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Current Transients Associated with BK Channels in Human Glioma Cells

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Abstract. We have previously demonstrated the expression of BK channels in human glioma cells. There was a curious feature to the whole-cell currents of glioma cells seen during whole-cell patch-clamp: large, outward current transients accompanied repolarization of the cell membrane following an activating voltage step. This transient current, $I_{transient}$, activated and inactivated rapidly (≈1 ms). The *I-V* relationship of $I_{transient}$ had features that were inconsistent with simple ionic current through open ion channels: (i) $I_{transient}$ amplitude peaked with a −80 mV voltage change and was invariant over a 200 mV range, and (ii) $I_{transient}$ remained large and outward at −140 mV. We provide evidence for a direct relationship of $I_{transient}$ to glioma BK currents. They had an identical time course of activation, identical pharmacology, identical voltage-dependence, and small, random variations in the amplitude of the steady-state BK current and $I_{transient}$ seen over time were often perfectly in phase. Substituting intracellular K^+ with Cs^+, Li^+, or Na^+ ions reversibly reduced $I_{transient}$ and BK currents. $I_{transient}$ was not observed in recordings of other BK currents (hbr5 expressed in HEK cells and BK currents in rat neurons), suggesting $I_{transient}$ is unique to BK currents in human glioma cells. We conclude that $I_{transient}$ is generated by a mechanism related to the deactivation, and level of prior activation, of glioma BK channels. To account for these findings we propose that K^+ ions are "trapped" within glioma BK channels during deactivation and are forced to exit to the extracellular side in a manner independent of membrane potential.

Key words: Large-conductance calcium-activated K^+ channel — Glia — Permeation — Patch clamp

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Introduction

The charge movements associated with many events in cells are detectable with electrical measurements. These include ionic currents through ion channels (Hille, 1992), the movement of charged amino acids in voltage-gated ion channels during gating (Armstrong, 1992; Aggarwal & MacKinnon, 1996), and electrogenic coupled transport (Thomas, 1972; Brew & Attwell, 1987). The currents resulting from these charge movements can be distinguished in the following ways. Ionic currents through open ion channels obey Ohm's law, and, for single ionic species, the equilibrium potential is predicted by the Nernst equation. Although some macroscopic ionic currents display non-Ohmic behavior across a specific range of voltages, due to complex gating and permeation properties, and fail to reverse direction due to rectification, at the single-channel level ion channels are Ohmic resistors across a range of potentials. Gating charge movements increase with increasing voltage changes until a maximum is reached, after which they plateau and remain voltage-independent (Aggarwal & MacKinnon, 1996). These charge movements are insensitive to ion substitutions (Armstrong, 1992). Failure of gating currents to reverse direction according to the Nernst equation and non-Ohmic behavior over large ranges of potentials distinguishes these currents from currents produced by ion flow across resistive pathways (ion channels) in cell membranes. Unlike ionic currents through resistive pathways, currents resulting from electrogenic coupled-transport are not associated with changes in input resistance.

Human glioma cells, like other inexcitable cells, possess a variety of ion channels, including classes of K^+ channels, voltage-gated Na^+ channels, and Cl^- channels (*see* Brismar, 1995). These ion channels participate in volume-regulation, migration, and cell proliferation (Chin et al., 1997; Soroceanu, Manning & Sontheimer, 1999). We are particularly interested in large-conductance, Ca^{2+}-activated K^+ channels

(BK channels) that are expressed by many human glioma cells (Ransom & Sontheimer, 2001). These channels belong to the superfamily of voltage-dependent K^+ channels and have the stereotypical voltage-sensing S4 segment with its alternating charged amino acids (Jan & Jan, 1999). In addition to some molecular details, BK currents are distinct from other voltage-gated K^+ channels because in the absence of $[Ca^{2+}]_i$, gating occurs only at aberrant membrane potentials (>+50 mV). They also have distinguishing pharmacology that includes inhibition by the scorpion venom peptide iberiotoxin and low concentrations (<500 μM) of tetraethylammonium ion (TEA). BK currents in glioma cells are mediated by a novel isoform, termed gBK, that contains a 34-amino-acid insert at splice site 2 (Liu et al., 2002).

In the present study, we describe transient currents in glioma cells that are intimately related to BK channel function. These currents are voltage-independent over a large range of potentials and do not reverse direction. Thus, this transient current may not represent simple ionic current flow across open ion channels (resistive pathways). The transient current was only observed following activation of glioma BK currents and the time course of activation, pharmacology, and voltage-dependence of transient currents and glioma BK currents were indistinguishable. We discuss possible mechanisms for the generation of this transient current.

Materials and Methods

Cell Culture

STTG-1 cells (an anaplastic astrocytoma cell line) were obtained from American Type Tissue Collection (Rockville, MD). D54-MG cells (a glioblastoma multiform cell line) were a gift of Dr. D. Bigner (Department of Pathology, Duke University). Vials of cells arrived frozen and were thawed and resuspended in our culture medium (*see* below). These cells were plated on four large culture flasks (Becton-Dickinson, Lincoln Park, NJ) and grown to confluency. Cells were detached from the flasks with a 1–2 min exposure of culture medium supplemented with trypsin (1.5 mg/ml). This suspension was added to an equal volume of culture medium and spun at 1200× *g* for 5 minutes in a centrifuge (Lab-Line Instruments Inc., Melrose Park, IL). We aspirated the supernatant and resuspended the pellet from three flasks in 30 ml of a freezing solution (culture medium with 5% DMSO). This suspension was divided into 180 0.5 ml aliquots and stored in liquid nitrogen for later use. Some cells were plated directly onto glass coverslips in 24-well plates (Becton-Dickinson) for experiments and into a culture flask (Becton-Dickinson) for future passage. Cells were used up to 100 passages before a new aliquot was thawed. Acutely-dissociated medial habenula neurons were provided by Dr. Robin Lester and were prepared as previously described (Lester & Dani, 1995).

Our culture medium was Dulbecco's Modified Essential Medium (Life Technologies, Inc., Grand Island, NY) with 10% fetal calf serum (Hyclone, Logan, UT). Cells were kept in an incubator (Lab-Line Instruments Inc.) at 37°C in a 90% O_2/10% CO_2 humidified environment. This resulted in a pH_o of 7.4.

Electrophysiology

Standard patch-clamp techniques were used to record whole-cell and single-channel membrane currents (Hamill et al., 1983). Patch pipettes were pulled on an upright puller (PP-83, Narishige Instruments, Tokyo, Japan) from thin-walled glass capillary tubing with filament (MTW150F-4, WPI, Sarasota, FL, USA) and had resistances of 3–5 MΩ. We used an Axopatch 200B amplifier (Axon Instruments, Redwood City, CA) controlled by a PC-compatible microcomputer (Dell Computers, Dallas, TX) running Axon Instruments software (pClamp7). Data were stored directly to disk using a Digidata 1200 A-D interface (Axon Instruments). Data were acquired at 10 kHz and filtered at 1 kHz and 2 kHz for patch and whole-cell recordings, respectively. Capacitance and series resistance, R_s, compensation was performed with the Axopatch amplifier. R_s was compensated up to 80%. We performed a post-hoc correction for the voltage errors associated with R_s in experiments looking at the voltage-dependence of the currents under study (i.e., data in Fig. 5). Cells were visualized with an inverted microscope (Nikon, Melville, NY). A three-axis micromanipulator (Newport, Irvine, CA) mounted onto a custom frame fitted to the microscope held the preamplifier headstage and pipette holder. The recording chamber had a volume of ≈300 μl and was constantly superfused with control extracellular solution at a rate of ≈0.5 ml/min. A triple-barreled microperfusion device with stepper motor (SF-77B perfusion fast-step, Warner Instruments, Hamden, CT) was used to apply test solutions directly to cells or patches. Two barrels were fed by 2-to-1 manifolds and one barrel was fed by a 4-to-1 manifold. Control solutions were continuously flowing in each barrel between applications of the five test solutions. The microperfusion flow pipes and stepper motor were mounted on a manual micromanipulator (MX-110, Soma Scientific Instruments, Irvine, CA) attached to our isolation table (Micro-g, Peabody, MA) with a magnetic base. Grounding the recording chamber via an agar salt bridge (4% agar, 1 M KCL) minimized liquid junction potentials.

Solutions

Our standard bath solution contained the following (in mM): 5 KCl, 135 NaCl, 1.6 Na_2HPO_4, 0.4 NaH_2PO_4, 1 $MgSO_4$, 10 glucose, 32.5 HEPES (acid). pH was adjusted to 7.4 with NaOH. The osmolarity was ≈300 mOsm. Drugs were added directly to this solution. In some experiments NaCl was substituted with an equimolar amount of KCl, Choline-Cl, or LiCl. Our standard pipette solution contained (in mM): 145 KCl, CsCl, NaCl, or LiCl, 1 $MgCl_2$, 10 HEPES (acid), 10 EGTA. pH was adjusted to 7.25 with Tris-base and Ca^{2+} was added from a stock solution to achieve a target free Ca^{2+} concentration of 20 nM. We calculated the calcium to add to our pipette solution in experiments with elevated free calcium concentrations with a software program based on equations provided in Marks and Maxfield (1991). This program takes into account ionic strength and pH. We corrected for EGTA purity. For a target free Ca^{2+} concentration of 0.1 μM we added 4.3 mM Ca^{2+}. All chemicals were purchased from Sigma unless otherwise noted. Iberiotoxin was purchased from Alomone labs (Jerusalem, Israel).

hbr5 Transfection of HEK Cells

To allow direct comparison of glioma BK currents to other BK currents we transiently transfected HEK cells (a gift from Dr. Michael J. Quick) with a BK channel cloned from human brain (hbr5, Tseng-Crank et al., 1996). We used FuGene 6 Transfection Reagent (Boehringer Mannheim) to transfect cells and followed the

manufacturer's procedures exactly. hbr5 was transfected in a plasmid vector (pcDNA3, Invitrogen). The hbr5 vector was a gift of Dr. Peter H. Reinhart. We co-transfected HEK cells with a plasmid containing the green fluorescent protein (GFP) gene. Only GFP-positive cells expressed BK currents.

Analysis

Data were analyzed off-line with the software package Origin (v.5.0, MicroCal Software, Northhampton, MA). All curve-fitting was performed using a least-squares curve-fitting routine provided by the software. To quantify the voltage dependence of currents under different conditions we fit normalized currents to a Boltzmann equation of the following form (Weiss & Magleby, 1990):

$$I/I_{max} = 1/[1 + \exp(-q(V - V_{0.5})/kT)]$$

where I/I_{max} is normalized current, q is the effective gating charge, $V_{0.5}$ is the apparent half-maximal voltage, k is the Boltzmann constant, and T is temperature in Kelvins. Under our conditions, the term kT was ≈25.6. Statistical analysis was performed with Excel (Microsoft, Bellevue, WA).

Values for the activation time constant of $I_{transient}$ were determined as the time to 63% ($1-^1$/e) of the peak amplitude of $I_{transient}$ following a deactivating voltage step. Because of the rapid time course of $I_{transient}$ and the coincident reduction of steady-state BK current during $I_{transient}$ activation, our current measurements may not reflect the true peak of $I_{transient}$ activation and lead to an underestimation of τ.

Results

As previously reported, with typical intracellular solutions (low $[Ca^{2+}]_i$), voltage-dependent currents in human glioma cells activated at large, positive (> +50 mV) potentials. These currents are mediated by a novel splice variant of large-conductance, Ca^{2+}-activated K^+ channels (BK channels), termed *gBK* for glioma BK channel (Ransom & Sontheimer, 2001; Liu et al., 2002). A curious feature of these currents was the appearance of large, outward current transients upon repolarization of the membrane from an activating test potential (*see* Fig. 1*A*). In response to repolarization to a constant voltage of −40 mV, the amplitude of this transient current, $I_{transient}$, increased with the amplitude of the steady-state *gBK* current (Fig. 1*B*, 1*C*). The relative amplitude of $I_{transient}$ (the ratio $I_{transient}/gBK$) also increased with the size of the voltage step (Fig. 1*D*, 1*E*). Thus, the relative amplitude of $I_{transient}$ was greatest at the largest positive potentials when BK channel activation was at its greatest.

There was no association between cell size or shape and the appearance of $I_{transient}$. $I_{transient}$ was present in small, completely spherical cells recorded from within 12 h of plating on coverslips. In addition, we have obtained examples of outside-out patches clearly displaying $I_{transient}$. These last findings argue against poor spatial voltage control as a source of $I_{transient}$ generation.

I-V Relationship of $I_{transient}$

To determine the *I-V* relationship of $I_{transient}$ we used the following voltage protocol; we stepped the membrane potential (V_m) to a maximally-activating value (+180 mV) for 35 ms and then stepped V_m to a range of potentials from +180 to −140 mV (V_h = 0 mV). We leak-subtracted currents by performing a point-by-point subtraction between currents recorded with and without a large blocking dose of tetraethylammonium ion (TEA; 1–3 mM) (Fig. 2*A*, 2*B*). To better illustrate $I_{transient}$, Fig. 2*C* shows the time frame before and after the step changes in membrane potential from the conditioning pulse of +180 mV on an expanded time scale (corresponding to the area within the dashed box in Fig. 2*B*). The amplitude of $I_{transient}$ peaked with a negative 80–140 mV voltage change and then reached a plateau, remaining relatively invariant over a 240 mV range. Most importantly, $I_{transient}$ was still large and outward at −140 mV, a potential at which all ions in our experimental solutions experience a driving force to produce inward current.

Activation Kinetics of $I_{transient}$

Although $I_{transient}$ amplitude nearly plateaus with increasing voltage changes, it was clear by inspection of records like those in Fig. 2*C* that $I_{transient}$ has voltage-dependent activation kinetics. The activation kinetics of $I_{transient}$ accelerate as the magnitude of the voltage change is increased. Such behavior is inconsistent with $I_{transient}$ representing some wholly passive membrane property. Capacitive currents would increase in amplitude without alteration of their time course. We quantified $I_{transient}$ activation kinetics by determining time constants, τ, at each test potential (Fig. 3). We determined time constants as the duration for $I_{transient}$ to reach 63% ($1-^1$/e) of its peak. On average, τ, values were 0.77 ± 0.07 ms at +100 mV, 0.51 ± 0.05 ms at +40 mV, and 0.31 ± 0.02 ms at −40 mV. The values and voltage-dependence of the activation time constants for $I_{transient}$ compare well with reported deactivation time constants for mouse and human BK channels (Cui, Cox & Aldrich, 1997; Brenner et al., 2000). Because our measurements of $I_{transient}$ occur under such dynamic conditions (rapid activation of $I_{transient}$ and coincident reduction in BK current amplitude) we may not record the true peak in $I_{transient}$ amplitude, leading to an underestimation of τ.

Because $I_{transient}$ was only observed immediately following prior activation of BK currents, there was reason to believe that $I_{transient}$ was associated with the deactivation of glioma BK channels. In the following sections we demonstrate the association of $I_{transient}$ and glioma BK channels in greater detail.

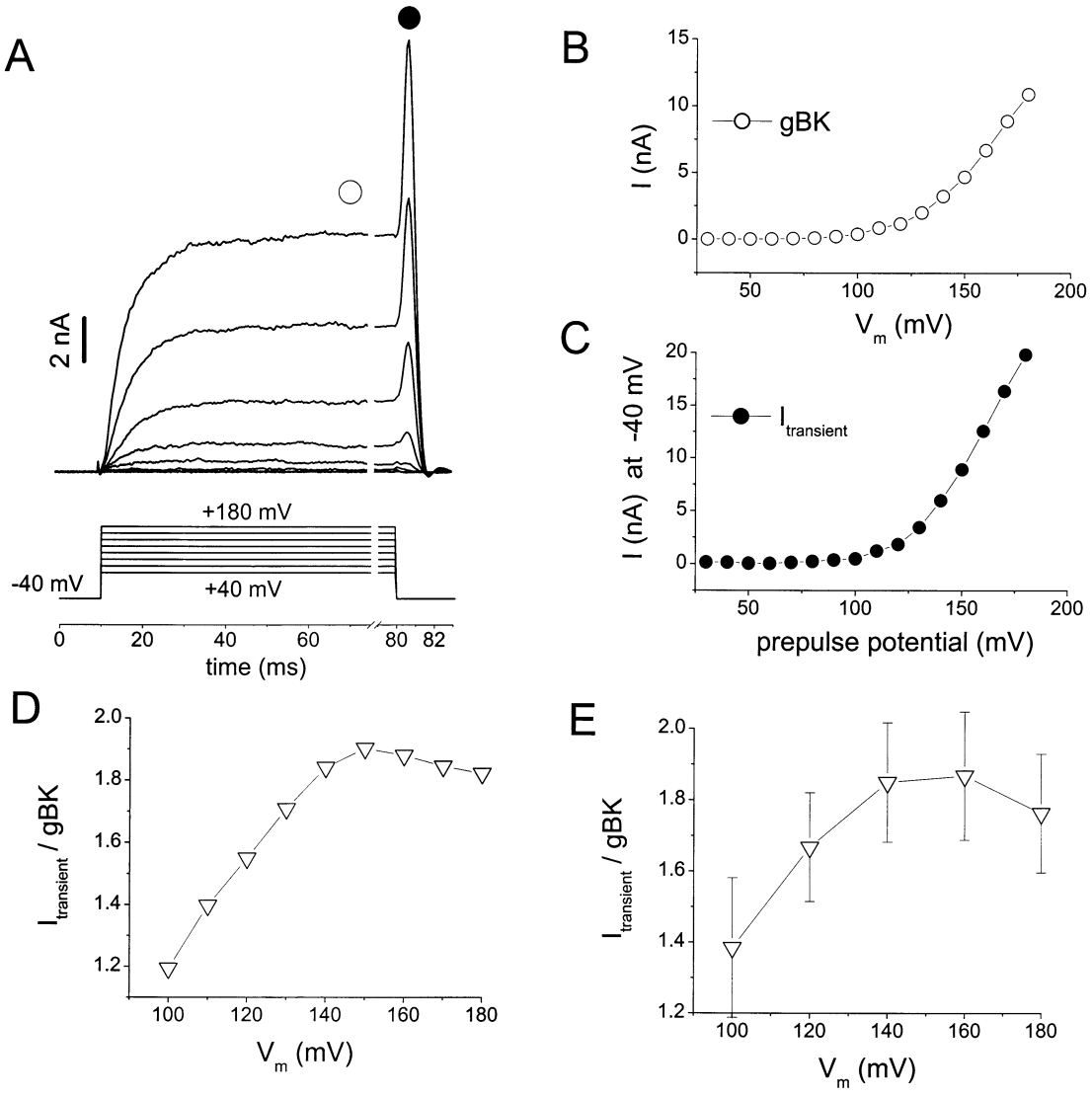

Fig. 1. Voltage-dependent currents in human glioma cells display a steady-state component and a transient component. (*A*) Whole-cell currents in response to the illustrated voltage steps. Note the expanded time scale at the end of these records. Depolarizations activated glioma BK currents (*gBK*). Repolarization of the membrane produced large, outward transient currents ($I_{transient}$). The amplitude of $I_{transient}$, at a constant voltage of −40 mV, increased with *gBK*. (*B*) *I-V* plot for the steady-state current. (*C*) $I_{transient}$ as a function of prepulse potential. (*D*) The ratio $I_{transient}/gBK$ as a function of V_m, from the data in (A). (*E*) Mean ± SE of $I_{transient}/gBK$ as a function of V_m ($n = 8$). The relative amplitude of $I_{transient}$ was greatest at the largest potentials, when *gBK* current activation was greatest.

Ionic Dependence of $I_{transient}$

The non-ohmic behavior of $I_{transient}$ (voltage-independence and failure to reverse direction) is inconsistent with current flow across a simple resistive pathway (open ion channels). We were therefore interested in what, if any, ionic dependence $I_{transient}$ possessed. We made serial patch-clamp recordings from single glioma cells with pipettes filled with KCl, CsCl, LiCl, and NaCl. Substitution of cations other than K^+ reversibly inhibits both $I_{transient}$ and *gBK*. An experiment with Cs^+ substitution is illustrated in Fig. 4. All recordings were made 5 min after establishing a whole-cell recording. Our previous work on these cells has shown that the effects of intracellular cation substitution and elevation of intracellular Ca^{2+} are complete within 3 min and often within 30 s (Ransom & Sontheimer, 2001). $I_{transient}$ was not equally inhibited by different cation replacements. As previously reported, *gBK* was reduced by cation substitution with an apparent selectivity sequence of $K^+ > Cs^+ > Li^+ > Na^+$ (Ransom & Sontheimer, 2001). However, Na^+ and Li^+ ions, but not Cs^+ ions, supported an $I_{transient}/gBK$ ratio greater than 1, albeit at greatly smaller amplitudes than seen with K^+ ions (*data not shown*). Substitution of extracellular Na^+ with $choline^+$ or Li^+ does not affect the amplitude of *gBK* or $I_{transient}$. Two manipulations that decrease the

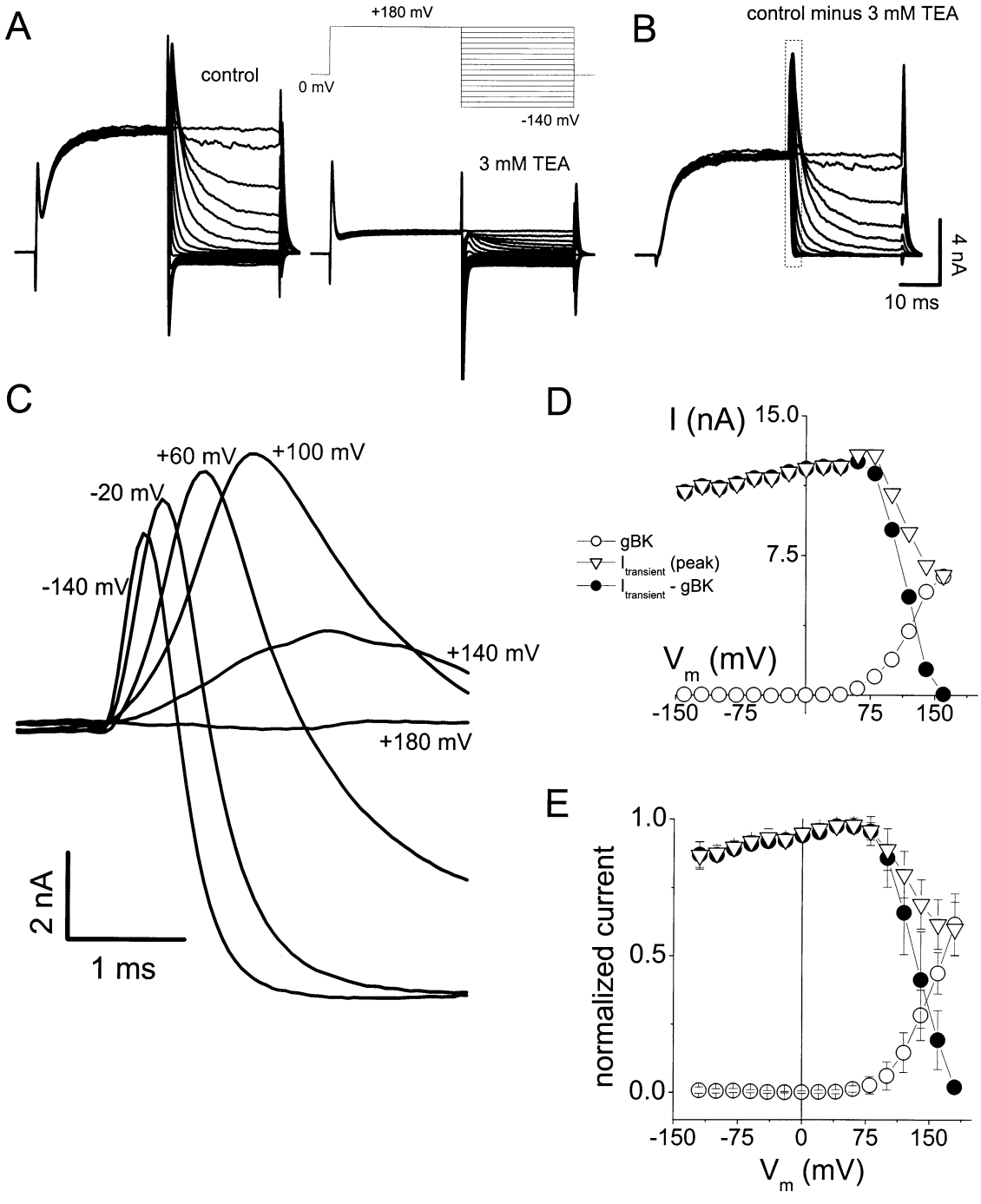

Fig. 2. *I-V* relationship of $I_{transient}$. (*A*) Whole-cell currents in response to the voltage protocol illustrated in the inset. Records were taken under control conditions and in the presence of a maximal blocking dose of TEA (3 mM). (*B*) Result of a point-by-point subtraction between the two data sets in (*A*) to isolate the voltage-dependent (TEA-sensitive) currents. (*C*) Expansion of the area enclosed in the dashed box in (*B*). For display purposes not all traces are illustrated. $I_{transient}$ developed with a −40 mV change in V_m from the conditioning potential of +180 mV. Larger voltage changes increased $I_{transient}$ amplitude and accelerated the activation kinetics. (*D*) *I-V* curve of the peak transient current and steady-state current. In addition, we have plotted the difference current, $I_{transient}$ - *gBK*, that, if we assume near instantaneous relaxation of *gBK* to its new value following the negative voltage change, represents the true *I-V* curve of $I_{transient}$. Features of this curve to note are that $I_{transient}$ amplitude is relatively invariant over the range +100 mV to −140 mV and $I_{transient}$ is still large and outward at −140 mV, a potential at which all ions in our experimental solutions experience an electrochemical gradient to produce an inward current. (*E*) Mean ± SD of the normalized currents analyzed as in *A-D* ($n = 6$). The pattern seen in (*D*) persisted across populations of cells.

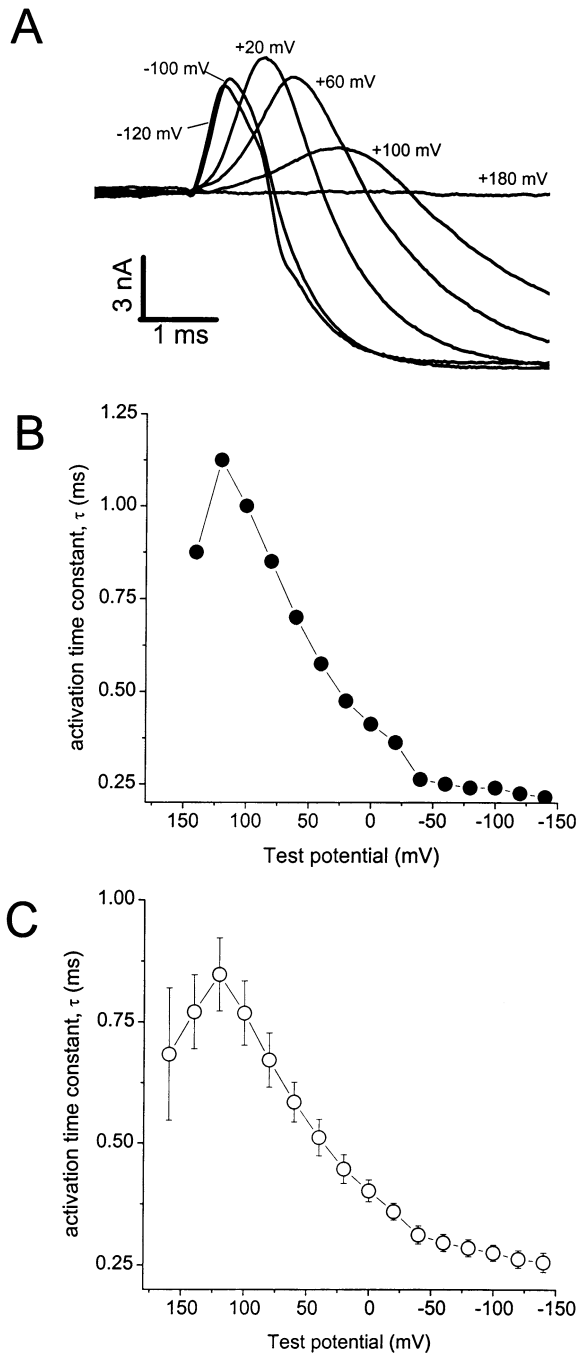

Fig. 3. Activation kinetics for $I_{transient}$. (*A*) Current traces in response to voltage steps as in Fig. 2*B* (different D54-MG cell). We measured activation time constants, τ, at 63% ($1-^1$/e) of the peak positive phase for each transient current. (*B*) Activation time constants as a function of test potential for the data in (*A*). (*C*) Mean activation time constants (±SE) as a function of test potential ($n = 6$ cells). In all cells examined, τ decreased with increasingly negative voltage steps. Because of the rapid time course of $I_{transient}$ and the concomitant reduction in steady-state BK current amplitude we are likely underestimating the true τ for $I_{transient}$.

prominence of $I_{transient}$ are elevation of extracellular K^+ and intracellular Ca^{2+} (>100 nM). We believe the effects of extracellular K^+ and intracellular Ca^{2+} on $I_{transient}$ are a result of slowed *gBK* deactivation kinetics (*see* Discussion, "Hypothesis for the generation of $I_{transient}$").

Time Course of Activation for $I_{transient}$ and *gBK*

To examine the time course of activation of $I_{transient}$ and *gBK* we gave voltage steps to +160 mV for increasing durations (*see* inset Fig. 5*A*). We measured *gBK* immediately before the end of the voltage step and the resulting $I_{transient}$ at its peak. For each trial, *gBK* and $I_{transient}$ amplitude were normalized to their maximum value and these normalized values were averaged and plotted as a function of voltage-step duration (*see* Fig. 5*B*). The amplitude of *gBK* and $I_{transient}$ increased with identical time courses following activating voltage steps of increasing duration. To quantify these data we plotted normalized current amplitude as a function of voltage step duration. The time constants of activation, τ, (determined with single-exponential fits to these data) for $I_{transient}$ and *gBK* were identical for each individual cell, but the absolute values varied across cells (2–6 ms). For the cell illustrated in Fig. 3, both *gBK* and $I_{transient}$ had a time constant of activation, τ, of 4.4 ms. On average, $\tau_{gBK} = 3.5 \pm 2$ ms and $I_{transient} = 3.4 \pm 2$ ms (mean ± SD, $n = 5$). For $I_{transient}$ the τ values presented in this section are not true time constants of activation (which will be constant in response to a constant voltage change, *see* Fig. 3) but provide a useful measure to quantitatively compare the time-dependence of *gBK* and $I_{transient}$ activation following voltage-steps of increasing duration.

Pharmacology of $I_{transient}$ and *gBK*

We have previously characterized the pharmacologic properties of the BK currents in human glioma cells (Ransom & Sontheimer, 2001). Currents were inhibited by scorpion venom peptides (iberiotoxin and charybdotoxin), tetraethylammonium ion (TEA), and quinine. We examined the effects of TEA and iberiotoxin on $I_{transient}$ and *gBK*. Both drugs inhibited *gBK* and $I_{transient}$ to a similar extent. An experiment with TEA is illustrated in Fig. 6. In Fig. 6*C*, the amplitudes of $I_{transient}$ and *gBK* were normalized to their initial values to demonstrate the similar degree of inhibition of both $I_{transient}$ and *gBK* by TEA. The reduction of $I_{transient}$ amplitude by BK channel inhibitors was unlikely a simple result of reducing the amplitude of the BK tail current at the end of a voltage step (i.e., $I_{transient}$ amplitude would be the sum of $I_{transient}$ and residual *gBK* current). This can be clearly inferred from cells in which $I_{transient}$ was much larger than *gBK*. From the experiment in Fig. 6 the mean amplitudes of $I_{transient}$ and *gBK* were 4.2 and 2.2 nA under control conditions and 0.8 and

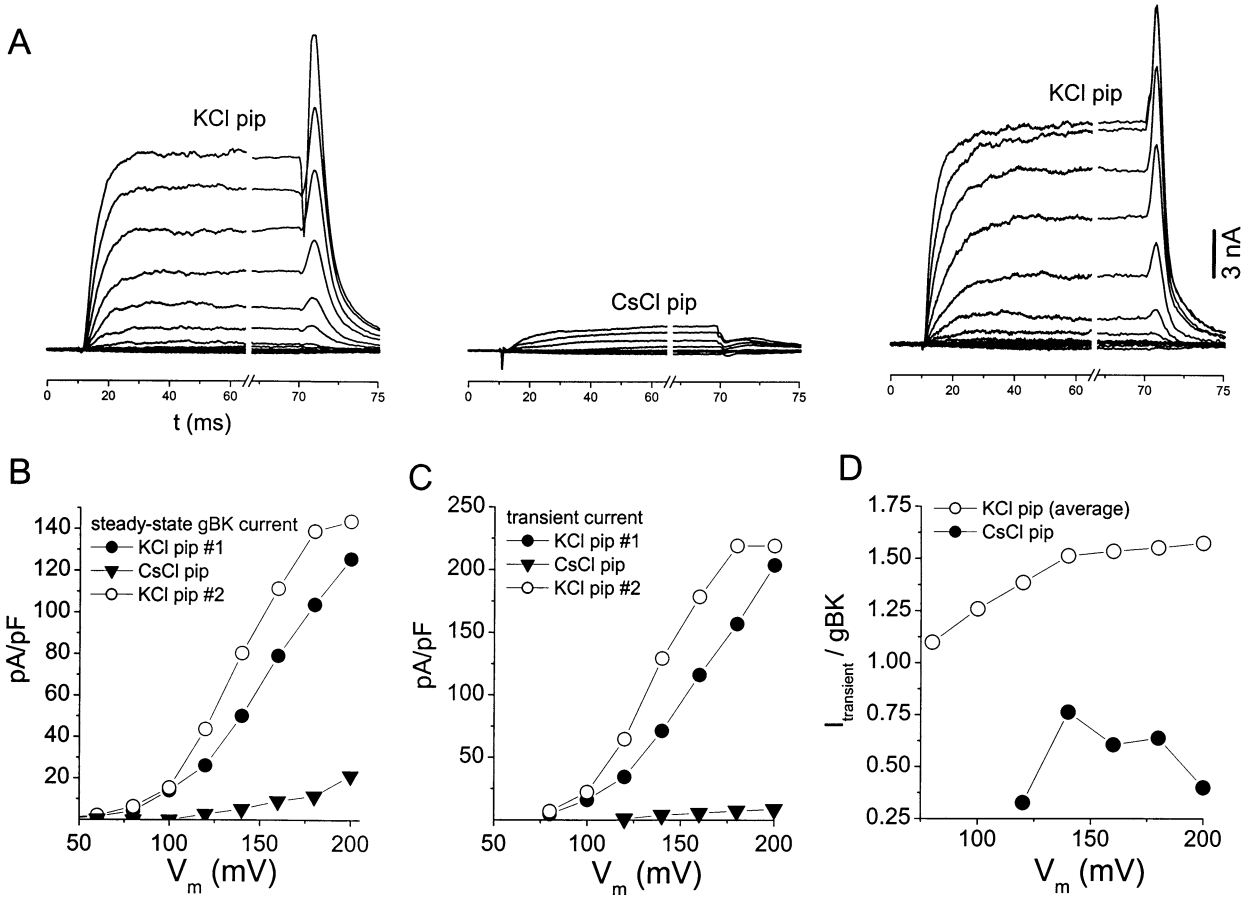

Fig. 4. $I_{transient}$ is dependent on intracellular K^+ ions. (*A*) Current traces from a single D54MG cell serially patch-clamped with three pipettes. The steady-state BK current and $I_{transient}$ were reversibly reduced in amplitude when Cs^+ was substituted for K^+. Currents evoked with voltage steps as in Fig. 1. (*B*) BK current density as a function of membrane potential for the data in (*A*). (*C*) $I_{transient}$ current density as a function of membrane potential for the data in (*A*). (*D*) The ratio $I_{transient}/gBK$ for the data in (*A*).

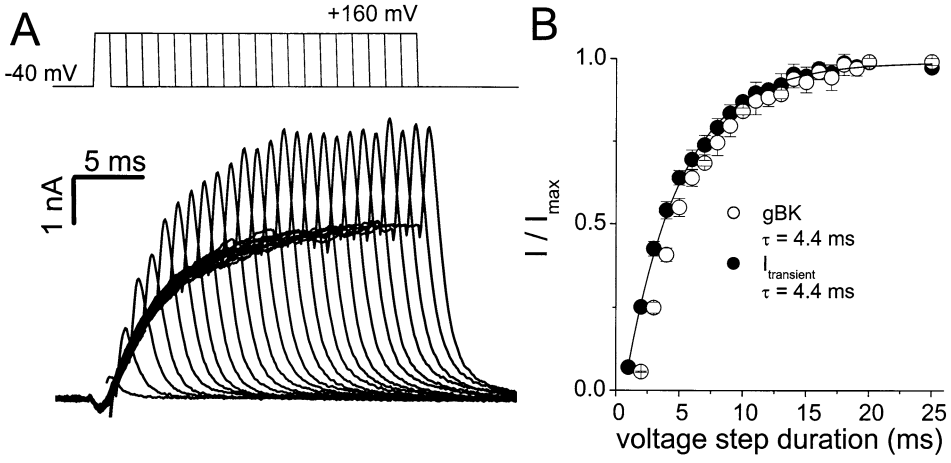

Fig. 5. $I_{transient}$ and gBK have identical time-dependence. (*A*) Whole-cell currents in response to voltage steps of increasing duration (*see* inset). $I_{transient}$ amplitude increased with BK current activation. (*B*) Normalized current as a function of voltage step duration. Single exponential fits (dashed lines) of data for $I_{transient}$ and gBK yielded identical time constants, τ. The τ value for $I_{transient}$ is not a true time constant for activation (*see* text). Each point is the mean ± SD of 5 trials such as in (*A*).

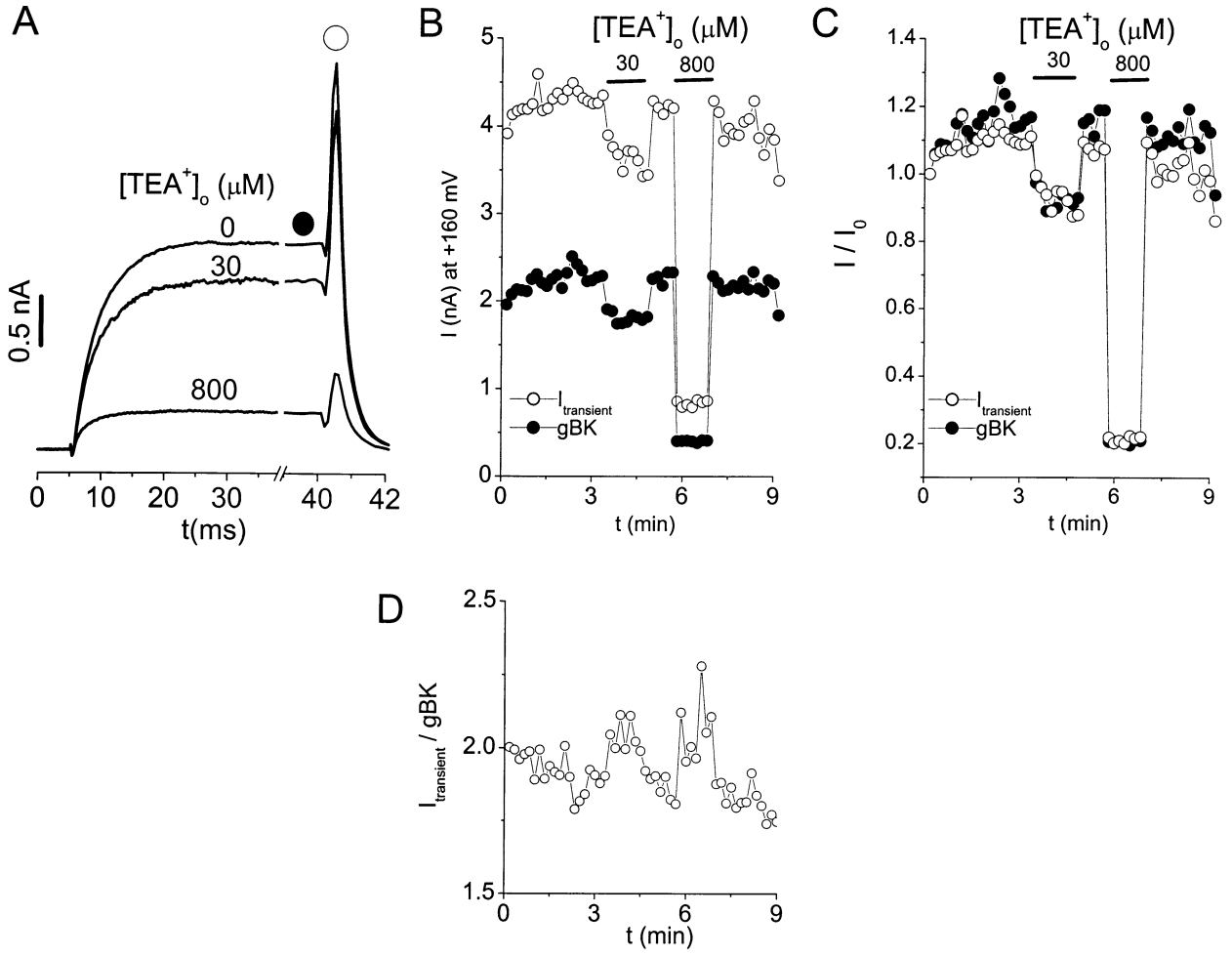

Fig. 6. $I_{transient}$ and *gBK* are similarly inhibited by tetraethylammonium ion (TEA). (*A*) Averaged current responses of 7–18 voltage steps to +160 mV with increasing concentrations of TEA. (*B*) Time course of TEA inhibition of $I_{transient}$ and *gBK*. Same cell as in (*A*). (*C*) Data in (*B*) normalized to the initial value. $I_{transient}$ and *gBK* were inhibited to a similar extent by 30 and 800 μM TEA. Note that the small, random variations in current amplitude of the two components during this time course are roughly in phase with one another. (*D*) Time course of change of the ratio $I_{transient}/gBK$ for this experiment.

0.4 nA in 800 μM. TEA, respectively. Even if the instantaneous *gBK* tail-current amplitude following return to the holding potential was the same as the steady-state *gBK* current (which is not expected because of the much smaller driving force at −40 mV), *gBK* tail current reduction by TEA would reduce $I_{transient}$ to 2.4 nA, only a 43% reduction. We observed ≈80% reduction of both *gBK* and $I_{transient}$ by 800 μM TEA (*see* Fig. 6*C*). It is worth noting here that during the time course of experiments the small, random variations in the amplitude of the two current components were often in phase (*see* Fig. 6*B*, 6*C*). If the production of $I_{transient}$ were somehow related to voltage errors associated with series resistance, reducing current amplitudes pharmacologically would be expected to modulate the ratio $I_{transient}/gBK$. We observed no significant changes in this ratio during application of 30 or 800 μM TEA (*see* Fig. 6*D*).

Voltage-dependence of $I_{transient}$ and *gBK*

If $I_{transient}$ were the result of some phenomenon related to the deactivation, and level of prior activation, of *gBK* currents these two components would be expected to have a similar voltage-dependence. To test this we measured steady-state *gBK* currents (averaged over the last 10 ms of a voltage step) and the corresponding transient currents at their peak. These data were normalized to their maximum, plotted as a function of voltage-step potential, and fit to the Boltzmann equation (*see* Fig. 7*A* and 7*B*). With 10 mM EGTA and no added Ca^{2+} in our pipette solution, the half-maximal voltage for channel activation, $V_{0.5}$, obtained from our Boltzmann fits, were very similar (within 1–5 mV) for both *gBK* and $I_{transient}$ in all cells analyzed in this fashion ($n = 10$). Elevating intracellular Ca^{2+} to 0.1 μM negative-shifted the activation curve of *gBK* and $I_{transient}$ (Fig. 7*C*, 7*D*).

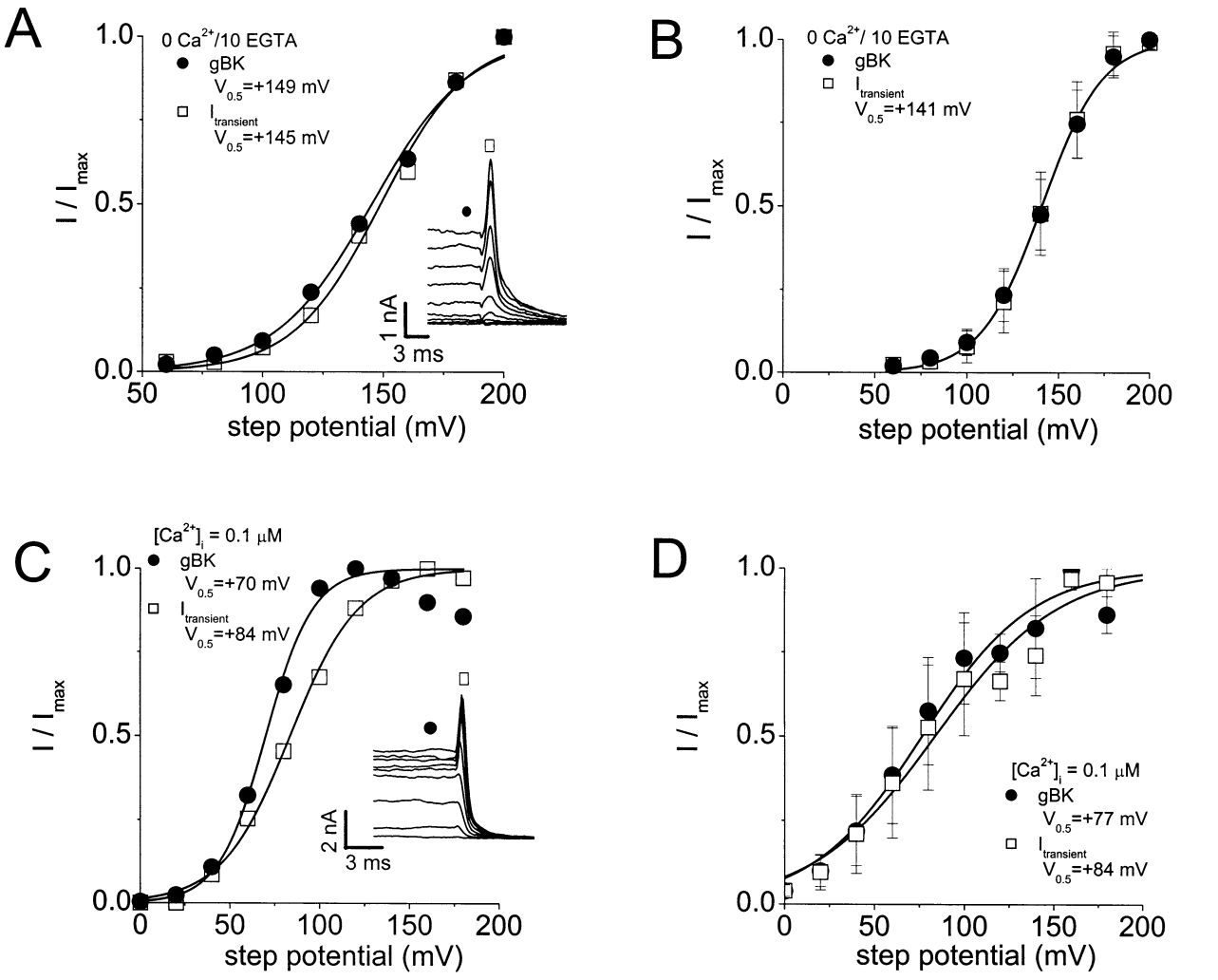

Fig. 7. $I_{transient}$ and *gBK* have similar voltage- and Ca^{2+}-dependencies. (*A*) Voltage dependence of activation of $I_{transient}$ and *gBK*. Normalized currents were plotted as a function of step potential and fit with the Boltzmann equation (*solid lines*) to determine the half-maximal voltage, $V_{0.5}$. $V_{0.5}$ was very similar for both $I_{transient}$ and *gBK*. The pipette solution contained 10 mM EGTA and no Ca^{2+} was added. Inset shows current traces (at the end of voltage steps) for the illustrated data. (*B*) Summary of voltage dependence of *gBK* and $I_{transient}$ with 0 Ca^{2+}/10 EGTA pipette solution. The data (mean ± SD; $n = 10$) were fit with a single Boltzmann equation. (*C*) Voltage dependence of *gBK* and $I_{transientt}$ with 0.1 μM $[Ca^{2+}]_i$. Inset shows current traces for the illustrated data. (*D*) Summary of voltage dependence of *gBK* and $I_{transient}$ with 0.1 μM $[Ca^{2+}]_i$ ($n = 5$). The $V_{0.5}$ values for *gBK* and $I_{transient}$ were both reduced by $[Ca^{2+}]_i$ but there were small quantitative differences; *gBK* had a lower $V_{0.5}$ than $I_{transient}$ in all cases.

With 0.1 μM $[Ca^{2+}]_i$ the activation curves for *gBK* and $I_{transient}$ did not match each other as well as we observed for the situation with no added Ca^{2+} and 10 mM EGTA; with 0.1 μM $[Ca^{2+}]_i$ the $V_{0.5}$ was smaller and the slope (q, apparent gating charge) was larger for *gBK* than for $I_{transient}$ in every cell examined. Boltzmann fits to the mean *gBK* data suggested a $V_{0.5}$ of +141 mV and +60 mV and an effective gating charge, q, of 1.6 and 2.1 for 0 Ca^{2+}/10 EGTA and 0.1 μM Ca^{2+}, respectively. Boltzmann fits to the mean $I_{transient}$ data suggested a $V_{0.5}$ of +141 mV and +73 mV and a q of 1.6 and 1.3 for 0 Ca^{2+}, 10 mM EGTA and 0.1 μM Ca^{2+}, respectively. The differences in $V_{0.5}$ with 0.1 μM Ca^{2+} were not statistically significant ($n = 5$ cells, $p = 0.36$).

Specificity of $I_{transient}$ for Glioma BK Currents

Two questions raised by the experiments described thus far are whether $I_{transient}$ is a general feature of glioma K^+ currents and if $I_{transient}$ is a general feature of BK currents.

We recorded currents from a human malignant glioma cell, U251-MG, that did not express BK currents. These cells had voltage-dependent currents, strongly reminiscent of delayed-rectifier type K^+ currents in terms of voltage dependence, kinetics, and TEA sensitivity. No currents resembling $I_{transient}$ were observed in U251MG cells (*data not shown*).

To evaluate the specificity of $I_{transient}$ for glioma BK channels we recorded BK currents from rat neu-

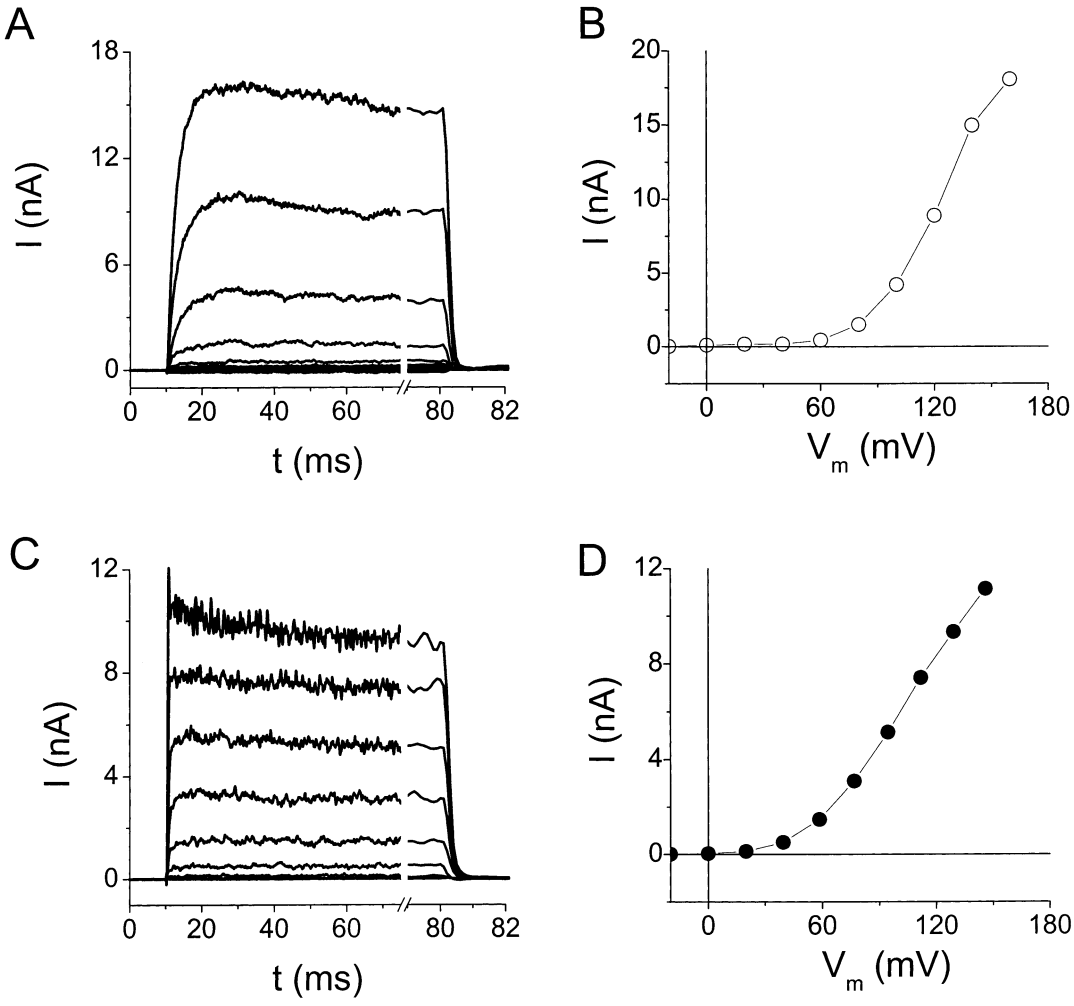

Fig. 8. BK currents in rat medial habenula neurons and a cloned BK channel do not display $I_{transient}$. (*A*) whole-cell ("TEA-sensitive") currents of an acutely dissociated rat neuron (medial habenula). Note break in records with different time base immediately preceding the end of the voltage step (same as in *C*). (*B*) *I-V* plot for the records in (*A*). (*C*) Whole-cell ("TEA-sensitive") currents in a HEK-293 cell expressing the BK clone, hbr5. (*D*) *I-V* plot for the records in (C). Neither habenular neurons nor hbr5-expressing HEK cells showed any evidence of $I_{transient}$ upon termination of activating voltage steps (compare to data in Fig. 1).

rons (acutely dissociated medial habenula cells) and a BK channel cloned from human brain (hbr5, Tseng-Crank et al., 1996) and expressed in HEK-293 cells (*see* Fig. 8). For these experiments, leak-subtraction was performed by subtracting records obtained in the presence of 10 mM TEA from control records ("TEA-sensitive" currents). Experiments on HEK cells and medial habenula neurons were performed under identical conditions as experiments on glioma cells. $I_{transient}$ was not observed in medial habenula neurons nor in cells expressing the hbr5 clone, suggesting that $I_{transient}$ is a unique feature of glioma BK channels.

Discussion

We have described a transient current ($I_{transient}$) that is intimately associated with the activity of large-conductance, Ca^{2+}-activated K^+ (BK) channels in human glioma cells. $I_{transient}$ was only observed following prior activation of glioma BK currents *gBK* and displayed intracellular K^+-dependence. The time-dependence, pharmacology, and voltage-dependence of the steady-state *gBK* currents and $I_{transient}$ were indistinguishable. In addition, intracellular Ca^{2+} shifted the activation curves of both $I_{transient}$ and *gBK* towards more negative potentials. There were small quantitative differences in the Ca^{2+}-dependence of $I_{transient}$ and *gBK*, but these were not statistically significant over the range of intracellular Ca^{2+} examined. Another compelling observation is that the small, random variations in the amplitude of the two components were roughly in phase with one another (Fig. 6*C*). These data indicate $I_{transient}$ is due to a mechanism dependent on the deactivation, and level of prior activation, of glioma BK channels. The activation time constants of $I_{transient}$ are voltage-dependent and in the same range as reported deacti-

vation time constants for BK channels determined with identical voltage steps (Cui et al., 1997; Brenner et al., 2000), supporting a direct relationship between BK channel deactivation and the generation of $I_{transient}$. $I_{transient}$, with unclear functional significance, is intriguing for its unusual biophysical properties (*see* below) and its absence in recordings of BK channels in cells other than human glioma. Specificity of $I_{transient}$ for glioma BK channels suggests glioma BK channels have unique molecular/biophysical properties. These unique properties could result from differences in primary structure, altered phosphorylation/kinetic state, and/or molecular-molecular interactions. Consistent with these conclusions, molecular studies have demonstrated the expression of a novel BK channel isoform with enhanced calcium sensitivity in the glioma cells under study (Liu et al., 2002). The pharmacology of glioma BK currents suggests expression of auxiliary β subunits by glioma cells (Ransom, Liu & Sontheimer, 2002).

Our data do not demonstrate the mechanism underlying generation of $I_{transient}$. We will begin by discussing the unusual biophysical characteristics of $I_{transient}$ and then discuss possible mechanisms for the generation of $I_{transient}$.

Biophysical Characteristics of $I_{transient}$

$I_{transient}$ has features that are inconsistent with it representing simple ionic current through open ion channels. First, $I_{transient}$ amplitude was relatively invariant over a 200 to 240 mV range (*see* Fig. 2*C*). For this to be accounted for by an ion channel, assuming no change in permeability characteristics, the channel must have a voltage-dependent single-channel conductance that is so exquisitely tuned to driving force that its *I-V* relationship is a flat line. This seems unlikely because modulation of unitary conductance does not occur over such large ranges (Mulle, Lena & Changeux, 1992; Newman, 1993; Berdiev et al., 1996). Secondly, and more to the point, $I_{transient}$ was still large and outward at −140 mV, a potential at which all ions in our experimental solutions experience an electrochemical gradient to produce inward current. An attractive interpretation of the lack of current reversal and voltage-independent amplitude of $I_{transient}$ is that $I_{transient}$ is generated by some mechanism unrelated to ion flow across the membrane through a resistive pathway (ion channels).

The central tenet for this conclusion is that our control of membrane potential is accurate. However, we have serious concerns about our ability to rapidly (within 1–3 ms) change membrane potential over such large ranges (up to 320 mV). In the ideal situation of an excised membrane patch, voltage changes are complete within 30–50 μs (Hilgemann, 1995). It is unlikely that our voltage-clamp of glioma cells during whole-cell recordings is this fast and voltage changes may not be complete over the 300 to 1000 μs duration of $I_{transient}$ activation. In addition to temporal concerns, the voltage errors associated with series resistance for such large currents, despite 80% compensation, can be in the range of 10–30 mV. In spite of this, the magnitude of the voltage change during the period $I_{transient}$ is active should increase progressively with increasing negative changes in command potential. This is supported by the observed acceleration of $I_{transient}$ activation kinetics and *gBK* deactivation kinetics. We observed <10% change in $I_{transient}$ amplitude from its peak at +60 mV to −140 mV (values given as command potentials; refer to Fig. 2*C*,*D*,*E*). Thus, despite concerns over the speed of our voltage clamp we can conclude that $I_{transient}$ exhibits non-Ohmic behavior.

Possible Mechanisms Underlying the Generation of $I_{transient}$

As noted above, our experiments do not demonstrate the mechanism underlying the production of $I_{transient}$. Below we discuss possible mechanisms of $I_{transient}$ generation including artifactual sources.

Two potential artifactual sources of $I_{transient}$ are voltage errors associated with series resistance and poor voltage-clamp control of membrane potential. If the generation of $I_{transient}$ were somehow related to voltage errors associated with series resistance, lessening these voltage errors by pharmacological reduction of current amplitudes would be expected to modulate the ratio of $I_{transient}/gBK$. This was not observed (*see* Fig. 6*D*). Poor spatial and temporal voltage control during a patch-clamp experiment implies that the membrane potential at distant parts of cells never reaches the command potential. The membrane potential of distal cell parts will always be less than that of proximal cell parts. Thus the driving force for K^+ ions would never be greater than it is during the activating voltage step. It is difficult to imagine how this situation could produce an $I_{transient}/gBK > 1$. It is conceivable that glioma BK currents could be relieved of some voltage-dependent block during the return to the holding potential, leading to the generation of $I_{transient}$. Removal of channel block during return to the holding potential could explain the $I_{transient}/gBK$ ratio >1 but cannot explain the large, outward current at −140 mV. We performed experiments with and without intracellular Mg^{2+} and observed no alteration of the ratio $I_{transient}/gBK$.

We must note that $I_{transient}$ was observed in our previous studies on BK currents in human glioma cells during whole-cell recordings but not during single-channel recording from cell-attached patches (Ransom & Sontheimer, 2001). Ensemble average currents (constructed from single-channel currents) had a small, inward (tail) current following activating voltage steps in contrast to the large, outward current transients seen following activating voltage steps during whole-cell

recording (Ransom & Sontheimer, 2001). The small, inward tail current of ensemble averages resulted from reversal of current through single BK channels but no correlate of $I_{transient}$ was observed. This could relate to the use of KCl pipette solution for cell-attached recordings and the slowing of deactivation in the presence of high $[K^+]_o$ (*see* Fig. 8, Ransom & Sontheimer, 2001). Outside-out patches expose BK channels to identical ionic conditions as during whole-cell recording. We have obtained two examples of outside-out patches with clear evidence of $I_{transient}$ (Ransom and Sontheimer, unpublished observations). The significance of the low frequency of $I_{transient}$ observation in outside-out patches is not clear to us. One possibility is that the generation of $I_{transient}$ is dependent on the kinetic state of BK channels and that the kinetic state is affected during patch excision, although previous studies have reported no effect of patch excision on the kinetic state of BK channels in glioma cells (Palotta et al., 1987). Regardless, the unequivocal presence of $I_{transient}$ in a subset of outside-out patches assuages our concerns over space-clamp errors as a potential source of $I_{transient}$ generation. Likewise, $I_{transient}$ was universally present in small, spherical glioma cells recorded from shortly (within 12 h) after plating on coverslips.

Is $I_{transient}$ due to gating charge movement? The large amplitude of $I_{transient}$ (often >10 nA) excludes this possibility. We only observed $I_{transient}$ during negative voltage changes following BK channel activation, while gating currents are observed at the onset and offset of voltage steps (Hille, 1992). This asymmetry is another peculiar aspect of $I_{transient}$. In addition, the charge movements associated with movement of gating particles during negative voltage changes should give rise to inward not outward currents.

A further consideration is whether transient changes in ion concentrations in the immediate microenvironment of the membrane could contribute to the non-Ohmic and non-Nernstian behavior of $I_{transient}$. If anything, the large outward BK current generated during depolarizations would be predicted to transiently increase $[K^+]_o$ and decrease $[K^+]_i$ in the membrane's microenvironment. These changes are exactly opposite to the changes in ion concentration necessary to explain the failure of $I_{transient}$ to reverse direction and its voltage-independence over a 200 mV range.

If $I_{transient}$ were some phenomenon directly related to channel deactivation, one might expect that the ratio $I_{transient}/gBK$ would be constant across a range of potentials in any given cell (i.e., each open channel contributes a constant amount of charge to $I_{transient}$). This was not the case. The increase in the ratio $I_{transient}/gBK$ with increasing depolarization is consistent with the idea that the generation of $I_{transient}$ depends on the degree of activation of BK channels and the resultant kinetic states (McManus & Magleby, 1988). That is, the predominant kinetic state(s) at low levels of activation (low P_o) are incapable of generating $I_{transient}$ during deactivation, while at higher levels of activation (high P_o) a different kinetic state(s) predominate and deactivation of BK channels produces the charge movements responsible for $I_{transient}$. Although we were unable to obtain patches with few enough channels to permit a detailed kinetic analysis (*see* Ransom & Sontheimer, 2001), there may be large differences in the kinetic behavior of these channels during whole-cell recordings and in excised patches (but *see* Palotta et al., 1987). If this were the case, one possibility for the low frequency of $I_{transient}$ in patches is that a transition between specific open states is required for the generation of $I_{transient}$ and the fraction of time spent in these specific open states is much, much lower in patches than it is during whole-cell recordings.

The exact nature of $I_{transient}$ remains unclear. One hypothesis is that conformational changes related to activation of the channel proteins responsible for the steady-state current (BK channels) exposes an ion-binding epitope(s) of the channel to the cytosolic or extracellular medium. Retraction to an intramembranous site during channel closure would force bound ions off of the epitope, producing a capacitive-like current which follows a time course not dependent on the time constant of the lipid bilayer but dependent on the time constant of channel closure. The slow activation of *gBK* predicts that the voltage is constant as conformational changes related to channel activation begin to occur. This would explain why ion interactions at these hypothetical sites do not contribute to any capacitive-like current at the beginning of a depolarizing voltage step. It is also conceivable that movement of highly charged macromolecules associated with glioma BK function contribute to $I_{transient}$ generation.

Hypothesis for the Generation of $I_{transient}$

We favor another possibility for the generation of $I_{transient}$ that is consistent with the data. K^+ ions lined up within the channel or in the cytosolic vestibule of the channel could be trapped there during deactivation and are subsequently forced to exit to the extracellular side. This implies that $I_{transient}$ is produced by ions traversing the pores of nonconducting, deactivated BK channels in a manner independent of membrane potential. Such a scenario is consistent with the effects of channel blockers, intracellular substitution of cations other than K^+ on $I_{transient}$ amplitude, and the non-Ohmic behavior of $I_{transient}$. To account for the $I_{transient}/gBK$ ratio greater than one we must assume that only a fraction of the BK channels competent to produce $I_{transient}$ upon deactivation is actually open and conducting K^+ ions during the preceding steady-state *gBK* current. The voltage-dependent deactivation of BK channels adequately explains the increasing amplitude of $I_{transient}$ seen with negative voltage changes of increasing magnitude. As *gBK* deactivation kinetics ac-

celerate, deactivation becomes more synchronous, as does whatever process underlies $I_{transient}$ production, generating faster activation and larger amplitudes of $I_{transient}$. In our previous studies the ratio of $I_{transient}$/*gBK* was reduced by increasing intracellular Ca^{2+} and elevating extracellular K^+, two maneuvers that slow BK deactivation kinetics and therefore the synchronicity of deactivation (Ransom & Sontheimer, 2001).

Specificity of $I_{transient}$ for Glioma BK Currents

To the best of our knowledge, ours is the first description of transient currents associated with the deactivation of BK channels. $I_{transient}$ was not observed with BK channels in two other systems, rat neurons and a cloned channel expressed in HEK cells. Comparison of the BK channel cloned from glioma cells (*gBK*) and hbr5 in oocytes showed no evidence of $I_{transient}$ (Liu et al., 2002). This suggests that $I_{transient}$ is dependent on some factor present in glioma cells but absent in rat neurons, HEK cells, or oocytes and that $I_{transient}$ is not an intrinsic characteristic of the novel BK channel expressed by glioma cells. Glioma cells are believed to express auxiliary β subunits for BK channels (Ransom et al., 2002). It is possible that β subunits represent the extrinsic factor for $I_{transient}$ production.

Summary

We have described a current transient that is intimately related to BK channel deactivation. This transient current was not observed in association with BK channels in other systems and may be dependent on factors unique to glioma cells but extrinsic to BK channels themselves. The non-Ohmic behavior of $I_{transient}$ could be explained by ion trapping within the pore or in a cytosolic vestibule with voltage-independent exit to the extracellular space during deactivation.

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References

Aggarwal, S.K., MacKinnon, R. 1996. Contribution of the S4 segment to gating charge in the *Shaker* K^+ channel. *Neuron* **16:**1169–1177

Armstrong, C.M. 1992. Voltage-dependent ion channels and their gating. *Physiol. Rev.* **72:**S5–S13

Berdiev, B.K., Prat, A.G., Cantiello, H.F., Ausiellio, D.A., Fuller, C.M., Jovov, B., Ismailov, I.L. 1996. Regulation of epithelial sodium channels by short actin filaments. *J. Biol. Chem.* **271:**17704–17710

Brenner, R., Jegla, T.J., Wickenden, A., Liu, Y., Aldrich, R.W. 2000. Cloning and functional characterization of novel large-conductance calcium-activated potassium channel β subunits, hKCNMB3 and hKCNMB4. *J. Biol. Chem.* **275:**6453–6461

Brew, H., Attwell, D. 1987. Electrogenic glutamate uptake is a major carrier in the membrane of axolotl retinal glial cells. *Nature* **327:**707–709

Brismar, T. 1995. Physiology of transformed glial cells. *Glia* **15:**231–243

Chin, L.S., Park, C.C., Zitmay, K.M., Sinha, M., Dipatri, A.J., Perillan, P., Sinard, J.M. 1997. 4-Aminopyridine causes apoptosis and blocks an outward-rectifier K^+channel in malignant astrocytoma cell lines. *J. Neurol. Res.* **48:**122–127

Cui, J., Cox, H.D., Aldrich, R.W. 1997. Intrinsic voltage-dependence and Ca^{2+} regulation of *mSlo* large-conductance Ca-activated K^+ channels. *J. Gen. Physiol.* **414:**647–673

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high-resolution recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391:**85–100

Hilgemann, D.W. 1995. The giant membrane patch. *In:* Single Channel Recording. B. Sakmann and E. Neher, editors. pp. 307–327. Plenum Press, New York

Hille, B. 1992. Ionic Channels of Excitable Membranes (2nd ed.)., Sinauer, Sunderland, MA

Jan, L.Y., Jan, Y.N. 1997. Ways and means for left shifts in the MaxiK channel. *Proc. Natl. Acad. Sci. USA* **94:**13383–13385

Lester, R.A., Dani, J.A. 1995. Acetylcholine receptor desensitization induced by nicotine in rat medial habenula neurons. *J. Neurophysiol.* **74:**195–206

Liu, X., Chang, Y., Reinhart, P., Sontheimer, H. 2002. Cloning and characterization of glioma BK, a novel BK channel isoform highly expressed in human glioma cells. *J. Neurosci.* **22**(5):1840–1849

Marks, P.W., Maxfield, F.R. 1991. Preparation of solutions with free calcium concentrations in the nanomolar range using 1,2-bis(o-aminophenoxy)ethane-N,N,N′N′-tetraacetic acid. *Anal. Biochem.* **193:**61–71

McManus, O.B., Magleby, K.L. 1988. Kinetic states and modes of single large-conductance calcium-activated potassium channels in cultured rat skeletal muscle. *J. Physiol.* **402:**79–120

Mulle, C., Lena, C., Changeux, J.P. 1992. Potentiation of nicotinic receptor response by external calcium in rat central neurons. *Neuron* **8:**937–945

Newman, E.A. 1993. Inward-rectifying potassium channels in retinal glial (Muller) cells. *J. Neurosci.* **13:**3333–3345

Palotta, B.S., Hepler, J.R., Oglesby, S.A., Harden, T.K. 1987. A comparison of calcium-activated potassium channel currents in cell-attached and excised patches. *J. Gen. Physiol.* **89:**985–997

Ransom, C.B., Sontheimer H. 2001. BK channels in human glioma cells. *J. Neurophysiol.* **85:**790–801

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

Soroceanu, L., Manning, T.J., Sontheimer, H. 1999. Modulation of glioma cell migration and invasion using Cl^- and K^+ ion channel blockers. *J. Neurosci.* **19:**5942–5954

Thomas, R.C. 1972. Electrogenic sodium pump in nerve and muscle. *Physiol. Rev.* **52:**563–594

Tseng-Crank, J., Foster, C.D., Krause, J.D., Mertz, R., Godinot, N. Dichiara, T.J., Reinhart, P.H. 1996. Cloning, expression, and distribution of functionally distinct Ca^{2+}-activated K^+ channel isoforms from human brain. *Neuron* **13:**1315–1330

Weiss, D.S., Magleby, K.L. 1990. Voltage-dependence and stability of the gating kinetics of the fast chloride channel from rat skeletal muscle. *J. Physiol.* **426:**145–176