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Role for Calcium-Activated Potassium Channels (BK) in Growth Control of Human Malignant Glioma Cells

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Abstract

Voltage-dependent large-conductance Ca^{2+} -activated K^{+} channels, often referred to as *BK channels*, are a unique class of ion channels coupling intracellular chemical signaling to electrical signaling. BK channel expression has been shown to be up-regulated in human glioma biopsies, and expression levels correlate positively with the malignancy grade of the tumor. Glioma BK channels (gBK) are a splice variant of the *hsl* gene, are characterized by enhanced sensitivity to $[\text{Ca}^{2+}]_i$, and are the target of modulation by growth factors. By using the selective pharmacological BK channel inhibitor iberiotoxin, we examined the potential role of these channels in tumor growth. Cell survival assays examined the ability of glioma cells to grow in nominally serum-free medium. Under such conditions, BK channel inhibition by iberiotoxin caused a dose- and time-dependent decrease in cell number discernible as early as 72 hr after exposure and maximal growth inhibition after 4–5 days. FACS analysis shows that IbTX treatment arrests glioma cells in S phase of the cell cycle, whereupon cells undergo cell death. Interestingly, IbTX effects were nullified when cells were maintained in 7% fetal calf serum. Electrophysiological analysis, in conjunction with biotinylation studies, demonstrates that serum starvation caused a significant translocation of BK channel protein to the plasma membrane, corresponding to a two- to threefold increase in whole-cell conductance, but without a change in total gBK protein. Hence, expression of functional gBK channels appears to be regulated in a growth-factor-dependent manner, with enhanced surface expression promoting tumor cell growth under conditions of growth factor deprivation as might occur under in vivo conditions.

Keywords

BK channel; glioma; proliferation; iberiotoxin; cell growth

Gliomas are primary brain tumors believed to originate from normal glial cells or their progenitors. They account for 20% of all brain malignancies and are characterized by relentless growth and aggressive invasion into the brain parenchyma. These features make surgical treatment of these cancers difficult, resulting in a dim prognosis for affected patients. Much has been learned regarding the unusual growth characteristics of gliomas. Most notably, it has been demonstrated that these tumors can grow under conditions that would be adverse to growth of nonmalignant cells (Rouzaire-Dubois et al., 1993). Thus, whereas normal cell growth depends on the continuous presence of growth factors, glioma cells have developed autocrine and paracrine signaling cascades that support their growth in the absence of exogenous growth-stimulating factors (Rozengurt, 1999; Heasley, 2001; Gerber and Ferrara, 2003; Yu et al., 2003). Such factors include, for example, neuregulin-1, which is synthesized and released by

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gliomas (Pun et al., 1997) and which signals through heterodimeric erb-B2/erb-B4 receptors that are overexpressed in glioma cells (Westphal et al., 1997). In at least 50% of human gliomas, the epidermal growth factor receptor (EGF-R, also known as *erb-B1*) is up-regulated and serves as a receptor for transforming growth factor- α (TGF α) and EGF, and several experimental therapeutic approaches attempt to disrupt EGF signaling (Chakravarti et al., 2002; Li et al., 2003). The erb-B receptors are tyrosine kinases that activate well-delineated downstream signaling cascades, the first step being an increase in cytoplasmic $[Ca^{2+}]_i$ (Munaron, 2002). It is in this light that we became interested in the role that growth factor signaling may play in the modulation of large-conductance, calcium-activated K^+ channels and the possible interdependence of these channels and cell growth.

The channels of interest are often termed “big conductance” (BK) channels, specifically because of the fact that they are characterized by single-channel conductances >100 pS. These channels give rise to outwardly rectifying potassium currents and are unique in that they respond not only to changes in membrane voltage but also to changes in $[Ca^{2+}]_i$. Indeed, changes in intracellular Ca^{2+} have been demonstrated to cause concurrent changes in resting membrane potential (Bordey et al., 2000). Hence, it appears that BK channels may endow glioma cells with a mechanism to translate effectively changes in intracellular Ca^{2+} into changes in K^+ conductance. Changes in both $[Ca^{2+}]_i$ and K^+ conductance have been shown to accompany the proliferation of numerous cell types, including lymphocytes (Premack and Gardner, 1991; Khanna et al., 1999), macrophages (DeCoursey et al., 1996), and various glial cell types (Baran, 1996; Attali et al., 1997; Knutson et al., 1997; Ghiani et al., 1999; MacFarlane and Sontheimer, 2000; Moll et al., 2002; Soliven et al., 2003). However, in most instances, these effects were due to modulation of channels from either the Kv family of voltage-gated K^+ channels or the family of intermediate-conductance Ca^{2+} -activated K^+ channels (IK). Examples in which BK channels have been implicated in growth control include Muller glial cells and I321N astrocytoma cells (Bringmann et al., 1999; Basrai et al., 2002). In both preparations cell proliferation could be inhibited by the BK channel inhibitors iberiotoxin (IbTX) or charybdotoxin (ChTX). These same blockers inhibit the ATP-stimulated DNA synthesis in Müller cells (Moll et al., 2002), and similarly carbachol, which activates glioma BK channels (Chavis et al., 1998), stimulates DNA synthesis in glioma cells (Ashkenazi et al., 1989).

A careful evaluation of biopsy tissues from patients with malignant gliomas revealed significant expression of BK channel protein (Liu et al., 2002; Ningaraj et al., 2002), and studies of human glioma cell lines established that BK channels are the predominant K^+ channel type expressed in these cells (Ransom and Sontheimer, 2001; Redekop and Naus, 1995). This prompted a molecular cloning effort, which identified the glioma BK channel as a novel splice variant of the *hSlo* gene with enhanced Ca^{2+} sensitivity (Liu et al., 2002; Ransom et al., 2002) that was named *gBK*. In light of the above-cited references, work suggesting a possible link between BK channels and proliferation in other inexcitable cell types, and the unusually high expression levels of BK channels in glioma cells, we set out to examine whether BK channels may function to support the unusual growth of these tumors. By using a combination of cell biological and physiological assays, we show that, under conditions of growth factor deprivation, glioma BK channels translocate to the cell membrane. BK channel activity is required to allow cells to survive under such conditions, suggesting an important role for BK channel function in these tumors.

MATERIALS AND METHODS

Cell Culture and Drugs

The glioma cell line D54-MG (World Health Organization grade IV, glioblastoma multiforme) was a gift from Dr. D. Bigner (Duke University, Durham, NC). Cells were maintained in

Dulbecco's modified Eagle's medium (DMEM/F12; Media Tech, University of Alabama at Birmingham Media Preparation Facility) supplemented with 2 mM glutamine (Media Tech) and 7% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT) at 37°C and 10% CO₂. Unless otherwise stated, all reagents were purchased from Sigma (St. Louis, MO). The BK channel antibody G18 was a gift of Dr. Yi Zhou (University of Alabama at Birmingham).

Cell Proliferation Assay

Cells were plated onto six-well plates (Becton Dickinson, Bedford MA) at a density of 5×10^4 cells/ml. On day 0, medium was removed, and cells were washed twice with sterile phosphate-buffered saline (PBS; Gibco, Grand Island, NY) and treated with either 7% serum-containing, "control," media or serum-free media. Tetraethylammonium (TEA) or IbTX was added directly to the wells at final concentrations of 2 mM and 100 nM, respectively, and the medium was changed daily throughout the course of the experiment. Cells were trypsinized and resuspended in phosphate-buffered saline (PBS), and cell numbers were determined using a Coulter counter (Beckman, Miami FL). For dose-response experiments, cells were incubated in serum-free media containing 0, 1, 10, and 100 nM IbTX. For time course experiments, cell numbers were determined daily. All experiments that attempted to compare growth of cells under different serum or drug conditions were performed on sister cultures.

Live/Dead Assay

This protocol was modified and adapted for use with fluorescence-activated cell sorting (FACS) from the Live/Dead Viability/Cytotoxicity Assay Kit (Molecular Probes, Eugene, OR). Briefly, cells were plated and drug was added as in the cell proliferation assay. On days 1-5, the media were collected, and cells were trypsinized, spun down, and washed once with sterile PBS. Ethidium homodimer and calcein were added to the cells at a concentration of 16 μ M and 1 μ M, respectively, and incubated for 30 min at room temperature in the dark. Both dyes were excited at 488 nm and detected at 530 nm (calcein) and 585 nm (ethidium homodimer). For dose-response studies, cells were treated with 0, 1, 3, 10, 30, 100, and 300 nM IbTX and ethidium homodimer incorporation was measured on day 5 only.

Cell Cycle Analysis

Cells were plated and drug was applied as described above for the proliferation assay. Beginning 24 hr after treatment, cells were trypsinized, harvested, and resuspended in 0.5 ml sterile PBS. Cell suspensions were transferred to 2 ml of 95% ethanol (EtOH) by pipetting slowly while vortexing the EtOH. Samples were stored at 4°C, and this process was repeated every day for the duration of the experiment. After collection, samples were resuspended in sterile PBS and allowed to rehydrate for 30 min. Cells were washed three times with PBS and then treated with ribonuclease (RNase; 100 μ g/ml) for 15 min. Propidium iodide (50 μ g/ml in PBS) was then added and allowed to incubate for an additional 30 min. DNA content was measured by exciting propidium iodide at 488 nm and measuring the emission at 580 nm. Modfit (Verity Software House, Topsham, ME) was then used to fit the data to a mathematical model for relating DNA content to cell cycle stage.

Electrophysiology

Recordings of whole-cell currents were made using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) following standard recording techniques (Hamill et al., 1981). Patch pipettes were made with thin-walled borosilicate glass (TW150F-4; World Precision Instruments, Sarasota, FL) by using an upright puller (PP-830; Narishige Instruments, Tokyo, Japan) and typically had resistances of 3-5 M Ω . Current recordings were low-pass filtered at 2 kHz and digitized online at 10 kHz with a Digidata 1200 (Axon Instruments). pClamp 8.2 (Axon Instruments) was used to acquire and store data. Series resistance (R_s) was compensated

to 80%, reducing voltage errors, and cells with a compensated R_s above 10 M Ω were omitted. D54 cells were plated on glass coverslips and allowed to adhere overnight. On the next day, media were aspirated; cells were washed once with sterile PBS and were fed either serum-containing or serum-free medium. Cells were then used for recordings for the next 5 days.

Solutions

Our standard bath solution consisted of the following (in mM): 125 NaCl, 5 KCl, 1.2 MgSO₄, 1 CaCl₂, 1.6 Na₂HPO₄, 0.4 NaH₂PO₄, 10.5 glucose, and 32.5 HEPES acid; pH was adjusted to 7.4 with NaOH, and osmolarity was ~300 mOsm. IbTX and TEA were added directly to these solutions at concentrations of 100 nM and 2 mM, respectively. Pipette solutions contained the following (in mM): 145 KCl, 1 MgCl₂, 10 EGTA, 10 HEPES sodium salt, with pH adjusted to 7.3 with Tris base. CaCl₂ was added directly to pipette solution on the day of use at a concentration of 0.2 mM, resulting in a free Ca²⁺ concentration of 1.9 nM. Standard bath solution was continuously exchanged at a rate of ~1 ml/min.

Western Blotting

A confluent dish of cells grown in either serum-containing or serum-free media was lysed with RIPA buffer [50 mM TrisCl, pH 7.5, 150 mM NaCl, 1% Nondet P-40 (NP-40), 0.5% sodium deoxycholate, 1% sodium dodecyl sulfate (SDS)] supplemented with protease and phosphatase inhibitor cocktail (Sigma) for 30 min. Protein was collected by centrifugation at 12,000g at 4°C. The supernatant was transferred to a new tube, and protein levels were quantified using the DC protein assay kit from Bio-Rad (Hercules, CA). An equal volume of 2× Laemmli-SDS sample buffer containing 600 mM β -mercaptoethanol was added to 10 μ g of protein collected from control or serum-free samples. Both samples were loaded into their own individual well of a 7.5% precast SDS-PAGE gel (Bio-Rad). Protein separation was performed at a constant 100 V for ~90 min. Gels were then transferred at 200 mA for 90 min at room temperature onto PVDF paper (Millipore, Bedford, MA). Membranes were blocked in blocking buffer [5% nonfat dried milk, 2% bovine serum albumin, 2% normal goat serum (NGS) in TBS plus 0.1% Tween 20 (TBST)]. The primary antibodies antiactin and G18 (BK channel antibody) were used at dilutions of 1:25,000 and 1:4,000, respectively. Blots were incubated in primary antibody for 1 hr, followed by a wash period (6 $\times$ 5 min). Membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hr, followed by another wash period (6 $\times$ 5 min), and developed using enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL) on Hyperfilm (Amersham).

Biotinylation

The entire process was carried out at 4°C. Cells were washed twice in standard bath solution containing 1 mM Ca²⁺. After washing, Sulfo-NHS-Biotin (1.5 mg/ml; Pierce, Rockford, IL) was added and allowed to incubate for 30 min, with occasional gentle rocking. Biotinylation was quenched by washing cells twice with standard bath solution containing 100 mM glycine plus an additional 15-min wash. Cells were then rinsed once in bath solution, collected, spun down, and resuspended in 0.5 ml RIPA buffer supplemented with protease and phosphatase inhibitors (1:100; Sigma). The suspension was rotated for 30 min, followed by centrifugation at 12,000g for 5 min. At this point, protein analysis was performed as in the Western analysis, and 10 μ g of protein were set aside as total lysate. The remainder of the sample was incubated with agarose streptavidin beads (400 μ l beads/ml supernatant; Pierce) for 2 hr. Once again, beads were centrifuged at 10,000g for 10 sec, and the supernatant (representing the unbound fraction) was collected. The beads were gently washed four times with RIPA and resuspended in 100 mM glycine, pH 2.8, for 3 min with occasional vortexing to separate surface protein from the beads. Sample buffer was added immediately to neutralize pH. Additionally, 2× sample buffer was added to the total lysate fraction, and all three samples were loaded onto a

7.5% precast SDS-PAGE gel, separated, and transferred according to the Western protocol. All blots were probed with antiactin as a negative control to ensure that the surface protein fraction was not contaminated with intracellular protein.

Immunocytochemistry

Cells were plated on coverslips (12 mm round; Macalaster Bicknell, New Haven, CT) and after 5 days of treatment were washed once in PBS. After being washed, cells were fixed in 4% paraformaldehyde for 10 min at room temperature. Cells were then washed twice in PBS and blocked and permeabilized in PBS containing 0.3% Triton and 5% NGS for 30 min. Once again, cells were washed in PBS containing 5% NGS and incubated overnight with primary antibody at 4°C. On the following day, cells were washed four times in PBS, reblocked for 30 min in PBS containing 5% NGS, and labeled with secondary antibody for 1 hr in the dark. After labeling, cells were washed with PBS and incubated with DAPI for 5 min. Cells were washed twice more and mounted onto slides with Gel/Mount (Biomedica Corp., Forest City, CA). Images (32×) were acquired with a Zeiss Axiovert 200M (Zeiss, Göttingen, Germany).

Data Analysis

Results were analyzed with Origin 6.0 (MicroCal Software, Northampton, MA) and Excel 2000 (Microsoft, Seattle, WA). Significance was determined by one-way ANOVA, because all data showed a normal distribution. All data reported are mean $\pm$ SEM, and an asterisk denotes $P < 0.05$ unless otherwise stated.

RESULTS

BK Channel Activity Regulates Glioma Cell Growth

One of the notable differences between glioma cells and nonmalignant brain cells is their unusual resistance to cell death even under conditions that are adverse to growth of normal cells (Rouzaire-Dubois et al., 1993). This has been attributed to the activation of PI3 kinase and subsequent phosphorylation of its downstream target, AKT (Joy et al., 2003). Glioma cell resistance to cell death is critical for cells found at the core of a tumor: those that often find themselves without a constant replenishing supply of nutrients. To mimic these tumor growth conditions, we have compared population growth in serum-containing media (cells on the surface of a tumor) with that in serum-free growth medium (cells at the core of a tumor), as is customary for cell survival studies (Tsai et al., 1996; Orike et al., 2001). We treated D54-MG glioma cells, a widely used cell line derived from a grade IV glioblastoma multiforme, both acutely and chronically with the BK channel blockers TEA or IbTX. Under standard culture conditions (DMEM/F12 with 7% FBS and 2 mM glutamine), acute treatment with either 2 mM TEA or 100 nM IbTX, which have been shown to inhibit glioma BK channels (Ransom and Sontheimer, 2001), did not show any effect on cell growth (data not shown). Likewise, chronic treatment under these culture conditions showed no effect on the population growth of these glioma cells over a 5-day culture period (Fig. 1A). However, glioma cells treated with TEA or IbTX grown in media lacking FBS showed a marked decrease in overall cell number. Specifically, chronic application of BK channel blockers decreased cell number to $88.5\% \pm 3.0\%$ (TEA) and $36.4\% \pm 3.8\%$ (IbTX; percentage decrease $\pm$ SEM) compared with drug-free controls (Fig. 1A). Representative photomicrographs of control (left) and IbTX treated cells (right) illustrate the marked reduction in cell number following a 5-day treatment with IbTX (Fig. 1C). These effects of IbTX on cell growth were dose dependent, with a half-maximal inhibitory effect at ~ 10 nM (Fig. 1B). This correlates well with previous electrophysiology data showing an IC_{50} of about 8 nM for IbTX (Ransom and Sontheimer, 2001). Although both TEA and IbTX were able to elicit this effect, we performed the remainder of our analysis solely in the presence of IbTX because it has been shown to be a selective inhibitor of large-conductance, calcium-activated, voltage-dependent (BK) potassium channels (Galvez et al.,

1990), whereas TEA has the potential to block other K^+ (Hille, 2001) and Cl^- channels (Sanchez and Blatz, 1992, 1994) as well.

BK Channel Inhibition Affects Tumor Cell Growth in a Time-Dependent Manner

To better understand the development of this growth-inhibitory effect, we obtained 5-day growth curves of glioma cells under serum-containing and serum-free conditions (Fig. 2). For these experiments, sister cultures were plated, and media with or without drug were replaced daily. As described earlier, for the course of the experiment, cells cultured in normal serum-containing media with IbTX continued to proliferate at the same rate as control cultures (Fig. 2A). Cultures in serum-free media grew at a rate about half that of cells maintained in serum. Under both conditions, growth curves plateau after 4-5 days, with log growth occurring during the initial 3 days in culture only (Fig. 2B). Under serum-free conditions, IbTX caused a marked reduction in cell growth compared with control cells maintained under serum-free conditions in the absence of IbTX (Fig. 2B), which was evident after 3 days of treatment. Notably, cell numbers declined at 5 days, suggesting that cells were actually dying. To examine the potential reversibility of this growth inhibition by IbTX, we performed a second set of experiments in which cultures were treated with IbTX for the initial 3 days in the absence of serum (Fig. 2C). After 3 days, IbTX was removed and fresh serum-free medium was added daily for the remainder of the experiment. Cells that had experienced IbTX in the preceding 3 days did not recover their growth in the following days, suggesting that IbTX effects were no longer reversible. These experiments were also repeated with the CellTiter cell proliferation assay (Promega, Madison, WI) by measuring the 490-nm absorbance of the MTS formazan product, which is an indicator of cell growth. This assay provided similar results (data not shown).

Serum Withdrawal Increases Surface Expression of gBK

The fact that BK channel blockers inhibited cell growth only after removal of serum from the media raised the question of whether there was a change in the expression of gBK channels. To address this, we obtained whole-cell voltage-clamp recordings from cells that had been cultured in either serum-containing or serum-free media for 5 days (Fig. 3). Whole-cell BK currents were noticeably larger under serum-free conditions, as shown for two representative examples in Figure 3A. The sensitivity of currents to IbTX was unaltered (Fig. 3), however, allowing us to isolate the true BK currents as the IbTX-sensitive current component by subtraction of whole-cell currents in the presence and absence of IbTX. To compare quantitatively the effects of serum withdrawal on expression of functional BK channels, we then isolated the IbTX-sensitive currents for each cell as described above, divided currents by the driving force to obtain whole-cell conductance values, and then normalized to the whole-cell capacitance, yielding specific conductance values (in pS/pF), which allowed comparison independent of cell size. Mean values for these data (Fig. 3B) show a 240% increase in specific gBK conductance following serum withdrawal.

Because $[Ca^{2+}]_i$ was identical for these experiments (1.9 nM free Ca^{2+}), it seemed an unlikely candidate for the increase in specific conductance even though glioma BK channels are highly sensitive to changes in $[Ca^{2+}]_i$. Nevertheless, we used Fura-II Ca^{2+} imaging experiments to determine the resting $[Ca^{2+}]_i$ in glioma cells maintained in the presence and absence of serum. $[Ca^{2+}]_i$ was monitored for up to 12 cells per field over a 1-min period. Because no significant changes in $[Ca^{2+}]_i$ occurred during this time, we averaged all samples and reported these values as resting $[Ca^{2+}]_i$. Free $[Ca^{2+}]_i$ was 118.0 nM (SEM = 7.3, $n = 58$) in the presence of serum and 107.6 nM (SEM = 6.8, $n = 71$) in the absence, an insignificant difference ($P < 0.05$). As a second indication that differences in $[Ca^{2+}]_i$ did not account for these changes, channel activation was measured by plotting the normalized currents (I/I_{max}) as a function of applied voltage (Fig. 3C). Mean values from 13 cells were used for these curves, again showing a congruence of this biophysical feature. An increase in $[Ca^{2+}]_i$ would have been manifested in

a leftward shift of this curve (Ransom et al., 2002), which we did not observe. Taken together, the above data suggested an up-regulation of functional BK channels leading to the development of sensitivity to IbTX upon serum withdrawal.

To investigate whether the enhanced whole-cell currents were due to changes in protein expression, we next examined total channel protein levels by Western blotting. Whole-cell lysates from sister cultures grown in either serum-containing or serum-free media were separated by SDS-PAGE, and BK channel protein levels were compared side by side (Fig. 4A). Moreover, densitometric analysis of the protein band of interest was performed and is displayed in Figure 4B. Surprisingly, these experiments showed no difference in overall channel protein expression under the two experimental conditions. The possibility remained that BK protein was selectively up-regulated in the cell surface by a shift of protein from intracellular compartments to the surface membrane, as has been demonstrated for BK channels in chick ciliary ganglion neurons (Lhuillier and Dryer, 2002). To examine this possibility, we used a surface biotinylation approach in which only protein present on the cell membrane becomes labeled with biotin and can be selectively retrieved over avidin beads. Equal amounts of protein from both control and serum-starved glioma cells were incubated with streptavidin beads to make a comparison between protein levels, because a loading control was not available. After isolation, blots were probed with BK-channel-specific antibodies. To ensure that the biotinylation procedure isolated only cell surface proteins and was not contaminated with cytoplasmic proteins, we also probed these blots with actin antibodies. A representative blot is shown in Figure 4C. Qualitative analysis of these blots suggested a two- to threefold increase in surface expression of gBK, and densitometric analysis (Fig. 4D) showed an increase of ~80%. These values are consistent with and sufficient to explain the increase in BK currents observed in the biophysical experiments described above.

These findings were surprising; they suggest that BK channels exist both in intracellular compartments and in the cell membrane and that trafficking between these compartments allows the regulation of functional BK channels in the plasma membrane. Using BK-channel-specific antibodies, we examined several glioma cells by three-dimensional confocal microscopy to determine the spatial distribution of BK channels in these cells. Representative examples are shown in Figure 5. BK channels were indeed present throughout the cell cytoplasm in all glioma cells examined, with a variable amount of staining concentrated in cell processes and the cell surface. Interestingly, cells cultured in the presence of serum appear to have a high concentration of perinuclear immunostaining, suggesting that the majority of BK channel protein is localized to the endoplasmic reticulum (ER) or Golgi. Serum withdrawal markedly reduced the perinuclear staining while enhancing immunoreactivity in the cell surface and on cell processes as predicted from our biochemical studies. Greater than 80% of all cells cultured under serum-free conditions for 5 days exhibited this marked change in staining pattern. These experiments suggest that glioma cells maintain a large pool of functional BK channels in the ER or Golgi apparatus.

gBK Block by IbTX Causes S-Phase Arrest and Induces Programmed Cell Death

Five-day growth curves indicated that cells treated with IbTX show a failure to grow beginning around day 3. Because cell growth is a balance between both cell proliferation and cell death, we examined the effect of IbTX on these two processes separately. Cultures were again grown in media lacking serum with or without drug. On each day for 5 days, cell death was measured by incorporation of ethidium homodimer into the DNA of cells lacking membrane integrity, and DNA content was determined by FACS (Fig. 6A,B). IbTX induced significant cell death that was time and concentration dependent, with 40.2% ($\pm 1.7\%$, mean $\pm$ SEM, $n = 5$) of cells dying compared with control cells, with no significant cell death ($4.4\% \pm 0.6\%$, control, $n =$

5; Fig. 6C). Increasing concentrations of IbTX increased the percentages of cell death (Fig. 6D).

We also examined the possibility that IbTX induced cell-cycle arrest by using FACS analysis. Cells were maintained under conditions identical to those for all prior experiments and were labeled with propidium iodide to examine the DNA content of each cell. By using Modfit to translate DNA content to cell-cycle information, we were able to determine cell cycle distribution for each population of cells under the defined treatment conditions. Representative data are illustrated in Figure 7A,B. After 5 days in culture, the vast majority of control cells had accumulated in the G1/G0 phase ($95.25\% \pm 0.65\%$; Table I, Fig. 7C), which is typical of cells undergoing serum withdrawal (Rouzaire-Dubois et al., 2000). By comparison, >20% of cells treated with IbTX accumulated in S phase (Table I, Fig. 7C). However, insofar as overall cell number declined, the enhancement of S-phase cells can only be interpreted as IbTX leading to S-phase arrest, followed by cell death.

DISCUSSION

This study was undertaken to examine the effect of chronic BK channel blockade by using iberitoxin on the population growth of human malignant glioma cells. Two independent findings provided the motivation for this study. 1) We have recently characterized a novel splice variant of the *hsl* gene in glioma cells that gives rise to Ca^{2+} -activated BK currents displaying an enhanced sensitivity to physiological concentrations of $[\text{Ca}^{2+}]_i$ (Liu et al., 2002). 2) Several studies have implicated Ca^{2+} -activated K^+ channels of either the IK or the BK variety in the growth control of keratinocytes, fibroblasts, T lymphocytes, Müller glial cells, and astrocytomas (Khanna et al., 1999; Pena and Rane, 1999; Kodali et al., 2000; Basrai et al., 2002; Koegel et al., 2003). Whereas in all previous studies BK channel blockade simply decreased the rate of cell proliferation, our studies revealed a much more complex biology. IbTX was without any effect on the growth of glioma cells when cells were maintained in high serum (7% FBS). Surprisingly, however, IbTX retarded growth and actually induced cell death when cells were maintained in medium lacking serum. We ruled out the possibility that IbTX lacked effect on cells in serum-containing media resulting from binding by one or more serum proteins by repeating the growth curve experiments in the presence of 2 mM TEA, a nonpeptide inhibitor that is selective for BK channels in these cells at that concentration (Ransom and Sontheimer, 2001). These experiments confirmed that blockade of BK channels in serum-free and not serum-containing media resulted in an inhibition of cell growth (data not shown). There was no difference in intracellular resting Ca^{2+} concentration under different serum conditions, and currents displayed identical biophysical properties. However, the sensitivity of cell growth to IbTX that was apparent after 3 days in culture correlated with a twofold up-regulation of BK channel activity. This enhanced BK channel activity was caused by the translocation of BK channels from an intracellular pool to the plasma membrane upon serum withdrawal. After serum removal, total BK channel protein levels remained unchanged, although the number of BK channels on the surface increased approximately twofold as determined biochemically through a biotinylation approach.

It is well established that the growth factors found in serum support cell growth and survival for all cells. Removing these factors from the growth media is a technique used frequently to induce growth arrest and ultimately cell death. Tumor cells, however, proliferate at such a rapid rate that cells at the core of a tumor are not likely to receive fresh supplies of nutrients and growth factors very often. Many of these have overcome this hurdle by producing and secreting growth factors not normally produced by differentiated, nonmalignant cells of the same type (Pun et al., 1997). It is this autocrine and paracrine stimulation that allows tumors to grow under conditions of nutrient exhaustion. By removing serum from the culture media, we were able to simulate this physiological state of growth factor exhaustion that glioma cells may find

in vivo. If one extrapolates from our finding, one would expect BK channels to be highly up-regulated at the surface of glioma cells at the core of a tumor in vivo. This is indeed what previous examinations of patient biopsies suggest (Liu et al., 2002).

Growth factors are known directly to elevate intracellular calcium and activate protein kinases that have been shown to modulate BK channel expression. For example, glial cell line-derived neurotrophic factor has been shown to enhance expression of BK channels in chick lumbar motoneurons (Martin-Caraballo and Dryer, 2002). This effect can be inhibited by anisomycin, a protein synthesis inhibitor, indicating that the increase in channel expression in these neurons is due to de novo synthesis of channel protein. The same group also showed that TGF β 1 can cause membrane insertion of BK channels in chick ciliary ganglion neurons as a result of activation of PI3 kinase and that β -neuregulin can regulate BK channel expression posttranslationally in the parasympathetic neurons of chickens (Lhuillier and Dryer, 2002). These studies demonstrate that growth factors are capable of modulating expression of functional BK channels. Our studies suggest that, in glioma cells, no increase in total BK channel protein occurs but, instead, that growth factors alter the relative distribution of BK channels in the cells' cytoplasm vs. the plasma membrane.

Previous studies in other cell systems have added exogenous growth factors to examine changes in expression or function of BK channels. Our study did the opposite, namely, deprived the cells of growth factors in an attempt to put the cells into stress. From these previous studies, which reported an up-regulation of BK channels following growth factor stimulation, we would have expected to see a decrease in BK channel expression in glioma cells. Our findings, however, suggest a much different biology. Growth factors do not appear to alter BK channel protein expression but, instead, regulate the compartmentalization of these channels. How this, in turn, enhances the ability of these cells to survive remains a matter of speculation at best. Cell resting potential changes have been shown to be an important correlative with cell cycle progression (Bubien et al., 1990; Takahashi et al., 1994; Arcangeli et al., 1995; Baran, 1996). Clearly, our cell cycle analysis data illustrate that cells in which BK channels are inhibited with IbTX arrest in S phase and ultimately undergo cell death. In Muller glial cells, it has been demonstrated that BK channel blockade inhibits DNA synthesis. If this were also the case in glioma cells, it might not be surprising to see cells dying slowly as necessary cellular proteins can no longer be synthesized.

The results presented in this study suggest that gBK channels enhance glioma cell survival under stress conditions. Cell survival is a balance between cell proliferation and cell death. Our data suggest that BK channel inhibition results in growth arrest of cells in S phase, followed by apoptosis. The latter has been shown to depend actively on potassium channel function (for review see Remillard and Yuan, 2004). Most notably, the first step of apoptosis, cell shrinkage, requires the activation of potassium channels that aid in osmolyte and water removal from the cell (Bortner et al., 1997; Maeno et al., 2000). Activation of BK channels as a result of $[Ca^{2+}]_i$ increases caused by a disruption of the mitochondrial proton gradient has been shown to induce apoptosis in vascular smooth muscle cells (Krick et al., 2001). These studies therefore suggest that activation of potassium channels, rather than their inhibition, induces apoptosis, which is contrary to our findings. However, Chin et al. (1997) have shown that inhibition of an outwardly rectifying potassium current with the K⁺ channel blocker 4-aminopyridine induced apoptosis in the malignant astrocytoma cell line U87. Similarly, porcine granulosa cells treated with the potassium channel blocker clofilium undergo apoptosis (Manikkam et al., 2002). Hence, the interdependence of K⁺ channel function and apoptosis of glioma cells requires further study.

Oligodendrocyte progenitor cells have been shown to up-regulate outwardly rectifying potassium currents in response to growth factor stimulation (Chittajallu et al., 2002), whereas

we find that glioma cells actually down-regulate BK currents under this same condition. However, blockade of these channels when they are at their peak expression has similar consequences in both cell types. It is interesting that two highly proliferative cell types, a glial progenitor cell and a glial-derived tumor, would regulate their outwardly rectifying potassium currents in such a contrasting manner, and this might have important functional implications for these cells. In the same vein, it should be emphasized that the role of gBK channels in normal glioma biology is poorly understood. These channels give rise to unusually large outward K^+ currents and are highly sensitive to ligands that elevate $[Ca^{2+}]_i$. Oscillations in Ca^{2+} that occur as glioma cells migrate lead to concurrent changes in resting potential as a result of activation of BK channels (Bordey et al., 2000). For this reason, gBK channels may also play important roles in the context of glioma cell migration/invasion. Further studies investigating the function of BK channels in fundamental cellular processes utilizing knock-down strategies may be able to shed light on the role of BK channels in glioma cell biology.

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Fig. 1.

BK channel blockers inhibit glioma cell growth in a dose-dependent manner after growth factor withdrawal. D54 glioma cells were treated with TEA or IbTX for 5 days. **A:** Relative cell numbers were measured after 5 days of treatment in the presence or absence of 7% FBS and normalized to the respective values at day 1. **B:** Dose-dependent inhibition of cell growth by IbTX after 5 days of treatment in media lacking serum. **C:** Images of cells after 5 days under serum-free conditions with (right) or without (left) IbTX. * $P < 0.05$.

Fig. 2. BK channel inhibition affects tumor cell growth in a time-dependent and irreversible manner. Five-day growth curves were obtained from cells receiving no treatment or IbTX in media containing 7% FBS (**A**) or serum-free (SF) media (**B**). **C**: Cells were treated as described above in the absence of serum. After 3 days of treatment, cells were washed and cultured in media lacking serum for the remainder of the experiment. Cell number was determined daily with a Coulter counter.

Fig. 3.

Whole-cell BK currents are up-regulated after serum withdrawal. **A:** Representative whole-cell currents evoked after voltage steps from 0 to +160 mV from a holding potential of -40 mV. All traces have been normalized to whole-cell capacitance to compare currents under different conditions regardless of cell size. Representative traces evoked with the same protocol as described above in the presence of 100 nM IbTX illustrating that sensitivity to drug has remained the same. Subtracted traces showing specifically the IbTX-sensitive BK current.

B: Bar graph illustrating an approximately two-fold increase in the whole-cell specific conductance of the BK current. Specific conductances were determined by dividing current (I) by the driving force ($E - E_K$) and normalizing these values to whole-cell capacitance (pF).

C: An $I/I_{\max}$ plot comparing cells cultured in media with or without serum illustrates that increases in current size were not due to changes in $[Ca^{2+}]_i$, which would have caused a leftward shift in the activation curve.

Fig. 4. BK channel protein is translocated to the membrane after chronic serum withdrawal. **A:** Western blot analysis reveals a prominent band at ~120 kDa, which is consistent with the expected BK channel protein. Actin was used as a loading to control in order to analyze qualitatively the BK protein levels in control (right lane) and serum-free (left lane) cultured cells. **B:** Densitometric analysis of the blot shown in A reveals no difference in protein levels between control and serum-free cells. **C:** Blot from biotinylation study indicating an approximate twofold increase in BK protein in the membrane-bound fraction lane of the serum-free cells. HEK-293 cells were used as a negative control, and the blot was probed with actin to ensure that membrane fractions were not contaminated with cytoplasmic protein. **D:** Densitometric analysis of three biotinylation assays revealed a 77% increase in the level of BK channel protein expressed on the plasma membrane as represented by the membrane-bound fraction.

Fig. 5.

Immunostaining revealing translocation of BK channels to the membrane of serum-starved cells. Cells maintained in the presence (**A-C**) or absence (**D-F**) of 7% FBS were fixed and probed with the BK channel antibody G18 (**A,D**) or DAPI (**B,E**). **C,F**: Merge of previous image with G18 (green), and DAPI (blue). The majority of cells grown in media containing serum showed prominent immuno-reactivity for BK around the nucleus of the cell (**A**), indicative of protein in the ER or Golgi, whereas cells grown in the absence of serum (**D**) were virtually devoid of the same perinuclear staining, with more labeling occurring on the plasma membrane.

Fig. 6.

FACS analysis of cells stained with ethidium homodimer. Representative example of an FACS analysis of cells cultured in media lacking serum (**A**) and treated with IbTX (**B**). Ethidium homodimer was used to label dying cells, those cells that occupy the upper left quadrant in the analysis plot. **C**: Analysis of the percentage of cells stained positively for ethidium homodimer after 5 days of treatment compared with control. Results were taken from five experiments, all performed in triplicate. **D**: Cell death caused by IbTX was dose dependent.

Fig. 7.

Cell cycle analysis of cells treated with IbTX. Representative FACS assays of cells left untreated (**A**) and treated with 100 nM IbTX (**B**). Propidium iodide, which stains DNA, was used to analyze DNA content of each cell, which is directly dependent on cell cycle stage. Using Modfit software, we were able to translate fluorescence into DNA content and thus cell cycle stage for each cell. Cells from five independent experiments, performed in triplicate, were used to determine cell cycle stage of cells receiving IbTX treatment. **C**: Relative percentages of cells in G₁/G₀ phase of the cell cycle after 5 days of serum-free (control) and serum-free + 100 nM IbTX (IbTX) conditions. **D**: Relative percentages of cells in S phase of the cell cycle under the same conditions as for C. **P* < 0.05.

TABLE 1
Cell Cycle Distribution of Cells in the Absence of Serum With or Without IbTX [Results Are Mean (%) ± SE]

	Day 1		Day 2		Day 3		Day 4		Day 5	
	G_1/G_0	S	G_1/G_0	S	G_1/G_0	S	G_1/G_0	S	G_1/G_0	S
Serum Free	76.4 ± 5.7	17.0 ± 3.8	84.1 ± 1.4	10.6 ± 1.3	91.8 ± 3.0	5.4 ± 0.2	95.4 ± 0.5	3.4 ± 1.1	95.2 ± 0.6	4.2 ± 1.2
SF + IbTX	74.5 ± 7.0	12.9 ± 0.1	76.5 ± 1.9	11.8 ± 5.4	88.3 ± 0.5	7.3 ± 0.5	76.1 ± 7.5	16.0 ± 4.7	67.0 ± 10.1	20.0 ± 2.5