Glioma Cells Release Excitotoxic Concentrations of Glutamate

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INTRODUCTION

L-Glutamate is an important nutritional amino acid involved in a number of biochemical pathways. Glutamate is also the main excitatory amino acid transmitter in the mammalian central nervous system. [Glu]i concentrations are normally maintained at low micromolar levels to assure proper synaptic function and to prevent excitotoxic injury of neurons (1, 2). This is accomplished through the activity of several Na+-dependent glutamate transporters expressed by neurons and astrocytes. Of the five glutamate transporter subtypes cloned (3–7), two (namely GLAST and GLT-1) appear to be located predominantly on glial cells (8, 9). It is believed that astrocytes, which comprise a large portion of the total cell population in the mammalian nervous system, are particularly important in maintaining glutamate homeostasis (10–13) because their processes closely encapsulate synapses, and they are normally invulnerable to glutamate challenge. Like neurons, astrocytes maintain a large transmembrane glutamate gradient, with [Glu]i concentrations of 2–10 mM (14–16), whereas the [Glu]o concentration is approximately 1 μM (2, 17). Because astrocytic glutamate transport is electroneutral and uses the transmembrane electrochemical gradients for Na+, K+, and H+ (18, 19), severe disruption of these gradients or membrane depolarization under conditions of energy failure (ischemia and hypoglycemia) can lead to glutamate release from astrocytes by reversal of glutamate transport (20–22). Glutamate excitotoxicity has been proposed to be the final common pathway in a number of nervous system diseases, including stroke, amyotrophic lateral sclerosis, Huntington’s disease, Alzheimer’s disease, and AIDS dementia (1, 23). Here we report a role for glutamate toxicity associated with the progression of brain tumors, a disease not previously implicated to involve glutamate toxicity.

Unlike neurons, glial cells retain the ability to proliferate postnatally. Uncontrolled, cancerous proliferation of glial cells results in primary brain tumors, collectively termed gliomas. The vast majority of these originate through the neoplastic transformation of astrocytes. Astrocyte-derived tumors often develop over the course of many months to years, beginning as slowly growing, low-grade astrocytomas and progressing toward more aggressive astrocytomas that can eventually give rise to glioblastoma multiforme, the most aggressive glial-derived tumors. The cellular and functional changes that accompany the malignant transformation of astrocytes are poorly understood. As in other cancers, a number of genetic alterations precede the malignant phenotype. Up-regulation of growth factor receptors, changes in extracellular matrix molecules (24) and focal adhesion sites (25) are conspicuous features. Neovascularization and focal necrosis are also a consistent features of high-grade gliomas (26). It is not clear if or how the growing tumor mass causes neuronal cell death along the growing tumor margins. However, epileptic seizures, as an indicator of compromised neural function, are a common occurrence associated with brain tumors (27). We show here that glioma cells are impaired in their ability to remove glutamate from the extracellular space; in addition, they release glutamate at concentrations that can induce widespread neurotoxicity. This finding suggests that tumors may actively induce neuronal death at the growing tumor margins and that glutamate release by tumors may contribute to seizure activity arising from peritumoral brain regions.

MATERIALS AND METHODS

Materials. The enzymes NAD(P)H:FMN oxidoreductase and glutamate dehydrogenase were purchased from Boehringer Mannheim (Indianapolis, IN). General cell culture supplies were obtained from Becton Dickinson (Franklin Lakes, NJ) and Corning (Corning, NY). Earle’s MEM and DMEM were obtained from Life Technologies, Inc. (Grand Island, NY). FBS was purchased from Hyclone (Logan, UT). S-4CPG was obtained from RBI (Natick, MA). t-AP5, (R,S)-3,5-dihydroxyphenylglycine, (S)-3-carboxy-4-hydroxyphenylglycine, (S)-4-carboxy-3-hydroxyphenylglycine, R-4-CPG, MK-801, (R,S)-ethyl-4-carboxyphenylglycine, (RS)-2-chloro-5-hydroxyphenylglycine, (RS)-2-cyclopropyl-4-phosphonophenylglycine, (S)-o-methyl-4-carboxyphenylglycine, (RS)-o-methyl-4-tetrazolylphenylglycine, and CNQX were purchased from...
Tocris Cookson (Bristol, United Kingdom). All other enzymes and chemicals were purchased from Sigma (St. Louis, MO) unless stated otherwise.

**Cell Lines and Primary Cultures of Human Astrocytomas.** Glioma cell lines used in these studies included STTG-1 (from American Type Culture Collection, Manassas, VA) and U-138 MG, U-251 MG, U-373 MG, CH-235 MG, 0-54 MG, and 0-65 MG [all from Dr. D. D. Bigner (Duke University, Durham, NC)]. These cell lines were cultured in DMEM supplemented with 10% heat-inactivated FBS. Glioma cells were used 2–5 days after plating, at which time they had reached over 80% confluence. The majority of the excitotoxicity data reported here were collected from STTG-1 cells but were subsequently confirmed using other glioma cell lines.

Primary cultures of human astrocytoma were established from freshly resected brain tumor biopsy tissues. Briefly, tumor tissues were freed of necrotic/hemorrhagic portions and minced to small pieces aseptically. This was followed by a 20-min digestion with 20 units/ml papain (Worthington, Freehold, NJ) in an oxygen-saturated solution consisting of 137 mM NaCl, 5.3 mM KCl, 1 mM MgCl₂, 25 mM glucose, 10 mM HEPES, 3 mM CaCl₂, 0.5 mM EDTA, and 0.2 mg/ml l-cysteine. Tissues were centrifuged and then triturated in culture media supplemented with 1.5 mg/ml trypsin inhibitor and BSA. Cells were cultured in the same media as glioma cell lines and used after they had reached confluence. Some cells were cultured on 12-mm round glass coverslips for staining of glial fibrillary acidic protein (Incastar, Stillwater, MN).

**Primary Cultures of Rat Astrocytes and Neurons.** Hippocampal astrocytes and neurons were prepared from Sprague Dawley rats as previously described (28). Briefly, hippocampi were removed from the decapitated rat pups (P0-P2) and freed of meninges, minced into 1-mm³ pieces, and digested in papain solution for 20–30 min. Cells were plated in 24-well plates or flasks in MEM supplemented with 10% FBS, 20 mM glucose, 10 units/ml penicillin, and 10 μg/ml streptomycin. Culture media for astrocyte cultures were changed biweekly, and astrocytes were used after 10 days in culture, at which time >90% of cells were glial fibrillary acidic protein positive and essentially free of neurons.

For neuronal cultures, culture plates were coated with polyornithine [0.1 mg/ml in 50 mM borate solution (pH 7.6) for 1 h]. As demonstrated previously (28), serum-borne glutamate is excitotoxic to neurons. Thus, astrocytes cultured in 75-cm² flasks were used for preparing GDM, and neuronal cultures were prepared and maintained in GDM instead of untreated serum-containing media. Cells were usually plated at a density of 5–10 × 10⁶ cells/cm² for 24 h, followed by a 36-h treatment with 15 μM 1-β-D-arabinofuranosylcytosine, which eliminates proliferating cells (mainly astrocytes). Thereafter, neurons received new GDM every 4–5 days. Cultures were used at 14–25 days in vitro, and neuronal purity was >90%.

**Glutamate Uptake.** Uptake procedures were similar to those described previously (29), with minor modifications. [3H]-Glutamate was used as a tracer to study high-affinity, Na⁺-dependent glutamate uptake. The solution used for uptake consisted of 125 mM NaCl, 3.0 mM KCl, 2.0 mM CaCl₂, 1.25 mM NaH₂PO₄, 23 mM NaHCO₃, 10 mM glucose, and 2.0 mM MgSO₄ and was warmed to 37°C and saturated with 5%–95% CO₂–O₂. Cells were washed twice with the uptake solution described above immediately before the experiments commenced. [3H]-Glutamate (0.4 μCi/ml) was mixed with 5–400 μM glutamate in the uptake solution, and the velocity of glutamate uptake was determined over a 5-min period, during which the uptake rate was linear. Glutamate uptake was terminated by three washes with ice-cold uptake solution. Cells were dissolved in 0.3 M NaOH and aliquoted. [3H] Activity was detected in a liquid scintillation counter (Beckman Instruments, Fullerton, CA). Protein concentration was determined by the Bradford method (30) using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA), and total uptake was normalized to protein content.

**Sampling and Determination of [Glu], and [Glu] Levels.** Cells were washed twice and incubated in GDM with various testing agents for variable periods, and the supernatant was collected for [Glu] measurement. Cells were washed twice with PBS, harvested in 0.3 M NaOH, and then neutralized with 0.3 M HCl. Aliquots were stored at −20°C for protein and [Glu] determination. Samples containing serum and samples with glutamate levels higher than 20 μM were diluted (range, 1:20 to 1:100) with distilled water before measurement.

We used the bioluminescence method for detection of glutamate in solution as described by Fosse et al. (31), with minor modifications. Briefly, the glutamate-specific reagent mixture contained 25 mM potassium phosphate (pH 7.0), 40 μg/ml Triton X-100, 100 μM DTT, 30 μM myristyl aldehyde, 2 mM β-NAD, 250 μM ADP, 2.5 mM FMN, 300 milliunits/ml NAD(P)H:FMN oxidoreductase, and 0.5 mM glutatione dehydrogenase. Samples (10 μl) were transferred to a 96-well white cliniplate (Labsystem, Franklin, MA). The glutamate-correlated luminescence was measured by a luminescence plate reader equipped with automatic solution pumps capable of delivering defined volumes to individual wells (LUMIstar; BMG LabTechnologies, Durham, NC). The reagent mixture was kept on ice, covered with foil during experiments, and injected just before measurement (80 μl/well). This method can reliably detect glutamate concentrations of >20 nM. We determined that all drugs used in these studies did not interfere with the bioluminescence assay at the concentration used. Glutamate standards used for calibration were prepared in glutatione solutions (0.15 N NaCl for [Glu]) and measured at both the beginning and end of each plate. [Glu] was calculated from the amount of [Glu], normalized to the total amount of protein and expressed in nmol/mg protein. [Glu] was either expressed as an absolute concentration (μM) or multiplied by the volume and then normalized to cellular protein levels and expressed in nmol/mg protein.

**Ratiometric [Ca²⁺] Measurements.** To evaluate the effects of GCM on neuronal [Ca²⁺], cultured hippocampal neurons were plated on polystyrene-coated 22 × 22-mm² square glass coverslips. After 14–25 days, cells were loaded in culture media (GDM) for 60 min with the ratiometric Ca²⁺ dye Fura-2-acetoxymethylester (10 μM; TEFLABS, Austin, TX). Subsequently, neurons were rinsed with HBSSA containing 126.25 mM NaCl, 3.0 mM KCl, 2.0 mM CaCl₂, 1.25 mM NaHPO₄, 25 mM HEPES, 10 mM glucose, and 2.0 mM MgSO₄ (pH 7.40). After allowing the dye to de-esterify for 10 min in the fresh HBSSAS, coverslips were placed in a Series 20 Microperfusion chamber (Warner Instruments, Hamden, CT) on the stage of a Nikon Diaphot 200 epifluorescence microscope. Neurons were constantly perfused at a rate of 2.0 ml/min with HBSSA heated to 37°C with a TC-344 Dual Heater Controller and a SH-27A in-line heater (Warner Instruments). GCM was either slowly diluted with 5%–95% CO₂–O₂ or diluted in HBSSAS. Recordings were obtained with a fluorescent imaging setup (Photon Technology International, Monmouth Junction, NJ) in which cells were alternately excited at 340 and 380 nm using a monochromatic light source. Emitted light was collected at >520 nm using a Hamamatsu intensified charge-coupled device camera. Images were digitized online, and 340:380 nm ratios were obtained every 10 s. The 340:380 nm ratio was converted to absolute [Ca²⁺] by the following equation:

\[
[Ca^{2+}] = \frac{K_d \times S_{340}/S_{380} \times (R - R_{min})/(R_{max} - R)}{(32)}. \quad (32)
\]

Rmax and Rmin were determined experimentally as described previously (33).

To study the effects of glioma cells on neurons, we used a modified time-lapse video microscopy system. Neurons were cultured on 35-mm Petri dishes with a glass bottom (MatTek, Ashland, MA), loaded with leakage-resistant calcium indicator Fura-PE3-acetoxymethylester (10 μM; TEFLABS), and then placed in a Leiden chamber (34) mounted on top of a Nikon Diaphot microscope. The Leiden chamber was maintained in a humidified environment at 37°C and was supplied with H₂O-saturated 5%–95% CO₂–air mixture through the gas duct of the chamber. Changes in osmolarity of the medium were <5% after 48 h (28). Cells were alternately excited at 340 and 380 nm through a Lambda 10-2 filter wheel driver (Sutter Instrument, Novato, CA) controlled by Axon Imaging Workbench 2.1 (Axon Instrument, Foster City, CA). Emitted light was collected at >520 nm using an intensified charge-coupled device camera. Images were digitized online, and 340:380 nm ratios were obtained every 10 s. These values were again converted to the absolute value of [Ca²⁺] as described above. All media changes and reagent applications were made through two sterile perfusion tubes. Fresh GCM was either applied directly to the cultured neurons or diluted with GDM before application. All tested reagents were also prepared in GDM to the final concentration and equilibrated with CO₂ and temperature for at least 1 h in the incubator before application. Glialoma cells were detached from 10-cm Petri dishes with trypsin and suspended in GDM, centrifuged, and resuspended in fresh GDM at a density of 1.0 × 10⁶ cells/ml. The time delay between the final suspension of glioma cells and the application to neurons was approximately 1 min.

**Assaying Excitotoxic Effects of Glioma Cells on Neurons.** To assess the effects of glioma cells on the survival of neurons, we performed three sets of experiments. (a) To ascertain the potential excitotoxicity of GCM, GDM was conditioned in glioma cultures for 6–24 h, and the resulting GCM was applied to neurons, with the identical batch of GDM used as a control. Neuronal
survival was determined by trypan blue exclusion. (b) For coculturing of neurons and glioma cells, glioma cells were harvested from a culture flask or a 6-well plate with trypsin, spun down, washed, and resuspended in GDM. Glioma cells were applied to neuronal cultures at a density of \( \sim 1 \times 10^5 \) cells/cm\(^2\), where they settled down within 1 h and formed direct contact with neurons. Neurons could be readily distinguished from glioma cells by their long tapered processes and round cell bodies. Surviving neurons, excluding trypan blue, were counted 40 h after coculturing. (c) For coculture of neurons and glioma cells without direct contact, glioma cells were cultured on 22 \( \times \) 22-mm\(^2\) glass coverslips with wax spots on each corner. Once the cultures had reached confluence, the coverslips were placed upside down on top of neurons cultured in 6-well plates, with glioma cells facing the neurons but separated by the wax spots as spacers with approximately 1 mm of clearance. In all experiments, neuronal death was quantified by determining the ratio of trypan blue containing dead cells after 35–40 h of coculture. At least three to six wells of neurons each with six to eight randomly selected fields were included for each experiment.

**Statistics.** All data were expressed as mean ± SE. Control values were derived from untreated sister cultures. Statistical evaluations of the data were performed using Student’s \( t \) test or ANOVA as appropriate. Significance levels (as shown in the figures) were as follows: *, \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.001 \).

**RESULTS**

**Glioma Cells Show Compromised Glutamate Transport.** We studied uptake of glutamate from the extracellular environment by assessing the Na\(^+\)-dependent influx of glutamate. These studies included glioma cells isolated from five surgically removed human glioma tissues, seven commonly studied glioma cell lines, and, for comparison, normal rat astrocytes. As summarized in Fig. 1A, the rates of glutamate uptake into glioma cell lines and normal astrocytes were assessed over a range of glutamate concentrations (5–400 \( \mu \)M). At every glutamate concentration tested, the rates of glioma glutamate uptake were remarkably lower than those of normal astrocytes. The mean values of glutamate uptake (at a concentration of 50 \( \mu \)M) for glioma cell lines (seven different lines) and primary human glioma cultures (five different glioblastoma multiforme tissues) were averaged and plotted along with the glutamate uptake of normal astrocytes in Fig. 1B. These data showed an approximately 10-fold difference in glutamate uptake between normal astrocytes and glioma cells. The uptake rates determined in our control rat primary astrocytes are close to those reported for normal human astrocytes (35), thus eliminating the possible concern that species differences may account for the large decrease in glioma cell uptake. Determination of the \( V_{\text{max}} \) and \( K_m \) values for glutamate uptake suggests that the observed difference was due mainly to a >10-fold reduction in the \( V_{\text{max}} \) of glutamate uptake into glioma cells (data not shown). At a physiological [Glu], concentration (\( \sim 1 \mu \)M), glutamate uptake into glioma cells is approximately 16.4 ± 5.4% (\( n = 7 \)) of that in normal astrocytes.

**Glioma Cells Release Neurotoxic Concentrations of Glutamate into the Culture Media.** As we reported previously (28), astrocytes can very effectively deplete glutamate from serum-containing media, thereby promoting the survival of neurons. This is most likely a key factor in maintaining [Glu], concentrations in the brain below neurotoxic concentrations at \( \sim 1 \mu \)M. In light of the above findings, we set out to investigate whether the reduced glutamate transport rate in glioma cells would also lead to compromised capability in maintaining low [Glu],. Therefore we monitored the [Glu], of glioma cells cultured in fresh media and compared it with that of normal astrocytes. As reported previously, astrocytes depleted serum-containing glutamate (\( \sim 92 \mu \)M) within 3 h and reduced [Glu], to \( \sim 1 \mu \)M. Surprisingly, glioma cells did not reduce the glutamate content in the media; instead, [Glu], in glioma cultures increased 3-fold over a 12-h period (Fig. 2A).

Because this increase must have been due to glutamate release from glioma cells, we assessed this more quantitatively by placing glioma cultures into media in which glutamate had previously been depleted by a 4–6-h preincubation with hippocampal astrocytes. This GDM contained glutamate concentrations of \( \sim 1 \mu \)M. Glioma cells very rapidly elevated [Glu], and within 5 h, [Glu], exceeded 100 \( \mu \)M (Fig. 2B). To evaluate the possibility that the glutamate was spontaneously generated from precursor molecules like glutamine by enzymes released from glioma cells, [Glu], was also monitored in cell-free GDM or in GDM with cells disintegrated by 0.1% digitonin (36). [Glu], only marginally increased under those conditions (Fig. 2B, dotted lines). The elevation of [Glu], in glioma culture was almost linear during the first 10 h. The velocity of glutamate release could thus be expressed as the amount of glutamate released into the media (0.5 ml), normalized for cell protein. This yielded a value of 4.94 nmol/min/mg protein for glioma cells, a value 44-fold higher than the glutamate generated by disintegrated cells (0.11 nmol/min/mg, 30% of which may come from GDM alone, and 70% was accounted for by the released enzymes). These data confirmed that the increases in [Glu], were due to release from glioma cells rather than glutamate synthe-
Fig. 2. Glioma cells release glutamate. A, astrocytes depleted serum glutamate levels from 92 μM to 1 μM within 3 h, whereas STTG-1 glioma cells induced a 3-fold increase in glutamate concentration in the media after a 12-h incubation. B, STTG-1 glioma cells induced [Glu]o elevation in GDM, whereas cells disintegrated by digitonin (0.1%) or GDM alone resulted in only small increases in [Glu]o. Other glioma cell lines induced similar [Glu]o elevations. C, the glutamate transport inhibitor THA (1 mM for 70 min) in GDM significantly increased [Glu]o in astrocyte cultures (A+THA) as compared to astrocytes in GDM alone, but without significant changes in [Glu]i. After 38 h in GDM, [Glu]o in STTG-1 cell cultures reached levels of ~0.5 mM, but [Glu]i remained at 10 mM. D, the same conditions were used as described in C, but the total amount of glutamate released into the media was compared to the intracellular glutamate content. In all cases, released glutamate from THA-treated astrocytes or glioma cells greatly exceeded the intracellular content (mean ± SE; n = 6).

Glutamate Release from Glioma Cells Can Be Inhibited by S-4CPG-like Phenylglycine Derivatives. We recently found that several mGluR agonists/antagonists can reduce [Glu]i, in astrocytic cultures (38). Among the various glutamate analogues tested, S-4CPG was the most effective. Therefore, we tested the effects of S-4CPG on glutamate release from glioma cells. Glioma cells were incubated in GDM with S-4CPG at concentrations ranging from 3–300 μM, and [Glu]o was sampled over a period of 24 h (Fig. 3A). S-4CPG markedly and dose-dependently reduced the elevations of [Glu]o, with an apparent EC50 of 1–2 μM. At 24 h, 100 μM S-4CPG reduced [Glu]o from 616.0 ± 40.9 to 18.8 ± 0.6 μM (n = 4) with a 32-fold reduction. The effect of (S,R)-4-carboxyphenylglycine was stereospecific, and the R-4CPG isomer was 1000-fold less potent (Fig. 3B). Other structurally related phenylglycine derivatives were also tested. Most of them are characterized as mGluR agonists/antagonists. Among the tested reagents, S-4CPG showed the highest efficiency, followed by S-4C3HPG and S-3C4HPG. It appears that the presence of a carboxyl group in the phenyl ring dramatically increases the efficiency [e.g., S-4CPG, (S)-4-carboxy-3-hydroxyphenylglycine, and S-3C4HPG versus phenylglycine, S-3-hydroxyphenylglycine, and (RS)-3,5-dihydroxyphenylglycine], whereas replacing the hydrogen that is attached to the α-carbon with a larger group dramatically reduced the efficiency [e.g., (S)-α-methyl-4-carboxyphenylglycine and (RS)-α-ethyl-4-carboxyphenylglycine].

Coculture of Hippocampal Neurons with Glioma Cells or GCM Elicits Large Calcium Responses in Neurons. Activation of glutamate receptors commonly leads to elevations of [Ca2+], in neurons (39, 40), and prolonged exposure to glutamate can induce Ca2+-dependent neurotoxicity (1, 41). Therefore, glutamate released by glioma cells would have the capacity to trigger a calcium response in
neurons in the proximity of glioma cells. To demonstrate this, we first exposed cultured hippocampal neurons briefly to GCM. As shown in Fig. 4A, a switch from HEPES-buffered solution to GDM elicited only a small calcium response that immediately returned to the basal level after the removal of GDM. This calcium response was likely caused by the residual glutamate (~1 μM) and serum factors, including growth factors and lysophosphatidic acid (33), contained in this medium. Subsequently, we exposed hippocampal neurons to GCM that had subsequently been conditioned for 6 h by primary hippocampal astrocytes to deplete glutamate. This GDGCM also elicited a rever-

Fig. 4. Neuronal [Ca$^{2+}$]$_i$ responses to GCM or glioma cell suspensions. A, hippocampal neurons (14 DIV) in HBSAS responded to GDM with a transient [Ca$^{2+}$]$_i$ increase, but a 3-min perfusion with GCM (conditioned by STTG-1 cells for 8 h) elicited a sustained [Ca$^{2+}$]$_i$ elevation. A 5-min application of GDGCM only triggered smaller and transient [Ca$^{2+}$]$_i$ increases. B, hippocampal neurons cultured in GDM exhibit spontaneous [Ca$^{2+}$]$_i$ oscillations. The addition of glioma cells (STTG-1) suspended in GDM induced a large sustained [Ca$^{2+}$]$_i$ elevation followed by membrane disintegration (arrows). C, both D-AP5 and CNQX inhibited the spontaneous [Ca$^{2+}$]$_i$ oscillations and blocked the sustained [Ca$^{2+}$]$_i$ elevation by glioma cells. D, glioma cells (STTG-1) pre-treated with S-4CPG and in the continuous presence of S-4CPG did not induce significant [Ca$^{2+}$]$_i$ elevation in hippocampal neurons.
network-forming neurons displayed spontaneous \([Ca^{2+}]_i\), response of larger amplitude than that observed with astrocyte-conditioned medium. Lastly, these neurons were exposed for 3 min to GCM in which glutamate was not predepleted. This led to a large and persistent increase in \([Ca^{2+}]_i\), from which the cells did not recover (Fig. 4A). This \([Ca^{2+}]_i\), increase must have been caused by molecules, most likely glutamate, secreted into the medium by glioma cells. We repeated these experiments with a variety of glioma cell lines and primary glioma cells cultured from patient brain tumor tissues, all of which yielded similar results.

By using the combination of time-lapse video microscopy and ratiometric calcium imaging, we also studied the calcium response of neurons to the direct application of glioma cells freshly suspended in GDM. Cultured hippocampal neurons maintained in vitro for 2 weeks were imaged on the stage of a microscope and incubated with fresh GDM (neurons were cultured in GDM, see "Materials and Methods"), which did not induce any sustained \([Ca^{2+}]_i\), increases. However, these network-forming neurons displayed spontaneous \([Ca^{2+}]_i\), oscillations as reported previously (42, 43). Superfusion of a cell suspension of STTT-1 glioma cells onto neuronal cultures elicited a large sustained calcium response in neurons (Fig. 4B). Neurons did not recover from these large \([Ca^{2+}]_i\), increases but instead began to die after a variable delay of 90–150 min. This is evident in our recordings by abrupt drops of the ratiometric signal (arrows in Fig. 4B), indicating disruption of the plasma membrane and leakage of calcium dye out of the cells. Both the spontaneous \([Ca^{2+}]_i\), oscillations and the glioma cell-induced \([Ca^{2+}]_i\), increases could be blocked if 100 \(\mu M\) D-AP5 and 20 \(\mu M\) CNQX were included in the media (Fig. 4C). Similar \([Ca^{2+}]_i\), elevations were observed when using other glioma cell lines or biopsy-derived glioma cells. Treatment with D-AP5 and CNQX also completely prevented neurotoxicity, suggesting that the glioma cell-induced neuronal \([Ca^{2+}]_i\), response might be due to the activation of glutamate receptors on neurons.

Because S-4CPG was found to profoundly reduce glutamate efflux from glioma cells (Fig. 3A), we tried to identify whether pretreatment of glioma cells with S-4CPG could reduce their ability to elicit excitotoxicity. As shown in Fig. 4D, exposure of hippocampal neurons to a cell suspension of glioma cells pretreated for 6 h with S-4CPG and in the continued presence of 50 \(\mu M\) S-4CPG did not induce any sustained increases in neuronal \([Ca^{2+}]_i\). This is consistent with the fact that glutamate release from glioma cells could be inhibited by S-4CPG. When exogenous glutamate was applied to neurons as a control, no difference in the neuronal \([Ca^{2+}]_i\), response was introduced by the presence or absence of 50 \(\mu M\) S-4CPG (data not shown). These results suggest that the absence of a \([Ca^{2+}]_i\), response in S-4CPG-treated glioma cells is due to the suppression of glutamate release from glioma cells. Interestingly, the addition of glioma cells alone seems to be able to trigger a transient neuronal calcium response that is blocked neither by D-AP5 + CNQX (Fig. 4C) nor by diminishing glutamate release from glioma cells (Fig. 4D). This transient response is unlikely to be artifactual because a media change alone (without glioma cells; Fig. 4C) could not trigger it.

**Glioma Cells Kill Neurons through the Release of Glutamate, and This Toxicity Can Be Prevented by S-4CPG-like Phenylglycine Derivatives.** Loss of intracellular Ca\(^{2+}\) homeostasis has been demonstrated to be a common pathway in excitotoxic neuronal death (1, 23). We assessed the ability of glioma cells and GCM to induce excitotoxicity in hippocampal neurons in vitro. Cultured hippocampal neurons were incubated for 16 h in GCM (prepared by incubating GDM with STTT-1 for 5 h) in the presence or absence of several glutamate receptor blockers (Fig. 5A). The percentage of viable neurons was determined by trypan blue exclusion and compared to neurons incubated in GDM. Application of GCM essentially wiped out the entire neuronal cell population. Neuronal loss was inhibited by >95% in the presence of the NMDA antagonists MK-801 (10 \(\mu M\)) or D-AP5 (50 \(\mu M\)). The AMPA/KA receptor antagonist CNQX (20 \(\mu M\)) was without effect, suggesting that the toxicity of GCM was caused primarily by the activation of neuronal NMDA receptors. If we used GDGC prepared by a 6-h incubation of GCM with primary hippocampal astrocytes to deplete glutamate (28), neurotoxicity was completely removed (Fig. 5A).

Because we showed that S-4CPG greatly reduced glutamate release from STTT-1 glioma cells (Fig. 3A), we also tested the effects of S-4CPG on the excitotoxicity induced by GCM. STTT-1 cells were incubated in GDM for 8 h with S-4CPG at different concentrations. Subsequently, the medium was harvested and applied to neurons, and neuronal survival was assessed 16 h later. The results (Fig. 5B) show a dose-dependent protective effect of S-4CPG that is in line with the dose-dependent inhibition of glutamate release from glioma cells by this compound.

To more closely mimic the conditions that neurons in the proximity of a glioma may encounter in vivo, we performed several glioma-neuronal coculture experiments in which cells were in direct contact with each other (Fig. 6) or neurons and glioma cells were in close apposition but without physical contact (Fig. 7). For direct coculture experiments, STTT-1 cells (1 × 10^5 cells/well) were suspended in GDM and plated onto neuronal cultures. Within 40 h after the application of glioma cells, substantial neuronal death was observed (Fig. 6B), with approximately a 70% loss in viability (Fig. 6F). Again, this toxicity could be largely prevented by the addition of 10 \(\mu M\) MK-801 or 50 \(\mu M\) D-AP5 to the media (Fig. 6, D and E).
In the second set of experiments, glioma cells were cultured on coverslips and placed upside down in the culture well containing hippocampal neurons but separated by 1 mm wax spacers to prevent cell-cell contact. In this system, glioma cells and neurons merely shared a common extracellular space (Fig. 7). These coculture experiments displayed similar neurotoxicity, with a 60% loss of neurons.

Under these conditions, we observed two major differences from the above coculture experiments or those using GCM: (a) albeit less effective than MK-801, which afforded complete protection, 20 μM CNQX in these cocultures was neuroprotective, reducing excitotoxicity by 50% (Fig. 7E). This suggests that at rather low glutamate concentrations (around 10 μM) in coculture conditions as compared to GCM (around 100 μM or higher), constant activation of the AMPA/KA receptor may significantly contribute to glutamate-induced neuronal damage; and (b) significant reduction in neurotoxicity was observed in the outside margins (edge) of the neuronal culture, specifically in areas where glioma cells were at a greater distance (Fig. 7F). This suggests that a concentration gradient existed for glutamate released from glioma cells. Again, as can be expected from the observation that S-4CPG inhibits glioma glutamate release, S-4CPG was very effective in preventing neurotoxicity (Fig. 7, D and E).

**DISCUSSION**

We demonstrate that glioma cells release sufficient amounts of glutamate to induce widespread toxicity in cultured neurons. These data were obtained not only in seven established glioma cell lines but also in short-term primary cultures of glioma cells prepared from biopsy tissues removed from glioma patients. Even when maintained for only minutes in an extracellular volume that exceeded the cellular volume by 1000-fold, glioma cell-released glutamate was sufficient to activate NMDA and AMPA/KA receptors on hippocampal neurons and to induce delayed Ca$^{2+}$-dependent cell death. This *modus operandi* is much different from that of normal astrocytes, which are poised to maintain low [Glu], thereby protecting neurons from glutamate toxicity.

Glutamate concentrations in the culture medium of glioma cells can increase from ~1 μM to more than 100 μM within 5 h. The toxicity of this medium or of glioma cells to neurons could be completely prevented by the NMDA-specific inhibitors MK-801 or D-AP5. Interestingly, toxicity was still prominent in glioma-neuronal coculture experiments where cells were grown without contact but shared the same extracellular environment. This indicates that even the chronic release of glutamate by glioma cells into a relatively large extracellular space is sufficient to induce neuronal glutamate toxicity. Glutamate released by glioma cells appears to be primarily from metabolizing glutamine, although several pathways exist for the *de novo* synthesis of glutamate from other precursors. We observed alterations in the speed of glutamate release after changes in the concentrations of several amino acids in the media. Because the tumor tissues are well vascularized and the blood-brain barrier in tumor tissues is hyperpermeable, glioma cells have ample access to glutamine and other nutrition factors for synthesizing glutamate. Interestingly, there is evidence to suggest an increased ratio of glutamine:glutamate in glioma tissues (44, 45). The decrease of the total glutamate amount in tumor tissues as observed previously (44) is most likely due to the loss of neurons in these tissues because neurons contain high levels of glutamate.

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Unpublished observations.
glutamate (\(10^{-10} \text{ to } 100 \text{ mM}\)). In addition, although changes in [Glu] in vivo may determine the neuronal fate, they would not significantly influence the total amount of glutamate in the whole tissue because the extracellular space accounts for only \(15\%\) of the total tissue volume (46), and [Glu] is generally lower than [Glu] without an exogenous supply.

It is difficult to directly extrapolate our in vitro data to an in vivo situation. However, our coculture studies using glioma-neuronal cocultures were aimed at closely mimicking in vivo conditions. In these cocultures, a balance exists between glutamate release from glioma cells and glutamate uptake into glioma cells and neurons. Because neurons possess a much higher uptake capability than glioma cells, the neuronal uptake of glutamate may play an important role in limiting the [Glu] elevation in the cocultures. This may also explain why the excitotoxicity by glioma cells in the coculture was not as complete as that by GCM, where [Glu] had been allowed to build up freely by the release from glioma cells. Because the total glutamate uptake capability is proportional to the number of cells, we observed that neurons cultured at higher density were more resistant to glioma cells. Interestingly we also found that if there was a \(>20\%\) contamination of normal astrocytes in our neuron/glioma cocultures, neurotoxicity was much less pronounced than in cocultures essentially free of astrocytes. This is consistent with the neuroprotective role of astrocytes (10) and their ability to remove glutamate very effectively (28).

Based on the above reasoning, the combined astrocytic and neuronal glutamate uptake would be expected to greatly retard the process of glioma-induced excitotoxicity as determined in vitro. On the other hand, because the in vitro cocultures used an extracellular space \(\times 10,000\)-fold larger than that in vivo, we may have greatly underestimated the true speed and extent of [Glu] accumulation in vivo. It is conceivable that glutamate released from glioma cells could fill up the narrow in vivo extracellular space much faster than it does in vitro, especially for the extracellular space inside glioma tissue or in the immediate vicinity of the tumor. Elevations of [Glu] may thus be sufficient to overactivate neuronal glutamate receptors in peritumoral tissue and induce neuronal death or contribute to the seizure activity that is often observed in patients with gliomas (27).

The cause of the impaired glutamate transport by glioma cells remains to be determined. We recently found that glioma cells express GLAST transporters in levels comparable to normal astrocytes. However, the majority of the immunohistochemically identified transporters are localized to the cell nucleus, with very little labeling of the plasma membrane. It is thus conceivable that these transporters are not properly targeted to the plasma membrane. This mislocalization of transporters may account for the observed deficiencies in glutamate transport.

The effect of phenylglycine derivatives to selectively inhibit glutamate release by glioma cells offers exciting clinical potential for this research procedure.
class of drugs. Reductions in glutamate release from gliomas may reduce neurotoxicity and slow tumor growth. Moreover, if our reasoning holds true, that glutamate release from tumors into the peritumoral brain tissue may contribute to seizures, then phenylglycine compounds may reduce the likelihood that seizures may be triggered from peritumoral neurons. This possibility is particularly exciting in light of the fact that some of the phenylglycine derivatives that were potent inhibitors of glioma glutamate release do not activate synaptic mGluR receptors and thus should exert very specific effects on the glutamate transport. In a recent study, we investigated the effect of these compounds on glial glutamate transport and were unable to unequivocally delineate the pathway by which they interact with the transporter (38). However, their structural similarity to glutamate suggests that they may bind directly to the glutamate transporter or cell surface proteins that may mediate glutamate release from the cells. Recently, we found that another type of glutamate transport system, the Na"+-independent cystine-glutamate exchange, is likely to be the primary mediator of glutamate efflux from glioma cells and the target of S-4CPG-like compounds.²

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REFERENCES

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