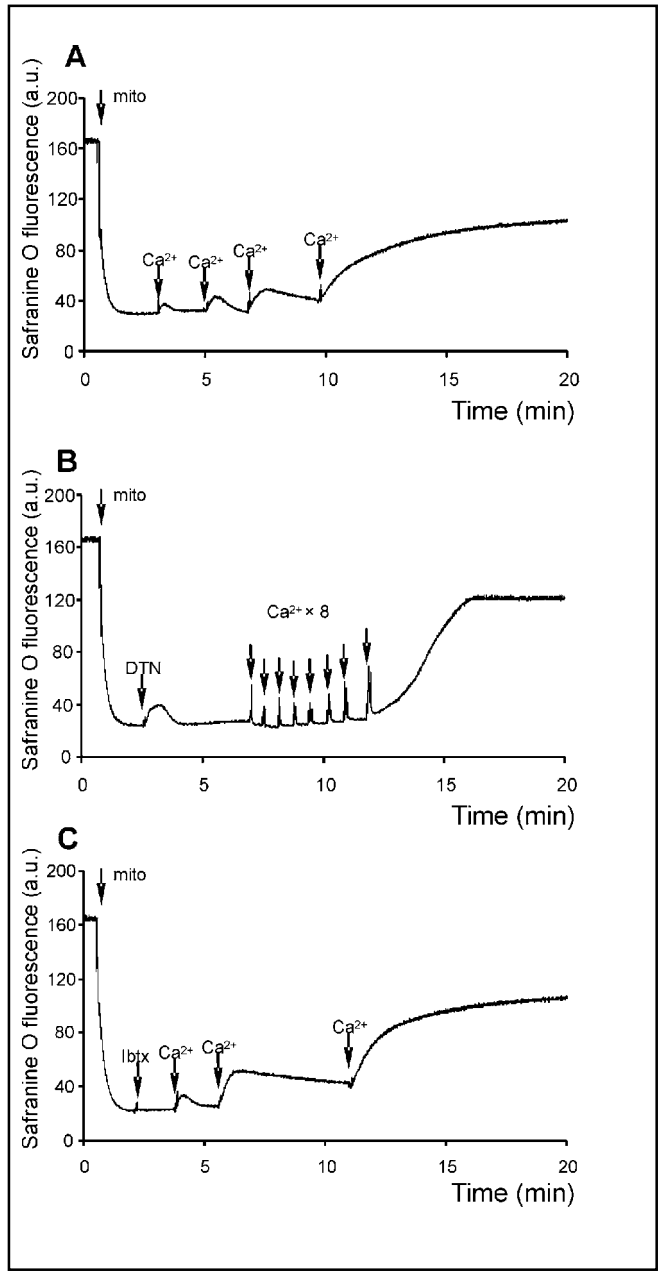


**Fig. 7.** Recording of membrane potential of intact rat brain mitochondria by means of potential-dependent safranine O uptake (in arbitrary units) demonstrates impaired opening of the PTP in DTN and improved opening after Ibtx-blockade of the mtBK. Increased extra-mitochondrial dye corresponds to depolarization. In control experiment (A) depolarization of the mitochondrial membrane was induced by 4 times application of 50 µM $Ca^{2+}$. After addition of 1 mM DTN more $Ca^{2+}$ (8 times application of 50 µM, B) was required to induce depolarization. After addition of the mtBK inhibitor Ibtx (100 nM) less $Ca^{2+}$ (3 times application of 50 µM, C) was required to induce depolarization. Each panel stands for 5 comparable experiments from different preparations with similar results.

that the decline in membrane potential was due to $Ca^{2+}$-activation of the PTP (not shown). After addition of 1 mM DTN also 8 aliquots of $Ca^{2+}$ corresponding to 400 µM were required to induce a continuing depolarization (Fig. 7B). After application of the BK-channel inhibitor Ibtx (100 nM), only 150 µM $Ca^{2+}$ were required to induce depolarization of mitochondrial membrane (Fig. 7C). The corresponding mean values of 5 experiments from 5 independent preparations were for the controls, 1 mM DTN, and 100 nM Ibtx: 190 ± 10 µM, 280 ± 33 µM ($P<0.05$), and 140 ± 10 µM ($P<0.01$), respectively. In summary, DTN increased the concentration of $Ca^{2+}$ required for opening of the PTP and the BK-channel inhibitor Ibtx reduced the $Ca^{2+}$ required for opening of the PTP.

## Discussion

Cells respond to hypoxia with pronounced responses, all of them leading to different forms of cell death. Sequentially, hypoxia and hypoxia-induced energy failure disrupt pH and ionic homeostasis [5]. However, little is known about the cellular sensors for hypoxia. Next to the enzymes of the mitochondrial respiratory chain and factors like HIF, other oxygen-depending systems have to be expected to account for the complex answers after e.g. ischemia/reperfusion injury or ischemic preconditioning. We therefore tested the response of two ion channels that were shown to be involved in the apoptosis signaling cascade and led to cell protection in animal models of stroke and cardiac infarction, namely the mtBK and the PTP [11, 15]. It was suggested that hypoxia inhibits the BK-channel in the plasma membrane [19], and hemoxygenase-2 (HO-2) which is part of the channel complex is involved in inhibition [6]. However, we find here that the mtBK activity was increased by hypoxia (Fig. 3) and DTN (Fig. 5) in astrocytes, similar to the situation in mitochondria of glioblastoma cells [13], while the PTP was inhibited (Fig. 6).

Hypoxia can be induced experimentally either chemically by application of an oxygen-quenching substance like DTN, or by carefully removing the oxygen contained in the solutions by extended bubbling of the solutions with nitrogen. For the single-channel experiments we used both methods. The DTN-method was preferred in intact mitochondria for technical considerations.

The use of cyanide did not seem appropriate for this study, as it would just inhibit oxygen binding to the heme-containing enzymes of the electron transport chain even at a sufficient oxygen supply. Therefore, cyanide leads to a mere shortage in ATP supply.

The increase of $P_o$ of the mtBK under DTN-induced hypoxia was larger than under hypoxia established by $N_2$. A possible explanation could be that $O_2$ in the DTN solutions was more completely deprived than in the $N_2$ solution. At room temperature (20 °C) the solubility of oxygen in water is 7.6 mg/l which is 0.24 mM so that one would need at least 0.48 mM DTN to bind the $O_2$ present in the solution (we used 1 mM DTN). Another possibility is that not only the lack of $O_2$ but also DTN itself can activate the mtBK by chemical interaction. As DTN is known to form free radicals ($H_2O_2$) [20], and as oxygen radicals are known to have an activating influence on PTP, it could well be that the DTN-induced radicals would modify the mtBK. Therefore, the single-channel experiments were always performed in either way, using $N_2$-bubbled solutions or applying DTN. However, free oxygen radicals are known to open the PTP. The outcome of either type of experiments is thus in accordance with an inhibiting influence of hypoxia on the PTP in that the PTP-activity is reduced directly or by activation of the mtBK. We hesitate to speculate how a reduced oxygen concentration could modify the gating mechanism of an ion channel. Our experimental evidence does not allow distinguishing between a negative allosterical effect e.g. of the free electrons of the oxygen molecule with the channel or a redox-effect at the voltage sensor of the channel.

Ischemia does not seem to cause PTP-opening, possibly due to the increased intracellular $H^+$-concentration [for review: 21]. However, reperfusion could be an ideal condition for PTP opening as respiration is recovering at both, high $Ca^{2+}$- and $P_i$-concentrations. Therefore, Bernardi et al. [21] state that the PTP should be considered as a target for protecting cells from reperfusion injury. Our patch-clamp conditions do not allow testing this statement as most of the mitochondrial membrane potential and probably many metabolites are lost in mitoplasts but it allows testing effects of hypoxia directly at the pore and at the mtBK controlling it. Interestingly, both channels respond to hypoxia in a way that would seem useful in terms of cell protection during ischemia, i.e. increasing the $P_o$ of the mtBK and closing of the pore. Also the $mtK_{ATP}$-channel seems to exert a protective effect on the PTP [9,22].

The experiments of Fig. 7 on intact mitochondria can answer some of the open questions. They show that a certain amount of $Ca^{2+}$ was required to induce a deregulation of the mitochondrial $Ca^{2+}$-content by opening the PTP as measured by a depolarization of the mitochondria. Under the influence of DTN-induced hypoxia there may appear a small hyperpolarization and more $Ca^{2+}$ was required to open the pore. Inhibiting the PTP by CSA increased $Ca^{2+}$ necessary for deregulation. Thus, the latter could be an indication of a few open PTPs which were closed by DTN-induced hypoxia. From these experiments it could not be concluded if the mtBK contributes to the increased demand for $Ca^{2+}$ to open the pore in hypoxia. This was answered by adding the specific BK-channel blocker Ibtx which by itself reduced the $Ca^{2+}$ required for deregulation.

We thus conclude from our single-channel experiments that hypoxia is able to increase the activity of the mtBK and to inhibit the PTP. Our potential measurements on intact mitochondria make it likely that these events appear in intact mitochondria as well, and that there is interaction of the mtBK with the PTP according to the hypothesis that an open mtBK tends to keep the PTP closed [11]. These observations would explain cell protective effects of BK-channel opener und PTP-inhibitors observed earlier [23, 24].

## Abbreviations

mtBK (big conductance potassium channel of the inner mitochondrial membrane); CSA (cyclosporine A); DTN (sodium dithionite); $E_H$ (holding potential); FCS (fetal bovine calf serum); HIF (hypoxia inducible factor); Ibtx (iberiotoxin); MSE (mannitol-sucrose-EGTA); $P_o$ (probability of being in the open state); PTP (permeability transition pore); RBM (rat brain mitochondria); RLM (rat liver mitochondria); ROS (reactive oxygen species).

## Acknowledgements

We are grateful to C. Höhne, K. Kaiser, and J. Witzke for excellent technical support. We are indebted to H. Goldammer and D. Koch for providing rat livers for dissection of mitochondria and to A. Szewczyk for reading the manuscript. Financial support by the State of Sachsen-Anhalt, and by the BMBF to D.S., by an EMBO Short Term Fellowship to P.B. (ASTF 114-06), and by NIH (PO1 HD-32573, RO1 NS-35918, and RO1 HL-66327 to GGH) is gratefully acknowledged.

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