Amiloride kills malignant glioma cells independent of its inhibition of the sodium-hydrogen exchanger.


Source

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Abstract

Previously, we demonstrated that malignant glioma cell lines have increased intracellular pH (pHi) as a result of increased activities of the type I sodium/hydrogen exchanger (NHE1). This alkalotic pHi of 7.2 to 7.4 is favorable for augmented glycolysis, DNA synthesis, and cell cycle progression. Conversely, reductions in pHi have been associated with reduced rates of proliferation in transformed cell types. The effects of reducing pHi directly and by NHE1 inhibition on human malignant glioma cells were systematically compared with those on primary rat astrocytes. Neither cariporide, nor direct acidification to pHi 6.9 altered the proliferative rates or viabilities of human U87 or U118 malignant glioma cell lines. However, amiloride significantly impaired glioma cell proliferation and viability while not affecting astrocytes at concentrations (500 microM) that exceeded its inhibition of NHE1 in glioma cells (IC50 = 17 microM). Preventing a reduction of pHi did not alter the drug's antiproliferative and cytotoxic effects on glioma cells. These findings indicated that amiloride's cytotoxic effects on glioma cells are independent of its ability to inhibit NHE1 or to reduce intracellular pHi. The amiloride derivative 2,4 dichlorobenzamil (DCB) inhibits the sodium-calcium exchanger (NCX) and was both antiproliferative and cytotoxic to glioma cells at low doses (20 microM). By contrast, KB-R7943 [(2-[2-[4-nitrobenzyl oxy]phenyl]ethyl)-isothiourea methanesulfonate] preferentially blocks sodium-dependent calcium influx by NCX (reverse mode) and was nontoxic to glioma cells. It is proposed that DCB (20 microM) and amiloride (500 microM) impair calcium efflux by NCX, leading to elevations of intracellular calcium that initiate a morphologically necrotic, predominantly caspase-independent glioma cell death.

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Amiloride Kills Malignant Glioma Cells Independent of Its Inhibition of the Sodium-Hydrogen Exchanger

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Abstract

Previously, we demonstrated that malignant glioma cell lines have increased intracellular pH (pH_i) as a result of increased activities of the type I sodium/hydrogen exchanger (NHE1). This alkalotic pH_i of 7.2 to 7.4 is favorable for augmented glycolysis, DNA synthesis, and cell cycle progression. Conversely, reductions in pH_i have been associated with reduced rates of proliferation in transformed cell types. The effects of reducing pH_i directly and by NHE1 inhibition on human malignant glioma cells were systematically compared with those on primary rat astrocytes. Neither cariporide, nor direct acidification to pH_i 6.9 altered the proliferative rates or viabilities of human U87 or U118 malignant glioma cell lines. However, amiloride significantly impaired glioma cell proliferation and viability while not affecting astrocytes at concentrations (500 μM) that exceeded its inhibition of NHE1 in glioma cells (IC_50 = 17 μM). Preventing a reduction of pH_i did not alter the drug's antiproliferative and cytotoxic effects on glioma cells. These findings indicated that amiloride's cytotoxic effects on glioma cells are independent of its ability to inhibit NHE1 or to reduce intracellular pH_i.

The amiloride derivative 2,4 dichlorobenzamil (DCB) inhibits the sodium–calcium exchanger (NCX) and was both antiproliferative and cytotoxic to glioma cells at low doses (20 μM). By contrast, KB-R7943 [(2-[4-nitrobenzyl]oxy)phenyl]ethyl)-isothioureamethanesulfonate] preferentially blocks sodium–dependent calcium influx by NCX (reverse mode) and was nontoxic to glioma cells. It is proposed that DCB (20 μM) and amiloride (500 μM) impair calcium efflux by NCX, leading to elevations of intracellular calcium that initiate a morphologically necrotic, predominantly caspase–independent glioma cell death.

Previous Section

High-grade malignant gliomas are the most common, lethal primary brain tumor in adults (Legler, 1999). Median survival is approximately 9 to 12 months after diagnosis (Stewart, 2002), and the tumors are usually refractory to aggressive multimodal therapy (Brandes et al., 1999). Glioma cells exhibit increased glycolytic fluxes associated with elevated lactate/pyruvate ratios (Miccoli et al., 1996) and would be expected to have an acidic
intracellular pH (pH). However, several \(^3\)P spectroscopic studies measured intra-cellular human glioma pH in situ and reported alkaline values (pH 7.12–7.24) compared with surrounding brain (pH 6.99–7.05) (Hubesch et al., 1990; Rutter et al., 1995). The alkaline pH is more optimal for obligate tumor glycolysis, DNA synthesis, and cell cycle progression (Hasuda et al., 1994; Miccoli et al., 1996; Katabi et al., 1999). In addition, a reduction in pH has been associated with reduced rates of proliferation and growth arrest in transformed cell types (Musgrove et al., 1987; Rotin et al., 1989; Horvat et al., 1992).

Our earlier investigation of four human and rat malignant glioma cell lines revealed an alkaline intracellular pH (pH of 7.31–7.48) compared with primary rat astrocytes (pH of 6.98 ± 0.01) (McLean et al., 2000). Increased activity of the type 1 Na\(^+\)-H\(^+\) exchanger (NHE1) in glioma cells maintained this alkaline pH under bicarbonate-free conditions. By contrast, NHE1 activity was negligible in primary astrocyte cultures at their physiological pH of 6.9 to 7.0, which was maintained primarily by sodium- and bicarbonate-dependent regulatory mechanisms (Bevensee et al., 1997a,b; McLean et al., 2000). The NHE1 inhibitors amiloride or cariporide (HOE694) rapidly reduced glioma pH from 7.4 to 6.9, but they did not alter the pH of primary astrocytes (McLean et al., 2000). These findings in malignant glioma cells are consistent with reports of increased NHE1 activity in other transformed cell lines, including colon and bladder (Boyer and Tannock, 1992; Bischof et al., 1996; Ramirez et al., 2000).

In the present study, we evaluated whether the elevated NHE1 activity and increased pH in malignant glioma cells are necessary for their proliferation and viability. We examined whether reducing pH, either directly or by NHE1 inhibition could impair the rate of growth or viability of human malignant glioma cells. Amiloride killed and inhibited the proliferation of human malignant glioma cells, but not astrocytes. However, mechanistic studies indicate that amiloride’s cytotoxicity toward glioma cells arises most likely from inhibition of the sodium–calcium exchanger (NCX), rather than from inhibition of NHE1. Consistent with this mechanism, the NCX inhibitor 2,4-dichlorobenzamil (DCB) was identified as having potent antiproliferative and cytotoxic effects upon glioma cells.

**Materials and Methods**

**Cell Culture.** Primary rat astrocyte cells were isolated from the cerebrum of newborn (~1–day-old) Sprague–Dawley rats using the method of Hertz et al. (1989). Astrocyte cultures were maintained at 37°C, 95% humidity, and 5% CO\(_2\) in Eagle’s minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (Hyclone Laboratories, Logan, UT), 5% horse serum, 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B, 1× vitamins, 1× amino acids, and 2 mM L-glutamine (Invitrogen). Human U118 and U87 glioblastoma cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown at 37°C and 5% CO\(_2\) in Dulbecco’s modified Eagle’s medium (Cellgro, Herndon, VA) and supplemented with 10% fetal bovine serum (Hyclone Laboratories; same lot as for normal astrocyte medium), 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B (Invitrogen). Medium was changed every 48 h. Passage numbers did not exceed 20 and were identical for samples within each experiment.

**Reagents.** Amiloride (Sigma–Aldrich, St. Louis, MO) or cariporide (HOE642, provided as a kind gift from Aventis Pharma, Frankfurt, Germany) was dissolved in dimethyl sulfoxide.
(DMSO; Sigma–Aldrich) at a stock concentration of 0.5 mM and diluted 1:1000 for experiments in either medium or HEPES–Ringer buffer at 37°C. Caspase inhibitors z–Asp(OMe)–Glu(OMe)–Val–Asp(OMe)–fluoromethyl ketone (zDEVD.fmk) and z–Val–Ala–Asp–fluoromethyl ketone (zVAD.fmk), and the negative control peptide z–Phe–Ala–fluoromethyl ketone (zFA.fmk) (Enzyme Systems Products, Dublin, CA) were dissolved in DMSO at a stock concentration of 20 mM 3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyltetrazolium (MTT; Sigma–Aldrich) was dissolved in distilled deionized water at a concentration of 5 mg/ml. Staurosporine (Sigma–Aldrich) was dissolved at a concentration of 0.5 μM in DMSO. All stock solutions were stored as aliquots in the dark at −20°C until use.

Glioma cell viability was determined using 1) metabolic conversion of a tetrazolium (MTT) dye by live cells, and 2) total cell counts with the dye–exclusion assays trypan blue and Sytox Green.

**MTT Assay.** Viable cells reduce MTT from a tetrazolium salt to an insoluble purple formazon that is detected spectrophotometrically (Mosmann, 1983). The correspondence of the MTT assay to cell number was determined after dilutions of either U118 glioma cells or primary astrocytes grown in a 96–well microtiter plate (Falcon Plastics, Oxnard, CA) at 10 wells/dilution (0.1 ml/well). Primary astrocytes (~12,000 cells/well) or U118 glioma cells (~10,000/well) were plated based upon the linear portion of their respective formazon–to–cell number curves and allowed to adhere overnight. Cells were treated for specified times with amiloride or vehicle [0.02% (v/v) DMSO]. MTT (0.5 μg/ml, final) was then added to the microtiter plates, and plates were incubated for 2 h under cell culture conditions. Cells were solubilized overnight in 100 μl/well of 10% SDS and 10 mM HCl in distilled deionized water. Absorbances were measured at 570 nm using a Power Wave microtiter plate reader with KC Junior 340 version 1.11 software (Bio–Tek Instruments, Winooski, VT). Mean background absorbance of cell–free media (630 nm) was subtracted from these values.

**Dye Exclusion Assays.** Identical concentrations of glioma cells were plated on 60–mm dishes and grown until 50% confluence (usually 24 h). At least four dishes were used for experimental conditions, whereas two were used for control conditions (n = 4 and n = 2, respectively). After drug treatments, media and floating cells were collected from each dish. Adherent cells were rinsed in calcium– and magnesium–free phosphate–buffered saline (PBS–CMF), incubated with 0.25% (v/v) trypsin for 5 min (37°C) and combined with the floating fraction. This ensured that all cells were collected from each dish. The mixture was centrifuged at 350g for 5 min, and the resulting pellet for each dish was resuspended in equal volumes of PBS–CMF, and 0.4% trypan blue. Aliquots (10 μl) of the suspension were plated on a hemocytometer, and cells were counted 5 min after staining. Two aliquots were taken from every pellet, and a minimum of 200 cells was counted for each determination so that every cell count was replicated twice. The sensitivity and specificity of the trypan blue exclusion assay in detecting cell death was compared with the fluorescent dye Sytox Green (Molecular Probes, Eugene, OR). Cells grown on coverslips (as described above) were rinsed in PBS–CMF and loaded with both 500 nM Sytox Green and 0.2% trypan blue for 5 min at room temperature (22°C). Simultaneous staining of cells with trypan and Sytox Green was visualized by switching between visible light and fluorescein green with a fluorescent microscope (Nikonophot).

**Caspase–3 Assay.** Caspase–3 activities were measured using a fluorometric caspase substrate, DEVD–AFC (ApoAlert caspase–3; BD Biosciences Clontech, Palo Alto, CA). Free
AFC released by the proteolysis of caspase–3 emits a yellow–green fluorescence at 505 nm. Cells were treated with amiloride (500 μM) in the presence of one of the following peptides (20 μM): the caspase–3 inhibitor zDEVD.fmk, the pan–caspase inhibitor zVAD.fmk, or the negative control peptide zFA.fmk. Each experiment contained duplicate plates for each treatment condition, and the results were tabulated from at least three experiments.

After treatments, floating cells were collected and combined with the residual adherent cells that were removed from the dish with trypsin. The combined fractions were resuspended in PBS–CMF and centrifuged at 350g for 5 min. The cell pellet was resuspended in 50 μl of chilled lysis buffer (BD Biosciences Clontech) and frozen at −80°C until all time points were collected. Samples were then thawed on ice for 10 min, centrifuged at 7500g for 3 min (4°C), and supernatant protein concentrations were determined using the DC Protein Assay (Bio–Rad, Hercules, CA). Aliquots of each sample (50 μl/μg protein) were combined with equivalent amounts of 2× reaction buffer (10 mM dithiothreitol, final) and 5 μl of 1 mM DEVD–AFC (50 μM final), and incubated with excess substrate for 1 h at 37°C. Product formation was detected in microtiter plates with a spectrofluorometer (excitation, 400 nm; emission, 505 nm; SLM Aminco Instruments, Rochester, NY).

**Morphological Studies of Cell Death.** U87 glioma cells in 60–mm dishes were harvested with trypsinization, and the resulting cell pellet was resuspended in Sytox Green stock diluted in 50 mM TBS (500 nM final; 5 × 10^5 cells/ml). Cell suspensions were stained for 20 min at room temperature, centrifuged, and the supernatant containing excess dye removed. Cold ethanol was then added to the pellet, and the cells were fixed overnight at −20°C. Ethanol was aspirated, and the pellet was resuspended in 50 mM TBS. Aliquots (300 μl) of cell suspension were then added to chambers attached to poly–L–lysine–coated glass slides (100 μg/ml; Sigma–Aldrich). Cells were affixed to the slides by centrifugation at 1600 rpm for 4 min using a Cytofuge 2 cytocentrifuge (StatSpin, Norwood, MA). Slides were then rinsed gently in 50 mM TBS (1 × 15 min, 3 × 5 min) followed by doubly distilled water for 5 min, mounted with glass coverslips, and visualized by both fluorescent and confocal microscopy.

**Confocal Microscopy.** Laser scanning confocal microscopy was performed on an Axioskop 2 FS Mot LSM 510 (Carl Zeiss, Thornwood, NY) equipped with argon and HeNe lasers. Sytox Green was excited at 488 nM and detected with a 500– to 530–nm band pass filter. Sequential 1.0–μm images were collected in a 4–μm optical section with a 3–μm numerical aperture using either 20× or 60× objective lenses.

**Determination of pH.** Intracellular pH was measured as described in detail previously (McLean et al., 2000). Briefly, glioma cells were grown to 70 to 80% confluence on collagen–coated coverslips and loaded for 30 min at 37°C with the fluorescent ratiometric dye 2′,7′–bis(2–carboxyethyl)–5(6)–carboxyfluorescein (BCECF)–acetoxymethyl ester (Molecular Probes) in bicarbonate–free HEPES–buffered Ringer (HR), containing 125 mM NaCl, 5.5 mM KCl, 24 mM HEPES, 1 mM MgCl₂, 0.5 mM CaCl₂, and 10 mM NaOH. Nigericin (10 μM; Sigma–Aldrich) was used in conjunction with high–K⁺ calibration solutions of known pHext to calibrate the dye. High K⁺ calibration solution contained 130 mM KCl, 24 mM HEPES, 1 mM MgCl₂, 0.5 mM CaCl₂, and 10 mM N–methyl–D–glucamine–Cl. Coverslips were rinsed to remove extracellular dye, and BCECF fluorescence was measured in a flow–through, spectrofluorometric cell (Hitachi F–2000) at 37°C perfused with HR, pH 7.4 (emission, 535...
nm; excitation, 507 and 440 nm). Fluorescence data were processed to yield pH for a population of cells (Boyarsky et al., 1988, 1993). Experiments were replicated three to five times.

Statistics. Statistical significance for MTT assays, caspase–3 activation, or cell counts/trypan exclusion assay was determined using one–way analysis of variance and either Bonferroni’s or Dunnett’s test for multiple pairwise comparisons, using SigmaStat software version 2.00 (SPSS Science Inc., Chicago, IL).

Results

Effects of Reduced Intracellular pH on Glioma Cell Proliferation and Viability. The effects of pH, on U87 glioma cell viability and proliferation were evaluated by directly acidifying glioma cells with HEPES–buffered Ringers at pH 6.8. In separate experiments, pH was reduced with cariporide, a selective NHE1 inhibitor (Masereel et al., 2003). U87 glioma cells were loaded with BCECF–acetoxymethyl ester, and spectrofluorometry demonstrated that both conditions reduced the pH by 0.3–0.5 pH units within 120 min (Table 1). In separate experiments, manual cell counts coupled with the trypan blue exclusion assay demonstrated that acidified HEPES media at pH 6.8 did not alter U87 glioma cell proliferation nor increase cell death, compared with time–matched controls at pH 7.4 (Fig. 1A). Similarly, cariporide (75 μM) did not alter glioma cell proliferation or cell viability, compared with time–matched, vehicle–treated controls (Fig. 1B).

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TABLE 1

Intracellular pH manipulations pH was measured in populations of U87 glioma cells containing BCECF during 45 to 120 min of pH manipulation. The pH values are expressed as means of three to five independent experiments ± S.D.

Fig. 1.

Effects of intracellular pH change on glioma cell viability. Numbers of viable glioma cells are indicated by white or hatched columns; black regions indicate numbers of trypan blue–positive cells. Results are expressed as mean cell counts (n = 6). A, U87 glioma cells were
grown in either pH 7.4 HEPES Ringer (white columns) or pH 6.8 HEPES Ringer (hatched columns). B, U87 glioma cells were treated with 75 μM cariporide (hatched columns) or vehicle [0.02% (v/v) DMSO, white columns]. U87 (C) or U118 (D) glioma cells were treated with either vehicle (white columns) or 500 μM amiloride (hatched columns). The asterisk (*) indicates significance in viable and trypan–positive cell numbers at $p < 0.05$, compared with time–matched, vehicle–treated controls. Error bars indicate standard deviations for viable cells (upward columns) and trypan–positive cells (downward columns).

**Effects of Amiloride on Glioma Cell pH, Proliferation, and Viability.** Amiloride is a less selective inhibitor of sodium–hydrogen exchange than cariporide and has been reported to have antiproliferative effects on several cancer cell lines (Horvat et al., 1992; Hasuda et al., 1994; García–Cañero et al., 1999), including U118 glioma cells (Szolgy–Daniel et al., 1991). Amiloride at 50 or 500 μM maximally reduced pH, in U87 glioma cells loaded with BCECF from 7.37 to 7.05 within 50 min (Table 1). The MTT assay was then used to identify a concentration range of amiloride that reduced numbers of glioma cells or astrocytes, compared with time–matched, vehicle–treated controls. U118 glioma cells and primary rat astrocytes were treated with amiloride with concentrations ranging from 10 to 1000 μM. The total number of metabolically active U118 glioma cells, as measured by formazan production, decreased by 40% with 100 μM amiloride and by 75% with 500 μM amiloride after 48 h of treatment (Fig. 2A). By contrast, the total number of metabolically active astrocytes after 48 h of amiloride treatment did not significantly differ from vehicle–treated controls after 48 h (Fig. 2B). Higher concentrations of amiloride (1000 μM) did not further reduce the number of metabolically active glioma cells compared with vehicle–treated U118 cells and did not affect the viability of primary astrocyte cultures (data not shown). The MTT data indicated that 500 μM amiloride optimally reduced the number of metabolically active glioma cells, whereas not affecting astrocytes.

**Fig. 2.**

MTT cell assay of U118 glioma cells and primary rat astrocytes during amiloride treatment. A, U118 glioma cells treated with either 100 μM (dashed line) or 500 μM (solid line) of amiloride for 48 h. The number of metabolically active glioma cells is expressed as a percentage of vehicle–treated, time–matched glioma cells ± S.D. B, primary rat astrocytes cultures are treated with either 100 μM (dashed line) or 500 μM (solid line) amiloride. The
number of metabolically active cells is expressed as a percentage relative to vehicle-treated, time-matched astrocytes ± S.D. Dilutional studies demonstrated that formazan production for astrocytes and U118 glioma cells, respectively, was proportional to manual cell counts (see text for details).

Manual cell counts were coupled with the trypan blue exclusion assay to directly measure proliferation and cell death in U87 and U118 glioma cells, and in primary astrocytes after amiloride treatment (Fig. 1, C and D). Based upon MTT results, glioma cells were treated with either 500 μM amiloride or with 0.02% (v/v) DMSO vehicle over 72 h. Floating and adherent cells were combined, stained with trypan blue, and counted. The numbers of vehicle–treated U87 and U118 glioma cells (Fig. 1, C and D) increased 400 to 600% by 72 h, respectively, with less than 10% exhibiting trypan blue uptake (Table 2). In contrast, the number of glioma cells treated with 500 μM amiloride did not increase over 4 to 16 h, and cell counts decreased significantly compared with vehicle–treated controls at 24, 48, and 72 h (Fig. 1, C and D). The number of trypan blue–positive glioma cells increased by 23 to 26% at 16 h and by 81 to 86% after 72 h of amiloride (Table 2). The magnitude of cell death of the amiloride–treated glioma cells was significantly greater than that observed in vehicle–treated, time–matched controls (Table 2; Fig. 1, C and D). Primary astrocytes clump after amiloride treatment, making manual cell counts technically difficult. However, astrocytes did not demonstrate an increase in the number of trypan blue cells after amiloride treatment (data not shown).

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### TABLE 2

Trypan blue uptake in dying and dead U87 glioma cells Glioma cells were treated with amiloride (n = 4) or vehicle (n = 2). Dying and dead cells that stain with trypan blue are expressed as a mean percentage of the total cell count ± standard deviation.

<table>
<thead>
<tr>
<th>Amiloride–Associated Morphological Changes in Malignant Glioma Cells.</th>
<th>Morphological changes in U87 glioma cells were first observed after 6 to 8 h of 500 μM amiloride using diffraction interference contrast microscopy. Affected cells changed from their normal elongated spindly appearance to a spherical shape with reduced adherence, and became permeable to trypan blue. Increasing numbers of glioma cells acquired these morphological characteristics during 12 to 24 h of amiloride treatment. Subcellular debris began to occur in amiloride–treated glioma cells at 48 h and increased markedly by 72 h.</th>
</tr>
</thead>
</table>

The fluorescent dye Sytox Green (Molecular Probes) permeates dying and dead cells and binds irreversibly to nucleic acids (Dailey and Waite, 1999; Hirt et al., 2000). The sensitivity of Sytox Green (500 nM) was first compared with trypan blue using manual cell counts and by switching between fluorescence and visible light on a fluorescent microscope. The number of dying or dead cells staining with Sytox Green was found to be comparable with that of trypan blue (data not shown; Nguyen et al., 2002). Sytox Green was then used in confocal microscopy to delineate the nuclear morphology of dying and dead cells. A portion of U87 cells treated with 500 μM amiloride for 24 h contained swollen nuclei with diffuse chromatin (Fig. 3D). This necrotic nuclear morphology (“karyolysis”) became increasingly
prevalent at the later treatment times of 36 to 48 h. A small number of amiloride–treated glioma cells exhibited shrunken nuclei and condensed chromatin that are consistent with apoptosis (Fig. 3B, arrow). As a positive control, this apoptotic nuclear morphology was widespread in glioma cells treated with the staurosporine (500 nM; Fig. 3, A and C). Vehicle–treated, time–matched glioma cells demonstrated negligible Sytox Green staining (data not shown).

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**Fig. 3.**

Glioma cell morphologies during treatment with staurosporine or amiloride. U87 glioma cells were stained with Sytox Green and visualized with confocal microscopy. Glioma cells exhibited condensed nuclear chromatin and reduced cytoplasmic volume after treatment with staurosporine (A and C). Diffuse staining with swollen nuclei, reticulated chromatin, and increased cytoplasmic volume was observed in cells treated with amiloride (B and D). Rare glioma cells treated with amiloride exhibited condensed chromatin (arrow, B). Scale bar, 50 μm (A and B) or 20 μm (C and D).

**Caspase Activation in Glioma Cells Treated with Amiloride.** Caspase–3 is an “executioner” aspartate–specific cysteinyl protease that is activated in apoptotic glioma cells (Narita et al., 2000) and in astrocytes (Schiffer et al., 2001). Caspase–3 activities increased in amiloride–treated U118 glioma cells and in primary rat astrocytes by 574 and 353%, respectively, as normalized to time–matched, vehicle–treated cells (Fig. 4). However, significant cell death after caspase–3 activation at 24 h was observed in treated glioma cells, but not in treated primary astrocytes (Fig. 2). Coincubation of glioma cells with 20 μM of either a pan–caspase inhibitor (zVAD.fmk) or a caspase–3 inhibitor (zDEVD.fmk) prevented increased caspase–3 activities that are associated with 24 h of amiloride treatment (Fig. 4). The inclusion of the zFA.fmk as a negative control did not prevent the increased caspase–3 activities in glioma cells or primary astrocytes treated with amiloride. The number of trypan blue–positive glioma cells, compared with time–matched, amiloride–treated controls, was not altered by the inclusion of zVAD.fmk during the first 48 h of drug treatment (Table 3). However, the presence of zVAD.fmk significantly reduced the number of trypan blue–positive cells to 44%, compared with 69% in amiloride–treated controls after 72 h (Table 3).
Fig. 4.

Caspase-3 activities in glioma cells and rat primary astrocytes after 24 h of amiloride. U118 glioma cells or primary astrocytes were treated with amiloride in the absence (black columns) or presence of a sham peptide (zFA.fmk, stippled columns), caspase-3 inhibitory peptide (zDEVD.fmk, striped columns), or pan-caspase inhibitory peptide (zVAD.fmk, checkered columns). Caspase-3 activities per milligram of protein are expressed as percentages of the specific caspase-3 activities measured in vehicle-treated, time-matched controls (white columns) ± S.D. The asterisk (*) indicates significance at \( p < 0.05 \), compared with caspase-3 activities in time-matched, amiloride-treated cells.

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TABLE 3
Nonviable cell counts of amiloride-treated glioma cells in the presence of caspase inhibitors Human U87 glioma cells were treated with 500 μM amiloride in the absence (\( n = 2 \)) or presence of either a pan-caspase inhibitor (20 μM zVAD.fmk, \( n = 4 \)) or sham peptide (20 μM zFA.fmk, \( n = 2 \)). Nonviable cells staining with trypan blue are expressed as a mean percentage of the total cell count ± standard deviation.

Alkaline Buffering of the Amiloride-Associated pH Reduction in Glioma Cells. We then investigated whether the decrease in glioma cell pH produced by amiloride was necessary for the drug’s cytotoxicity. Glioma cells were buffered against the acidifying effects of amiloride by preincubation in pH 8.0 HR for pH, measurements or in pH 8.0 HEPES-buffered media for manual cell counts. The pH of U87 glioma cells increased from 7.50 to 7.90 when perfused with pH 8.0 HR (Table 1). The subsequent inclusion of amiloride to the alkaline perfusate reduced glioma cells’ pH from 7.90 to 7.32 (Table 1). This demonstrated that the alkaline perfusate was effective in buffering glioma cells against amiloride’s acidifying effects.

Amiloride-associated effects on glioma cell proliferation and cell death were measured in separate experiments using HEPES-buffered media at alkaline (8.0) or normal (7.4) pH. U87 glioma cells treated with amiloride exhibited decreasing viable cell numbers over 48 h that
did not differ significantly between alkaline (pH 8.0) and control (pH 7.4) conditions (Fig. 5). Similarly, the increasing number of dying and dead cells staining with trypan blue did not differ between alkaline and control conditions (Fig. 5). Vehicle–treated cells in alkaline (8.0) and normal (7.4) pH media were compared with a negative control, and no significant differences were observed (data not shown).

**Fig. 5.**

Effects of alkaline pH$_{\text{ext}}$ on glioma cells treated with amiloride. U87 glioma cells were treated with amiloride in pH$_{\text{ext}}$ 7.4 or pH$_{\text{ext}}$ 8.0 HEPES Ringer buffer. The number of viable glioma cells after treatment with amiloride at pH$_{\text{ext}}$ 7.4 is depicted with white columns; the viable cell number of those treated at pH$_{\text{ext}}$ 8.0 is shown with hatched columns. Black regions depict the number of trypan blue–positive cells. Results are expressed as mean cell counts ($n = 6$). Error bars indicate standard deviations for viable cells (upward columns) and trypan–positive cells (downward columns).

**Amiloride Inhibition of the Sodium–Calcium Exchanger.** The pH$_i$ manipulations and cariporide experiments described earlier strongly indicated that amiloride's effects on glioma cells' viability are likely to be independent of its inhibition of NHE1. Amiloride inhibits the NCX with an IC$_{50}$ of 500 μM (Frelin et al., 1988). We hypothesized that amiloride's inhibition of the forward mode of NCX could impair sodium–dependent calcium extrusion and increase [Ca$^{2+}$]$_i$, levels over the ensuing 24 to 48 h, leading to calcium–mediated necrotic cell death. U87 glioma cells treated with the more potent NCX inhibitor DCB (20 μM) did not proliferate over 48 h, and approximately 60% of cells were trypan–positive (Fig. 6A). In contrast, the number of vehicle–treated cells more than tripled during this period, with only 6% exhibiting trypan uptake (Fig. 6A). Caspase inhibition did not significantly alter the magnitude of the glioma cytotoxicity at 48 h that was produced by DCB. Sixty–seven percent of DCB–treated U87 cells were trypan–positive in the presence of the pan–caspase inhibitor zVAD.fmk (20 μM), compared with 83% of treated cells that were coincubated with the control peptide zFA.fmk (Fig. 6A). All DCB treatment conditions (with or without the aforementioned peptides) produced a statistically significant decrease in viable cell number, and a statistically significant increase in trypan–positive cells at 48 h, compared with time–matched vehicle–treated cells.
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Fig. 6.

Effects of inhibiting the sodium–calcium exchanger on glioma cells. U87 glioma cells were treated with DCB, and black regions indicate the number of trypan blue–positive cells. A, U87 glioma cells were treated with DCB in the absence (gray columns) or presence of a pan–caspase inhibitor (zVAD.fmk, striped columns), or a sham peptide (zFA.fmk, stippled columns). White bars indicate numbers of vehicle–treated, time–matched cells. B, glioma cells were treated with KB–R7943 (gray columns) or vehicle (white columns). Results are expressed as mean cell counts \((n = 3)\). The asterisk (*) indicates significance in viable and trypan–positive cell numbers at \(p < 0.05\), compared with time–matched, vehicle–treated controls. Error bars indicate standard deviations for viable cells (upward columns) and trypan–positive cells (downward columns).

KB–R7943, in the presence of extracellular sodium, preferentially inhibits sodium–dependent calcium influx (reverse mode) of NCX (Iwamoto et al., 2002; Kimura et al., 2002). Treatment of U87 glioma cells with 10 μM KB–R7943 over 48 h did not produce significant cell death nor alter cell proliferation, compared with vehicle–treated, time–matched cells (Fig. 6B). These results are consistent with the idea that inhibition of the forward mode of NCX activity contributes toward the glioma cytotoxicity produced by DCB and by the higher concentrations of amiloride.

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Discussion

Previously, we reported that the increased intracellular pH measured in four human and rat malignant glioma cell lines resulted from increased NHE1 activity (McLean et al., 2000). NHE1 inhibition was found to reduce glioma pH whereas not affecting the pH of primary astrocytes (McLean et al., 2000). In this study, we examined the consequences of NHE1 inhibition and pH reduction on glioma cell proliferation and viability.

Glioma pH was comparably reduced by amiloride, cariporide, and with incubation in acidified HEPES buffer of pH 6.8 (Table 1). Cariporide reduced pH nearly to the same extent as amiloride, consistent with other reports of this drug’s pH–reducing effects in glioma cell lines (Glunde et al., 2002). Only amiloride reduced glioma proliferation and viability but at concentrations that significantly exceeded the 17 μM needed to inhibit 50% of the NHE1
activated in U118 gliomas after pH reduction in bicarbonate–free conditions (McLean et al., 2000). These findings indicated that moderate pH reduction was not sufficient in replicating the antiproliferative and cytotoxic effects of amiloride on glioma cells. U87 and U118 human glioma cell lines are p53 wild type, and the failure of their growth to be impaired by mild pH reduction contrasts with reports of glioma cell line growth arrest under acidic conditions (Reichert et al., 2002). These investigators found that pH 7.0 inhibited the growth of glioma cell lines that retained wild–type p53 activity, whereas not affecting the growth of cell lines that lacked p53 function. In our investigation, incubating U87 glioma cells in alkalinized HEPES media (pH 8.0) further corroborated the pH–independent effect of amiloride on glioma cells. The buffering of pH at 7.3 did not alter the antiproliferative or cytotoxic effects of amiloride and further indicated that pH reduction is not required for the anti–proliferative and cytotoxic effects of amiloride.

NHE1 inhibition by the more specific antagonist cariporide (HOE694) did not reduce U87 glioma cell proliferation or viability. Our findings are similar to those of Tannock and coinvestigators who reported that cariporide was nontoxic to cells at doses that inhibited the regulation of pH, but impaired cell growth under conditions of reduced extracellular pH of 7.0 to 6.8 (Wong et al., 2002). Our cariporide data further indicated that the selective glioma cytotoxicity associated with 500 μM amiloride is likely to be independent of amiloride's NHE1 inhibitory effects.

Amiloride has antiproliferative and cytotoxic effects on U87 and U118 human glioma cells, as determined by manual cell counts combined with the trypan blue exclusion assay. The number of viable glioma cells treated with amiloride failed to increase over 72 h, in contrast to stage–matched, vehicle–treated controls. As an alternate explanation to this antiproliferative effect, the rate of cell death and fragmentation of treated glioma cells could have exceeded the rate of concurrent proliferation. However, this latter explanation is unlikely, because the decline in viable cell numbers preceded increased numbers of trypan–positive glioma cells that were first observed after 16 h of amiloride treatment. Glioma cell death, as measured by trypan blue, exceeded 80% after 72 h of amiloride treatment, whereas the number of astrocytes staining with trypan blue did not increase significantly.

Amiloride induces self–aggregation of trypsinized astrocytes, which precluded the use of cell counts to assess its antiproliferative effects. However, the MTT data of amiloride–treated astrocytes did not demonstrate a reduction in the number of metabolically active cells, compared with vehicle–treated, time–matched controls. In support of these MTT studies, direct intracerebral infusion of amiloride (276 pmol/24 h) into rats for 12 days is not associated with any observable neuropathological changes to normal brain cell types, whereas affecting U87 intracerebral glioma xenografts (F. Gorin and W. Harley, unpublished observations). In this current study, the increased concentrations of amiloride required to affect glioma cells raised concerns about nonspecific toxicity. However, amiloride's selective cytotoxicity in glioma cell cultures and in vivo increases the likelihood that the drug uses specific mechanism(s) to kill glioma cells.

Amiloride–associated glioma cell death seems to be predominantly necrotic, based upon morphological and biochemical criteria. Glioma cell morphology was assessed with Sytox Green, a fluorescent nucleic acid stain that penetrates dead and dying cells. Amiloride–treated glioma cells seemed swollen with enlarged reticulated nuclei, which is consistent with necrotic or oncotic cell death (Majno and Joris, 1995). In contrast, glioma cells treated
with the apoptosis–inducing agent staurosporine (Narita et al., 2000) demonstrated the expected bright staining, highly condensed chromatin consistent with apoptosis (Majno and Joris, 1995).

Biochemical analyses also are consistent with necrosis rather than caspase–mediated apoptosis. Caspase–3 activities increased nearly 6–fold in U87 glioma cells and 3.5–fold in primary astrocytes after 24 h of treatment with 500 μM amiloride, but only glioma cells died in significant numbers. Other investigators have also reported that caspase activation in astrocytes may be associated with gliosis rather than with cell death (Viviani et al., 2000). The pan–caspase inhibitor zVAD.fmK prevented caspase–3 activation in amiloride–treated glioma cells, but did not reduce the magnitude of cell death until 72 h. Furthermore, the necrotic morphologies of dying glioma cells treated with zVAD.fmK and amiloride were the same as dying cells only treated with amiloride (Sytox Green staining; data not shown). These results indicate that caspase activation is not necessary for amiloride–induced glioma cell death during the initial 48 h; consistent with necrotic cell death. The modest caspase dependence of glioma cell death by amiloride at 72 h was associated with cells that demonstrated necrotic morphologies and is consistent with secondary activation of caspase–3 by calpain–mediated necrotic mechanisms (Neumar et al., 2001).

High–dose amiloride has other pharmacological effects, including inhibition of urokinase plasminogen activator (Jankun and Skrzypczak–Jankun, 1999), of sodium and calcium channels (Allen and Xiao, 2003), and of the sodium–calcium exchanger (Kleyman and Cragoe, 1988). We hypothesized that amiloride’s inhibition of the NCX could elevate intracellular calcium and trigger the subsequent necrotic glioma cell death. Therefore, we assessed the effects of an NCX inhibitor, DCB, on U87 glioma cell viability. DCB was antiproliferative and cytotoxic to glioma cells at lower concentrations (20 μM) than amiloride (500 μM). As with amiloride, glioma cell death from DCB is not altered with the inclusion of the pan–caspase inhibitor zVAD.fmK. This model proposes that inhibition of sodium–dependent calcium efflux by NCX (forward mode) causes glioma demise from DCB and from high–dose amiloride. Currently, there is no selective inhibitor of the forward mode of NCX, but KB–R7943 preferentially inhibits the reverse mode of NCX (Kimura et al., 2002), although it is not entirely specific (Reuter et al., 2002). In contrast to DCB, KB–R7943 failed to reduce glioma cell proliferation or viability. The DCB and KB–R7943 data are consistent with a cell death model where high–dose amiloride inhibits the forward mode of NCX, leading to cytotoxic accumulation of $[Ca^{2+}]_i$ over a 24– to 48–h interval, and which initiates predominantly necrotic glioma cell death.

Amiloride’s selective cytotoxic and antiproliferative effects on glioma cells has led to intracellular free calcium measurements by our laboratory. Basal levels of $[Ca^{2+}]_i$ in glioma cells are 5–fold higher than those in normal primary astrocytes (Gorin and Floyd, unpublished results). Ongoing investigations are comparing the levels and regulation of intracellular calcium in glioma cells and astrocytes in the presence of NHE1 and NCX inhibitors.

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**ABBREVIATIONS:** pH, intracellular pH; NHE1, sodium hydrogen exchanger type 1; NCX, sodium calcium exchanger; DCB, 2,4 dichlorobenzamil; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PBS-CMF, phosphate-buffered saline–Ca\(^{2+}\) and Mg\(^{2+}\) free; TBS, Tris-buffered saline; BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-AM; HR, HEPES Ringer; pH\(_e\), extracellular (buffer) pH; [Ca\(^{2+}\)], intracellular calcium concentration.

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