Mitochondrial but not Plasmalemmal BK Channels are Hypoxia-Sensitive in Human Glioma

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Tumor cells are resistant to hypoxia but the underlying mechanism(s) of this tolerance remain poorly understood. In healthy brain cells, plasmalemmal Ca^{2+}-activated K^+ channels ($_{plasma}$BK) function as oxygen sensors and close under hypoxic conditions. Similarly, BK channels in the mitochondrial inner membrane ($_{mito}$BK) are also hypoxia sensitive and regulate reactive oxygen species production and also permeability transition pore formation. Both channel populations are therefore well situated to mediate cellular responses to hypoxia. In tumors, BK channel expression increases with malignancy, suggesting these channels contribute to tumor growth; therefore, we hypothesized that the sensitivity of $_{plasma}$BK and/or $_{mito}$BK to hypoxia differs between glioma and healthy brain cells. To test this, we examined the electrophysiological properties of $_{plasma}$BK and $_{mito}$BK from a human glioma cell line during normoxia and hypoxia. We observed single channel activities in whole cells and isolated mitoplasts with slope conductance of 199 ± 8 and 278 ± 10 pA, respectively. These currents were Ca^{2+}- and voltage-dependent, and were inhibited by the BK channel antagonist charybdotoxin (0.1 μM). $_{plasma}$BK could only be activated at membrane potentials $>+40$ mV and had a low open probability (NP_o) that was unchanged by hypoxia. Conversely, $_{mito}$BK were active across a range of membrane potentials (-40 to $+40$ mV) and their NP_o increased during hypoxia. Activating $_{plasma}$BK, but not $_{mito}$BK induced cell death and this effect was enhanced during hypoxia. We conclude that unlike in healthy brain cells, glioma $_{mito}$BK channels, but not $_{plasma}$BK channels are oxygen sensitive.

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Introduction

Cancer is the second leading cause of death in the United States (Jemal et al., 2010). Within the core of tumors, cancerous cells typically experience persistent hypoxic or anoxic environments due to irregular vascularization. Tumor cells are remarkably tolerant to low oxygen stress and thrive in hypoxia. Indeed, hypoxic cells in solid tumors are particularly resistant to anticancer drugs and radiation therapy (Gali-Muhtasib et al., 2002), making them critical targets in the study of anticancer treatments. Tumor cells express a variety of ion channels that have been linked to cancerous cell proliferation and malignant tumor progression (Fiske et al., 2006); however, few of these channels act as direct oxygen sensors. One particular channel, the Ca^{2+}-activated and voltage-dependent K^+ channel (BK channel), is sensitive to hypoxia and may play a key role in tumor progression (Weaver et al., 2006). BK channels are found on the plasma ($_{plasma}$BK) and inner mitochondrial membranes ($_{mito}$BK) of healthy nervous cells and also cancerous cells (Bordey and Sontheimer, 2000; Douglas et al., 2006; Seidel et al., 2011; Siemen et al., 1999; Sontheimer, 1994; Weaver et al., 2006), and differences in the response of either population of these channels to hypoxia may contribute to differences in sensitivity to hypoxic environments between healthy and cancerous cells. Indeed, $_{plasma}$BK expression is upregulated in biopsies from

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human gliomas and this change correlates with the malignancy grade of tumors (Liu et al., 2002). Based on this observation, BK channels have been suggested to play a leading role in tumor metastasis (Sontheimer, 2008); however, the mechanism via which they contribute to tumor formation and survival remains undiscovered.

In healthy cells, BK channels function as oxygen sensors. For example, in neocortical neurons $_{plasma}$BK activity is significantly inhibited by hypoxia (Liu et al., 1999). This sensitivity is thought to mediate key signaling pathways that communicate local changes in oxygen availability to cells. Conversely, $_{mito}$BK are activated under hypoxic conditions and this contributes to hypoxia/ischemia tolerance by delaying the opening of the mitochondrial permeability transition pore, a key stage in mitochondrial apoptosis (Cheng et al., 2008). Furthermore, in cardiac and brain models of hypoxia tolerance, activation of $_{mito}$BK has been linked to cytoprotective reductions in reactive oxygen species generation and reduced Ca^{2+} accumulation during anoxia, hypoxia, or ischemia (Gaspar et al., 2009; Heinen et al., 2007; Kulawiak et al., 2008; Pamenter et al., 2008).

In human glioma cells, $_{plasma}$BK have a slope conductance that is similar to that of $_{plasma}$BK found in healthy tissues (Ransom and Sontheimer, 2001); however, they also display unique characteristics. For example, glioma $_{plasma}$BK exhibit unusually high sensitivity to Ca^{2+} (Ransom et al., 2002) and contain an additional 34-amino-acid exon at splice site 2 in the C-terminus (Liu et al., 2002). We reported previously that $_{mito}$BK from human glioma cells are activated by hypoxia (Gu et al., 2007), but the response of glioma cell $_{plasma}$BK to hypoxia has not been assessed. Since tumors are generally very tolerant of hypoxia, we hypothesized that glioma BK channels may have altered sensitivity to hypoxia relative to BK channels in healthy brain cells. This variability may contribute to divergent tolerance to hypoxia between tumor cells and healthy brain cells. As a first step towards delineating a potential role for BK channels in the response to hypoxia of tumor cells, we asked if mitochondrial and plasmalemmal glioma BK channels function similarly to channels found in healthy brain tissue. Using human gliomas cells, the aims of the present study were to (i) examine the effects of hypoxia on $_{plasma}$BK, (ii) confirm our previous examinations of the effect of hypoxia on $_{mito}$BK, (iii) compare the kinetic components of channel activity between these two populations of BK channels, and (iv) investigate the impact of $_{plasma}$BK or $_{mito}$BK modulation on glioma cell viability during normoxia and hypoxia.

Methods

Cell Cultures

We used the human glioma cell line LN 229 (ATCC, Manassas, VA) since these cells express BK channels in their plasma membrane and mitoplasts (Siemen et al., 1999). Cells were grown in DMEM supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 50 IU/mL Penicillin, and 50 µg/mL streptomycin (Sigma, St. Louis, MO), in a 37°C incubator with 5% CO_2. Cells were split and reseeded once per week and culture media was changed twice per week. Samples were treated as specified below. Chemicals were purchased from Sigma unless otherwise indicated. BMS-191011 was purchased from Tocris (Bristol, UK).

Mitoplast Preparation

Mitochondria were separated and harvested after several centrifugation steps as described previously (Borecky et al., 1997), and stored at 4°C for up to 36 h (in storage solution; in mM: 150 KCl, 20 K-HEPES, and 1 K-EGTA). 10 μL of suspension was added to 2 mL of a hypotonic solution (in mM: 5 K-HEPES and 1 $CaCl_2$) to induce swelling, following which the outer membrane was broken. Hypertonic solution (0.5 mL, in mM: 750 KCl, 80 K-HEPES, and 1 $CaCl_2$) was then added to restore isotonicity.

Electrophysiology

All experiments were performed at room temperature (22–25°C). We recorded BK channel activity at LN229 plasma- and inner-mitochondrial membranes. For both experimental approaches, symmetrical solutions were used with pipette and external bath containing 1.0 mM Ca^{2+} and 171 mM K^+, unless otherwise indicated. For the 0 mM Ca^{2+} solution containing (in mM): 150 K^+, 5 K-HEPES, 0 Ca^{2+}, 15 K-EGTA (pH 7.4 with Tris base), the solution change was made in a microchamber with the recording pipette placed at the opening of the chamber. Mitoplasts were typically <1 μm in size and were visually identified as having one or more black caps on the surface, believed to be the contact points between inner and outer membranes of mitochondria (Sorgato et al., 1987).

Patch-clamp experiments were performed using an Axopatch 1C amplifier (Molecular Devices, Downingtown, PA, USA) or an EPC-7 amplifier (HEKA, Lambrecht, Germany). Recordings were made in mitoplast-attached or cell-attached, inside-out, or outside-out modes using electrodes pulled from borosilicate glass (WPI, Sarasota, FL) using a Sutter puller (P-87, Sutter instruments, Navato, FL). Electrodes typically had a resistance of 10–25 MΩ for on-mitoplast experiments and 5–8 MΩ for cell-attached experiments, respectively. The bath solution contained (in mM): 150 KCl, 20 K-HEPES with different Ca^{2+} concentrations. In some experiments, cells were treated with the Ca^{2+} ionophore ionomycin (1 µM) to increase the $[Ca^{2+}]$ in the cytosol and permit us to examine the impact of higher intracellular Ca^{2+} on $_{plasma}$BK activity in normoxia and hypoxia.

The pipette solution contained (in mM): 150 KCl, 20 K-HEPES 0 $CaCl_2$, and 1 K-EGTA. pH was adjusted to 7.2. 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). Plasma membrane BK recordings were low-pass filtered at a corner frequency of 2 kHz and sampled at a frequency of 4 kHz and recorded on hard disk. Some mitoplasts 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 analyzed

using pClamp 6 and pClamp 9 software (Molecular Devices, Downingtown, PA). Voltages are reported as at the inner side of the mitoplasts minus outside and inward currents always deflect downward. The open probability (NP_o) was obtained from pClamp 10 as $NP_o = T_o/(T_o + T_c)$, where N is the number of channels in the patch and $T_o = \Sigma L t_o$ where L is the level of the channel opening and t_o is the total time that the channel is in the open state.

ATP Luciferase Assay

Total ATP content [ATP] was assessed in solid-bottom, black 96-well microplates (Corning) using PerkinElmer ATPlite Luminescence Assay System kits as specified by the manufacturers protocol (PerkinElmer, MA, USA) and a Bio-Tek PowerWave 340 microplate spectrophotometer (Bio-Tek, Winooski, VT). Equal numbers of cells were seeded into each well (~50,000 cells/well). [ATP] was assessed following 12 and 24 h treatment in normoxia (21% O_2 or hypoxia (1% O_2) alone, or in combination with either the $_{mito}$BK-specific agonist NS-1619 (Lee et al., 1995) or the $_{plasma}$BK-specific agonist BMS-191011 (Hewawasam et al., 2003) (10 μM each). Standard curves were generated using serial dilutions of a known ATP standard provided in each kit. The sensitivity of the detector was calibrated to the luminescence of the highest [ATP] standard in each experiment. The results were normalized to ATP luminescence recorded from control cells assayed at $t = 0$ h. Microplate ATP luciferase experiments were repeated 5 times in parallel, and each plate contained at least 16 replicate wells of each treatment group. Blank wells and cell-free wells containing media-alone were also included on each plate, and the final data is corrected for these factors.

Propidium Iodide Exclusion Membrane Viability Assays

Membrane viability was assessed following 12 and 24 h treatment as the ability of cells to exclude the vital dye propidium iodide (PI). PI exclusion was assessed using a high-throughput 96-well microplate-based assay as described for the ATP luciferase assay (above). PI uptake was assessed at Ex/Em: 485/630 nm and analyzed using Gen 5 software (Bio-Tek).

Statistics

Data were analyzed using a two-tailed Student's t test. Significances were indicated if $P < 0.05$ assuming two groups had an equal variance. Statistical analysis was performed using Originlab Origin (Northampton, MA). Curves were fitted using the Boltzmann equation: $NP_o = NP_{o,\ max}/(1 + e^{(V_{50} - V/V_c)})$, where NP_o is open probability, V is membrane potential, V_{50} is the potential at which the current is half maximal, and V_c is the voltage required to change g e-fold.

Results

We recorded channel activity from LN229 plasma- and inner-mitochondrial membranes using the on-cell and on-mitoplast patch clamp configurations, respectively. Patches of plasma membrane preparations frequently contained multiple channels, whereas mitoplast patches rarely had more than one channel in any given recording. The mean slope conductance

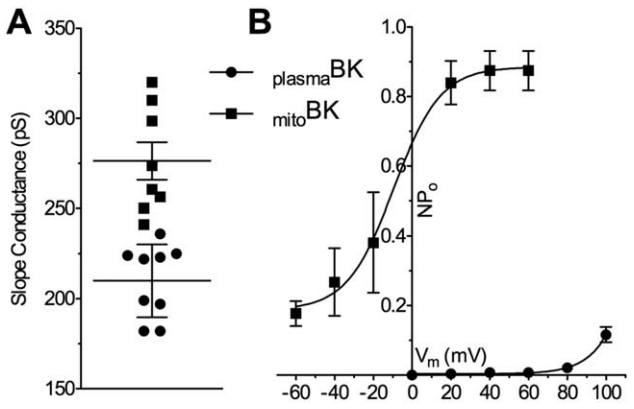

FIGURE 1: Mitochondrial and plasmalemmal BK channels have different slope conductance and open probability (NP_o) characteristics. (A) Comparison of single channel conductance between $_{mito}$BK and $_{plasma}$BK. Measurements were made using symmetrical 171 mM KCl in the pipette and bath solutions. (B) Comparison of NP_o of $_{mito}$BK and $_{plasma}$BK in normoxia. Data are mean ± SEM from 4–8 separate experiments for $_{mito}$BK and 3–13 experiments for $_{plasma}$BK.

of plasmalemmal channels was 199 ± 8 pS ($n = 13$), and conductance ranged from 125 to 236 pS (Fig. 1A). Conversely, the mean slope conductance of mitochondrial channels was 278 ± 10 pS ($n = 8$), and ranged from 241 to 320 pS (Fig. 1A). In the on-mitoplast configuration, channel activity could be readily observed at membrane potentials ranging from −40 to +40 mV (Fig. 2A, $n = 4$). Conversely at the plasma membrane, channel activity was rarely observed at membrane potentials below +40 mV, while even at the highest membrane potential tested (+100 mV), the $_{plasma}$BK NP_o remained low (0.12 ± 0.02, $n = 13$). When this data was fit to a voltage–NP_o curve using the Boltzmann's equation, the midpoint values were +109.5 mV ($n = 3$–13) and −11.4 mV ($n = 4$–8) for plasmalemmal and mitochondrial channels, respectively (Fig. 1B). Slope factor, on the other hand, was similar for the curves from channels in mitoplasts (9.8) and in plasma membranes (9.5). These values are in good agreement with previous electrophysiological examinations of BK channels in the mitochondrial and plasmalemmal membranes of healthy brain cells and glioma, (Cheng et al., 2008; Gu et al., 2007; Liu et al., 1999; Ransom and Sontheimer, 2001; Ransom et al., 2002, 2003; Siemen et al., 1999), and therefore we hypothesized that the channels we recorded from were also BK channels.

To better characterize the channels recorded from the two preparations as BK channels, we next examined channel activities at different membrane potentials and Ca^{2+} concentrations ($[Ca^{2+}]$) (Fig. 2). The Ca^{2+} dependence of $_{plasma}$BK was studied using the inside-out configuration to allow control of Ca^{2+} concentrations on the cytosolic face of BK channels. Mitochondrial BK channels were assessed in the on-

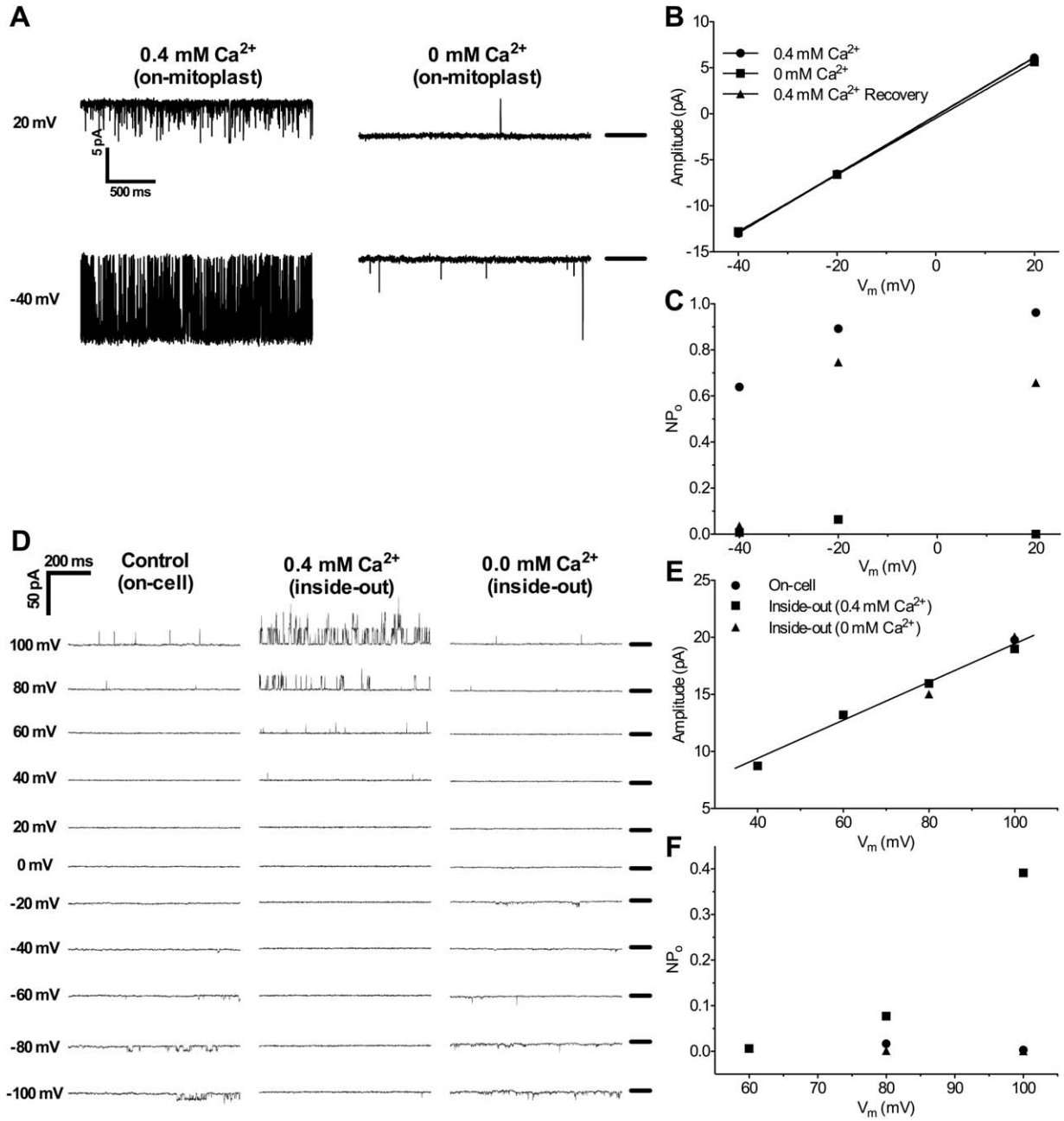

FIGURE 2: Mitochondrial and plasmalemmal BK channel activity is Ca^{2+}-dependent. (A) Raw sample recordings of $_{mito}$BK activity. Reducing bath [Ca^{2+}] from 0.4 to 0 mM reduced single BK channel activity. Horizontal lines indicate closed levels. (B) *I–V* curve of single $_{mito}$BK current amplitude vs. V_m at low or high [Ca^{2+}]. (C) Relationship between NP_o and V_m at different Ca^{2+} concentrations for $_{mito}$BK activities. (D) Raw sample recordings of $_{plasma}$BK activity. Reducing bath [Ca^{2+}] from 0.4 to 0 mM reduced single $_{plasma}$BK activity. (E) *I–V* curve of single $_{plasma}$BK current amplitude vs. V_m at low or high [Ca^{2+}]. (F) Relationship between NP_o and V_m at different Ca^{2+} concentrations for $_{plasma}$BK activities.

mitoplast configuration because obtaining inside-out patches of mitoplast membranes is not technically feasible. In these experiments, $_{mito}$BK activity was very high (Fig. 2A), whereas $_{plasma}$BK activity was very low (Fig. 2D). In both populations of channels, varying Ca^{2+} concentrations of the bath solution from 0.4 to 0.0 mM decreased channel NP_o without altering the amplitude of single channel events (Fig. 2A–F). For example, with 171 mM K^+ and 0.4 mM $CaCl_2$ on the bath side of the plasma membrane and in the recording pipette, $_{plasma}$BK activity occurred at V_m ranging from 0 to +100 mV, while replacing this high [Ca^{2+}] bath solution with a bath solution containing 0 mM Ca^{2+} and 15 mM K-EGTA substantially reduced NP_o (Fig. 2D,F). Reperfusing cells with the higher [Ca^{2+}] resulted in a partial recovery of NP_o (data

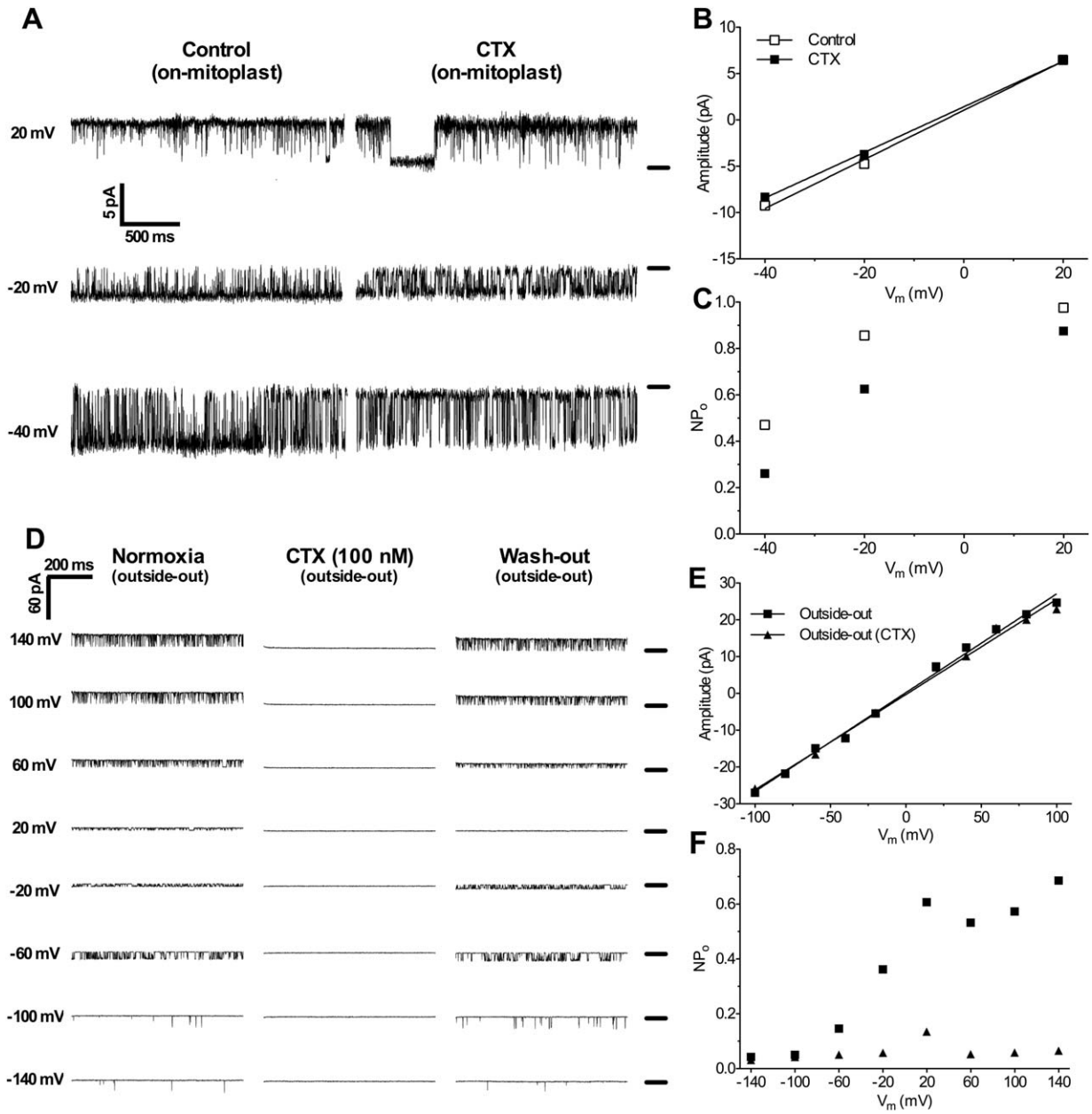

FIGURE 3: Charybdotoxin (CTX) inhibits mitochondrial BK and plasmalemmal BK channel activity. (A) Bath application of 100 nM CTX reduced raw single $_{mito}$BK activity (right) relative to untreated controls (left). Horizontal lines indicate closed levels. (B) *I–V* curve of single $_{mito}$BK current amplitude vs. V_m before and after CTX. (C) Relationship between NP_o and V_m before and after CTX for $_{plasma}$BK activity. (D) Bath application of CTX reduced raw single $_{plasma}$BK activity (middle) relative to untreated controls (left). (E) *I–V* curve of single $_{plasma}$BK current amplitude vs. V_m before and after CTX. (F) Relationship between NP_o and V_m before and after CTX for $_{plasma}$BK activity.

not shown). At negative V_m in plasma membrane preparations we observed activity from a second channel (Fig. 2D). We conclude that this activity was not due to BK channel opening because the amplitude of the single-channel events was smaller than those of BK channels.

To further support our conclusion that the identity of the channel activities we recorded were BK-mediated, we recorded channel activities at different V_m in the presence of the BK-channel antagonist charybdotoxin (CTX) in outside-out or on-mitoplast patch configuration. BK channel NP_o from $_{mito}$BK and $_{plasma}$BK was decreased by CTX (100 nM), but single channel slope conductance was not affected (Fig. 3).

Next, we examined the oxygen sensitivity of both channel populations. In response to hypoxia, $_{mito}$BK NP_o increased at all V_m tested (Fig. 4A,B). Conversely, $_{plasma}$BK NP_o decreased only slightly in response to hypoxia, while of 13 patch recordings collected, 4 showed a slight increase in

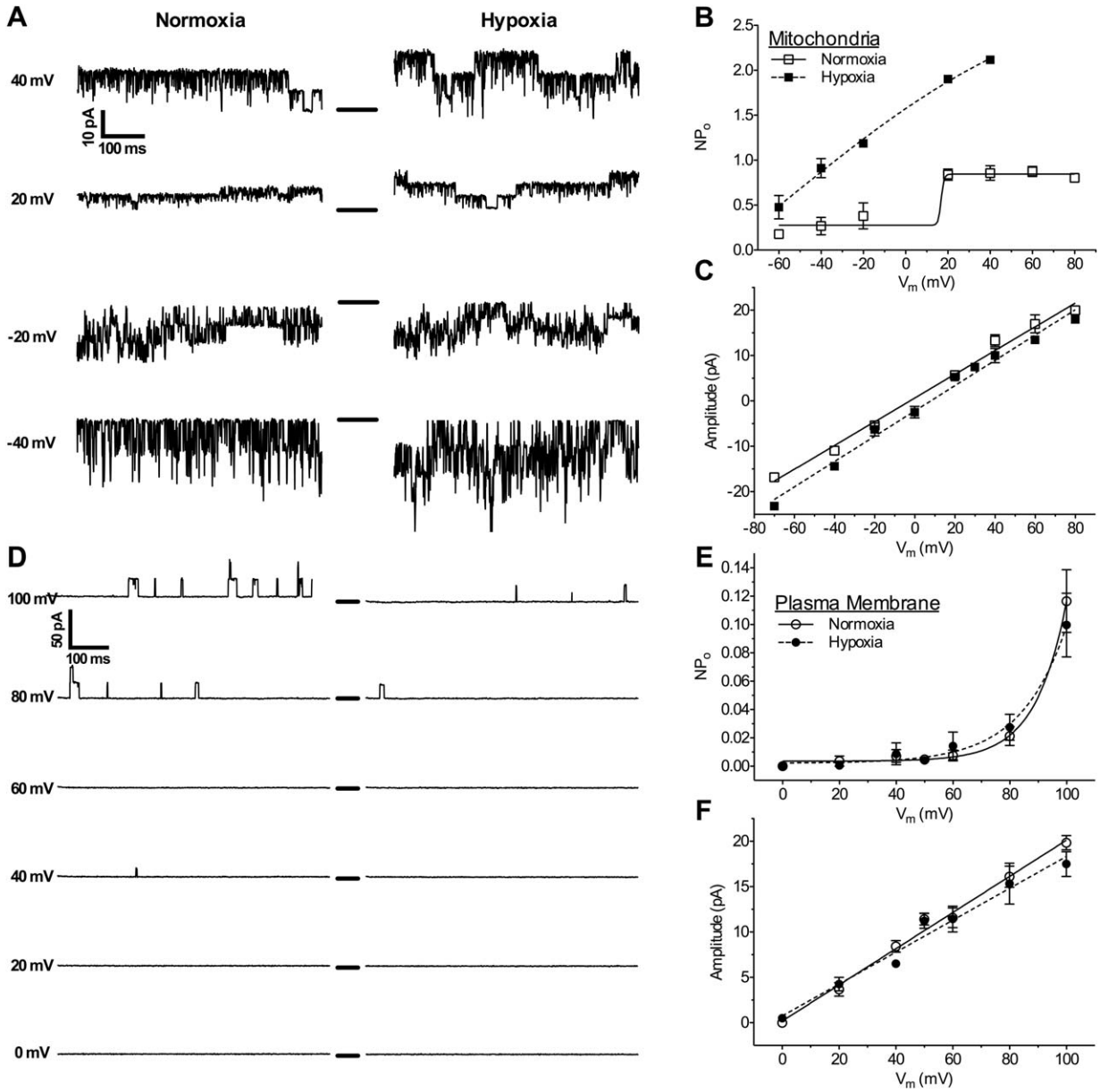

FIGURE 4: Mitochondrial but not plasmalemmal BK channels are hypoxia-sensitive. (A) Effect of hypoxia on single $_{mito}$BK activities. Data are raw single-channel current traces at different V_m under normoxia (left) and hypoxia (right). Data are raw single-channel current traces at different V_m under normoxia (left) and hypoxia (right). Horizontal lines between traces indicate closed channel levels. (B) Relationship between NP_o and V_m in normoxia and hypoxia for $_{mito}$BK activities. (C) *I–V* curve of single $_{mito}$BK current amplitude vs. V_m in normoxia and hypoxia. (D) Effect of hypoxia on single $_{plasma}$BK activities. (E) Relationship between NP_o and V_m in normoxia and hypoxia for $_{plama}$BK activity. (F) *I–V* curve of single $_{plasma}$BK current amplitude vs. V_m in normoxia and hypoxia.

NP_o (Fig. 4D,E). The averaged NP_o at +100 mV decreased from 0.12 ± 0.02 to 0.10 ± 0.03 ($n = 13$, $P > 0.05$). At other V_m tested (<+100 mV) NP_o was minimal. When we plotted I–V curves (single channel amplitude vs. V_m) to compare the effect of hypoxia relative to normoxia, we observed that in $_{plasma}$BK, the reversal potential was similar between hypoxia and normoxia (Fig. 4F), whereas the reversal potential was slightly depolarized in hypoxia relative to normoxia in mitoplasts (Fig. 4C). In on-cell preparations, $_{plasma}$BK activity was lower than $_{mito}$BK activity; therefore, to examine the effect of hypoxia on both populations of channels at similar open states, we treated LN229 cells with the Ca^{2+} ionophore ionomycin (1 μM, Fig. 5, $n = 9$). Ionomycin increased the mean NP_o of $_{plasma}$BK relative to patches from cells in ionomycin-free bath solution in both normoxia and hypoxia (Figs. 4E and 5B). However, similar to in ionomycin-free experiments, in the presence of ionomycin, hypoxia had no effect on NP_o (Fig. 5B) or amplitude (Fig. 5C) relative to normoxic controls.

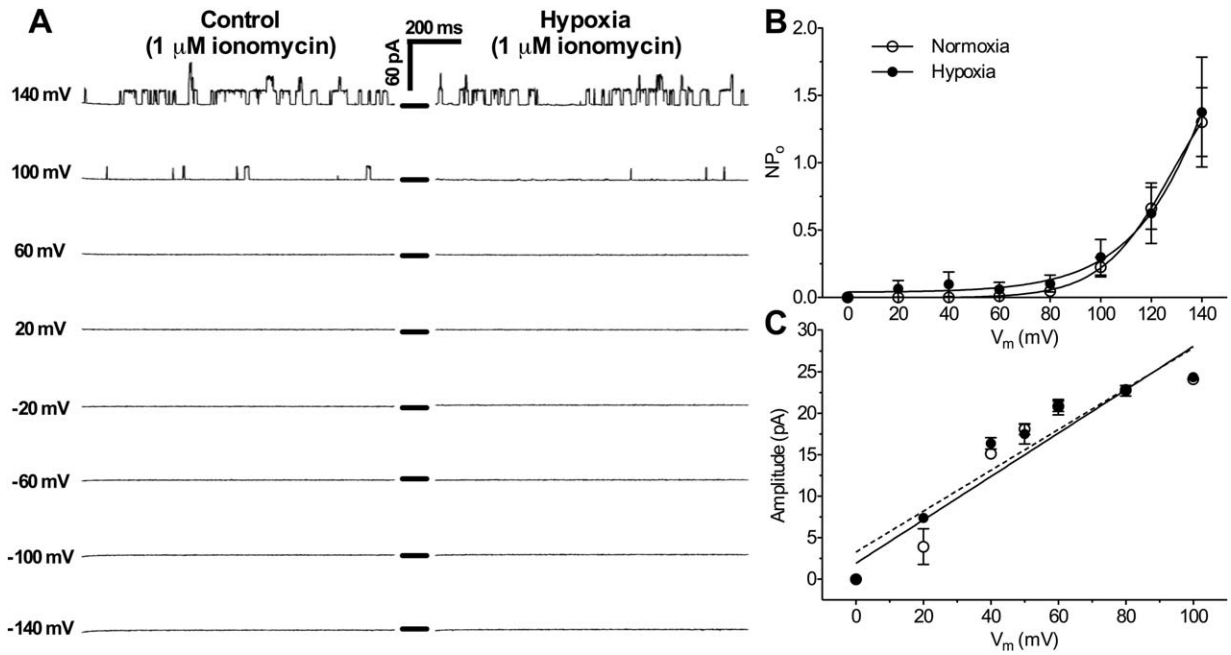

FIGURE 5: Plasmalemmal BK channels are not hypoxia-sensitive at higher intracellular [Ca^{2+}]. (A) Effect of hypoxia on single $_{plasma}$BK activities in the presence of 1 μM ionomycin. Data are raw single-channel current traces at different V_m under normoxia (left) and hypoxia (right). Horizontal lines between traces indicate closed channel levels. (B) Relationship between NP_o and V_m in normoxia and hypoxia for $_{plama}$BK activity in the presence of ionomycin. (C) *I–V* curve of single $_{plasma}$BK current amplitude vs. V_m in normoxia and hypoxia in the presence of ionomycin.

Finally, since $_{plasma}$BK were insensitive to hypoxia, we assessed the biological impact of both $_{plasma}$BK and $_{mito}$BK activation on cellular viability during both normoxia and hypoxia (1% O_2). Using microplate-based assays, we measured propidium iodide (PI) exclusion as a measure of cellular membrane integrity and also cellular [ATP] as a measure of metabolic health. Activation of $_{mito}$BK with the specific $_{mito}$BK agonist NS-1619 had no effect on cellular viability measured with either assay through 24 h of treatment (Fig. 6). Conversely, the $_{plasma}$BK agonist BMS-191011 induced significant cell death in normoxia as indicted by 9.4 ± 2.4 and $16.8 \pm 2.1\%$ increases in PI uptake at 12 and 24 h treatment, respectively, while cellular [ATP] decreased to 83.4 ± 3.1 at 12 h and further to $72.3 \pm 2.8\%$ at 24 h. During hypoxia, these effects increased ~2-fold in all measurements and time points. PI uptake increased to 15.1 ± 1.8 and $40.7 \pm 1.7\%$ at 12 and 24 h, respectively, and cellular [ATP] decreased further to 77.8 ± 1.9 at 12 h and to $43.3 \pm 3.4\%$ at 24 h.

Discussion

We studied the normoxic and hypoxic activation of single-channel events of BK channels from both plasma and inner mitochondrial membranes in a human glioma cell line. We conclude that the currents recorded are due to BK channel activation since (a) the single channel slope conductance in symmetrical K^+ solutions is in the range of the conductance of BK channels previously studied in glioma (Ransom et al., 2003; Siemen et al., 1999); (b) currents are voltage-dependent and Ca^{2+} sensitive; and (c) currents are inhibited by the BK channel antagonist CTX (Firth et al., 2011). Using similar patch clamp configurations and identical experimental solutions to those utilized in previous studies, we report here that glioma BK channels from plasma and inner mitochondrial membranes have very different properties in (i) single channel slope conductance; (ii) open probability in normoxic conditions; (iii) response to hypoxia, and (iv) the impact of channel opening on glioma cell viability in normoxia and hypoxia.

The single channel BK slope conductance we report is similar to previously published examinations using the same patch clamp configuration and pipette and external solutions in both plasma membrane (194 pS in Ransom et al., vs. our measurement of 210 pS) and inner mitochondrial membrane (295 pS in Siemen et al., vs. our measurement of 278 pS) in glioma cell lines (Ransom and Sontheimer, 2001; Siemen et al., 1999). This difference in slope conductance between BK channels located in different membranes likely reflects different channel molecular compositions since BK channels achieve functional diversity primarily through alternative splicing of the Slo1 mRNA and modulation by accessory β-subunits (Cui, 2010; Liu et al., 2002). For example, a cloned human glioma BK channel, termed gBK, is 97% identical to its closest homolog hbr5, but contains an additional 34-

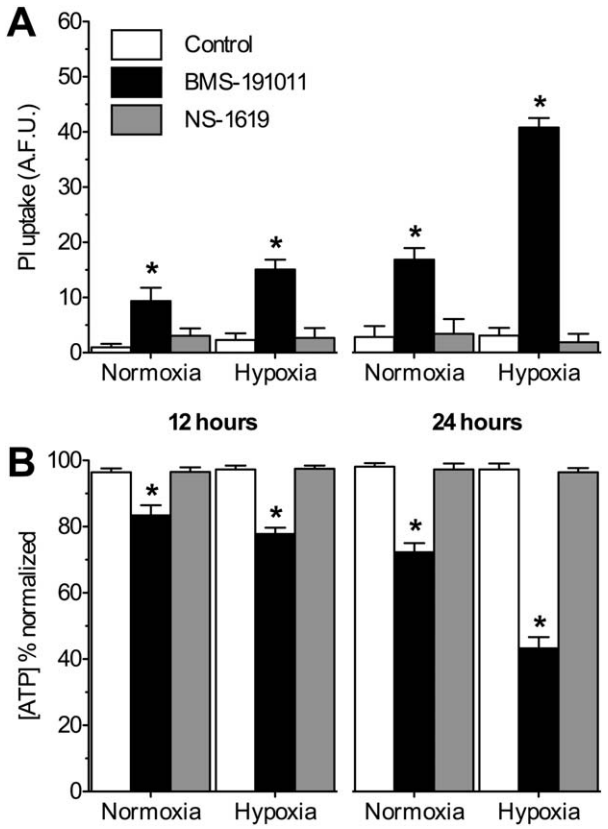

FIGURE 6: Activation of plasmalemmal but not mitochondrial BK channels induces cell death in human glioma cells in normoxia (21% O_2) and hypoxia (1% O_2). (A) Summary of change in propidium iodide (PI) uptake with time from glioma cells treated as indicated through 24 h. (B) Summary of change in [ATP] with time from glioma cells treated as indicated through 24 h. Data are mean ± SEM from five different experiments for each assay. Asterisks (*) indicate significant difference from normoxic controls at $t = 0$ h ($P < 0.05$). Treatments: NS-1619 or BMS-191011 (10 μM each).

amino acid exon at splice site 2 in the C-terminal tail of BK channels (Liu et al., 2002).

Under normoxic conditions, we found that $_{mito}$BK have a different NP_o than that of $_{plasma}$BK using the same pipette and bath solutions. BK channels from inner mitochondrial membranes have a larger NP_o and their activity can be observed at very negative membrane potentials, whereas $_{plasma}$BK exhibit a small NP_o and single channel events cannot be seen at negative membrane potentials, but rather can only be observed when the patch is held at +40 mV and above. However, upon obtaining an inside-out configuration, $_{plasma}$BK NP_o increases, and channel activity is observed at holding potentials as low as −100 mV, which is very similar to that of $_{mito}$BK. From these results, we conclude that the mitochondrial matrix of glioma cells has ∼171 mM K^+ (based on the reversal potentials of BK channels of ∼0 mV and our pipette solution of 171 mM K^+) and much higher concentration of Ca^{2+} (based on the higher NP_o of mitoplast BK); whereas the cytosol of glioma cells has a very low [Ca^{2+}] (which ranges from ∼70 to 150 nM in human glioma cells but is markedly lower than in mitochondria, which buffer cytosolic calcium, Ducret et al., 2002; Hartmann and Verkhratsky, 1998).

It is notable that $_{mito}$BK respond to changes in bath [Ca^{2+}] even though recordings were made in the on-mitoplast configuration. This finding is consistent with previous examinations of the sensitivity of $_{mito}$BK to changes in bath [Ca^{2+}] recorded using this technique (Gu et al., 2007; Siemen et al., 1999). In a previous study we demonstrated that the effects of bath Ca^{2+} on $_{mito}$BK activity, which take ∼5 min to fully manifest, are not accelerated by treating mitoplasts with the Ca^{2+}-ionophore A23187 (Siemen et al., 1999). This indicates that Ca^{2+} equilibration between the bath solution and the inner solution of the mitoplast is likely due to maintained activity of ion transport systems across the mitoplast membrane. In support of this, a recent study found that the cation current mediated by Ca^{2+} uptake into mitoplasts is largely abolished by bath application of the mitochondrial Ca^{2+}-uniporter antagonist ruthenium red, or by selective knockout of this uniporter in mitoplasts derived from HEK293 cell (Chaudhuri et al., 2013). The remaining current may be due to nonspecific ion movement through the inner membrane component of voltage-dependent ion channels, which are known to be permeable to large molecules.

A similar phenomenon may be involved in the varied response to CTX that we observed between $_{mito}$BK and $_{plasma}$BK. The CTX-binding site on BK channels is located on the outer membrane surface of $_{plasma}$BK. To access this site with bath-perfused CTX we employed outside-out patches of plasma membrane preparations. In these experiments, we observed near complete inhibition of BK channel activity. Conversely, the binding site of CTX to $_{mito}$BK has not been examined but is presumed to also be located on the outside (cytosolic side) of the mitochondrial membrane. In our on-mitoplast experiments, the patch pipette would therefore block the putative binding site for CTX. However, in our experiments, CTX partially blocked $_{mito}$BK activity, and this result is consistent with our previous experiments utilizing bath-applied CTX and on-mitoplast preparations (Gu et al., 2007; Siemen et al., 1999). Nonetheless, in these experiments CTX blocked BK channels, albeit less robustly than at the plasma membrane. It is possible that CTX was able to enter the mitoplasts via VDAC channels near the black caps of the mitoplast and it is possible that CTX may have acted on intramitochondrial binding sites to partially inhibit $_{mito}$BK channel activity. Further examination of the CTX-binding site on mitoplasts is warranted to better understand this interaction.

BK channels are an important component of the cellular response to hypoxia in other mammalian cell types. For

example, hypoxia is known to inhibit BK-channel activity in the plasma membranes of neurons (Liu et al., 1999; McCartney et al., 2005), chemoreceptor cells (Riesco-Fagundo et al., 2001), recombinant HEK-cells (Williams et al., 2004a, b), alveolar epithelial cells (Jovanovic et al., 2003), basilar and aortic myocytes (Navarro-Antolin et al., 2005), myocytes (Porter et al., 2001), and anterior pituitary AtT20 corticotropes (McCartney et al., 2005). Similarly, others and we have shown that hypoxia decreases BK NP_o in the cell-attached mode (Liu et al., 1999), but does not affect single-channel slope conductance (Jovanovic et al., 2003; McCartney et al., 2005; Porter et al., 2001; Riesco-Fagundo et al., 2001; Williams et al., 2004a, b). In contrast to this commonly observed hypoxic phenotype of $_{plasma}$BK located of healthy cells, we confirm our earlier reports that $_{mito}$BK of glioma cells increases its NP_o during hypoxia (Cheng et al., 2008; Gu et al., 2007), while single-channel conductance remains unchanged. Conversely, the major novel finding of our present study is that $_{plasma}$BK in gliomas cells do not exhibit a significant response to hypoxia. The reasons for this difference between $_{plasma}$BK and $_{mito}$BK responses to hypoxia might be related to: (1) differences in resting membrane potential between the two membranes (Xu et al., 2002); (2) a direct effect of hypoxia on membrane proteins (Jiang and Haddad, 1994); or (3) differences between Ca^{2+} levels inside the mitoplast when compared with regional Ca^{2+} levels in the cytosol (Miyata et al., 1992; Silverman, 1993), although this last possibility is unlikely as in the presence of ionomycin we also observe no effect of hypoxia on $_{plasma}$BK activity.

Another important finding in our study is that activation of $_{plasma}$BK, but not $_{mito}$BK is deleterious to glioma cells in normoxia and that the negative impact of $_{plasma}$BK activation is greatly increased during hypoxia. The effect of glioma BK channel agonism during hypoxia has not been assessed before but others have reported that activating $_{plasma}$BK (with CGS7181 or CGS7184, Debska-Vielhaber et al., 2009), but not $_{mito}$BK (with NS-1619 or NS-004, Debska et al., 2003), is deleterious to LN229 glioma cell viability in normoxic conditions, in good agreement with our findings in normoxia. These authors demonstrated that activation of $_{plasma}$BK induced glioma cell death via a mechanism that involved large-scale Ca^{2+} influx and calpain activation. Although we do not evaluate the specific mechanism of cell death in our experiments, it is likely that cell death during hypoxia is due to an enhancement of $_{plasma}$BK-mediated Ca^{2+}-dependent cell death mechanisms previously reported in normoxic glioma cells.

In general, tumor cells are known to tolerate hypoxic environments, but the mechanism of their hypoxic resistance is unknown. To our surprise, $_{plasma}$BK from LN229 glioma cells are generally unresponsive to hypoxia, which is unlike the large hypoxic decreases in NP_o that are typical of $_{plasma}$BK from healthy tissues. Interestingly, others have found that activating $_{plasma}$BK in tumor cells impairs survival and proliferation of cancerous cells (Han et al., 2008; Kraft et al., 2003); based on these reports and our present findings, we propose that the muted or absent hypoxic response of $_{plasma}$BK might represent a strategic adaptation of phenotypic tumor cells to hypoxia that may permit the tumor cell to survive in otherwise deleterious hypoxic environments. Enhancing $_{plasma}$BK activity or "re-activating" the sensitivity of these channels to hypoxia may offer therapeutic potential in the treatment of cancers.

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References

Bordey A, Sontheimer H. 2000. Ion channel expression by astrocytes *in situ*: Comparison of different CNS regions. Glia 30:27–38.

Borecky J, Jezek P, Siemen D. 1997. 108-pS channel in brown fat mitochondria might Be identical to the inner membrane anion channel. J Biol Chem 272:19282–19289.

Chaudhuri D, Sancak Y, Mootha VK, Clapham DE. 2013. MCU encodes the pore conducting mitochondrial calcium currents. Elife 2:e00704.

Cheng Y, Gu XQ, Bednarczyk P, Wiedemann FR, Haddad GG, Siemen D. 2008. Hypoxia increases activity of the BK-channel in the inner mitochondrial membrane and reduces activity of the permeability transition pore. Cell Physiol Biochem 22:127–136.

Cui J. 2010. BK-type calcium-activated potassium channels: Coupling of metal ions and voltage sensing. J Physiol 588:4651–4658.

Debska G, Kicinska A, Dobrucki J, Dworakowska B, Nurowska E, Skalska J, Dolowy K, Szewczyk A. 2003. Large-conductance K+ channel openers NS1619 and NS004 as inhibitors of mitochondrial function in glioma cells. Biochem Pharmacol 65:1827–1834.

Debska-Vielhaber G, Godlewski MM, Kicinska A, Skalska J, Kulawiak B, Piwonska M, Zablocki K, Kunz WS, Szewczyk A, Motyl T. 2009. Large-conductance K+ channel openers induce death of human glioma cells. J Physiol Pharmacol 60:27–36.

Douglas RM, Lai JC, Bian S, Cummins L, Moczydlowski E, Haddad GG. 2006. The calcium-sensitive large-conductance potassium channel (BK/MAXI K) is present in the inner mitochondrial membrane of rat brain. Neuroscience 139: 1249–1261.

Ducret T, Boudina S, Sorin B, Vacher AM, Gourdou I, Liguoro D, Guerin J, Bresson-Bepoldin L, Vacher P. 2002. Effects of prolactin on intracellular calcium concentration and cell proliferation in human glioma cells. Glia 38:200–214.

Firth AL, Remillard CV, Platoshyn O, Fantozzi I, Ko EA, Yuan JX. 2011. Functional ion channels in human pulmonary artery smooth muscle cells: Voltage-dependent cation channels. Pulm Circ 1:48–71.

Fiske JL, Fomin VP, Brown ML, Duncan RL, Sikes RA. 2006. Voltage-sensitive ion channels and cancer. Cancer Metastasis Rev 25:493–500.

Gali-Muhtasib HU, Diab-Assef M, Haddadin MJ. 2002. Hypoxic cells in tumors as a target for cancer therapy. J Med Liban 50:175–179.

Gaspar T, Domoki F, Lenti L, Katakam PV, Snipes JA, Bari F, Busija DW. 2009. Immediate neuronal preconditioning by NS1619. Brain Res 1285:196–207.

Gu XQ, Siemen D, Parvez S, Cheng Y, Xue J, Zhou D, Sun X, Jonas EA, Haddad GG. 2007. Hypoxia increases BK channel activity in the inner mitochondrial membrane. Biochem Biophys Res Commun 358:311–316.

Han X, Xi L, Wang H, Huang X, Ma X, Han Z, Wu P, Lu Y, Wang G, Zhou J, et al. 2008. The potassium ion channel opener NS1619 inhibits proliferation and induces apoptosis in A2780 ovarian cancer cells. Biochem Biophys Res Commun 375:205–209.

Hartmann J, Verkhratsky A. 1998. Relations between intracellular $Ca2^{+}$ stores and store-operated $Ca2^{+}$ entry in primary cultured human glioblastoma cells. J Physiol 513(Pt 2):411–424.

Heinen A, Aldakkak M, Stowe DF, Rhodes SS, Riess ML, Varadarajan SG, Camara AK. 2007. Reverse electron flow-induced ROS production is attenuated by activation of mitochondrial Ca2+-sensitive K+ channels. Am J Physiol Heart Circ Physiol 293:H1400–H1407.

Hewawasam P, Ding M, Chen N, King D, Knipe J, Pajor L, Ortiz A, Gribkoff VK, Starrett J. 2003. Synthesis of water-soluble prodrugs of BMS-191011: A maxi-K channel opener targeted for post-stroke neuroprotection. Bioorg Med Chem Lett 13:1695–1698.

Jemal A, Siegel R, Xu J, Ward E. 2010. Cancer statistics, 2010. CA Cancer J Clin 60:277–300.

Jiang C, Haddad GG. 1994. A direct mechanism for sensing low oxygen levels by central neurons. Proc Natl Acad Sci USA 91:7198–7201.

Jovanovic S, Crawford RM, Ranki HJ, Jovanovic A. 2003. Large conductance $Ca2^{+}$-activated K^{+} channels sense acute changes in oxygen tension in alveolar epithelial cells. Am J Respir Cell Mol Biol 28:363–372.

Kraft R, Krause P, Jung S, Basrai D, Liebmann L, Bolz J, Patt S. 2003. BK channel openers inhibit migration of human glioma cells. Pflugers Arch 446: 248–255.

Kulawiak B, Kudin AP, Szewczyk A, Kunz WS. 2008. BK channel openers inhibit ROS production of isolated rat brain mitochondria. Exp Neurol 212: 543–547.

Lee K, Rowe IC, Ashford ML. 1995. NS 1619 activates BKCa channel activity in rat cortical neurones. Eur J Pharmacol 280:215–219.

Liu H, Moczydlowski E, Haddad GG. 1999. O(2) deprivation inhibits $Ca(2^{+})$-activated $K(^{+})$ channels via cytosolic factors in mice neocortical neurons. J Clin Invest 104:577–588.

Liu X, Chang Y, Reinhart PH, Sontheimer H. 2002. Cloning and characterization of glioma BK: A novel BK channel isoform highly expressed in human glioma cells. J Neurosci 22:1840–1849.

McCartney CE, McClafferty H, Huibant JM, Rowan EG, Shipston MJ, Rowe IC. 2005. A cysteine-rich motif confers hypoxia sensitivity to mammalian large conductance voltage- and Ca-activated K (BK) channel alpha-subunits. Proc Natl Acad Sci USA 102:17870–17876.

Miyata H, Lakatta EG, Stern MD, Silverman HS. 1992. Relation of mitochondrial and cytosolic free calcium to cardiac myocyte recovery after exposure to anoxia. Circ Res 71:605–613.

Navarro-Antolin J, Levitsky KL, Calderon E, Ordonez A, Lopez-Barneo J. 2005. Decreased expression of maxi-K+ channel beta1-subunit and altered vasoregulation in hypoxia. Circulation 112:1309–1315.

Pamenter ME, Shin DS, Cooray M, Buck LT. 2008. Mitochondrial ATP-sensitive K+ channels regulate NMDAR activity in the cortex of the anoxic western painted turtle. J Physiol 586:1043–1058.

Porter VA, Rhodes MT, Reeve HL, Cornfield DN. 2001. Oxygen-induced fetal pulmonary vasodilation is mediated by intracellular calcium activation of K(Ca) channels. Am J Physiol Lung Cell Mol Physiol 281:L1379–L1385.

Ransom CB, Liu X, Sontheimer H. 2002. BK channels in human glioma cells have enhanced calcium sensitivity. Glia 38:281–291.

Ransom CB, Liu X, Sontheimer H. 2003. Current transients associated with BK channels in human glioma cells. J Membr Biol 193:201–213.

Ransom CB, Sontheimer H. 2001. BK channels in human glioma cells. J Neurophysiol 85:790–803.

Riesco-Fagundo AM, Perez-Garcia MT, Gonzalez C, Lopez-Lopez JR. 2001. O(2) modulates large-conductance $Ca(2^{+})$-dependent $K(^{+})$ channels of rat chemoreceptor cells by a membrane-restricted and CO-sensitive mechanism. Circ Res 89:430–436.

Seidel KN, Derst C, Salzmann M, Holtje M, Priller J, Markgraf R, Heinemann SH, Heilmann H, Skatchkov SN, Eaton MJ, et al. 2011. Expression of the voltage- and $Ca2^{+}$-dependent BK potassium channel subunits BKbeta1 and BKbeta4 in rodent astrocytes. Glia 59:893–902.

Siemen D, Loupatatzis C, Borecky J, Gulbins E, Lang F. 1999. $Ca2^{+}$-activated K channel of the BK-type in the inner mitochondrial membrane of a human glioma cell line. Biochem Biophys Res Commun 257:549–554.

Silverman HS. 1993. Mitochondrial free calcium regulation in hypoxia and reoxygenation: Relation to cellular injury. Basic Res Cardiol 88:483–494.

Sontheimer H. 1994. Voltage-dependent ion channels in glial cells. Glia 11: 156–172.

Sontheimer H. 2008. An unexpected role for ion channels in brain tumor metastasis. Exp Biol Med (Maywood) 233:779–791.

Sorgato MC, Keller BU, Stuhmer W. 1987. Patch-clamping of the inner mitochondrial membrane reveals a voltage-dependent ion channel. Nature 330: 498–500.

Weaver AK, Bomben VC, Sontheimer H. 2006. Expression and function of calcium-activated potassium channels in human glioma cells. Glia 54:223–233.

Williams SE, Wootton P, Mason HS, Bould J, Iles DE, Riccardi D, Peers C, Kemp PJ. 2004a. Hemoxygenase-2 is an oxygen sensor for a calcium-sensitive potassium channel. Science 306:2093–2097.

Williams SE, Wootton P, Mason HS, Iles DE, Peers C, Kemp PJ. 2004b. siRNA knock-down of gamma-glutamyl transpeptidase does not affect hypoxic K+ channel inhibition. Biochem Biophys Res Commun 314:63–68.

Xu W, Liu Y, Wang S, McDonald T, Van Eyk JE, Sidor A, O'Rourke B. 2002. Cytoprotective role of $Ca2^{+}$-activated K^{+} channels in the cardiac inner mitochondrial membrane. Science 298:1029–1033.