Chloride Accumulation Drives Volume Dynamics Underlying Cell Proliferation and Migration

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Habela CW, Ernest NJ, Swindall AF, Sontheimer H. Chloride accumulation drives volume dynamics underlying cell proliferation and migration. J Neurophysiol 101: 750–757, 2009. First published November 26, 2008; doi:10.1152/jn.90840.2008. During brain development, progenitor cells migrate over long distances through narrow and tortuous extracellular spaces posing significant demands on the cell’s ability to alter cell volume. This phenotype is recapitulated in primary brain tumors. We demonstrate here that volume changes occurring spontaneously in these cells are mediated by the flux of Cl\(^{-}\) along with obligated water across the cell membrane. To do so, glioma cells accumulate Cl\(^{-}\) to \(\sim 100\) mM, a concentration threefold greater than predicted by the Nernst equation. Shunting this gradient through the sustained opening of exogenously expressed GABA-gated Cl\(^{-}\) channels caused a 33% decrease in cell volume and impaired the ability of cells to migrate in a spatially constrained environment. Further, dividing cells condense their cytoplasm prior to mitosis, a phenomenon which is associated with the release of intracellular Cl\(^{-}\) as indicated by a 40-mM decrease in [Cl\(^{-}\)]. These findings provide a new framework for considering the role of intracellular Cl\(^{-}\) in glioma cells. Here, Cl\(^{-}\) serves as an important osmotically active regulator of cell volume being the energetic driving force for volume changes required by immature cells in cell migration and proliferation. This mechanism that was studied in CNS malignancies may be shared with other immature cells in the brain as well.

INTRODUCTION

Ion homeostasis is important to the normal functioning of all cells, however, in the brain, tight regulation of both intra- and extracellular ions is central to electrochemical signaling. Interestingly, Cl\(^{-}\), the major inorganic anion in the brain, is often overlooked. This is likely due to the fact that in most mature neurons, intracellular [Cl\(^{-}\)] is quite low (\(\sim 10\) mM) and primarily serves to stabilize the resting membrane potential on opening of GABA-gated channels (Kaila 1994). Yet mature astrocytes accumulate chloride to levels approaching four times what would be determined by passive distribution (Walz 2002). Further all proliferating cells in the brain, including the immature neurons of development and the neural stem cells (NSCs) in the adult actively accumulate chloride (Achilles et al. 2007; Kakazu et al. 1999; Kuner and Augustine 2000) such that these cells exhibit a characteristic depolarizing response to \(\gamma\)-aminobutyric acid (GABA)-mediated chloride channel opening. As these cells differentiate into neurons, this gradient is often extinguished and passive levels are established (Kakazu et al. 1999; Luhmann and Prince 1991). This indicates that chloride homeostasis may in fact be a key determinant of the mature versus immature phenotype of neural cells.

Outside of development and the normally dividing adult NSC population, Cl\(^{-}\) homeostasis seems to have a central role in various pathological states (De Koninck 2007). For example, hippocampal neurons from drug resistant temporal lobe epilepsy patients exhibit alterations in the expression of chloride transporters resulting in hyperexcitability (Palma et al. 2006) as also observed following cortical injury in pyramidal neurons (Jin et al. 2005), and astrocytes increase their resting chloride conductance in the case of reactive gliosis (MacVicar et al. 1989; Walz and Wuttke 1999). Additionally, cells of the major primary brain tumors, gliomas, have a significant resting conductance for Cl\(^{-}\), which has been shown to be important for both the extensive proliferation and invasive migration of these cells (Habela and Sontheimer 2007; Ransom et al. 2001). In the case of glioma cells, the high chloride conductance likely reflects the importance of volume changes to these processes.

In addition to regulating membrane voltage, the flux of ions across the cell membrane will also osmotically drive the movement of water. As, in most cells, there is both an outward directed gradient and a significant conductance for K\(^{+}\), volume changes are dictated by the levels and movement of the limiting ion, namely Cl\(^{-}\). In situations where isotonic volume decreases are required, as in the case of cell migration, this process would be facilitated by an outward directed gradient for chloride such as that found in NPCs. When intracellular Cl\(^{-}\) concentrations ([Cl\(^{-}\]\text{in}) are high, the opening of Cl\(^{-}\)-channels allows the efflux of Cl\(^{-}\), which, coupled to K\(^{+}\) efflux, would draw water out of the cell. Like NPC’s, glioma cells are highly migratory and proliferative, and a growing body of evidence indicates that they are in fact derived from NPCs (Hemmati et al. 2003; Ignatova et al. 2002; Ma et al. 2008; Rao et al. 2003; Singh et al. 2003). Whether they accumulate intracellular chloride to the same extent and the role that intracellular chloride plays in migration and proliferation in these cells have yet to be determined.

In the present study, we determined [Cl\(^{-}\]\text{in} from the reversal potentials of pure Cl\(^{-}\) currents using the gramicidin patch-clamp technique. The results of these experiments indicated that, in nondividing glioma cells, [Cl\(^{-}\]\text{in} was greater than threefold what would be expected for passive distribution. We previously observed that glioma cells undergo a significant volume condensation, termed premitotic condensation (PMC), in preparation for division (Habela and Sontheimer 2007) and that chloride-channel-mediated changes in cell volume are involved in migration (Ransom and Sontheimer 2001). We therefore determined whether the Cl\(^{-}\) gradient could provide...
the driving force for volume changes involved in migration and PMC in these cells. Strikingly, the sustained opening of exogenously expressed ligand activated chloride channels was sufficient to cause an approximate 33% volume decrease that could be further potentiated by inhibiting regulatory volume increase. We also demonstrate that in mitotic cells, which are smaller and have lower [Cl\(^-\)]\(_{\text{int}}\), cell volume is less dependent on modulation of [Cl\(^-\)]\(_{\text{int}}\) by alterations in transport. Finally, we establish that this gradient is critical to the volume changes occurring in migration as depleting the Cl\(^-\) gradient brought about a 40% decrease in cell migration. This is the first study to directly examine [Cl\(^-\)]\(_{\text{int}}\) in glioma cells and it demonstrates that changes in [Cl\(^-\)]\(_{\text{int}}\) have important consequences for cell migration and proliferation. As these cells may recapitulate early development, these findings may also apply to other immature cells in the brain.

METHODS

Glioma cell culture and pharmacology

D54-MG glioma cells (WHO IV, glioblastoma multiforme) were a gift from Dr. D. Bigner (Duke University, Durham, NC). These cells were maintained at 37°C, 10% CO\(_2\) in 1:1 DMEM-F12 (Mediatech, Hendon, VA) with 7% FBS and 2 mM Gln and were used for experiments within 20 passages. The stable GFP cell line used for Fig. 3, A and B, was derived from D54-MG cells (D54-MG-GFP), which were transfected with pEGFP-N1 (Clontech, Mountain View, CA). The plasmid insertion was maintained with 0.25 mg/ml G418 disulfate salt (GIBCO). All other experiments were performed with a stable line generated by transfection of D54-MG cells with a pRES-GFP vector (Clontech) containing a Rho 1 CDNA insert. Rho1-pcDNA3 encodes for a GABA-gated Cl\(^-\) channel and was a generous gift of Dr. David Weiss (University of Texas San Antonio Medical Center, San Antonio, TX). The rho 1 sequence was cut from the pcDNA3 vector by room temperature partial digest with EcoR1 and spliced into the EcoR1 site in the multiple cloning site of pIRES-GFP. Either the Rho1-pRES-GFP construct or the empty vector control was transfected into D54-MG cells using a Fugene 6 standard transfection protocol (Roche, Basel). Stable plasmid insertions were selected for and maintained with 0.25 mg/ml G418 disulfate salt.

All stock concentrations of drugs used were dissolved in dimethyl sulfoxide (DMSO) or water. Unless otherwise noted the DMSO stock was diluted 1:1,000 to obtain the final working concentration. Drug treatment media was composed of control media supplemented with DMSO or drugs dissolved in the same. RT/rtreatment media was composed of control media supplemented with 5 μg/ml of a mixture of gramicidin A–D from Bacillus brevis and 4 μg/ml Alexa 594 hydrazide sodium salt (Molecular Probes, Eugene, OR). Gramicidin patch recordings were achieved as described previously (Kyrozis and Reichling 1995). Normal extracellular NaCl bath solution contained (in mM): 130 NaCl, 5 KCl, 1 CaCl\(_2\), 10.5 glucose, and 32.5 HEPES acid. pH was adjusted to 7.4 and osmolarity ranged from 306 to 312 mosM/kg.

Intracellular chloride concentrations were calculated using the Nernst Equation: \(E_{\text{ion}} = RT/F \ln \left[\text{ion}\right]_{\text{out}}/\left[\text{ion}\right]_{\text{in}}\); where \(E_{\text{ion}}\) is the reversal potential for the chloride current, \([\text{ion}]_{\text{out}}\) is the known chloride concentration of the bath solution, and under our recording conditions RT/F is equal to ~60.

Two-dimensional time-lapse imaging

Glioma cells were plated on 0.17-mm glass-bottom, 35-mm dishes (Mattek, Ashland, MA). Imaging experiments were performed in normal culture media 24–48 h after plating for experiments in which cells were not chronically drug treated and 96 h after plating when 72-h drug treatments were applied. Time-lapse images were acquired with a Hamamatsu IEE1394 Digital CCD camera (325-6, Sunayama-Cho, Hamamatsu City, Japan) mounted on an Olympus IX81 motorized inverted microscope equipped with an Olympus Disk Scanning Unit (DSU; Olympus, Melville, NY) and controlled by Slidebook software (Intelligent Imaging Innovations, Denver, CO). The microscope was housed in a temperature- and CO\(_2\)-controlled humidified incubator (LIVE; Olympus, Melville, NY) with 5% CO\(_2\) in the mixed gas. Imaging experiments were performed in normal culture media 24–48 h after plating for experiments in which cells were not chronically drug treated and 96 h after plating when 72-h drug treatments were applied. Time-lapse images were acquired with a Hamamatsu IEE1394 Digital CCD camera (325-6, Sunayama-Cho, Hamamatsu City, Japan) mounted on an Olympus IX81 motorized inverted microscope equipped with an Olympus Disk Scanning Unit (DSU; Olympus, Melville, NY) and controlled by Slidebook software (Intelligent Imaging Innovations, Denver, CO). The microscope was housed in a temperature- and CO\(_2\)-controlled humidified incubator (LIVE; Olympus, Melville, NY) with 5% CO\(_2\) in the mixed gas.

INTENSITY MEASUREMENTS

Starting from baseline measurements prior to drug or vehicle addition. Each measurement indicates the mean cell volume of 5,000–10,000 cells. Relative volume measurements were calculated as a ratio to 10 baseline measurements prior to drug or vehicle addition.

Three-dimensional imaging

Laser scanning confocal images were obtained using an Olympus Fluoview 300 system equipped with a ×40/0.90 NA water-immersion lens (Olympus America, Center Valley, PA). GFP-expressing cells were grown in tissue culture on 35 mm glass-bottom dishes, and cells were imaged in normal culture media warmed to 37°C with a heated dish holder while continuously superfused with 5 CO\(_2\)-95% O\(_2\). GFP was excited by the argon (excitation 488) laser and Z spacing was set to a 0.325–0.5 μm. Image stacks were exported and volume measurements were made in Image Pro 3D Suite (Media Cybernetics, Bethesda, MD).

J Neurophysiol • VOL 101 • FEBRUARY 2009 • www.jn.org
Transwell migration assays

The bottoms of 8.0 μm pore PET track-etched membrane cell culture inserts (Becton Dickinson) were coated with 3 μg/ml vitronectin overnight at room temperature then washed and blocked with 1% fatty acid free (FAF) bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1–2 h. Cells were washed with PBS, lifted with 0.5 mM EGTA, pelleted, and resuspended in serum-free DMEM/1% fatty acid free (FAF) BSA. These were then counted and diluted to 1 × 10^6/ml and 400 μl were added to the top of the membrane insert. Cells were allowed 30 min to adhere then GABA was added to both the top and bottom of the insert. The Transwell assay was returned to a cell culture incubator, and cells were allowed 4 h to migrate then were fixed and stained with a cresyl violet solution [20 ml stock cresyl violet (1 g/99 ml 20% ethanol) 20 ml 95% ethanol, 150 ml H_2O] overnight at 4°C. Cresyl violet solution was washed three times with PBS, and the top side of the membrane was wiped clean with cotton tipped applicator so that the only remaining cells were those that had migrated through the pore. Five random fields per insert were imaged at 20× magnification, and all conditions were performed in duplicate.

Statistical analysis

Current subtractions and I-V relationships from electrophysiologically recorded observations were determined using Clampfit (Axon Instruments). For all experiments, raw data were analyzed and plotted using Origin 8.0 software (Microcal Software). Appropriate statistical tests were chosen according to the type of data analyzed and performed using Graphpad Instat (Graphpad Software, San Diego, CA). Unless otherwise stated, all measurements are reported with SE and number of observations (n).

RESULTS

To determine the role of intracellular chloride in the biology of glioma cells, we first sought to directly measure intracellular chloride concentrations ([Cl^-]_in) using D54-MG glioma cells, which are a well-characterized glioma line. The most accurate way to measure [Cl^-]_in is to calculate it from the reversal potential of a pure Cl^- current in a cell in which intracellular Cl^- is undisturbed. Unfortunately, high-grade glioma cells such as the D54-MG line do not express the major ligand-gated chloride channels (Labrakakis et al. 1998), and inhibitors of the voltage-gated anion channels present in these cells are relatively nonspecific.

To overcome this problem, we generated a stable cell line that expressed the rho 1 (ρ1) receptor driven by the CMV promoter as well as GFP linked by an internal re-entry site (RES) to ρ1 expression. The ρ1 receptor forms the homomeric GABA_2 channel, which conducts nondesensitizing chloride currents, has a high affinity for GABA (K_i = 0.8–2.2 μM), and is blocked by picrotoxin (Zhang et al. 2001). We confirmed channel expression in the ρ1-D54 cells under whole cell patch-clamp conditions. As shown by the example cell in Fig. 1A, top, in which the ρ1 expressing cell is clamped at −40 mV, application of 1 μM GABA produces a large inward current that is rapidly decreased by the application of 10 μM picrotoxin. This current is not present when ρ1-D54 cells are preincubated with picrotoxin (Fig. 1A, colored trace) or in cells transfected with the pRES-GFP vector alone (Fig. 1A, bottom). The ρ1-expressing cells therefore allowed us to control chloride currents by application or removal of micromolar concentrations of GABA.

To determine [Cl^-]_in, we required a technique that allowed us to record Cl^- currents under conditions in which native intracellular Cl^- remained undisturbed. The antibiotic, gramicidin, creates pores that are cation permeable but impermeable to Cl^-.

When gramicidin is included in a pipette that is sealed to a cell membrane, pores form and intracellular K^+ and Na^+ equilibrate with the pipette solution, but dialysis of chloride ions is prevented. We used this technique in combination with the ρ1-D54 cells. As a control to ensure that the cell membrane and, therefore intracellular chloride, remained intact, a fluorescent dye was included in the pipette solution and fluorescent images were taken before and after each recording. On membrane disruption and transition from the perforated to the whole cell configuration the cell is dialyzed with the dye as demonstrated by Fig. 1B.

Under perforated-patch conditions, current/voltage (I-V) relationships were determined either by ramping from −160 to +160 (400 ms duration) or by taking 100-ms voltage steps from −140 to +140 mV (20 mV increment). Chloride currents were maximally activated by application of 10 μM GABA and the subtracted I-V curve from before and after application was used to determine E_Cl^- (voltage at I = 0). As demonstrated by the I-V curve in Fig. 1C for an example cell, E_Cl^- under gramicidin patch was −6.82 mV. Solving the Nernst equation by substituting E_Cl^- and the known extracellular chloride concentration of 137 mM results in a calculated [Cl^-]_in of 105 mM. This approach was taken to determine [Cl^-]_in for n = 22 growth phase, nondividing cells (Fig. 1D). As a control, the same calculations were used to determine [Cl^-]_in from the reversal potentials of currents recorded under the whole cell configuration (Fig. 1C, black trace) in which case the [Cl^-]_in was determined by the pipette solution. The data in Fig. 1D indicate that intracellular chloride is very high in these cells. Growth phase D54 glioma cells contain 100.5 ± 11.1 mM Cl^-.

As passive distribution would set [Cl^-]_in at −29.5 mM for a membrane potential of −40 mV; this indicates an outwardly directed chloride gradient.

In the cell cycle of glioma cells, the transition to mitosis is accompanied by a significant reduction of cell volume, referred to as premitotic condensation (PMC), that is impaired by blocking chloride efflux (Habela and Sontheimer 2007). This suggests a net efflux of chloride between growth and M phase of dividing cells and prompted us to examine [Cl^-]_in in mitotic cells as compared with their nondividing counterparts. Using chromatin condensation and a rounded cell body as morphological criteria to distinguish mitotic cells from growth phase cells, we determined [Cl^-]_in to be 66 ± 12 mM (Fig. 1D, n = 10). Interestingly, although at 66 mM there is still an outwardly directed gradient for chloride, this is a significant (P < 0.05) decrease from the nondividing bipolar cells, suggesting that a net loss of chloride may drive the volume decrease observed in PMC.

We hypothesized that [Cl^-]_in is a determinant of cell volume. Further, we hypothesized that with such high concentrations of intracellular chloride found in glioma cells, opening of chloride channels would be sufficient to cause a volume decrease as the osmotically active Cl^- ions would drive the efflux of water. To determine the relationship between Cl^- and size, we therefore measured the in-suspension volumes of ρ1-D54 cells using a Coulter Counter. After establishing a baseline volume (V_o), the cells were exposed to 10 μM GABA and...
volume measurements were continued for 50 min. As demonstrated by the example experiment in Fig. 2A, the GABA application caused a significant decrease in cell volume (closed squares) compared with control cells not treated with GABA (open circles). The mean percent volume decrease from four independent experiments was 6.8 ± 0.2 (% \( P < 0.0001 \), 1-sample t-test). Albeit significant, it is likely that the volume decrease was underestimated due to the effects on volume of cytoskeletal disruption when adherent cells are suspended. We therefore used a second technique to determine the effects of chloride channel opening on cell volume while maintaining the cells in an adherent state.

The two-dimensional (2-D) time-lapse technique that we used to measure volume changes in adherent cells is based on the concentration or dilution of cytoplasmic molecules as a function of decreased or increased cytoplasmic water volume. In the case of fluorescent molecules, changes in concentration are reflected in changes in fluorescence intensity (Alvarez-Leefmans et al. 2006). We use the soluble GFP expressed by the \( \rho \)1-D54 cells to measure intensity over time in the presence or absence of GABA. The ratio of fluorescence at any given time \( (F) \) to the initial baseline fluorescence \( (F_i) \) was calculated for untreated and GABA-treated cells. The resultant GABA-treated curve was normalized to the control curve to generate the curve in Fig. 2B (closed symbol). These data demonstrate that application of GABA causes a 50% increase in GFP intensity suggestive of an approximate 33% decrease in cytoplasmic water volume. Further, this effect was almost doubled when we block the ability of these cells to compensate for this loss of intracellular chloride by inhibiting NKCC1, the cotransporter implicated in RVI in these cells, (Ernest and Sontheimer 2007) with 200 \( \mu \)M bumetanide (open symbol). These data show that under isotonic conditions, the sustained opening of chloride channels leads to an efflux of chloride sufficient to cause a significant decrease in cell volume and that, similar to their response to a hypertonic stress, these cells attempt to recover their volume through bumetanide-sensitive transporters.

These experiments indicate that under normal culture conditions opening of native chloride channels could potentially bring about a volume change similar to that which we observed for the opening of the GABAC channels in Fig. 2. Two biological situations where isotonic volume regulation is a central factor are cell division and cell migration. We previously reported that the transition into mitosis is accompanied by a cytoplasmic volume decrease consistent with the osmotically driven loss of water (Habela and Sontheimer 2007). The observed decrease in [\( \text{Cl}^- \)] in for mitotic cells (Fig. 1D) may be reflective of a channel-mediated efflux of chloride driving this volume change. We therefore compared the effects on cell volume of inhibiting chloride transport in normal growth phase bipolar cells versus rounded M phase cells (Fig. 3B). Individual cell volumes were determined using three-dimensional (3D) confocal microscopy for control cells and those treated with either 200 \( \mu \)M bumetanide or 40 \( \mu \)M DIOA, an inhibitor of the potassium chloride cotransporters (KCCs) responsible for chloride efflux. Interestingly, although treatment with DIOA sig-
significantly increased the volume of bipolar cells compared with controls, there was no difference between the M phase cells in any of the conditions.

As the transition from a bipolar cell to a rounded M phase cell requires a volume decrease (Habela and Sontheimer 2007), the increased volume of DIOA-treated bipolar cells suggests that these cells require a larger volume decrease to achieve the same volume at M phase as control cells. To determine whether chloride-efflux-mediated volume changes would be increased in the treated cells, we applied GABA to cells after 72 h of treatment with DIOA or bumetanide and analyzed relative volume changes by the 2-D time-lapse technique (Fig. 3C). The large DIOA-treated cells shrank to a greater extent than either the control or the bumetanide-treated cells. These data in combination with Fig. 3B suggest that the increase in cell volume for the bipolar cells is reflective of an increase in chloride and that an increased driving force and therefore efflux of chloride on channel opening decreases cell volume. As demonstrated in Fig. 1D, intracellular chloride is decreased in mitotic cells. Therefore cell volume at this stage would be less dependent on changes in chloride and may not be altered to the same extent as growth phase cells by treatment with transporter inhibitors. The elevated chloride prior to mitosis then provides the driving force for the mitotic volume condensation.

The second situation in which the ability to regulate cell volume is integral to the biological process is cell migration. As glioma cells invade through the confined extracellular spaces of the brain, either local or global decreases in cell volume are necessary to facilitate this. This can be conveniently modeled in Transwell invasion assays where cells have to transverse an 8 μm pore. We examined the effects of depleting intracellular chloride over a short period of time on the cells’ ability to migrate using this assay. Chloride was depleted by a 2-h treatment with 10 μM GABA prior to seeding cells for the Transwell migration assay. The ability of the cells to migrate was judged based on the number of cells in the control versus treated condition that migrated through 8 μm pores toward a chemotactic stimulus. As demonstrated by the
example frames in Fig. 4A, a greater number of control cells migrated. These results were quantified for four independent experiments and plotted in Fig. 4B. The 40% decrease in migration suggests that glioma cells require an outwardly directed gradient for chloride to undergo the volume and morphology changes involved in migration.

**DISCUSSION**

In this study, we show that glioma cells actively accumulate intracellular chloride to ~100 mM, which is well above comparable values of differentiated astrocytes (~40 mM) or neurons (~10 mM). Immature cortical (Achilles et al. 2007) and hippocampal (Kuner and Augustine 2000) neurons also accumulate [Cl$^-$]$_i$ to ~30 mM, with the latter study suggesting [Cl$^-$]$_i$ as high as 100 mM in P19 neurons. In glioma cells, this Cl$^-$ accumulation provides an outward directed driving force for the release of chloride that can be utilized to regulate cell volume in the processes of migration and proliferation. The opening of chloride channels allows glioma cells to rapidly decrease their volume when necessary while regulatory volume increase mechanisms, i.e., uptake of Cl$^-$ via transporters would allow them to regain homeostasis. The importance of this process is highlighted by the fact that glioma migration in our culture assay system is impaired by chloride depletion (as shown in Fig. 4) in addition to inhibition of native chloride channels (Ransom et al. 2001). These observations mechanistically link the required volume changes to the coordinated release of Cl$^-$ along with obligated water.

Our data examining mitotic cells suggest that, additionally, changes in [Cl$^-$]$_{in}$ are also required for cells to proliferate. As mitotic cells round up, they condense their cytoplasmic volume through the release of Cl$^-$ (Habela and Sontheimer 2007), and we were indeed able to show that during this stage [Cl$^-$]$_{in}$ is reduced almost twofold compared with the bipolar growth phase cells (Fig. 1D). Our data suggest that this volume decrease is caused by the efflux of chloride through ion channels because inhibitors of KCC transporter mediated efflux did not affect premiotic volume condensation but did cause interphase cells to gain in size. Chloride therefore determines both homeostatic volume and dynamic volume changes in glioma cells. Interestingly, the efflux of Cl$^-$ has also been implicated in volume changes that occur in conjunction with proliferation and invasion of human cervical cancer cells, yet in these cells, Cl$^-$ efflux was mediated by KCC1 (Shen et al. 2003) rather than channel mediated as is the case in gliomas (Habela et al. 2008).

The accumulation of intracellular chloride in glioma cells is reminiscent of that observed during neuronal development and in neural stem cells (NSCs) in the adult. In these cells, the differential activity of NKCC and KCC transporters during development leads to Cl$^-$ gradients that are outward in immature cells but become inward in mature neurons (Kakazu et al. 1999; Kuner and Augustine 2000; Overstreet et al. 2005), with the majority of Cl$^-$ uptake in immature cells attributable to the NKCC1 transporter (Achilles et al. 2007). The developmental change in intracellular [Cl$^-$]$_i$ also explains a switch from a depolarizing action of GABA caused by Cl$^-$ influx to the more common hyperpolarizing action of GABA caused by Cl$^-$ influx. The biological significance of this switch has been extensively discussed (Ben Ari et al. 2007; Galanopoulou 2007; Rivera et al. 2005; Zhao et al. 2008). In immature neurons, a depolarized resting potential appears essential for cell maturation (Cancedda et al. 2007; Wang and Kriegstein 2008). A role for Cl$^-$ in cell volume changes of migratory cells, however, has thus far not been considered. Like glioma cells, neural stem cells migrate extensively in the brain and one could speculate that they too utilize the Cl$^-$ gradient to facilitate volume changes as they migrate. Once neuronal migration ends and cells differentiate, an outward directed Cl$^-$ gradient is no longer needed to support this process. In this vein, Cl$^-$ may serve multiple roles one of which could be serving as the energetic driving force for cell volume changes in many migratory cells during development. On transformation, glioma cells may recapitulate this developmental phenotype, as this has been reported for other biological traits (Oliver and Wechsler-Reya 2004; Sanai et al. 2005).

Although we did not directly attempt to identify the transport mechanism responsible for the chloride accumulation in gliomas, the fact that chloride efflux is exacerbated when NKCC transporters were inhibited with bumetanide (Fig. 2B) suggests that this accumulation is actively maintained by a bumetanide-sensitive transporter. Although bumetanide inhibits the two known isoforms, NKCC1 and NKCC2 (Hannaert et al. 2002), a previous report suggest that only NKCC1 is expressed in

**FIG. 4.** An outwardly directed chloride gradient is required for glioma cell migration. A: example fields imaged from Transwell migration inserts from control cells (top) or cells treated with 10 mM GABA prior to and during the migration assay (bottom). B: migration through a Transwell 8 μm pore is impaired in cells treated with 10 μM GABA. Results are from 4 independent experiments performed in duplicate. For each assay, the number of cells that had migrated through the filter in 6 fields of view were counted and averaged. Paired t-test, *, $P < 0.05$. 

J Neurophysiol • VOL 101 • FEBRUARY 2009 • www.jn.org

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glioma cells (Ernst and Sontheimer 2007). Similarly, in developing brain as well as in the adult, NKCC1 is the principle transporter mediating neuronal chloride accumulation (Sung et al. 2000; Yamada et al. 2004). This strongly suggests that NKCC1 is likely responsible for chloride accumulation in glioma cells as has been demonstrated for immature neocortical neurons in rat (Achilles et al. 2007). In light of the critical role that this transporter plays in glioma migration, it presents a valuable pharmacological target to be explored in future therapeutic studies.

Although numerous papers have been published on the accumulation of intracellular chloride in immature neurons and NSCs, this is the first direct examination of [Cl\(^{-}\)] dynamics in human glioma cells and its functional relationship to the cells’ biology. Importantly, our results mechanistically implicate chloride accumulation and release as the energetic driving force for volume changes implicit to migration and proliferation. These results may apply to other immature growth competent cells in the nervous system, although a recent studies examining embryonic and neural stem cells suggest some remarkable differences regarding growth control of these cells compared with somatic cells (Andang et al. 2008).

Finally, while we used the nonactivating GABA\(_A\) receptor as a convenient tool to experimentally activate ligand-gated Cl\(^{-}\) channels in these glioma cells, natively expressed GABA\(_A\) channels may serve the same role in migratory cells. Indeed only in culture do glioma cells loose expression of GABA\(_A\) channels, yet when placed into the brain of a host animal, GABA channels are prominently expressed (Labarakakis et al. 1998). This is also the case in human gliomas in vivo where ~70% express GABA\(_A\)-R. Hence exposure to GABA may modulate cell volume and hence migration of these glioma cells in vivo via activation of GABA\(_A\) receptors. This may also apply to migratory neurons many of which also express prominent GABA receptors in vivo (Bolteus and Bordey 2004).

Consistent with this idea, application of nondesensitizing doses of GABA slowed the migration of neurons in the rostral-migratory stream (Bolteus and Bordey 2004) as this would be expected to shunt the Cl\(^{-}\) gradient. Clearly, further studies examining the role of ligand gated Cl\(^{-}\) channels in neuronal and glial migration appears to be warranted.

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