Gliomas malignos expressam canais de Na+ sensíveis ao amiloride

Malignant human gliomas expressan amiloride-sensitive Na+ conductance

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Abstract

Human astrocytoma cells were studied using whole cell patch-clamp recording. An inward, amiloride-sensitive Na+ current was identified in four continuous cell lines originally derived from human glioblastoma cells (CH235, CRT, SKMG-1, and U251-MG) and in three primary cultures of cells obtained from glioblastoma multiforme tumors (up to 4 passages). In addition, cells freshly isolated from a resected medulloblastoma tumor displayed this same characteristic inward current. In contrast, amiloride-sensitive currents were not observed in normal human astrocytes, low-grade astrocytomas, or juvenile pilocytic astrocytomas. The only amiloride-sensitive Na+channels thus far molecularly identified in brain are the brain Na+ channels (BNaCs). RT-PCR analyses demonstrated the presence of mRNA for either BNaC1 or BNaC2 in these tumors and in normal astrocytes. These results indicate that the functional expression of amiloride-sensitive Na+ currents is a characteristic feature of malignant brain tumor cells and that this pathway may be a potentially useful target for therapeutic intervention.

astrocytoma

glioblastoma

patch clamp

brain tumor cells

astrocyte-derived brain tumors comprise a diverse group of neoplasms that differ in their morphology, their central nervous system location, their degree of invasiveness, their tendency for progression, and their growth characteristics. Primary malignant brain tumors are the most common cause of cancer-related death in children 16 years of age and younger. Approximately 3 in every 100,000 children under the age of 16 are diagnosed with brain tumors, according to the Central Brain Tumor Registry (14a). The most common childhood brain tumors are primitive neuroectodermal tumors (including medulloblastomas), astrocytomas, ependymomas, and choroid plexus papillomas (2, 11). Due to the infiltrative nature of many of these tumors or because of their location, total surgical excision is not always possible. Conventional adjunct treatment modalities include chemotherapy and radiation therapy. Because unifying molecular events underlying tumor development and progression are only beginning to be understood (4), there is a need to define tumor-specific markers so as to develop potential therapeutic regimens that will target exclusively these unwanted cells.

There is increasing evidence that ion channels may be intimately involved in the cellular pathophysiology related to cancer. Several different laboratories have demonstrated that the

expression of certain oncogenes directly affects Na+(12, 15, 19), K+ (20, 21, 29, 31), and Ca2+ (12-14) channel function. For example, the rasoncogenes, known to be involved in metastasis (6), influence nerve growth factor-induced neuronal differentiation and voltage-sensitive Na+ channel expression (18, 26). Moreover, cell adhesion (5), motility (24, 30), interaction with extracellular matrix (7), and proliferation (12, 25, 27, 31, 33) all involve ion channel activity. Ion channels make attractive candidates for investigation of tumor-specific protein expression because they can be rapidly identified using whole cell patch clamp. If a specific current is noted, there must be a channel containing an extracellular protein epitope through which the current passed. This epitope could serve as a specific antigen target for the tumor. Thus it may be possible to develop a dual attack strategy by specific antibody binding and subsequent immunization and inhibition of function.

The ever-expanding degenerin/epithelial Na+ channel (ENaC) superfamily of Na+ channels contains to date >20 proteins having a similar topology: short intracellularly located amino and carboxy termini, two predicted transmembrane-spanning domains, and a large extracellular loop (9, 17). All channels that have been examined electrophysiologically are cation selective and blocked by the diuretic drug amiloride (8, 9, 17). Recently, another branch of this superfamily, the human brain Na+ channel (BNaC) family, has been identified (16, 28). BNaC1 is located on chromosome 17q11.2–12 and BNaC2 on 12q12 (16). The two thus far identified members of this family, BNaC1 and BNaC2, are only expressed in brain. In normal brain, BNaC1 and BNaC2 have been localized primarily to neurons, although BNaC2 has been detected in choroid plexus (16). Neither transcript has been detected in ependymal cells, anterior commissure, or corpus callosum.

Electrophysiological studies of primary brain tumor cells and the role of specific ion channels in growth control of gliomas are few. Both K+ and Cl− channels have been identified in astrocytoma-derived cell lines and, for the Cl− channels, in primary cell cultures obtained from human brain tumor resections (10, 34). In this work, we identified the specific expression of an inward amiloride-inhibitable Na+conductance in high-grade glioma cells. This conductance was not found in normal human astrocytes or in low-grade astrocytic tumors. This conductance appears to be associated with the functional expression of BNaC in these cells.

EXPERIMENTAL PROCEDURES

Cell culture. Primary cultures from human gliomas were established from fresh brain tumor tissue obtained at the time of surgery. Primary human astrocyte cultures were established by disaggregating normal brain from patients undergoing surgery for intractable epilepsy. The tissue was transported from the operating room in sterile, ice-cold tissue culture medium, and necrotic and/or hemorrhagic regions were dissected away. For brain tumor cultures, the remaining tissue was minced, pipetted repeatedly to disassociate the cells, and plated onto a 35-mm tissue culture dish in DMEM mixed in equal proportions with Ham's F-12 (DMEM/F-12) supplemented with 10 mM HEPES (pH 7.4), 2 mMl-glutamine, and 20% fetal bovine serum. Normal brain was enzymatically disaggregated with 0.1% trypsin/0.53 mM EDTA and plated onto 150-cm2 flasks in DMEM/F-12 plus 20% fetal bovine serum, 1× mito-X, and 10 ng epidermal growth factor/ml as previously described (3). Established cell lines derived from human glioblastoma tumors (CH235, CRT, SKMG-1, U251-MG, U373-MG) are carried in the University of Alabama at Birmingham (UAB) Brain Tumor Research Laboratory facilities and were all used at passages >100. A portion of a freshly resected medulloblastoma was prepared as described above, and the disassociated cells were used immediately for electrophysiological recording and RNA extraction. Brain tumor tissue was obtained in accordance with the human

tissue procurement procedures at the Medical Center as approved by the UAB Institutional Review Board (22 April 1998).

Whole cell patch-clamp studies. Cultured cells were mechanically scraped from 35-mm culture dishes, rinsed in serum-free RPMI medium, placed in the perfusion chamber, and allowed to adhere to the glass bottom. Once the cells had adhered (evidenced by lack of movement during bath superfusion), they were examined electrophysiologically by whole cell patch clamp. Micropipettes were constructed using a Narashigi PP-83 two-stage micropipette puller. The tips of these pipettes had an internal diameter of $\sim$0.3–0.5 μm. When filled with an electrolyte solution containing (in mM) 100 potassium gluconate, 30 KCl, 10 NaCl, 20 HEPES, 0.5 EGTA, and 4 ATP, as well as <10 nM free Ca^{2+}, at a pH of 7.2, the electrical resistance of the tip was 1–3 MΩ. The bath solution was serum-free RPMI 1640 cell culture medium. The solutions approximate the normal ionic gradients across the cell membrane. Pipettes were mounted in a holder and connected to the head stage of an Axon 200A patch-clamp amplifier affixed to a three-dimensional micromanipulator system attached to the microscope. The pipettes were abutted to the cells and slight suction applied. Seal resistance was continuously monitored (Nicolet model 300 oscilloscope) using 0.1-mV electrical pulses from an electrical pulse generator. After formation of seals with resistances >1 GΩ, another suction pulse was applied to form the whole cell configuration by rupturing the membrane within the seal but leaving the seal intact. Successful completion of this procedure was known by the sudden increase in capacitance with no change in seal resistance. The magnitude of the capacitance increase is a direct function of the membrane available to be voltage clamped (i.e., cell size). Typically, this capacitance was between 5 and 10 pF. Subsequently, the capacitive transients were compensated for with the use of the capacitive and resistance circuits of the Axon 200A amplifier.

Once the whole cell configuration was obtained, the pipette solution and the cellular interior equilibrated within 30 s. the cells were then held at a membrane potential of 0 mV for 1 s between each test voltage. This procedure induced inward Na^+ (at more hyperpolarized potentials) and outward K^+ (at more depolarized potentials) currents to flow across the membrane. 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 6 software (Axon Instruments, Sunnyvale, CA). For any given cell line or tumor preparation, 4–12 cells were examined.

RNA extraction and RT-PCR. Total RNA was extracted from tumor cell lines and freshly excised material using guanidinium thiocyanate cell lysis and phenol chloroform extraction. The integrity of the RNA was verified after electrophoresis through 1% agarose-formaldehyde denaturing gels. Two micrograms of total RNA were reverse transcribed at 42°C for 60 min using oligo(dT) as a primer (Pharmacia) and avian myeloblastosis virus RT (Promega). PCR was performed using Taq (Fisher) polymerase with 0.2, 0.6, or 1 mM $MgCl_2$ added to theTaq buffer and 2.5 μl of the RT-PCR product, in a total volume of 50 μl. The primers, corresponding to regions in either BNaC1 or BNaC2, were used at a concentration of 150 ng per reaction. BNaC1, forward and reverse primers, respectively, were as follows: 5'-TGCAAGTTCA AAGGGCAG-3' and 5'-TGGCTGATGT CTTGCTGG-3' and corresponded to bases 511–528 and 1136–1153. The BNaC2, forward and reverse primers, respectively, were as follows: 5'-TGCTCTCCTG CCACTTCC-3' and 5'-GCTTTGCTGG GGATCTTG-3' and corresponded to bases 735–752 and 1366–1383. PCR was performed beginning with a single cycle of 94°C for 4 min, followed by cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min, for 30 cycles. This was followed by a single cycle of

72°C for 60 min to facilitate addition of T and A overhangs to the PCR product. Parallel reactions were run using human β-actin as a control for the RT-PCR. Aliquots of each resulting reaction mixture were run on a 2% agarose gel using φX174Hae III cut DNA (Promega) as molecular size markers. Products of correct molecular size were subcloned into pCR-II using the TA cloning kit following the manufacturer's instructions (InVitrogen). Recombinants were selected by blue/white screening and restricted with EcoR I (Promega). Plasmids containing inserts of correct size were then subjected to automatic DNA sequencing (DNA Sequencing Facility, Iowa State University).

RESULTS

Whole cell patch-clamp experiments were performed on primary cultures of cells derived from eight human brain tumors, five established human glioblastoma cell lines, one freshly resected medulloblastoma, and two separate populations of normal human astrocytes (see Table1). All cells stained positively for glial fibrillary acidic protein. Representative voltage-clamp traces are shown in Fig. 1 for a cell obtained from a medulloblastoma. In the basal state, the current records were characterized by large "ragged" inward currents (Fig. 1 A), and these currents were completely inhibited within 30 s following superfusion with 100 μM amiloride (Fig. 1 B). Figure1 C shows the difference current (i.e., amiloride-sensitive component), and it can be seen that only the inward currents were inhibited by this drug. This result should be contrasted to the lack of inward current and lack of effect of 100 μM amiloride in juvenile pilocytic astrocytoma cells (Fig.2) and normal astrocytes (Fig.3). Comparable amiloride-sensitive inward currents were seen in four of five continuous cell lines (U373-MG being the exception), and in three primary cultures of freshly resected glioblastoma multiforme cells (Table 1). None of the nonneoplastic (i.e., normal) astrocytes or any of the four low-grade astrocytomas displayed any measurable amiloride-sensitive current. These results suggest the presence of an amiloride-inhibitable component to whole cell currents in only the high-grade, highly invasive glioblastoma multiforme tumors.
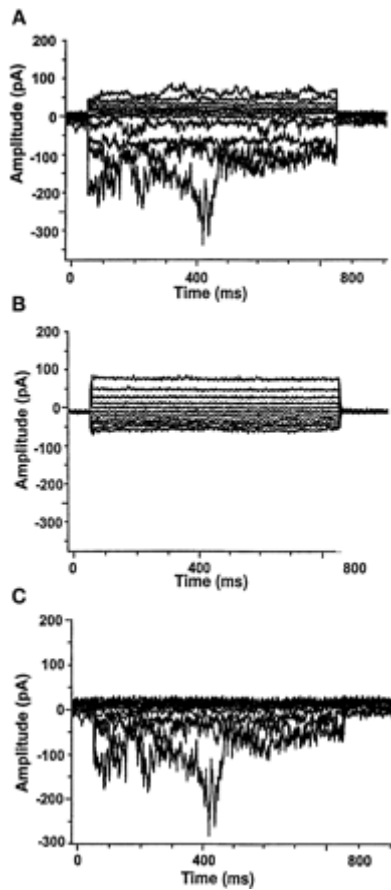
Fig. 1.

Whole cell patch-clamp recording from a representative primary cultured human medulloblastoma cell (Case AM30326). Cells were stepped between −100 and +100 mV in 20-mV increments from a holding potential of 0 mV. Cells were superfused with RPMI 1640 medium, and the pipette contained (in mM) 100 potassium gluconate, 30 KCl, 10 NaCl, 20 HEPES, 0.5 EGTA, 4 ATP, and <10 nM free Ca2+ (pH 7.2).A: basal recordings.B: after superfusion with RPMI 1640 containing 100 μM amiloride. C: difference current.
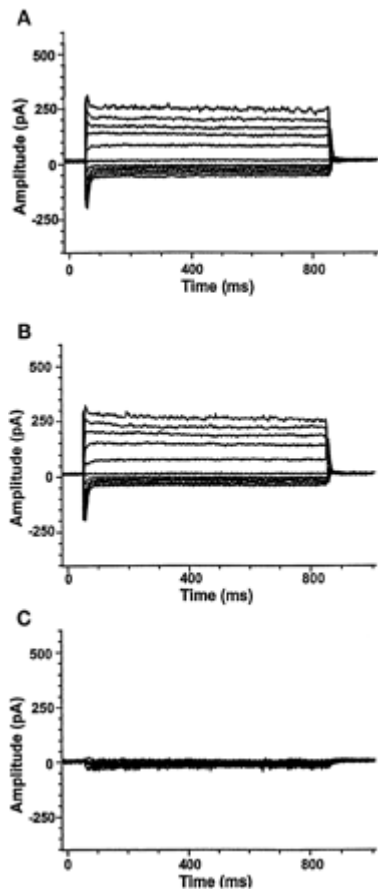
Fig. 2.

Whole cell patch-clamp recording from a representative primary cultured human juvenile pilocytic astrocytoma cell (JPA 501503, passage 2). Conditions were the same as indicated in legend to Fig. 1. A: basal recordings. B: after superfusion with RPMI 1640 containing 100 μM amiloride. C: difference current.
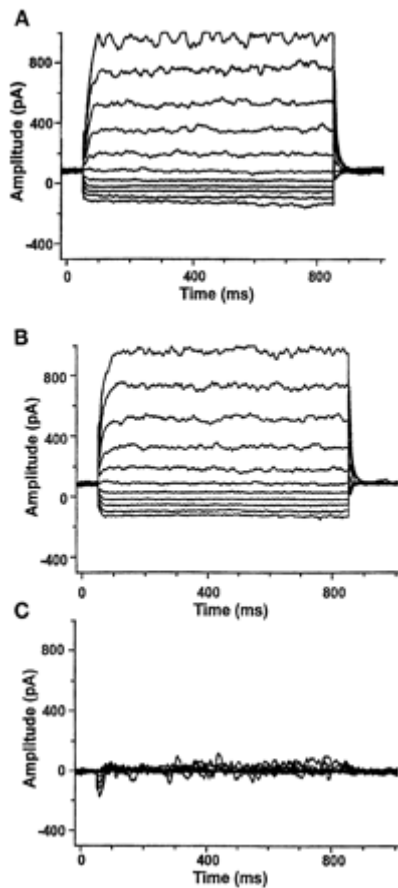
Fig. 3.

Whole cell patch-clamp recording from a normal human adult astrocyte obtained as a biopsy sample from surgery for intractable epilepsy (NB0925317, passage 3). Conditions were the same as indicated in legend to Fig. 1.A: basal recordings.B: after superfusion with RPMI 1640 containing 100 µM amiloride. C: difference current.

To assess the range of amiloride efficacy on the constitutively activated inward currents, primary GBM cells (0968662,passage 3) were superfused sequentially with solutions containing 0.1, 1, 10, and 100 µM amiloride, subsequent to establishing the whole cell configuration. Amiloride concentrations of 0.1 and 1 µM had no measurable effect. Ten micromolar amiloride partially inhibited the current in one of three cells examined, and 100 µM amiloride consistently produced the maximum amount of current inhibition in these trials and in trials on the other tumor cell types studied. From these studies, we estimated aK i (apparent equilibrium dissociation constant) of ~30 ± 20 µM for amiloride-mediated current inhibition in cells expressing this constitutively activated inward Na+ current.

To elucidate the molecular basis for this amiloride-sensitive current, we next performed RT-PCR on total RNA extracted from each of these cell samples. Specific primers were designed to amplify signals corresponding to messages for either BNaC1 or BNaC2, the only two amiloride-sensitive cation channels thus far identified in brain tissue. The primer pairs for BNaC1 and BNaC2 predicted products of 643 and 649 bp, respectively. Examples of these reactions are shown in Fig.4. The BNaC2 message was detected in SKMG-1, medulloblastomas, and normal astrocytes. The BNaC2 message was found in virtually all of the samples analyzed, with the notable exceptions of two glioblastoma multiforme primary cultures and the one anaplastic

astrocytoma (see Table 1). In these particular cases, the BNaC1 message was found instead. In the samples where the BNaC2 message was detected, no BNaC1 product was found (and vice versa). Direct sequencing of the PCR products confirmed their identity.
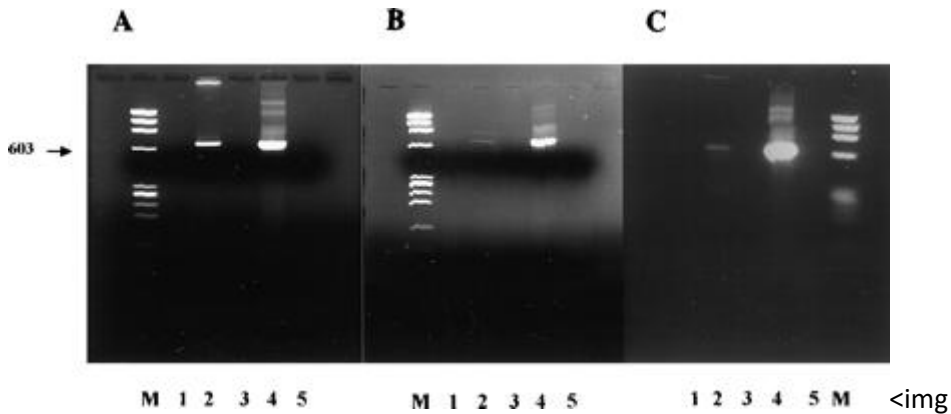
Fig. 4.

RT-PCR detection of human brain Na+ channel 2 (BNaC2) and actin from SKMG-1, medulloblastoma, and normal astrocytes. Optimization of reaction conditions was performed in separate experiments. All reactions were negative for genomic DNA (PCR without RT; not shown).Lane M, φX174HaeII molecular weight markers; lane 1, negative RT control;lane 2, complete reaction mixture (positive); lane 3, negative (water) control; lane 4, actin;lane 5, negative RT for actin.

DISCUSSION

We report the presence of amiloride-sensitive inward Na+ currents in human malignant brain tumor cells. These currents were seen in primary cultures of freshly resected tumors, as well as in established cell glioma lines. These currents were not present in normal astrocytes or in low-grade astrocytomas (e.g., pilocytic astrocytomas). Molecular biological analyses indicated the presence of either the BNaC1 or BNaC2 message in these cells. The level of expression was generally higher in the cells functionally expressing amiloride-sensitive current (for the same number of PCR cycles) but was, nonetheless, detected in all of the samples examined. The endogenous function and physiological role of the BNaC family of ion channels are not known. Their epithelial counterparts, the ENaCs, act as highly regulated Na+ channels in tissues designed to reabsorb Na+ (8, 9, 17). Like the degenerins and ENaCs, BNaCs may form regulated ion channels; such channels may be involved in cell volume regulation (1, 22, 23). The BNaCs may be involved in the small Na+ influx that occurs in neuronal cells and thus may contribute to the resting potential of the cell. Alterations in membrane potential, by either activating or inhibiting these channels, may have deleterious effects on cell survival (35).

It is difficult to detect currents following heterologous expression of either wild-type BNaC1 or BNaC2 (16, 28, 35). However, introduction of mutations into BNaC1, comparable with those

found in the nematode degenerins that produce neurodegeneration, results in easily measurable amiloride-blockable currents, but with a reduced Na+ permselectivity (35). These mutations were made in the second membrane-spanning domain. Because the PCR primers used in our experiments did not cover this region, we cannot state with any certainty that the basal-activated currents measured in the malignant tumor cells were due to mutated BNaC. However, the existence of this current in highly proliferative human tumor cells is significant, in that it may play a crucial role in tumor cell progression. Because normal glial cells do not express this current, it is unlikely that inhibition of the current would be fatal to the malignant cells. However, if, as these studies suggest, the expression of these channels is restricted to malignant tumor cells, then these channels represent a specific target that is exclusive. It may be possible to devise treatments based on this protein.

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