Requirement for cGMP in Nerve Cell Death Caused by Glutathione Depletion

Yonghong Li,* Pamela Maher,‡ and David Schubert*

*Cellular Neurobiology Laboratory, The Salk Institute for Biological Studies, La Jolla, California 92037; and ‡Department of Cell Biology, The Scripps Research Institute, La Jolla, California 92037

Abstract. Glutathione depletion occurs in several forms of apoptosis and is associated with Parkinson’s disease and HIV toxicity. The neurotransmitter glutamate kills immature cortical neurons and a hippocampal nerve cell line via an oxidative pathway associated with glutathione depletion. It is shown here that soluble guanylyl cyclase (sGC) activity is required for nerve cell death caused by glutathione depletion. Inhibitors of sGC block glutamate toxicity and a cGMP analogue potentiates cell death. Glutamate also induces an elevation of cGMP that occurs late in the cell death pathway. The resultant cGMP modulates the increase in intracellular calcium that precedes cell death because sGC inhibitors prevent calcium elevation and the cGMP analogue potentiates the increase in intracellular calcium. These results suggest that the final pathway of glutamate induced nerve cell death is through a cGMP-modulated calcium channel.
lar cystine and its reduction product cysteine, thereby causing a decrease in the level of GSH. This mechanism, called oxidative glutamate toxicity, has also been described in a hippocampus nerve cell line HT22 (Davis and Maher, 1994), in a nerve-glial hybrid cell line N18-RE-105 (Murphy et al., 1989), and in primary oligodendrocytes (Oka et al., 1993). When artificially depleted of GSH, immature cortical neurons also undergo programmed cell death (Ratan et al., 1994).

The steps linking GSH depletion to neuronal cell death are largely unknown. We have been studying this pathway using glutamate treatment of immature cortical neurons and a neuronal cell line. Recently, we found that depletion of GSH activates neuronal 12-lipoxygenase (12-LOX), which leads to the production of reactive oxygen species, the increase in intracellular Ca\(^{2+}\), and ultimately to cell death (Li et al., 1997). The experiments outlined below extend these studies to show that the soluble guanylyl cyclase (sGC) (Li et al., 1997). The experiments outlined below extend these studies to show that the soluble guanylyl cyclase (sGC)/cGMP pathway is involved in nerve cell death caused by glutamate toxicity. This paper reports the first evidence for a role of sGC in mediating programmed cell death caused by glutathione depletion.

### Materials and Methods

#### Cell Cultures

Mouse hippocampal HT22 cells (Davis and Maher, 1994) are derived from the HT4 cell line (Morimoto and Koshland, 1989) and are propagated in DME (Vogt and Dulbecco, 1963) supplemented with 10% FBS. Primary cortical neurons were prepared from embryonic day 17 Sprague-Dawley rats as described (Abe et al., 1996). After dissociation from brain tissues with trypsin and DNase I, cells were maintained in MEM supplemented with 30 mM glucose, 2 mM glutamine, 1 mM pyruvate, and 10% FBS.

#### Cell Viability Assay

Cell viability was determined by either visual cell counting or MTT assays in 96-well plates. The MTT assays measure the ability of cells to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and is often used to measure cell proliferation (Mosmann, 1983). In this system, it correlates with cell viability as determined by trypan blue exclusion and a colony-forming assay (Davis and Maher, 1994). The assay medium contained 5% dialyzed FBS for the glutamate toxicity assay or horse serum for the assay of buthionine sulfoximine (BSO) toxicity (Li et al., 1997). Fresh preparations of BSO were used. For the MTT assay, cells were dissociated and seeded into 96-well microtiter plates at a density of 2.5 \times 10^5 or 5 \times 10^5 cells per well in 100 \mu l medium for HT22 cells and cortical neurons, respectively. The next day cells were treated with various reagents according to the experimental design. 20 h after the treatment, 10 \mu l of MTT solution (5 mg/ml) was added to each well and incubated for 3 h. 100 \mu l of solubilization solution (50% dimethylformamide, 20% SDS, pH 4.8) was then added to the wells, and the next day the absorbance at 570 nm was measured. Results are expressed relative to the indicated controls and were analyzed using a Mann-Whitney test.

### Glutathione, cGMP, and Ca\(^{2+}\) Measurements

For the glutathione, cGMP, and Ca\(^{2+}\) measurements to be described below, HT22 cells were seeded at 5 \times 10^5 cells/100 mm and the next day were treated according to the experimental design. Cells were then collected at indicated times for the above assays. Glutathione was determined by the recycling assay based on the reduction of 5,5-dithiobis(2-nitrobenzoic acid) with glutathione reductase and NADPH (Tietze, 1969). Sample preparation and assay procedures were described elsewhere (Li et al., 1997).

For cGMP determination, HT22 cells were treated with glutamate for various times, collected, and resuspended in 0.1 N HCl. After 1 h on ice, the samples were centrifuged in a microfuge. The supernatant was neutralized and assayed for cGMP content using an EIA cGMP assay kit from Amersham International (Buckinghamshire, England). The pellet was dissolved in 0.1 N NaOH, and protein content was determined using a commercial kit from Pierce Chemical Co. (Rockford, IL). cGMP content was calculated per milligram protein and presented relative to the controls.

The intracellular ionized calcium concentration was determined by flow cytometry using ratiometric analysis. HT22 cells were loaded with 1 \mu M Indo-1 at 37°C for 30 min in the presence of 0.005% Pluronic F-127 in DME containing 10% FCS. After incubation, cells were collected, washed, and resuspended in phenol red-free Hepes-buffered DME supplemented with 2% dialyzed FBS. Cells were allowed a 15-min recovery period to hydrolyze the ester bond before being analyzed with a FACStar® flow cytometer (Becton Dickinson, Mountain View, CA). The fluorescence light from the two emission peaks of Indo-1, 410 nm (violet) and 485 nm (green), was collected, and the ratio of violet to green, which is proportional to Ca\(^{2+}\) concentrations (Gryniewicz et al., 1985), was obtained. 10,000 viable cells were analyzed in each assay.

### Reagents

Tissue culture reagents were obtained from GIBCO BRL (Gaithersburg, MD). The fluorescent dye Indo-1 acetoxymethylster was obtained from Molecular Probes (Eugene, OR). LY83583, N\(^6\)-methyl-t-arginine, nitro- dipine, N\(^6\)-nitro-t-arginine, 7-nitroindazole, and tin protophorphor IX were from LC Laboratories (Woburn, MA). 8-(4-chlorophenylthio) guanosine-3',5'-cylic monophosphate (CPT-cGMP) was obtained from Biolog (La Jolla, CA). Other reagents, including L-buthionine sulfoximine, hydroxylamine, methylene blue, N-methyl-hydroxylamine, and N\(^4\)-monomethyl-ethyl-t-arginine methyl ester, were purchased from Sigma Chemical Co. (St. Louis, MO).

### Results

#### Inhibitors of sGC Prevent Glutamate-induced Neuronal Cell Death

The addition of glutamate to the hippocampal cell line HT22 causes a rapid depletion of GSH, which in turn activates 12-LOX, leading to a form of programmed cell death that is similar to but distinct from apoptosis (Tan, S., M. Wood, and P. Maher, manuscript submitted for publication). It has recently been shown that the products of 12-LOX enzymatic activity are required for oxidative glutamate toxicity (Li et al., 1997). One target for LOX metabolites is sGC (Snider et al., 1984; Brune and Ulrich, 1991). To determine if sGC is involved in glutamate toxicity, we first tested the effect of various inhibitors of sGC on the survival of HT22 cells after exposure to glutamate. HT22 cells were incubated with 5 mM glutamate in the presence of several concentrations of the inhibitors for 20 h. Cell viability was then determined by MTT reduction, a viability assay that correlates in this system with trypan blue exclusion and colony formation assays (Davis and Maher, 1994). Under these conditions, glutamate alone caused the complete lysis of cells as assayed by both MTT reduction (Fig. 1 A) and microscopic examination. Treatment of the
**Inhibitors of sGC Also Prevent Neuronal Cell Death Induced by Buthionine Sulfoximine**

Since the activation of 12-LOX has been experimentally linked to the reduced level of intracellular GSH (Li et al., 1997), it was asked if GSH depletion is also sufficient to activate sGC-mediated cell death. To determine if sGC is also involved in neuronal cell death associated with GSH depletion, the effect of sGC inhibitors on BSO toxicity was examined. BSO, a specific inhibitor of γ-glutamylcysteine synthetase, the rate-limiting enzyme in GSH synthesis, depletes intracellular GSH and causes cell death in a time- and dose-dependent manner in both HT22 cells and primary cortical neurons (Li et al., 1997). An overnight exposure of HT22 cells or primary cortical neurons to 50 μM BSO causes glutathione depletion (Fig. 2) and a dramatic loss of cell viability (Fig. 1, C and D). sGC inhibitors LY83583, methylene blue, and N-methyl-hydroxylamine all block BSO toxicity in a manner similar to that of glutamate, suggesting that sGC is directly linked to neuronal cell death caused by GSH depletion. There are, however, differences in the pharmacology of sGC inhibitors that prevent glutamate- and BSO-induced cell death. These differences may be due to the fact that at least the early events are different for these two models of cell death, because glutamate interferes with the cystine uptake while BSO directly inhibits GSH synthesis.

**sGC Inhibitors Do Not Affect the Ability of Glutamate or BSO to Deplete GSH**

To determine whether sGC inhibitors block the toxicity of glutamate and BSO by affecting their ability to deplete GSH, we compared intracellular glutathione levels in HT22 cells treated with these agents as a function of time. Fig. 2 shows that sGC inhibitor LY83583 did not block the depletion of GSH by either glutamate or BSO. None of the other sGC inhibitors blocked glutathione depletion either (data not shown). In the case of BSO treatment, the addition of LY83583 accelerated the depletion of GSH (Fig. 2).
This is probably due to the fact that LY83583 is also capable of inhibiting glutathione reductase (Luond et al., 1995) and therefore the regeneration of GSH from GSSG. These data, showing that sGC inhibitors prevent cell death but not GSH depletion, support the previous argument that GSH depletion caused by glutamate is not in itself sufficient to cause cell lysis (Murphy et al., 1989).

The Activation of sGC Occurs Near the Time of Cell Death

Since sGC inhibitors block toxicity, it is likely that the enzyme is activated to produce cGMP. To determine if there is an increase in the level of cGMP, intracellular cGMP levels of HT22 cells treated with 5 mM glutamate for various times were assayed. Fig. 3A shows that the level of cGMP starts to increase 8 h after glutamate treatment. Under the conditions used, cells also begin to die at ~8 h after the addition of glutamate. The increase in cGMP was prevented by treatment of cells with 1 µM LY83583 (data not shown). Therefore, sGC activation occurs near the time of cell death.

To independently determine when in the cytotoxicity pathway sGC is activated, HT22 cells were treated with glutamate, and at various times afterwards LY83583 was added, followed by a cell viability assay at 20 h. Alternatively, glutamate was added and then removed at various times after its initial addition to the cultures. LY83583 was able to inhibit glutamate toxicity even when applied up to 8 h into the cell death process (Fig. 3B). The protection conferred by LY83583 started declining at 8 h after addition of glutamate, which is the same time that glutamate was no longer required for cell death (Fig. 3B). After ~8 h of exposure to glutamate, HT22 cells gradually initiate cell lysis, which is complete after ~15 h. However, surviving cells can still be rescued by LY83583 even during the late period of cell death. For example, if 1 µM LY83583 is added to cultures when 73% of cells are dead, the remaining 27% of the cells survive when examined 24 h later. These observations suggest that the activation of sGC occurs at a point that is very close to cell lysis.

A Cell-permeable cGMP Analogue Potentiates Glutamate Toxicity

If cGMP is involved in the cell death pathway, then exogenous cGMP should potentiate glutamate toxicity. The ability of CPT-cGMP, a cell permeable and phosphodiesterase-resistant cGMP analogue, to potentiate glutamate-induced cell death was examined. Overnight exposure of HT22 cells to 2 mM glutamate led to a 35% decrease in cell viability (Fig. 4A). Coapplication of CPT-cGMP resulted in further decreases in cell viability in a dose-dependent manner. For example, 2 mM glutamate caused a 55% cell death, while 300 µM CPT-cGMP together with 2 mM glutamate caused nearly a 90% decrease in cell viability (Fig. 4A). At 500 µM, CPT-cGMP alone was not significantly toxic to HT22 cells. However, marked cell death was observed when these cells were treated with CPT-cGMP at concentrations over 1 mM (Fig. 4A, inset). Application of the less stable cGMP analogues, dibutyl-cGMP and 8-bromo-cGMP, did not result in cell death at concentrations up to 5 mM. The potentiation by CPT-cGMP was dependent on glutamate concentration (Fig. 4B) but was largely unaffected by the time when CPT-cGMP was added up to 12 h after glutamate addition (Fig. 4C). This result is consistent with the above data, suggesting that cGMP functions near the time when cells die.

cGMP-dependent Protein Kinase Is Not Involved in Glutamate Toxicity

Since the molecular targets of cGMP action include cGMP-gated ion channels and cGMP-dependent protein kinases (PKG) and phosphodiesterases (Goy, 1991), it is expected...
that the inhibition of sGC will affect the activity of these targets. If they are in the cell death pathway, then their inhibition will also block cell death. We examined whether PKG is involved in cell death using KT5823, a highly selective inhibitor of PKG with an IC₅₀ of 0.23 μM (Ito and Karachot, 1990). Fig. 4 D shows that KT5823 was unable to block cell death even at concentration up to 100 μM, suggesting that PKG is not involved in the cell death caused by GSH depletion.

**sGC/cGMP Regulates the Elevation of Ca²⁺**

Another potential target for cGMP is the activation of Ca²⁺ channels, which play an important role in oxidative glutamate toxicity. The removal of extracellular Ca²⁺ or the addition of the Ca²⁺ channel blocker CoCl₂ inhibits glutamate toxicity (Murphy et al., 1989; Davis and Maher, 1994), and glutamate induces the elevation of intracellular Ca²⁺ (Li et al., 1997). These data suggest that the increased intracellular Ca²⁺ is derived from extracellular medium rather than intracellular calcium pools. Initial experiments were done to identify the specific channels involved in cell death. The influx of Ca²⁺ may be via voltage-dependent channels, cGMP-regulated channels, Na⁺/Ca²⁺ exchangers, or nonspecific membrane leakage. Membrane leakage is unlikely since CoCl₂ prevents cell death (Murphy et al., 1989; Davis and Maher, 1994). Replacement of Na⁺ by Li⁺ did not prevent cell death (data not shown), which excludes the involvement of a Na⁺/Ca²⁺ exchanger. Various inhibitors of Ca²⁺ channels were then tested for their ability to prevent cell death induced by glutamate. These included the L-type channel inhibitors nifedipine, diltiazem, verapamil, and nimodipine, and the N-type channel inhibitor ω-conotoxin GVIA. None of these was protective at concentrations up to toxic doses (data not shown). Therefore, it is unlikely that L- or N-type channels or Na⁺/Ca²⁺ exchangers are involved in the cell death. The involvement of a cGMP-regulated Ca²⁺ channel can not be tested biochemically because no specific inhibitors exist (Finn et al., 1996). Although pimozide and D600 can block these channels in some cases (Finn et al., 1996), they were not effective in preventing cell death caused by glutathione depletion (data not shown). If, however, it was possible to demonstrate that CPT-cGMP potentiates the increase in intracellular Ca²⁺ in a manner similar to toxicity, then it is likely that cGMP-gated Ca²⁺ channels are opened. Fig. 5 A shows that CPT-cGMP indeed potentiates Ca²⁺ elevation. CPT-cGMP greatly enhances the level of intracellular Ca²⁺ in cells exposed to marginally toxic (2 mM) concentrations of glutamate.

It was then asked if there is a temporal relationship between sGC activation and Ca²⁺ elevation. Cells were exposed to LY83583 and CoCl₂ at various times after the addition of glutamate, and cell viability was assayed 20 h after the initial addition of glutamate. Fig. 3 B shows that LY83583 and CoCl₂ function in parallel in terms of maximal protection. Both agents maintain maximal protection up to 8 h past the addition of glutamate, after which cell lysis occurs gradually over the next few hours. Therefore, both sGC activation and Ca²⁺ elevation occur near the time of cell death.

If sGC activation is required for Ca²⁺ elevation, then its inhibition should block the glutamate-induced accumulation of intracellular Ca²⁺. We therefore investigated the effect of sGC inhibitors on the intracellular Ca²⁺ profile during the glutamate treatment. The intracellular Ca²⁺ response to glutamate was first determined by flow cytometry using ratiometric analysis. Intracellular Ca²⁺ content is proportional to the ratio of fluorescence intensities from the two emission peaks of the Ca²⁺-bound and unbound Indo-1 dye (Gryniewicz et al., 1985) and was arbitrarily defined as low (the ratio is less than 50), medium (the ratio is between 50 and 100), and high (the ratio is between 100 and 250). In control cells, only about 2% of the cells had medium to high levels of Ca²⁺, with very few cells containing the high Ca²⁺ level (Fig. 5 B; percentiles of cells in each class were derived from the scatter plots shown). Treatment with 5 mM glutamate for 12 h resulted in a dra-
matic increase in the percentages of cells containing medium to high levels of Ca\(^{2+}\) (Fig. 5B). 12 h after the exposure of cells to glutamate, \(\sim 20\%\) of the cells were alive. Of these, only 10–20% had medium to high levels of Ca\(^{2+}\) (Fig. 5B and data not shown). The fact that the high Ca\(^{2+}\)-containing cells accounted for such a minority of cells at the time when cells were dying rapidly again suggests that Ca\(^{2+}\) elevation occurs very close to cell lysis. The rise of Ca\(^{2+}\) level during the incubation of cells with glutamate was prevented by sGC inhibitors, while these inhibitors had no effect on control cells (Fig. 5B and data not shown). Therefore, sGC inhibitors block glutamate-induced Ca\(^{2+}\) elevation in HT22 hippocampal cells. It was not, however, possible to do these experiments in primary cultures because of unstable baselines due to spontaneous cell death in the short term cultures.

**sGC-mediated Glutamate Toxicity Is through a Mechanism Independent of NO and Carbon Monoxide**

Since several of the inhibitors used above do not inhibit the basal activity of sGC (see Discussion), the activation of sGC is probably not due to its transcriptional upregulation, but rather due to the induction of sGC activators. NO and carbon monoxide are activators of sGC. If they are responsible for the sGC activation observed in the cell death, these activators should also be a target for blocking glutamate toxicity. Therefore, we determined if the inhibition of their synthesis is able to block glutamate toxicity.

Various inhibitors of NO synthase (NOS) were tested at several concentrations. N\(^{G}\)-monomethyl-L-arginine methyl ester, a specific inhibitor of NOS, did not attenuate glutamate or BSO toxicity in HT22 cells or in primary cortical neurons (Fig. 1). Other NOS inhibitors, including 7-nitroindazole (a specific inhibitor for neuronal NOS-1) (up to 0.25 mM), N\(^{G}\)-methyl-L-arginine (up to 1.0 mM), and N\(^{G}\)-nitro-L-arginine (up to 1.0 mM), also did not show any protective effect on glutamate toxicity (data not shown). Similarly, tin protoporphyrin, an inhibitor of heme oxygenase and carbon monoxide production, only had minimal effects on glutamate toxicity at near toxic concentrations (data not shown).

**Discussion**

The data presented above indicate a requirement for sGC/cGMP in nerve cell death caused by GSH depletion. First, three structurally unrelated inhibitors of sGC all block glutamate or BSO toxicity (Fig. 1) at concentrations that inhibit sGC activity or cause cGMP reduction in vitro. Second, glutamate induces an increased production of cGMP (Fig. 3). Third, a membrane-permeable cGMP analogue, CPT-cGMP, potentiates glutamate toxicity (Fig. 4). Lastly, sGC inhibitors block the glutamate-induced increase in intracellular Ca\(^{2+}\) required for toxicity (Fig. 5). Therefore, the generation of cGMP and the subsequent activation of a Ca\(^{2+}\) channel are events occurring in GSH depletion–induced nerve cell death.

Of the sGC inhibitors used here, hydroxylamine and its analogue, N-methyl-hydroxylamine, competitively bind to the heme group associated with sGC, inhibiting sGC acti-
vation (Deguchi et al., 1978). However, the other sGC inhibitor, LY83583, appears to be relatively specific for sGC in various studies (Schmidt et al., 1985; Mulsch et al., 1988). The mechanism by which LY83583 inhibits sGC is not completely known, but the inhibitory effect appears to require the intracellular reduction of the compound (Mulsch et al., 1988). However, LY83583 inhibits glutathione reductase, thereby causing an increase in the level of GSSG (Luond et al., 1993). This effect may contribute to the ability of LY83583 to inhibit sGC since high levels of GSSG can cause an irreversible loss of sGC activity (Graff et al., 1978; Frey et al., 1980; Wu et al., 1992; Mayer et al., 1995).

Both cytoprotective and cytotoxic roles for cGMP have been described in the nervous system. For example, cGMP prevents motor neuron degeneration (Weill and Greene, 1984) and protects against excitatory amino acid–induced damage in cerebellar slices (Garthwaite and Garthwaite, 1988). cGMP may also be involved in the excitoprotective activities of the secreted forms of β-amyloid precursor in hippocampal neurons (Barger et al., 1995) and mediate the survival effect of NO on trophic factor-deprived PC12 cells and sympathetic neurons (Farinelli et al., 1996).

In summary, it is shown in a clonal cell line and primary cortical neurons that the elevation of cGMP, which likely results from sGC activation, is a critical step in the nerve cell death caused by GSH depletion. The activation of sGC is via an NO-independent mechanism. cGMP then induces Ca2+ influx, which immediately precedes cell death. If the observation can be extended to in vivo situations, then intervention of the sGC/cGMP pathway could be beneficial to individuals suffering from PD or other pathologies associated with GSH depletion.

We would like to thank Drs. H. Kimura, Y. Liu, Y. Sagara, and S. Tan for their helpful suggestions during the course of this study and critically reading the manuscript, and Dr. D. Chambers for expert assistance in the FACS® analysis.

This work was supported by National Institutes of Health grants R01 NS09658 (to D. Schubert) and 5POI-NS28121 (to D. Schubert and P. Maher). Y. Li was supported in part by a postdoctoral fellowship from the de Hoffmann Foundation and a National Research Service Award F32 AG 05769-01.

Received for publication 28 July 1997 and in revised form 25 September 1997.

References


Herzenberg, L.A., S.C. De Rosa, J.G. Dubs, M. Roederer, M.T. Anderson,