Surface Expression of ASIC2 Inhibits the Amiloride-sensitive Current and Migration of Glioma Cells*

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Gliomas are primary brain tumors with a complex biology characterized by antigenic and genomic heterogeneity and a propensity for invasion into normal brain tissue. High grade glioma cells possess a voltage-independent, amiloride-inhibitable, inward Na⁺ current. This current does not exist in normal astrocytes or low grade tumor cells. Inhibition of this conductance decreases glioma growth and cell migration making it a potential therapeutic target. Our previous results have shown that the acid-sensing ion channels (ASICs), members of the epithelial Na⁺ channel (ENaC)/degenerin (DEG) family of ion channels are part of this current pathway. We hypothesized that one member of the ENaC/DEG family, ASIC2, is retained intracellularly and that it is the lack of functional expression of ASIC2 at the cell surface that results in hyperactivity of this conductance in high grade gliomas. In this study we show that the chemical chaperone, glycerol, and the transcriptional regulator, sodium 4-phenylbutyrate, inhibit the constitutively activated inward current and reduce cell growth and migration in glioblastoma multiforme. The results suggest that these compounds induce the movement of ASIC2 to the plasma membrane, and once there, the basally active inward current characteristic of glioma cells is abolished by inherent negative regulatory mechanisms. This in turn compromises the ability of the glioma cell to migrate and proliferate. These results support the hypothesis that the conductance pathway in high grade glioma cells is comprised of ENaC/DEG subunits and that abolishing this channel activity promotes a reversion of a high grade glioma cell to a phenotype resembling that of normal astrocytes.

Acid-sensing ion channels (ASICs) are proton gated cation channels of the epithelial Na⁺ channel/degenerin (ENaC/DEG) superfamily. ASICs have been discovered in sensory neurons originating from dorsal root ganglia in many parts of the body including the heart (1). Not only have ASICs been implicated in pH detection, but they also play a role in normal touch sensation (2). These same channels have also been found in the brain, although their function there is less clear. ASICs are present in hippocampal neurons and, because of the pH fluctuations that are known to occur during synaptic activity (3), this channel has been hypothesized to be involved in synaptic plasticity (4–10) and neuronal communication (11). In situ hybridization studies in mouse brain revealed ASIC expression in the Purkinje, the granular cell layers of the cerebellum, dentate regions of hippocampus, and the olfactory bulb (12). Thus, these channels are primarily found in neurons. One exception to this generalization is the presence of ASICs in cells obtained from high grade brain gliomas (13). These tumors are of astrocyte cell origin. In grade IV gliomas, also called glioblastoma multiforme (GBM), there exists a constitutively activated, amiloride-sensitive inward Na⁺ current. This glioma cation current is mediated by mixed ASIC and ENaC components, including ASIC1 and ASIC2 (14). Interestingly, this current is absent in normal astrocytes and low grade gliomas. We hypothesize that GBM cells express this basal current because of the lack of ASIC2 in the plasma membrane. If this current could be blocked either pharmacologically or by manipulation of its regulatory pathway, the high rate of migration of the GBM cells, proliferation, and invasiveness could be corrected.

The chemical chaperone, glycerol, and the transcriptional regulator, sodium 4-phenylbutyrate, are known to stabilize protein conformation, increase the rate of protein refolding, and accelerate oligomeric protein assembly (15–20). They have been used successfully to promote the membrane insertion of a misfolded mutant of the cystic fibrosis transmembrane conductance regulator (CFTR), ΔF508-CFTR (19, 21, 22). Sodium 4-phenylbutyrate has also been shown to have inhibitory effects on glioma cell proliferation, migration, and invasiveness (23, 24) and to promote differentiation of tumor cells in vivo and in vitro (25). However, the mechanism underlying the action of sodium 4-phenylbutyrate in glioma cells is unknown.

Using a combination of electrophysiological, cell biological, and biochemical techniques, we have tested the hypothesis that glycerol and sodium 4-phenylbutyrate promote the delivery of ASIC2 to the surface of tumor cells. Electrophysiological studies demonstrated that the movement of ASIC2 to the surface...
inhibits the basally active, amiloride-sensitive, inward cation current of glioma cells. Cell proliferation and migration assays demonstrated that the translocation of ASIC2 to the plasma membrane inhibits the proliferation and mobility of GBM cells. The results obtained in this study support the hypothesis that a basally active, multimeric ASIC/ENaC constitutes the conduction pathway responsible for inward Na⁺ current seen in high grade gliomas.

EXPERIMENTAL PROCEDURES

Cell Culture—Primary cultures of normal human astrocytes and primary cultured human glioblastoma cells (GBM) were obtained from the University of Alabama at Birmingham, Neurosurgery Brain Tissue Bank (Institutional Review Board approval X030403011). U87-MG cells were purchased from ATCC. SK-MG-1 and D54-MG cells were gifts from Dr. Gregory Caincross, University of Calgary and Dr. Darell Bigner, Duke University, respectively. The cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 (1:1) supplemented with 10% fetal bovine serum. All cells were maintained at 37 °C in a 95% O₂ and 5% CO₂ humidified incubator.

RT-PCR—Total RNA was isolated from tissue culture cells in log phase using TRIzol (Invitrogen) following the manufacturer’s instructions. The ASIC1 and ASIC2 forward and reverse primer sequences were as follows: ASIC1 5’-GGACTCGGATTTGATT-3’ (forward primer) and 5’-TGTTGGCAGCCTATGT-3’ (reverse primer); ASIC2, 5’-CCGTCACTGTGACAA-3’ (forward primer) and 5’-ATCCTCGCCGTAGTTAACATG-3’ (reverse primer). These primers skip introns allowing us to distinguish between products derived from mRNA as opposed to genomic DNA. RT-PCR was carried out under standard conditions (50 °C, 30 min × 1; 95 °C, 15 min × 1; 72 °C, 15 min × 20) following the manufacturer’s instructions.
Inhibition of Gliomas

1; 95 °C, 1 min, 54 °C, 1 min, 72 °C, 1 min

The identity of all products was confirmed by sequencing of the PCR product.

Antibodies, Immunoprecipitation, SDS-Polyacrylamide Gel Electrophoresis, and Western Blots—Rabbit anti-ASIC1 and -ASIC2 antibodies raised against the peptide sequences DVKRNPCESLRGHP and DLKESPSEGSLQPSSIQC, respectively, or the rabbit anti-ASIC1 and rabbit anti-ASIC2 antibodies from Alomone (Jerusalem, Israel) were used for immunoprecipitation and Western blot detection. Briefly, normal astrocytes, GBM, and glioblastoma cell lines were washed twice with phosphate-buffered saline (PBS) (138 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, 1 mM MgCl₂, and 0.1 mM CaCl₂, pH 7.4). The cells were then lysed with 1 ml of radioimmune precipitation assay buffer consisting of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA, and 0.1% SDS, supplemented with Complete Protease Inhibitors (Roche Applied Science). Cells

FIGURE 3. Western blot of ASIC1 and ASIC2 immunoprecipitated from SK-MG-1 cells and digested with Endo H. SK-MG-1 cells were immunoprecipitated with anti-ASIC1 or anti-ASIC2 antibody and incubated overnight with Endo H at 37 °C followed by Western blot analysis. A, Western blot with anti-ASIC1 antibody shows two bands following digestion with Endo H, equivalent to native and partially deglycosilated ASIC1, suggesting that ASIC1 is processed through the trans-Golgi. B, Western blot with anti-ASIC2 antibody shows that ASIC2 is sensitive to Endo H, equivalent to a shift in its molecular weight, suggesting that ASIC2 is being trapped in the ER and/or cis-Golgi.

FIGURE 4. Effects of glycerol on cell viability. A, representative dose-response curve for glycerol in D54-MG cells on cell viability measured with the MTT assay. B, LD50 values in glioma cell lines at different cell densities. Glycerol was effective in the range between 100 mM and 1 mM. The percentage of loss of mitochondria function equals the percentage of non-viable cells. They represent mean values from three independent experiments ± S.D.

FIGURE 5. Effects of sodium 4-phenylbutyrate on cell viability. A, representative dose-response curve for sodium 4-phenylbutyrate in D54-MG cells measured with the MTT viability assay. B, LD50 values for glioma cell lines at different cell densities. Sodium 4-phenylbutyrate was effective in the range of 1–10 mM. They represent mean values from three independent experiments ± S.D.
were scraped and placed into microcentrifuge tubes where they were sheared with a 23-gauge needle. Supernatant from the lysate after centrifugation (15,800 g for 20 min at 4 °C) was incubated with 2 μg of either anti-ASIC1 or anti-ASIC2 antibody, overnight at 4 °C on a rotator followed by the addition of protein A-agarose beads. Beads were washed with PBS and pelleted five times. Samples were resuspended 1:1 (v/v) with Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, 5% -mercaptoethanol) and heated at 95 °C for 5 min. Standard electrophoresis and blotting protocols were followed. Briefly, proteins were run on 8% SDS-PAGE minigels with 4% stacking gels. Gels were transferred onto polyvinylidene difluoride membranes (Bio-Rad). Blots were blocked with 10% nonfat dry milk/Tris-buffered saline-Tween, overnight at 4 °C, and probed with the appropriate anti-ASIC antibodies. After washing, blots were reacted with either a horseradish peroxidase-conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories) or an Alexa 680-conjugated anti-rabbit antibody (Molecular Probes, Inc.) and visualized using either chemiluminescent reagents (Super Signal West Pico, Pierce) imaged onto Kodak X-Omat AR film or the Odyssey infrared imaging system (Li-Cor Biosciences). Specificity of the anti-ASIC antibodies for immunoprecipitation was demonstrated by substitution of ChromoPure non-immune rabbit IgG (Jackson ImmunoResearch Laboratories) for the primary antibodies.

**Cell Surface Biotinylation**—SK-MG-1 and normal astrocytes were grown on 100-mm plates for 48 h before biotinylation experiments. Cells were washed three times with ice-cold PBS and incubated 15 min with 1.5 mg/ml EZ-link TM Sulfo-NHS-biotin (Pierce) in cold biotinylation buffer (10 mM triethanolamine, 2 mM CaCl2, 150 mM NaCl, pH 7.5) with gentle agitation. Cells were washed once with quenching buffer (100 mM glycine in PBS) and incubated for 10 min in quenching buffer with light agitation. Cells were then rinsed twice with PBS, scraped in cold PBS, and pelleted at 2000 rpm at 4 °C. They were lysed in radioimmuno precipitation assay buffer and incubated on ice 30 min before centrifugation, 10 min at 15,800 × g at 4 °C. Supernatants were transferred to a new tube and after the addition of 50 μl of a 50% slurry of streptavidin-agarose beads (Pierce) were rotated for 2 h at 4 °C. Beads were pelleted by brief centrifugation, and aliquots of the supernatant were taken to represent the unbound, intracellular pool. Beads were then washed three times with HNTG (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol). Biotinylated proteins were eluted by boiling in Laemmli sample buffer, separated on 8% SDS-PAGE, and immunoblotted.

**Endoglycosidase Treatment**—SK-MG-1 cells were immunoprecipitated either with anti-ASIC1 or anti-ASIC2 antibodies. Samples were treated with Endo H (New England Biolabs)
A.  

**Human Primary GBM**

![Graphs showing untreated and treated cells](image)

**FIGURE 8.** Representative whole-cell patch clamp recordings and *I*-*V* curves. A–E, human primary GBM (A), D54-MG (B), SK-MG-1 (C), U87-MG (D), and normal human astrocytes (E) were treated with either 500 mM glycerol or 5 mM sodium 4-phenylbutyrate for 48 h. They were voltage-clamped between −160 and +40 mV in 20 mV increments from holding potential of −60 mV for 800 ms. Top traces, basal current; middle traces, currents following superfusion with 100 μM amiloride; bottom traces, difference current (amiloride-sensitive). *I*-*V* curves represents the mean ± S.D. of three cells under each experimental condition.

according to manufacturer’s protocol and incubated overnight at 37 °C.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Viability Assay**—The MTT cell viability assay investigates the number of viable cells by measuring the mitochondria-dependent reduction of water-soluble tetrazolium into insoluble formazan product. The absorbance obtained is directly proportional to the activity of functional mitochondria and represents the number of viable cells. Cells were counted with a hemocytometer, resuspended in phenol red-free medium, and seeded at various densities (1250, 2500, and 5000 cells/well for human primary GBM, SK-MG-1, and D54-MG cells, respectively, and 5000 cells/well for human normal astrocytes) in 96-well plates. After 24 h cells were treated with different concentrations of either glycerol or sodium 4-phenylbutyrate. The MTT viability assay (Vybrant, Molecular Probes) was performed 48 h later. The cell culture medium was replaced with 100 μl of phenol red-free medium mixed with 10 μl of a 12 mM MTT stock solution in PBS. The cells were incubated for 4 h before adding 100 μl of 100 mg/ml SDS dissolved in 0.01 N HCl. The absorbance was measured at 570 nm in a microplate reader. The absorbance of the wells with no cells was subtracted as background. This experiment was repeated three times per cell line.

**Proliferation Assay**—SK-MG-1, D54-MG, and GBM cells were seeded in 60-mm culture dishes at a density of 2.5 × 10^5 cells/dish (24 h). After 1 day (0 h), one dish was harvested and stained with 0.2% trypan blue, and viable cells (excluding trypan blue) were counted using a hemocytometer. The other dishes were treated with glycerol (final concentration 500 mM) and sodium 4-phenylbutyrate (final concentration 5 mM). Cells were harvested at 24 and 48 h after treatment, stained, and counted as described above. This experiment was repeated four times per cell line.

**Wound-healing Migration Assay Time-lapse Videomicroscopy**—SK-MG-1 cells were plated on 60-mm culture dishes at a density of 4 × 10^4 cells/dish. Once they reached confluence, they were scarred with a 1-ml pipette tip and washed three times with cell culture medium. They were treated with either 500 mM glycerol, 5 mM sodium 4-phenylbutyrate, or 100 μM amiloride and placed in a chamber mounted on the stage of a
Nikon TE 200 inverted microscope attached to a CoolSNAP ES monochrome camera (Photometrics) linked to IPLab software (Scanalytics, Inc.). Cells were maintained under cell culture conditions, i.e. Dulbecco’s modified Eagle’s medium/F-12 (1:1) supplemented with 10% FBS at 37 °C using a temperature controller B600 (20/20 Technology, Inc.) and an atmosphere of 95% O₂ and 5% CO₂ using an IC₅₀ controller (20/20 Technology, Inc). Cells were visualized with phase contrast optics at ×10 magnification. Images were recorded every 5 min for 24 h. We measured the remaining area at the end of the 24-h period and subtracted it from the area at the beginning of the experiment and normalized to the control to obtain the percentage of cell migration. This experiment was repeated three times per each condition.

Plasma Membrane Preparation—D54-MG cells were seeded in 100-mm culture dishes and treated with either 500 mM glycerol or 5 mM sodium 4-phenylbutyrate for 48 h. They were scraped and collected by centrifugation at 600 × g for 5 min at 4 °C. The plasma membrane protein extraction kit (BioVision) was used according to the manufacturer’s protocol to isolate specifically the plasma membrane from the total cellular membranes. The plasma membrane fraction was dissolved in 0.5% Triton X-100 in PBS, and plasma membrane protein concentration was measured using the BCA protein assay (Pierce).

Whole-cell Patch Clamp—Cells were treated with either 500 mM glycerol or 5 mM sodium 4-phenylbutyrate for 48 h. They were placed in a chamber filled with RPMI 1640 culture medium (133 mM Na⁺, 5.3 mM K⁺, and 108.3 mM Cl⁻) and mounted on the stage of an inverted microscope. Once the cells adhered to the glass bottom of the chamber, micropipettes that were connected to an Axon 200A patch clamp amplifier and filled with an electrolyte solution containing (in mM) 100 potassium gluconate, 30 KCl, 10 NaCl, 20 HEPES, 0.5 EGTA, <10 free Ca²⁺, and 4 ATP at a pH of 7.2 were abutted against the plasma membrane of individual cells. Gigaohm resistance seals between the pipette tips, and the cells were obtained by applying suction to the pipettes. Once the pipette tips were sealed to the cells, a sharp suction pulse was applied to rupture the membrane within the sealed area, leaving the seal intact. This procedure produced a low resistance electrical connection to the cellular interior. This is the whole-cell patch configuration. In this configuration, the entire plasma membrane can be voltage clamped and specific ionic conductance pathways can be identified. Once the whole-cell configuration had been achieved,
the stability of each preparation was monitored between voltage clamp protocols and during drug superfusions by clamping the membrane to positive and negative potentials continuously. The cells were held at a membrane potential of \(-60\) mV and clamped sequentially for 800 ms each to membrane potentials of \(-160\) to 40 mV at intervals of 20 mV, returning to the holding potential of \(-60\) mV for 800 ms between each test voltage. The continuous monitoring shows, in real time, the effects of inhibitors, such as amiloride, and acts as a quality control for the stability of the preparations. The currents were recorded digitally and filed in real time using the pCLAMP 6 software (Axon Instruments). Each recording under a given experimental condition was done on at least three different cells.

**Transwell Migration Assay**—To measure U87-MG cell mobility, the transwell migration assay was used. Transwell chambers were obtained from B-D Falcon. The bottom of the filters (8 \(\mu\)m pore diameter) was coated overnight with 5 \(\mu\)g/ml vitronectin in PBS. As a control, migration was measured in chambers with filters coated with 1% bovine serum albumin. Fifty-thousand cells were added to the upper chamber. The cells were incubated at 37 °C for 5 h, fixed with 4% paraformaldehyde in PBS, washed with PBS, and stained with crystal violet. Cells on the upper side of the filter were removed with a cotton swab. Cells that had migrated through the pores to the lower side of the filter were counted manually in five random fields per filter. Five filters equal an \(n\) of 1. This experiment was repeated three times.

### RESULTS

**Expression of ASIC1 and ASIC2**—One of our goals was to test the hypothesis that ASIC1 and ASIC2 are present in astrocytes and glioma cells. To this end, we used RT-PCR (Fig. 1A) and Western blotting (Fig. 1B) to test for the presence of both ASIC1 and ASIC2 in normal human astrocytes, human primary GBM cells, and three continuous cell lines; namely, SK-MG-1, D54-MG, and U87-MG. Our results showed that both ASIC1 and ASIC2 mRNA and protein are present in normal astrocytes, human primary GBM, SK-MG-1, and D54-MG cells, but only ASIC1 was found in U87-MG cells.
ASIC2 Is Retained in an Intracellular Pool in Glioma Cells—
To provide evidence that ASIC1 and/or ASIC2 were expressed in the plasma membrane, surface biotinylation, followed by immunoprecipitation of both ASIC1 and ASIC2, were performed on normal human astrocytes, SK-MG-1, D54-MG, and human primary GBM tumor cells. From Western blot analysis of the intracellular fraction, both ASIC1 and ASIC2 can be detected (Fig. 2). However, in the cell surface fraction of SK-MG-1 (Fig. 2A), D54-MG (Fig. 2B), and human primary GBM (Fig. 2C) only ASIC1 is present. In human normal astrocytes, both ASIC1 and ASIC2 are present at the plasma membrane (Fig. 2A).

ASIC2 Is Trapped in the ER/Golgi in GBM Cells—To analyze the processing of ASICs, cells were lysed and ASIC immunoprecipitated. The precipitate was incubated with the endoglycosidase enzyme, Endo H, and separated by SDS-PAGE. We compared the glycosylation pattern of ASIC1 and ASIC2 in SK-MG-1 cells. The extent of glycosylation allowed us to determine how far ASIC1 and ASIC2 progress along the biosynthetic pathway. As shown in Fig. 3B, Endo H, which cleaves the saccharide chains characteristic of the ER and/or cis-Golgi but cannot cleave processed chains or other terminal sugars added later in the Golgi, caused a shift in the molecular mass of ASIC2 immunoprecipitated protein, consistent with ASIC2 being trapped in the ER and/or cis-Golgi. However, ASIC1 was resistant to Endo H (Fig. 3A), suggesting that it is processed through the trans-Golgi.

Effect of Glycerol and Sodium 4-Phenylbutyrate on Cell Viability—The MTT cell viability assay was used to test the effect of glycerol and sodium 4-phenylbutyrate on cell viability for 48 h. The percentage loss mitochondrial function is proportional to non-viable cells remaining in the population. We found that glycerol was effective above 100 mM (Fig. 4), whereas sodium 4-phenylbutyrate worked above 1 mM (Fig. 5). For further experiments we used 500 mM glycerol and 5 mM sodium 4-phenylbutyrate.

Effects of Glycerol and Sodium 4-Phenylbutyrate on Cell Proliferation—Based on the results obtained from the MTT viability assay, we tested whether 500 mM glycerol and 5 mM sodium 4-phenylbutyrate induced cell death or just decreased cell proliferation of glioma cells. We found that both compounds slowed down proliferation at 24 h and stopped it after
48 h in each cell type examined (Fig. 6). Thus while both glycerol and sodium 4-phenylbutyrate stopped proliferation, neither compound induced cell death after 48 h at these concentrations. Normal human astrocytes grow extremely slowly and exhibited no proliferation within 48 h. As measured with the MTT viability assay, treatment with glycerol affected normal astrocytes viability to much lesser extent than glioma cells (Fig. 7D). Furthermore, sodium 4-phenylbutyrate had almost no effect (Fig. 7E).

**Glycerol and Sodium 4-Phenylbutyrate Treatments Eliminate the Amiloride-sensitive Current**—The membrane permeability properties of the cells was assessed using the whole-cell patch clamp technique. Electrophysiological experiments were done in D54-MG, SK-MG-1, human primary GBM, U87-MG, and normal human astrocytes after 48 h treatment with 500 mM glycerol or 5 mM sodium 4-phenylbutyrate. In all cells, inward currents that are amiloride-sensitive were measured. However, after glycerol or sodium 4-phenylbutyrate treatments, the amiloride-sensitive current in the human primary GBM, D54-MG, and SK-MG-1 cells was no longer present (Fig. 8, A–C). In contrast, glycerol and sodium 4-phenylbutyrate had less effect on amiloride-sensitive current in the U87-MG cells, which do not express ASIC2 (Fig. 8D). We have previously shown that the constitutively active, amiloride-sensitive current in glioma cells is permeable to both Na⁺ and K⁺ (P_{Na⁺}/P_{K⁺} ~ 0.15–0.36) (26). Therefore, the amiloride-sensitive outward current present in these traces most likely corresponds to K⁺ currents (Fig. 8D). Additionally, normal human astrocytes, which do not have this amiloride-sensitive current, did not present any change in current in the presence of glycerol or sodium 4-phenylbutyrate (Fig. 8E).

**Effects of Glycerol and Sodium 4-Phenylbutyrate on Cell Migration**—We monitored the migration and proliferation ability of SK-MG-1 cells treated with 500 mM glycerol, 5 mM sodium 4-phenylbutyrate, or 100 μM amiloride by time-lapse videomicroscopy, using a scratch migration assay. Untreated cells migrate into an artificially created scratch and cover it after 24 h (Fig. 9). However, SK-MG-1 cells exposed to glycerol, sodium 4-phenylbutyrate, or amiloride only filled half of the scratch after 24 h (Fig. 9). To test the hypothesis that the presence of ASIC2 plays a role in the rapid migration and proliferation of glioma cells, we studied U87-MG cells,
which lack ASIC2 mRNA and protein. Transwell migration assays were used to measure U87-MG cell migration in the presence of 500 mM glycerol, 5 mM sodium 4-phenylbutyrate, or 100 μM amiloride. This migration assay showed that glycerol and sodium 4-phenylbutyrate do not have an effect on U87-MG cells migration (Fig. 10). However, amiloride is an effective inhibitor of migration in all cell types examined.

**Glycerol and Sodium 4-phenylbutyrate Treatment Promotes the Delivery of ASIC2 to the Plasma Membrane**—To further substantiate that surface delivery of ASIC2 is occurring subsequent to glycerol and sodium 4-phenylbutyrate treatment, we performed Western blots on a purified plasma membrane fraction of D54-MG cells. As shown in Fig. 11A, ASIC2 was absent from the plasma membrane fraction of non-treated (control) cells. However, after a 48-h exposure to 500 mM glycerol or 5 mM sodium 4-phenylbutyrate, ASIC2 appeared in the plasma membrane fraction of D54-MG cells (Fig. 11A). ASIC1 was present at the plasma membrane of all untreated and treated cells (Fig. 11A). These results support the hypothesis that both compounds stabilize newly synthesized ASIC2 and lead to the delivery of ASIC2 to the plasma membrane. The polyclonal antibody to ASIC2 was stained with Coomassie Blue to confirm that equal amounts of protein were added to each well of gel (45 μg) (Fig. 11B). To validate that we were working with plasma membrane proteins, Western blots were probed for Na+/K+ ATPase, a marker of the plasma membrane. The Na+/K+ ATPase was detected in all membrane samples (Fig. 11C). The plasma membrane fraction obtained from cells treated with sodium 4-phenylbutyrate showed an increase in the glycosylated form of Na+/K+ ATPase consistent with previous results, which demonstrated that sodium 4-phenylbutyrate increased Na+/K+ ATPase expression (27). To corroborate that the obtained plasma membrane fraction was not contaminated with other membranes, control Western blots were done using a mouse anti-GM130 antibody (BD Transduction Laboratories), which is a marker of Golgi membranes (Fig. 11D), and a rabbit anti-SERCA2 ATPase antibody (Abcam), specific for the sarcoplasmic/endoplasmic reticulum calcium ATPase 2 of 115 kDa (Fig. 11E). Plasma membrane blots were negative for both proteins, while a total cellular membrane fraction reacted positively with both antibodies. These results demonstrate that our sample only contained plasma membrane proteins.

**DISCUSSION**

The trafficking of proteins to their final cellular location can be influenced by several different factors (28). Many ion channels such as ENaC and CFTR undergo regulated trafficking as a means of controlling their plasma membrane density. Furthermore, the ER is not only the site where newly synthesized proteins acquire their tertiary and quaternary structures but is also the starting point for eventual insertion into the plasma membrane. There are many quality control mechanisms in the ER to ensure that these nascent proteins are folded or oligomerized correctly. If they are not, they are rapidly degraded (29). A notable example of this type of quality control occurs with the major mutation in CFTR (ΔF508) that results in the failure of the
newly synthesized protein to move out of the ER to the plasma membrane (15). A number of strategies such as low temperature, glycerol, and sodium 4-phenylbutyrate have been used to stabilize the ΔF508-CFTR protein, thereby allowing some of it to reach the plasma membrane where it retains some chloride channel function (16, 18, 19).

The results obtained in this study support the hypothesis that ASIC2 is not present in the plasma membrane of GBM cells, even those in which both message and protein exist. In contrast, both ASIC1 and ASIC2 are present in the plasma membrane of normal human astrocytes, cells in which no basal amiloride-sensitive current can be detected. We also tested the hypothesis that lack of plasma membrane expression of ASIC2 is a consequence of intracellular retention of this protein. Endoglycosidase digestion showed that ASIC2 is not processed beyond the ER and/or cis-Golgi in glioma cells.

In this study, we provide both biochemical and electrophysiological evidence suggesting that exposure of human primary GBM, SK-MG-1, and D54-MG glioma cells that were originally derived from high grade tumors, which contain ASIC2 message, to glycerol and sodium 4-phenylbutyrate results in the delivery of functional ASIC2 to the plasma membrane. It is not known if ASIC2 is packaged intracellularly into functional channel complexes and then delivered to the surface membrane or if ASIC2 reaches the plasma membrane and then intercalated into pre-existing channels, as has been suggested for components of the ENaC family (30). Regardless of the mechanism, the net effect is to convert an active channel characteristic of high grade gliomas into an inactive one, characteristic of non-transformed, normal human astrocytes. Thus, these results are consistent with the hypothesis that abolishing the amiloride-sensitive inward Na⁺ current promotes a reversion of a high grade glioma cell to a more normal (i.e. non-malignant) astrocytic phenotype. The results obtained from the glioma cell lines, showing that amiloride was without effect in the presence of glycerol and sodium 4-phenylbutyrate, suggest that the cells acquired an electrophysiological phenotype resembling a normal astrocyte cell. Moreover, the results also show that the presence of ASIC2 plays a role in the inhibition of the amiloride-sensitive current because the U87-MG cells, which lack ASIC2, were not affected by glycerol or sodium 4-phenylbutyrate. The results suggest that in the absence of ASIC2, glycerol and sodium 4-phenylbutyrate failed to promote a reversion of glioma cell to a more normal astrocytic phenotype as assayed by migration and proliferation.

The data presented in this study are consistent with the
hypothesis that the movement of Na$^+$ ions through channels assists the growth and migration of glioma cells. Moreover, these results reconcile our previous observations that a significant proportion (~40%) of high grade gliomas, while expressing message for ASIC2, still display constitutively activated inward currents (14). Our results are also consistent with the hypothesis that inhibition by any means of these glioma-specific, up-regulated, amiloride-sensitive ion channels will diminish growth and decrease migration rate. Because gliomas are invasive tumors that diffusely infiltrate the normal brain, we hypothesize that the expression of these ion channels represents an intrinsic adaptation that facilitates tumor cell movement through the narrow extracellular spaces in the brain, probably because of their involvement in tumor cell volume regulation. Ultimately, either the ion channel itself or its regulatory pathways may provide a target or targets for elimination or containment of these cells.

Acknowledgments—We thank Melissa McCarthy (Department of Physiology and Biophysics, University of Alabama at Birmingham) and Cathy Langford (Division of Neurosurgery, University of Alabama at Birmingham) for cell culture assistance.

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