Acid-sensing Ion Channels in Malignant Gliomas

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High grade glioma cells derived from patient biopsies express an amiloride-sensitive sodium conductance that has properties attributed to the human brain sodium channel family, also known as acid-sensing ion channels (ASICs). This amiloride-sensitive conductance was not detected in cells obtained from normal brain tissue or low grade or benign tumors. Differential gene profiling data showed that ASIC1 and ASIC2 mRNA were present in normal and low grade tumor cells. Although ASIC1 was present in all of the high grade glial cells examined, ASIC2 mRNA was present in less than half of the cells. The main purpose of our work was to examine the molecular mechanisms that may underlie the constitutively activated sodium currents present in high grade glioma cells. Our results show that 1) gain-of-function mutations of ASIC1 were not present in a number of freshly resected and cultured high grade gliomas, 2) syntaxin 1A inhibited ASIC currents only when ASIC1 and ASIC2 were co-expressed, and 3) the inhibition of ASIC currents by syntaxin 1A had an absolute requirement for either γ- or δ-ENaC. Transfection of cultured cells originally derived from high grade gliomas (U87-MG and SK-MG1) with ASIC2 abolished basal amiloride-sensitive sodium conductance; this inhibition was reversed by dialysis of the cell interior with Munc-18, a syntaxin-binding protein that typically blocks the interaction of syntaxin with other proteins. Thus, syntaxin 1A cannot inhibit Na+ permeability in the absence of adequate plasma membrane ASIC2 expression, accounting for the observed functional expression of amiloride-sensitive currents in high grade glioma cells.

Primary intracranial neoplasms remain a significant cause of mortality and morbidity in both children and adults. In patients with malignant gliomas of World Health Organization Grades III and IV, disease progression is uniformly rapid despite aggressive surgical and adjunctive therapies. Median survival of optimally treated individuals with the most aggressive of these tumors, glioblastoma multiforme, is 12 months, and this statistic has not varied for more than 30 years. The ability to treat these tumors successfully has been hampered by a fundamental lack of understanding of the control of the growth and differentiation of glial cells, how the transformed phenotype evolves, and how glioma cells modify their environment to support their increased energy demands. We have identified a novel amiloride-sensitive inward Na+ current that appears to be constitutively activated in malignant gliomas but not in low grade or normal astrocytes (1). In addition, glioma cells display up-regulation of Cl− and K+ channels not found, at least functionally, in normal glia (2, 3). Thus, it is reasonable to hypothesize that ion transport systems specifically expressed by glioma cells are intimately related to and indeed may define the unique growth and migratory ability of these cells.

The main objective of the present study was to explore the molecular mechanisms that underlie the constitutively activated Na+ currents present in high grade glioma cells. We assume, based on our previous electrophysiological, pharmacological, and molecular biological studies, that the brain Na+ channels, also known as acid-sensing ion channels (ASICs),1 may comprise the core conduction element of these channels. To date, six members of the ASIC family have been cloned in mammals (4).

These channels share the common property of generating excitatory currents in response to acidic pH when studied in heterologous expression systems, except for ASIC2b that, at least in its homomeric form, does not appear to respond to low pH (5). Moreover, ASIC4 is inactive by itself and hence is not thought to encode a proton-gated ion channel (6, 7). Although the subunit composition of brain Na+ channels in native tissues is unknown, evidence for heteromultimeric channel formation with distinctive functional characteristics has been obtained (7–10). These brain Na+ channels, like their epithelial counterparts, can be inhibited by amiloride and its analogs, although with a much lower affinity (11). A role for chemical pain sensation has been proposed for these channels in sensory neurons (12, 13), but their role in the brain is obscure. Proton-activated neuronal currents have been identified in different brain regions (13, 14). Hence, these channels may function as acid pH sensors in normal brain and in pathophysiological states such as ischemia or epilepsy where tissue acidification occurs (15, 16). Gain-of-function mutations of ASIC1 and ASIC2 have also been detected and have been proposed to participate in neurodegenerative disease (7, 17, 18).

1 The abbreviations used are: ASIC, acid-sensing ion channel; CFTR, cystic fibrosis transmembrane conductance regulator; ENaC, epithelial Na+ channel; GBM, glioblastoma multiforme; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; GST, glutathione S-transferase; RT, reverse transcriptase; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; PKC, protein kinase C.
The function of these channels in gliomas remains a mystery, yet functional amiloride-sensitive Na+ current expression selectively characterizes high grade gliomas.

There are a number of potential mechanisms that may produce a constitutive activation of this class of channel. We tested two hypotheses. First, as a consequence of oncogenic transformation, gain-of-function mutations of ASIC1 may occur. Second, the hypothesis that syntaxin 1A may regulate ASIC1 activity by analogy with the effects of syntaxin 1A on other ion channel activities, such as voltage-dependent Ca2+ channels, CFTR, and ENaC. Our results show that gain-of-function mutations of ASIC1 are not present in a multitude of freshly resected and cultured high grade gliomas (glioblastoma multiforme or GBM, World Health Organization classification Grade IV). However, syntaxin 1A inhibits ASIC currents, but only when both ASIC1 and ASIC2 are co-expressed. Moreover, the inhibition of ASIC currents by syntaxin 1A requires either γ- or δ-ENaC. Furthermore, message for ASIC2 could be detected in 30–40% of high grade gliomas. We suggest that it is the failure of syntaxin 1A to inhibit ASIC1 activity in the absence of plasma membrane-localized ASIC2 that accounts for the functional expression of amiloride-sensitive Na+ currents in astrocytoma cells.

**MATERIALS AND METHODS**

**DNA Constructs**—The cDNAs encoding full-length γ-ENaC subunits, ASIC1, and ASIC2 are described elsewhere (19–21). The human ASIC 1a (ASIC1) and ASIC2a (ASIC2) cDNAs were a gift from Drs. David Corey and Jaime Garcia-Agoveros of the Harvard Medical School. The human ENaC subunits were given to us by Dr. Michael J. Welsh of the University of Iowa.

**In Vitro Transcription and Translation**—cDNAs were transcribed and translated in vitro using the TNT transcription/translation system (Promega) as previously described (22). To test for protein–protein interaction between different ENaC subunits and syntaxin 1A or between ASIC1 and ASIC2, we translated these constructs either with radioactive or nonradioactive methionine, immunopurified them, and reconstituted them in different combinations in proteoliposomes as previously described (22). Proteoliposomes were solubilized in precipitation buffer that contained 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS. All of the precipitation reactions were carried out in this buffer. As a rule, antibodies directed against nonlabeled proteins were used, and the presence of co-precipitated radioactively labeled proteins were detected using SDS-PAGE and autoradiography.

**ASIC Expression in Xenopus Oocytes**—DNA samples were in vitro transcribed using the SP6 or T3Message Machine kits (Ambion, Austin, TX). The integrity of the cRNA was assessed by running the samples under denaturing conditions on a formaldehyde-agarose gel. RNA concentration and purity were determined by UV spectrophotometry at 260 nm. The oocytes were removed from appropriately anesthetized adult female *Xenopus laevis* (Xenopus Express, Beverly Hills, FL) using standard techniques (23). Folllicular cells were removed by the addition of collagenase to calcium-free medium as described (23). Defolliculated oocytes were washed in OR-2 medium (82.5 mM NaCl, 2.4 mM KCl, 1.0 mM MgCl2, 1.8 mM CaCl2, and 5.0 mM HEPES, pH 7.4) and allowed to recover overnight in half-strength Liebovitz medium at 18 °C. Groups of stage V oocytes were injected with cRNA in a total volume of 1.0 μl containing 12.5 ng of ASIC and/or ENaC subunits depending upon the experiments. Standard two-electrode voltage clamp procedures were performed at room temperature on the oocytes 24–72 h post-injection.

The oocytes were impaled with two 3 M KCl-filled electrodes, each held at 100 mV in 20-mV increments, returning to the holding potential of 0 mV between each test voltage. The currents were recorded digitally and filed in real time. The entire procedure was performed using a DOS Pentium computer modified for A/D signals with pCLAMP 8 software and with an A/D interface controlled by pCLAMP (Axon Instruments, Union Vale, CA).

**Antibodies, Immunoprecipitation, Gel Electrophoresis, and Western Blots**—The anti-ASIC2a antibodies (Alamone, Jerusalem, Israel), anti-ASIC1 antibodies (Chemicon International, Temecula, CA), anti-γENaC antibodies (raised against peptide; CNTLR...DRAFSSQLT), and anti-syntaxin 1A antibodies (25) were used for immunoprecipitation and Western blot detection. Crucial membrane fractions from the glioblastoma cell line SK-MG1 were prepared as previously described (26). Immunoprecipitation and co-purification from SK-MG1 cell lysates were performed using the size X protein A immunoprecipitation kit from Pierce according to the manufacturer’s instructions. Briefly, affinity-purified ASIC2 antibody (280 μg) was bound to protein A-Sepharose 4B beads. After washing, the beads were added to the SK-MG1 cell lysates (1.5 ml) and incubated overnight at 4 °C. The cells were lysed with 1.5 ml of 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5, containing protease inhibitors. Immunoprecipitated proteins were eluted and analyzed by electrophoresis and Western blotting. Standard electrophoresis and blotting protocols were followed. The gels were stained with Coomassie brilliant blue R-250 and then transferred to nitrocellulose. The nitrocellulose membranes were incubated overnight in 2% SDS-PAGE mini gels with 4% stacking gels for about 1 h at 200 V in a Bio-Rad Minisorb apparatus. The gels were transferred onto polyvinylidene fluoride, treated with 5% nonfat dry milk/Tris-buffered saline/ Tween, and probed with the appropriate antibodies. Secondary antibody was conjugated to horseradish peroxidase, and visualization was performed using chemiluminescent reagents (Amersham, Arlington Heights, IL). Controls included substitution of nonimmune rabbit IgG for primary antibodies.

**ASIC2 Transfections**—To determine whether expression of hASIC2 could restore the normal astrocyte Na+ current phenotype to astrocyte-
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Fig. 1. Representative whole cell patch clamp recordings from freshly isolated normal human astrocytes and GBM (World Health Organization Grade IV) and primary cultures of different World Health Organization Grade astrocytomas. The cells were voltage-clamped between −100 and +100 mV in 20-mV increments from a holding potential of 0 mV, and the resulting currents were recorded. The cells were superfused with RPMI 1640 medium, and the pipette contained 100 mM potassium gluconate, 30 mM KCl, 20 mM HEPES, 0.5 mM EGTA, 4 mM ATP, and < 1 μM CaCl2 at a pH of 7.2. 

Traces A show whole cell inward currents from freshly resected normal brain tissue and GBM in response to changes in extracellular calcium concentration. The cells were superfused with RPMI 1640 medium, and the pipette contained 100 mM potassium gluconate, 30 mM KCl, 20 mM HEPES, 0.5 mM EGTA, 4 mM ATP, and < 1 μM CaCl2 at a pH of 7.2. Bottom traces, basal currents. Middle traces, currents following superfusion with 100 μM amiloride. Top traces, difference currents (amiloride-sensitive). This experiment was repeated at least four separate times for each cell type.

Freshly isolated normal brain tissue and GBM were superfused with RPMI 1640 medium, and the pipette contained 100 mM potassium gluconate, 30 mM KCl, 20 mM HEPES, 0.5 mM EGTA, 4 mM ATP, and < 1 μM CaCl2 at a pH of 7.2. Bottom traces, basal currents. Middle traces, currents following superfusion with 100 μM amiloride. Top traces, difference currents (amiloride-sensitive). This experiment was repeated at least four separate times for each cell type.

Preparation of Syntaxin 1A and Syntaxin 3 Proteins—Defined regions of syntaxin 1A and syntaxin 3 (amino acids 1–266; referred to as ΔC as they lack the C-terminal membrane anchor) were generated by polymerase chain reaction. Restriction sites (EcoRI in forward and XhoI in reverse), 5’ overhangs (TATA), and a stop codon were introduced into the primers. The PCR product was cloned into pGEXKKX-1 vector (Pharmacia Corp.) and transformed in a protease deficient E.coli strain (BL21-DE3). The GST fusion protein (GST-Syn1AC and GST-Syn3AC) was purified on glutathione-Sepharose beads (Pharmacia Corp.). The protein was eluted using 20 mM reduced glutathione in phosphate-buffered saline, pH 7.4, and dialyzed extensively at 4 °C to remove any contaminating GST. Recombinants were selected by blue/white screening and retransformed with EcoRI (Promega) to determine molecular size. Products of the correct molecular size were isolated from the gel using the QIAquick gel extraction kit (Qiagen) and subcloned into the pCR-2.1 vector using the TOPO-TA cloning kit (Invitrogen) following the manufacturer’s instructions. Recombinants were selected by blue/white screening and retransformed with EcoRI (Promega) to verify incorporation of insert of correct size. ASIC1 sequences were verified by further restriction enzyme digested analysis and automated DNA sequencing (DNA Sequencing Facility, Iowa State University).

RESULTS

We previously reported that there was an amiloride-sensitive component to whole cell inward currents in high grade, highly invasive brain tumors (1). These currents were seen in primary cultures of tumors as well as in established glioma cell lines. This amiloride-sensitive current was not present in low grade brain tumors or normal brain tissue. To verify these initial findings and to extend these results to freshly resected GBM tumors, we performed whole cell patch-clamp experiments on freshly excised normal astrocytes and freshly resected and primary cultured brain tumor tissue samples (Fig. 1). In the basal state, the current records for both freshly resected and primary cultures of World Health Organization Grade III and IV tumor cells were characterized by large inward currents (Fig. 1A), and these results were completely...
inhibited following superfusion with 100 μM amiloride (Fig. 1B). Fig. 1C shows the difference current (i.e. the amiloride-sensitive component). These results should be contrasted to the lack of effect of 100 μM amiloride in normal astrocytes and Grades I and II astrocytoma cells. The absolute magnitudes of the outward currents at +40 mV (Fig. 2A) and inward currents at −60 mV (Fig. 2B) in the absence and presence of amiloride for normal astrocytes, different grade gliomas, medulloblastoma, and two continuous GBM cell lines are summarized in Fig. 2. Although there was no discernible pattern to the magnitudes of either the outward or inward currents, amiloride only blocked inward currents in the more aggressive, higher grade tumors (Grades III and IV and medulloblastoma). Amiloride likewise blocked inward currents in SK-MG and U87-MG cells, both originally derived from GBM. A summary of the current-voltage (I–V) characteristics of both freshly resected normal astrocytes and GBM cells is presented in Fig. 3. It is apparent that the GBM cells are depolarized by an average of 31 mV compared with normal astrocytes under these recording conditions. The depolarized zero current membrane potential is due to the presence of an enhanced Na+ conductance. These results confirm our previous findings and establish that this amiloride-inhibitable component to whole cell currents is present in freshly excised GBM cells.

Because these currents were inhibited by amiloride and because the apparent amiloride inhibitory constant (K_i) was high (i.e. ~10 μM), we hypothesized that the channels underlying these currents were of the ASIC variety. Thus, to begin to test this hypothesis, we performed RT-PCR on total RNA extracted from human brain tissue samples obtained during craniotomy for epilepsy (normal tissue, lanes 1–3) or primary GBM resection (lanes 4–6) and from continuous cell lines derived from anaplastic astrocytoma (lane 7), a gliosarcoma (lane 8), and 7 different GBMs (lanes 9–15) (Fig. 4A). Specific primers were designed to amplify a 482-bp product for ASIC1a and a 447-bp product for ASIC2a. We also designed primers to identify ASIC3, ASIC4, and α-, β-, γ-, and δ-hENaC subunits. The ASIC1a product was detected in all of the samples (both normal and tumor), including the pancreatic carcinoma cell line BxPC-3 (lane 16). In contrast, the ASIC2a message was found in the
three normal samples and in only one of the freshly resected GBMs (lane 5) and in the D54MG, SK-MG1, and U373MG cell lines (lanes 9, 11, and 15). Direct sequencing of the PCR products confirmed their identity. ASIC 3 was present in many of the glioma cell lines and in both normal tissue and GBM tumors, whereas ASIC 4 was present in most of the fresh tissue samples but in none of the cultured cell lines. α-hENaC was found in the majority of the tumor cell lines, in one freshly excised GBM tumor but not in any of the normal samples. β-hENaC was found in one of the freshly resected GBMs (lane 4) and in the pancreatic carcinoma cell line but not in any other samples. Also, either δ- or γ-hENaC were observed in most of the freshly excised GBM tissues as well as in the tumor cell lines. The γ-hENaC was also present in the normal tissue as compared with δ-hENaC.

Another independent set of experiments was performed to examine specifically the distribution of ASIC1 and ASIC2 message between different tumor samples (Fig. 4B). Total RNA was extracted from tissue obtained from resected GBM tumors (lanes labeled G), primary cultures of GBM cells (lanes labeled P), cultured normal human astrocytes (Astrocyte), and continuous GBM cell lines. Examining in toto the data in Fig. 4,
clamp conditions. The oocytes were voltage-clamped from −100 mV to +100 mV in 20-mV increments. The currents were activated by reduction in pH to 4.0. Between voltage clamp levels, the extracellular pH was returned to pH 7.5 for 45 s prior to the next voltage clamp level and return in pH to 4.0. Between voltage clamp levels, the extracellular pH (pH0) was 7.5. However, during the voltage clamp protocol. There was no measurable inward sodium currents in oocytes. The experimental conditions were the same except that experiments were all conducted at pH 7.5. We used −Xenopus oocytes to express ASIC1, ASIC2, or ASIC1/2 cRNA. Moreover, this gain-of-function mutant (ASIC2 G430F) expressed in oocytes and subsequently assessed sodium currents (Fig. 5A). Voltage clamp traces in the absence and presence of amiloride are shown in the upper half and the inward currents are sensitive to amiloride. The bottom half of the figure shows the inhibition in response to amiloride. These currents were recorded at −100 mV. Almost all of the inward current was inhibited by amiloride. The reversal potential for this gain-of-function mutation was +3 mV. No amiloride-sensitive currents could be elicited in oocytes (n = 18, 3 frogs) injected with the gain-of-function ASIC1a mutant cDNA (i.e. G433F). However, this mutant channel could be recorded in planar bilayers and displayed the same biophysical and pharmacological characteristics as the G430F ASIC2a channel (not shown). We have previously shown that the protein actin, when added to the presumptive cytoplasmic surface of ENaC incorporated into planar lipid bilayers, can functionally interact with the channel reducing single channel conductance as well as altering cation selectivity (28). Addition of actin to the gain-of-function ASIC1 or ASIC2 channel had no effect on the single channel signatures. However, when vesicles were prepared from channels containing a mixture of gain-of-function ASIC1
plus ASIC2, actin reduced single channel conductance to ~9 pico siemens, and the channel was almost always open (not shown). Therefore, both our bilayer and oocyte expression studies show that ASIC1 and ASIC2 can be functionally studied, and more importantly, co-expression of ASIC1 plus ASIC2 results in channels that are functionally distinct from their homomeric counterparts.

Gain-of-function Point Mutations in ASIC1 Isolated from Brain Tumor Samples—To test the hypothesis that gain-of-function mutations may underlie the appearance of an amiloride-sensitive conductance in malignant tumor cells, we isolated total RNA from malignant gliomas following resection or in established cell lines. The results of these experiments are presented in Table I. Using RT-PCR and the appropriately designed primers, we sequenced the entire coding region of ASIC1 from these cells. From the sequence analysis, we determined that there was no gain-of-function in positions that have previously been described, namely, Gly433 (ASIC1) or Gly430 (ASIC2) (7, 17, 18). The results of these experiments demonstrate that distinct gain-of-function mutations do not occur in ASIC1. The ASIC1 primary sequence, for the most part, was identical to the wild type channel. However, during these analyses we did note some other random mutations in ASIC1, namely, F197S, D212G, E219K, and N395T. We constructed ASIC1 mutants containing these specific amino acid changes and expressed them in oocytes. None of these mutant constructs displayed gain-of-function activity (not shown). Thus, from these results we conclude that gain-of-function mutations in ASIC1 do not contribute to the constitutive amiloride-sensitive sodium conductance measured in high grade glioma cells.

**SNARE Protein Interactions with ASIC**—Fig. 6 presents data obtained from our previous differential gene profiling studies on human brains showing that syntaxin 1A is present in normal and high grade glioma tumor cells. A more extensive survey by RT-PCR demonstrates the presence of syntaxin 1A in normal human astrocytes, freshly resected GBMs, and GBM cell lines (Fig. 7). Moreover, γ-hENaC appears to be present in all these samples as well. We tested the hypothesis that syntaxin 1A may down-regulate ASIC activity by analogy with the effects of syntaxin 1A on other ion channel activities such as voltage-dependent calcium channels, CFTR, and ENaC (25, 29–32). The results of these experiments are shown in Fig. 8 and summarized in Table II. GST-Syn1AΔC (0.175 μM) had no effect on the activity of channels comprised of ASIC1 plus ASIC2 (either alone or in combination) when the γ subunit of ENaC was not present (Fig. 8A). However, if γ-hENaC was simultaneously reconstituted with ASIC1 plus ASIC2, the same concentration of GST-Syn1AΔC significantly inhibited channel open probability (Fig. 8A). The concentration dependence of this inhibitory effect is shown in Fig. 8B (KD = 207 ± 31 nm). If the syntaxin 1A-binding protein Munc-18 is added to the bath, subsequent addition of GST-Syn1AΔC had no effect (Fig. 8C). GST by itself did not have any effect (Fig. 8D). Truncation of either the N or C termini of γ-hENaC rendered GST-Syn1AΔC ineffective (Table II). Interestingly, δ-hENaC can substitute for γ-hENaC. We conclude that syntaxin 1A can specifically down-regulate ASIC activity only if ASIC1 plus ASIC2 and γ (or δ) hENaC are present. Essentially the same results were found in oocyte expression studies, i.e. inhibition of ASIC1 plus ASIC2 by syntaxin required γ-hENaC (Fig. 8E). This effect was specific for syntaxin 1A, because syntaxin 3 was without effect (not shown). Based on our molecular biological data (Figs. 4 and 6), it appears that in normal cells ASIC1, ASIC2, and γ (or δ)-hENaC are present, but in most tumor cells ASIC2 is lacking or attenuated. Thus, inhibition of channel activity by syntaxin 1A would be relieved in tumor cells because of the absence or reduced levels in the plasma membrane of one necessary component of the complex, namely, ASIC2.

Biochemical Evidence of ASIC1a, and ASIC2a, and γ-ENaC Interactions—The results of our preliminary functional studies in oocytes and bilayers imply an interaction between ASIC1, ASIC2, and γ-ENaC. To provide biochemical evidence of such an interaction, we did a co-precipitation study in a glioma cell line (SK-MG1). The strategy was to immunoprecipitate ASIC2 and test for the co-precipitation of ASIC1 and γ-ENaC by Western blot. The results are shown in Fig. 9. ASIC2 can be detected by Western blot after precipitation with anti-ASIC2 antibodies, but not if nonimmune IgG was used for precipitation (Fig. 9A), demonstrating the specificity of the anti-ASIC2 antibodies. Fig. 9 (B and C) shows co-precipitation of ASIC1 and γ-ENaC with ASIC2 and lack of co-precipitation if nonimmune IgG was used for precipitation. These biochemical results demonstrate that ASIC1, ASIC2, and γ-ENaC can associate to form heteromeric complexes in an intact glioma cell line.

To provide additional evidence for these potential interactions, co-immunoprecipitation experiments have been performed with in vitro translated and proteoliposome-reconstituted ASIC1, ASIC2, and the voltage gated chloride channel 5 (CIC5) as a control peptide (Fig. 10A). Transcripts of ASIC1 and ASIC2 were radioactively labeled (two left lanes). These transcripts were immunopurified and reconstituted either with the unlabeled conjugated ASIC partner or with unlabeled CIC5. The strategy was to immunoprecipitate one partner to detect the presence of the other by autoradiography. Our results dem-
onstrated that ASIC1 and ASIC2 can indeed associate to form heteromeric complexes, and co-precipitation between ASIC1 and ASIC2 is specific because of the lack of co-precipitation of ASIC1 and ASIC2 with ClC5. We used the same strategy to test for protein-protein interactions between /H9253-hENaC and syntaxin 1A, between /H9254-hENaC and syntaxin 1A, and between /H9252-hENaC and syntaxin 1A (Fig. 10B). We used in vitro translated proteins for these co-precipitation experiments because of a lack of good anti-/H9254-hENaC antibodies. In vitro translation allowed us to test co-precipitation between two proteins, even if only one antibody is available, because the co-precipitated protein is radioactively labeled and can be detected without antibodies. In Fig. 10B, the results show that immunoprecipitation with anti-syntaxin 1A antibodies resulted in co-precipitation of both γ-hENaC and δ-hENaC but not β-hENaC.

Transfection of U87-MG and SK-MG-1 Tumor Cells—If the hypothesis is correct, namely, that the absence of an amiloride-sensitive current in normal cells is due to the presence of ASIC1, ASIC2, γ (or δ)-hENaC, and syntaxin 1A in the membrane and that the basally activated amiloride-sensitive conductances seen in high grade glioma cells is due to a relief of inhibition by syntaxin 1A because of the absence of ASIC2, then transfecting ASIC2 back into a tumor cell (displaying amiloride-sensitive currents) should re-establish the nontumor phenotype (i.e. no inward amiloride-sensitive current). The results of such experiments are shown in Fig. 11A. These experiments were performed in two different cultured glioma cell lines (U87MG and SK-MG1) that were originally derived from GBMs. The cells were transfected with an empty expression vector and green fluorescence protein (left panels). Superfusion of 100 μM amiloride abolished the inward current in these GBM-derived tumor cells, as reported earlier (Ref. 1 and Fig. 2). In contrast, in the right panels, tumor cells that were transfected with both green fluorescence protein and the vector containing ASIC2 resulted in a phenotype in which there was no amiloride-sensitive inward current. Likewise, inclusion of Munc-18, a syntaxin 1A-binding protein that blocks syntaxin interaction with other proteins (25), in the patch pipette of
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Single channel characteristics of ASIC in the absence and in the presence of syntaxin 1A in planar lipid bilayers

$G$, single channel conductance; $P_g$, the probability of the channel being in open state; Na/K, sodium and potassium permeability ratio calculated under bionic conditions (100 mM NaCl$_{cis}$/100 mM KCl$_{trans}$); $K_{Amil}$, Amiloride inhibitory constants; ND, not determined. Bilayer bathing solution in the syntaxin 1A experiments were buffered with 100 µM EGTA, and the levels of free Ca$^{2+}$ were 25–50 nM.

| Construct | $G$ (µS) | $P_g$ | Na/K | $K_{Amil}$ (µM) |
|-----------|----------|-------|------|-----------------|
| ASIC1     | 19 ± 2   | 0.88 ± 0.09 | 1.00 ± 0.14 | 0.93 ± 0.15 |
| ASIC2     | 20 ± 1.6 | 0.90 ± 0.08 | 1.00 ± 0.13 | 0.85 ± 0.12 |
| ASIC1 + γENaC | 19 ± 3.0 | 0.90 ± 0.06 | ND | ND |
| ASIC2 + γENaC | 21 ± 1.9 | 0.91 ± 0.06 | ND | ND |
| ASIC1 + ASIC2 | 20 ± 2.0 | 0.90 ± 0.08 | 1.00 ± 0.14 | 0.90 ± 0.20 |
| ASIC1 + ASIC2 + γENaC | 19 ± 2.0 | 0.90 ± 0.07 | ND | ND |
| ASIC1 + ASIC2 + βENaC | 19 ± 1.3 | 0.90 ± 0.08 | ND | ND |
| ASIC1 + ASIC2 + δENaC | 21 ± 1.7 | 0.90 ± 0.07 | ND | ND |
| ASIC1 + ASIC2 + γδENaC | 20 ± 1.5 | 0.90 ± 0.07 | ND | ND |
| ASIC1 + ASIC2 + γγENaC | 20 ± 1.8 | 0.90 ± 0.07 | ND | ND |
| ASIC1 + ASIC2 + γγγENaC | 19 ± 2.0 | 0.90 ± 0.08 | ND | ND |
| ASIC1:ASIC2 10:1 | 18 ± 2.0 | 0.87 ± 0.08 | ND | ND |
| ASIC1:ASIC2 5:1 | 21 ± 2.0 | 0.90 ± 0.06 | ND | ND |
| ASIC1:ASIC2 3:1 | 19 ± 1.7 | 0.91 ± 0.07 | ND | ND |
| ASIC1:ASIC2 2:1 | 20 ± 2.2 | 0.94 ± 0.06 | ND | ND |

**Fig. 9.** Analysis of the interaction between ASIC2, ASIC1, and γ-ENaC. Crude membrane fractions from SK-MG cells were used for precipitation and co-precipitation. Anti-ASIC2 antibodies or nonimmune IgG cross-linked with protein A beads were used for precipitation. Precipitated and co-precipitated proteins were probed with anti-ASIC2 (A), anti-ASIC1 (B) or anti-γ-ENaC antibodies (C). ASIC2 can be detected by Western blot after precipitation with anti-ASIC2 antibodies but not if nonimmune IgG was used for precipitation (A). ASIC1 and γ-ENaC co-precipitated with ASIC2 but not if nonimmune IgG was used for precipitation (B and C).

ASIC2-transfected U87MG cells rapidly restored the inward amiloride-sensitive Na$^+$ current (Fig. 11B). These results are consistent with the hypothesis that SNARE protein interactions (specifically syntaxin 1A) with ASIC may underlie the lack of channel activity seen in normal astrocytes and the appearance of such activity in the malignant state.

**Induction of Amiloride-sensitive Currents in Normal Astrocytes**—In normal astrocytes, no amiloride-sensitive inward currents are present. We hypothesize a tonic inhibition of these currents by two independent, convergent mechanisms: inhibition by PKC-β (33) and inhibition by syntaxin 1A. To test this hypothesis, we performed whole cell patch clamp experiments on primary cultured normal human astrocytes treated with charybdothrine chloride, a generalized PKC inhibitor, and Munc-18 (to disrupt syntaxin 1A-protein interactions) (Fig. 12). Charybdothrine treatment alone or inclusion of Munc-18 in the patch pipette had no influence on macroscopic currents. However, if astrocytes were pretreated with charybdothrine prior to entering the whole cell mode with Munc-18 included in the pipette, activated, amiloride-sensitive inward currents resulted. These results support the hypothesis that both PKC and syntaxin 1A inhibit ASIC currents in astrocytes.

**DISCUSSION**

Our previous results indicated that an inward sodium conductance is present in high grade gliomas but not in low grade tumors or normal astrocytes (1). Moreover, messenger RNA analysis (either by gene microarray or RT-PCR) indicated that ASIC1 message was present in normal and GBM tissues and that ASIC2 mRNA was only present in normal and not in the majority of glial cells examined thus far. In the present work, we addressed several potential molecular mechanisms that may account for the constitutive presence of an inward Na$^+$ conductance in high grade glioma cells that is absent in normal astrocytes and lower grade astrocytic tumors. There are three main conclusions that can be drawn from our experiments: 1) gain-of-function mutations of ASIC1 do not account for the constitutive activation of sodium currents seen in high grade glioma tumor cells; 2) interactions between an ASIC-based channel complex and a SNARE protein (syntaxin 1A) result in channel inhibition; and 3) the molecular composition of functional brain amiloride-sensitive sodium channels is complex and may include both brain and degenerin/ENaC subunits.

Gain-of-function mutations in both ASIC1 and ASIC2 have been described. Using RT-PCR, we sequenced the entire coding region of ASIC1 from malignant tumor cells obtained either from freshly resected tissue or from established cell lines. From the sequence analysis, we determined that there were no gain-of-function mutations in positions that have been previously described (8, 17, 34). Although we detected polymorphisms of ASIC sequence in positions other than those previously described for gain-of-function mutations, these ASIC mutants were not basally activated when heterologously expressed in *Xenopus* oocytes. Thus, we postulate that gain-of-function mutations of ASIC1 do not underlie the activated current seen in high grade gliomas. However, if the amiloride-sensitive currents are derived from a combination of subunits of ASIC and ENaC, as we suggest, our results do not exclude the possibility of gain-of-function mutations occurring in other channel com-
subunit is required (32). There is disagreement as to whether the inhibitory effect is due to altering the number of channels at the cell surface or to an alteration of the gating properties of the channel (31, 32). Our data demonstrate that syntaxin 1A can also affect ASIC. Based upon our gene profiling RT-PCR and functional data, we hypothesized that syntaxin 1A inhibits ASIC, that the C-terminal anchor is not required and that ASIC1, ASIC2, and γ-ENaC must be present within the channel complex for this inhibition to occur. These results were confirmed in our single channel bilayer experiments, indicating a direct interaction between syntaxin and the channel. Moreover, our RT-PCR results indicate that γ-ENaC is not present in all tumors (Fig. 2). However, δ-ENaC has a widespread distribution. Our functional studies do, in fact, indicate that δ-ENaC can substitute for γ-ENaC in this regard. Biochemically, we show that syntaxin 1A can immunoprecipitate both γ- and δ-ENaC but not β-ENaC (Fig. 10). Our results show that ASIC1 + ASIC2 + γ (or δ)-ENaC exists in normal brain tissue or low grade tumors and that ASIC2 is not present in more than half of the higher grade glioma cells that we have examined. In the other subset of tumors where ASIC2 message is present, we hypothesize that ASIC2 protein is not present in the plasma membrane. Thus, in the absence of plasma membrane ASIC2, syntaxin 1A, which is present in all cells of astrocytic origin (Fig. 7), cannot inhibit channels comprised primarily of ASIC1. In the absence of inhibitory inputs, the conductance would be constitutively active because of the heterogeneous combination of ENaC/degenerin components and/or low prevailing [Ca^{2+}] (27). Our biochemical and electrophysiological experiments are consistent with this hypothesis.

Our working paradigm for amiloride-sensitive Na^+ current regulation in normal astrocytes and glial tumors is as follows. Two sodium channels, ASIC1 and ASIC2, are co-expressed in normal cells; however, in cells derived from high grade astrocytic tumors, ASIC2 is either not expressed at all or is retained within the cell such that it is absent from the plasma membrane. ASIC1 is present in the plasma membrane of both normal and tumor cells. The consequence of this difference in expression and cellular localization between ASIC1 and ASIC2 is that astrocytic tumor cells can be characterized by a large inward Na^+ current that is sensitive to the Na^+ channel blocker, amiloride. In contrast, in normal cells, this current is not expressed; thus, the role of ASIC2 in this context is to suppress the otherwise rampant Na^+ conductance. Moreover, in normal cells, two independent inhibitory pathways are operative: inhibition by syntaxin 1A and inhibition by PKC-β (33). Addition of a broad spectrum PKC inhibitor (chelerythrine chloride) to normal astrocytes, in combination with a disruption of syntaxin 1A/Na^+ channel interaction with Munc-18, results in the appearance of a large, amiloride-sensitive Na^+ conductance.

Although our results provide a plausible explanation for the constitutive activation of sodium conductance seen in malignant glioma cells, there is yet another possibility to consider, namely, that ASIC2 is not expressed in normal astrocytes. Our gene profiling studies were performed on tissue specimens obtained in surgery for intractable epilepsy (39). We recognize that there are limitations in the use of any source of intact human tissue. Even though we obtained tissue samples from white matter and not cortex and the number of astrocytes exceeds the number of neurons by at least 1 order of magnitude in these samples, these tissue specimens, nonetheless, contain other cell types such as neurons. Even though RT-PCR results indicate the presence of ASIC2 in primary cultures of human astrocytes (Fig. 4B), we have not yet measured protein. Indeed, recent work from Johnson et al. (16) using triple-labeling im-

![Figure 10](http://www.jbc.org/...)}
munofluorescence suggest that ASIC2 is not expressed in normal astrocytes. But even in the absence of ASIC2, and thus the elimination of the syntaxin 1A inhibitory arm, ASIC1-based amiloride-sensitive currents would still be blocked because of the presence of PKCα and PKCβII in normal astrocytes, PKCα and PKCβII are not present in GBM (40). The experiments presented in

![Representative whole cell patch-clamp recordings from transfected U87-MG and SK-MG-1 cells.](image)

**Fig. 11.** Representative whole cell patch-clamp recordings from transfected U87-MG and SK-MG-1 cells. Voltage clamp conditions were identical to those described in the legend to Fig. 1. **A,** top traces, basal currents. **Middle traces,** currents following superfusion with 100 μM amiloride. **Bottom traces,** difference currents (amiloride-sensitive). At least six cells under each experimental condition were measured. **B,** upper panel, current record from a whole cell clamped ASIC2-trasfected U87-MG cell. Munc-18 was added to the pipette solution. **Middle panels,** current records from a whole cell clamped U-87-MG cell that was pretreated with amiloride (100 μM). The pipette solution also contained Munc-18. **Lower panel,** Average current voltage relations of the current activated by Munc-18 compared with the currents recorded after the same amount of time in the presence of amiloride.

![Whole cell patch-clamp recordings of normal human astrocytes.](image)

**Fig. 12.** Whole cell patch-clamp recordings of normal human astrocytes. Conditions were the same as described in the legend to Fig. 1. The cells used in the end rows were pretreated with 10 μM chelerythrine chloride for 20–30 min prior to experimentation, and chelerythrine was included in both the bath and pipette solutions throughout the experiment. The experiments are representative of at least three under each condition.
Fig. 12 are consistent with this postulate. Our findings of the presence of constitutively activated, amiloride-sensitive sodium channels in glial-derived tumor cells run counter to the suggestions of Horimoto et al. (40), who show that transferring a gain-of-function ASIC mutation into cancer cells using tumor-specific promoters can produce cell death and suggest that this may be a new strategy for cancer gene therapy. Although this may be the case for certain somatic cell cancers, it cannot be so for brain tumors because a functionally activated sodium conductance is operative in the glial tumors that we have examined.

Thus, our work demonstrates that constitutive amiloride-sensitive currents are a specific feature of the more aggressive brain tumors. At present, little is known about the function of ASIC in the brain and less about how the composition and activity of these channels are regulated. Our results provide new insights into the cellular mechanisms that control ASIC function in the brain and how these controlling mechanisms integrate channel function with the other physiologic demands of both normal and malignantly transformed glial cells. Moreover, this work demonstrates that amiloride-sensitive sodium channels cannot easily be classified based on simple biophysical parameters, such as single channel conductance and/or sensitivity to amiloride. This class of ion channel, both in the brain and in epithelial tissues, appear to have a variable composition, and hence tissue-specific differences in biophysical parameters may result from different channel compositions in different tissues. In any case, the novel findings concerning the regulation and biophysical properties of ASICs that have been presented in this work are important in defining the cell biology of brain tumor cells. Understanding the molecular targeting or regulatory sites of ASIC will undoubtedly promote the development of new strategies to circumvent the unwanted activation of these channels and thus have important therapeutic ramifications.

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