Increased Production of Extracellular Glutamate by the Mitochondrial Glutaminase following Neuronal Death*

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Robert Newcomb‡§, Xiaoyun Sun†, Lynn Taylor‡, Norman Curthoys¶, and Rona G. Giffard¶

From ‡Neurex Corporation, Menlo Park, California 94025, the ¶Department of Anesthesiology, Stanford University Medical School, Stanford, California 94305, and the §Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, Colorado 80523

Elevated extracellular concentrations of the excitatory transmitter glutamate are an important cause of neuronal death in a variety of disorders of the nervous system. The concentrations and rates of clearance and production of extracellular glutamate were measured in the medium of primary cultures from mouse neocortex containing neurons, astrocytes, or both cell types. Measurements were performed in the presence and absence of 2 mM glutamine with or without neuronal injury caused by 5-h exposure to hypoxia or 500 μM N-methyl-D-aspartate or a freeze-thaw cycle. High rates of glutamate generation (0.5–0.8 μM/min in the 0.4-ml culture well) occurred if neurons were both damaged and exposed to glutamine. Intact neurons or glia exposed to glutamine generated only small amounts of glutamate (0.05 μM/min). Glutamate generation by damaged neurons was dependent on the presence of glutamine, activated by phosphate, and inhibited by 6-diazo-5-oxo-L-norleucine and p-chloromercuriphenylsulfonic acid (pCMPS), strongly implicating the mitochondrial glutaminase. Following 5-h exposure to 500 μM N-methyl-D-aspartate, the glutaminase was localized to fragments of damaged neurons and was accessible to inhibition by the membrane-impermeant pCMPS. The glutaminase activity from damaged neurons is sufficient to account for the neurotoxic concentrations of glutamate in hypoxic mixed neuronal-glial cultures exposed to 2 mM glutamine. Finally, pCMPS is neuroprotective and also prevents the increased rate of generation of glutamate observed in neuronal cultures after prolonged exposure to glutamine. The cumulative data indicate the following: 1) excitotoxic neuronal death activates the hydrolysis of extracellular glutamate by the mitochondrial glutaminase, and 2) the glutaminase in damaged neurons is sufficient to cause neuronal death in in vitro models of neuronal injury.

Excess extracellular concentrations of the neurotransmitter glutamate contribute to neuronal damage in such conditions as cardiac arrest, stroke, and seizures (1–4). Although this is well established, the sources of glutamate responsible for pathological effects are incompletely described. In the early phase of hypoxia (minutes), transmitter glutamate is released in vivo (5, 6). However, there is increasing evidence for a delayed component to glutamate-mediated neuronal damage in brain ischemia. Glutamate receptor antagonists are neuroprotective even if given hours after reversible focal ischemia (7, 8) as well as global ischemia (9). This is consistent with the large delayed increase in extracellular glutamate observed after both reversible and permanent focal occlusion of the middle cerebral artery (10, 11). The cellular sources responsible for this delayed glutamate production remain to be characterized.

The mitochondrial glutaminase, which catalyzes the enzymatic conversion of glutamine to glutamate, is both an important contributor to transmitter pools of glutamate (12, 13) and the predominant glutamine-utilizing enzyme of the brain (14). In brain, the enzyme is present in higher amounts in neurons than in glia (15, 16). The glutaminase is highly compartmentalized, being localized to the inner mitochondrial membrane (17–19). Glutamine is the amino acid in highest concentration in the brain extracellular fluid (20) and provides an abundant substrate for the enzyme in vivo.

Several previous studies have suggested a role for glutamine and glutamate hydrolysis in excitotoxicity in cell culture. When neurons were cultured with reduced numbers of astrocytes, glutamine was found to be toxic coincident with increased extracellular glutamate. Both glutamine toxicity and glutamate generation were blocked by an NMDA receptor antagonist (21). The medium of damaged neuronal cultures (22) was found to possess glutamine hydrolysis activity, which is particulate and blocked by the glutamine affinity-labeling reagent 6-diazo-5-oxo-l-norleucine (23). Finally, glutamine potentiated hypoxic neuronal death in mixed glial/neuronal cultures (24), and toxicity was again inhibited by glutamate antagonists.

The above studies provide reasons to form the hypothesis that the mitochondrial glutaminase may contribute to glutamate generation and neuronal injury in vitro. However, further information is required to evaluate the relative importance of the enzyme in these processes. Likewise, the cellular location of the enzyme that contributes most significantly to glutamate production has not yet been determined in neuronal injury paradigms. In this study, experiments are performed that test the hypothesis that 1) The production of glutamate from glutamine following neuronal death is significant by comparison with other cellular sources of glutamate in primary cultures of mouse cortex, and 2) The mitochondrial glutaminase is the enzyme responsible for this activity. Additional experiments address the cellular compartment of active glutaminase after neuronal death, as well as the role of the enzyme in in vitro neuronal injury paradigms.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Eagle’s minimal essential medium, containing 1 mM phosphate but not bicarbonate or glutamine, was purchased from Life Sciences.

§ To whom correspondence should be addressed: Neurex Corp., 3760 Haven Ave., Menlo Park, CA, 94025. Tel.: 415-853-1500; Fax: 415-853-1538.

‡ To whom correspondence should be addressed: Neurex Corp., 3760 Haven Ave., Menlo Park, CA, 94025. Tel.: 415-853-1500; Fax: 415-853-1538.

¶ The abbreviations used in the text are: NMDA, N-methyl-D-aspartate; DON, 6-diazo-5-oxo-l-norleucine; pCMPS, p-chloromercuriphenylsulfonic acid; LDH, lactate dehydrogenase; HPLC, high pressure liquid chromatography.
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Technologies, Inc. Horse and fetal bovine serum were obtained from Hyclone Laboratory Inc. All other tissue culture reagents were from Sigma.

Primary cultures of cerebral cortical cells were prepared from Swiss Webster mice (Simonsen Laboratories). All animal use procedures were in agreement with the National Research Council’s Guide for the Care and Use of Laboratory Animals (41); all protocols were approved by the Stanford University Institutional Animal Care and Use Committee.

Cultures of pure cortical neurons and mixed cultures of neurons and astrocytes were prepared by modifications of the procedures of Dichter (25) and Choi et al. (26) as described previously (27). Astrocytes were cultured from cortical cell suspensions from 1–2-day-old Swiss mouse pups. Mixed cultures of neurons and astrocytes were obtained by plating cortical neurons from embryonic day 15 or 16 mice on confluent astrocyte monolayers. Pure neuronal cultures were made from the same embryonic cortical cell suspensions; astrocyte growth was inhibited by adding cytosine arabinoside and decreasing serum in the medium. For survival, neuronal cultures were fed with glial conditioned medium. Neuronal cultures were used for experiments at 11–15 days in vitro, and mixed cultures were used at 13–15 days after plating the neurons.

Hypoxia—Hypoxia was carried out at 37 °C in an anoxic chamber (<0.2% oxygen; Forma Scientific). Before each experiment, the medium was preequilibrated with an anoxic gas mixture containing 5% CO2, 85% N2, and 10% H2 and incubated at 37 °C. Cells were deprived of oxygen by triple exchange (approximately 1:1000 dilution) of the culture medium with an oxygen-free medium containing 5% CO2, 85% N2, and 10% H2, or without the addition of glutamate, to the indicated concentration. The oxygen tension in the anoxic chamber was monitored using an oxygen electrode (model MI-730; Microelectrodes Inc.). After 5 or 7 h of hypoxia, the cultures were returned to the normoxic incubator.

Glutamine—Glutamine was purchased from Bachem or from Life Technologies, Inc. To avoid possible complications from glutamate toxicity, glutamate was removed from the Bachem glutamine by passage of the culture medium over a 3 × 100-cm column of diethylaminoethyl-Sepharose (Pharmacia Biotech Inc.) at pH 5.0. The resulting glutamine stock contained less than 0.2 μmol glutamate per μl. Stock contained less than 0.2 μmol glutamate per μl and was stored at concentrations of 50 mM or greater at —80 °C. The resulting glutamate was rapidly removed. Glutamine solutions were stored previously (28), and a gradient of 0–40% methanol in 1.25 mM sodium phosphate, pH 7.2 or 80 °C. Chromatography was over a 4.5 × 250-mm Phenomenex Primesphere “HC” octadecylsilica column (dp 5 μm) using a gradient of 0–35% methanol in 15 mM sodium phosphate, pH 6.2 (0.13% tetrahydrofuran), over 65 min at 1 ml/min. It was shown for each of the experimental conditions (i.e., glial and control damaged neuronal cultures) that both analytical systems gave results for glutamate and glutamine measurements that were, within error, identical.

Western Blots—Neuronal cultures were exposed to 500 μM NMDA or control conditions for 5 h, at which time the medium was removed from the cellular layer and both were stored at —80 °C. The media from single wells (300 μl) were centrifuged at 100,000 × g for 40 min. The resulting pellets were resuspended in 40 μl of sample loading buffer and subjected to SDS-polyacrylamide gel electrophoresis. The cells from a single well were resuspended in 100 μl of sample loading buffer, and 20 μl were used. Gel electrophoresis and Western blotting was performed as described (31) except that the immune complex was detected using the enhanced chemiluminescence system (Amersham). The relative intensities of the resulting bands were determined by densitometric analysis using a Microscan 2000 densitometer.

Measurement of Neuronal Death—Neuronal death was measured by assay of lactate dehydrogenase (LDH) activity released into the culture medium (32) and was also independently assessed by light microscopy for all experiments. The amount of LDH released was expressed as the value of LDH activity in the medium to the amount released from the same cultures after 24-h exposure to 500 μM NMDA. Neuronal cultures, neuronal LDH release was measured after freeze-thaw. In the neuronal cultures, LDH release by the NMDA exposure method is normally about 85% of that obtained by freeze-thaw. In the neuronal cultures, LDH release by the NMDA exposure method is normally about 85% of that obtained by freeze-thaw.

Expression of Glutamate Amounts and Production Rates—Glutamate was measured in units of pmol/μl of culture medium, or μM. In the absence of cellular uptake (i.e. in damaged neuronal cultures), the rate of glutamate production was obtained directly from the measurements of the amounts of glutamate in culture medium at various times.

Experiments on the clearance of glutamate from culture medium showed first order kinetics in intact glial, neuronal, and mixed cultures. This allows expression of medium glutamate concentrations according to the equation (see Ref. 33), d(Glu)/dt = S − k(Glu), where [Glu], in units of μM, is the concentration of glutamate in the 0.4 ml of medium in a culture well, S (in μM/min) is the rate of addition of glutamate to the culture medium from cellular sources, and k (in min −1) is the empirically determined rate constant for clearance. Pseudo-first order kinetics are expected for the clearance of glutamate at concentrations significantly below the Kc for cellular uptake (about 45 μM; Refs. 34 and 35). Although the value of k is proportional to cell (carrier) density, little variation is expected in cultures containing glia, since glial cultures are confluent.

In cultures where glutamate concentrations are (within error) constant, d(Glu)/dt is close to 0, and cellular production of medium glutamate is balanced by its removal, such that glutamate production can be calculated from the steady state equation, S = k[Glu].

Calculations were performed using the slopes of the regression lines that describe the rate constants for glutamate removal (Fig. 1) and the mean values for culture medium glutamate concentrations. Because of the relevance to data on glutamate toxicity, rates of glutamate production are given with units of concentration over time. For comparative purposes, glutamate production rates are also given in the text as the pmol of glutamate produced per min in each culture. These values were obtained after multiplication by the volume of culture medium.

Measures of culture medium and total cellular lactate dehydrogenase activity (above) were performed for all experiments and were used to verify that systematic variations in neuronal density did not affect the results; essentially identical results were obtained for data in Tables I–III if normalized to mean total culture LDH release. The mean of the total neuronal LDH was 259 units with pure neuronal cultures (n = 116) and 776 units with the mixed cultures (n = 99). The coefficient of variation of the LDH measurements was 0.38 in both culture systems.

Statistical Procedures—All data are expressed as the mean ± S.E. Each value represents data from one culture well. Where not given in the text, values for n are given in the tables. Unless otherwise indicated, an n value of 4–6 culture wells was used for each condition in each experiment, with repetitions carried out using at least three independent dissections for each experiment. Statistical comparisons were performed by the standard t test for the comparison of the means of two populations.

RESULTS

Glutamate Production by Neuronal Death—Initial experiments were performed to measure the rates of glutamate uptake and production in intact and damaged cultures of pure neurons. The steady state concentration of glutamate in the medium of neuronal cultures was 0.35 ± 0.05 μM. In preliminary experiments, neuronal cultures were subjected to a freeze-thaw cycle to completely lyse the cells. The medium glutamate concentration was increased to 4.5 ± 0.5 μM (n = 8). Thus, lysis of the neuronal cultures released approximately 1 μmol of glutamate into the 0.4 ml of culture medium. Much of this presumably represents the large amount of glutamate that is stored as a neurotransmitter. Subsequently, the lysed cultures were incubated with 50 μM glutamate. In all cases (n = 8), the concentration of glutamate in the medium after 2 h was altered by less than 5%. The stability of glutamate in the medium of
damaged cultures indicated that the enzymatic degradation of glutamate in the culture medium was not significant. Thus, the rates of glutamate clearance and production could be measured directly.

As shown in Fig. 1, the clearance of glutamate from the medium of intact neuronal cultures could be described by a first order process with an apparent kinetic constant of 0.012 min⁻¹. Thus, the intact neuronal cultures exhibit a relatively slow rate of glutamate uptake. Fig. 2 illustrates the time course of appearance of glutamate in the cell culture medium of intact pure neuronal cultures or of cultures lysed by a freeze-thaw cycle, both in the presence and absence of 2 mM glutamine. In the absence of added glutamine, glutamate concentrations in intact neuronal cultures are maintained at below 1 μM. Under these conditions, the rate of glutamate production calculated from the steady state equation is 0.0046 μM/min (1.8 pmol/min). Thus, the production of glutamate by neurons cultured in the absence of added glutamine is also very slow and is small in comparison with the total cellular content of glutamate.

If 2 mM glutamine is added to intact neuronal cultures, the rate at which glutamate accumulates in the culture medium increases with time (Fig. 2). Since the clearance of medium glutamate by neuronal cultures is extremely slow, one estimate of the rate of glutamate production by neurons exposed to glutamine can be obtained from the initial rate of glutamate accumulation. Using the difference in glutamate concentrations at 1 and 15 min after the glutamine addition, a value of 0.032 ± 0.006 μM/min (12.8 pmol/min) was obtained. Since glutamate exposure itself eventually causes neuronal death and high rates of glutamine hydrolysis, this estimate of glutamate production should be viewed as an upper limit. However, this value is similar to that obtained by comparing glutamate production by glial and mixed neuronal-glial cultures exposed to glutamine (see below).

In contrast, the rate of glutamate production by damaged cultures of pure neurons exposed to glutamine is very rapid and is linear with time. In the absence of added glutamine (i.e. in the 0.15 mM glutamine manufactured by the neuronal cultures), glutamate production into the medium of cultures lysed by freezing and thawing occurred at a rate of 0.035 ± 0.006 μM/min (14 pmol/min). However, the addition of 2 mM glutamine to the medium of freeze-thawed cultures resulted in the much higher rate of glutamate production of 0.79 ± 0.08 μM/min (316 pmol/min). Similarly, neuronal cultures that were subjected to “excitotoxic” injury by treatment for 5 h with 500 μM of the glutamate receptor agonist NMDA and then incubated with 2 mM glutamate produced glutamate in the culture medium at a rate of 0.54 ± 0.09 μM/min (216 pmol/min). Thus, damaged neurons incubated in 2 mM glutamate produce within 6–8 min an amount equal to that released by freeze-thawing. The various rates and rate constants measured with neuronal cultures are summarized in Table I.

Glutamate Production by Glial Cultures—When incubated with 15 μM glutamate, pure cultures of intact astrocytes exhibit a rapid uptake of glutamate that is described by a first order rate constant of 0.083 min⁻¹ (data not shown). This is consistent with previous reports (34, 35) and shows that uptake of glutamate by the glial cultures is about 7 times faster than that of the neuronal cultures. The glutamate concentration of glial cultures was maintained below 1 μM when cells were maintained in the absence or presence of 2 mM added glutamine or when subjected to hypoxic conditions (see below). The medium glutamate concentration remained unchanged between 7 and 24 h after medium change and was 0.14 ± 0.04 μM and 0.36 ± 0.06 μM in medium containing 0.15 mM or 2 mM glutamine, respectively. Based on these steady state concentrations and the measured rate of glutamate uptake, the rates of glutamate production in the absence and presence of added glutamate were calculated to be 0.012 μM/min (4.8 pmol/min) and 0.030 μM/min (12 pmol/min), respectively. Thus, the calculated rates of glutamate production by glial cultures are only slightly affected by the addition of glutamine and are similar to those observed in intact neurons cultured in the absence of added glutamine. The various rates and rate constants measured for cultures of pure glial cells are summarized in Table II.
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The glutamate concentration was measured 5 h after adding fresh medium and was constant within experimental error for at least 9 h. The glutamate production by neurons in the absence of added glutamine was calculated from the steady state equation. Following the addition of glutamine, the rate of glutamate production was estimated to be 0.0046 μmol/min. The latter method was used to assess glutamate production in damaged neurons. n values are given in parentheses.

| Glutamate uptake (k) | Glutamate concentration | Glutamate production (S) |
|----------------------|-------------------------|-------------------------|
| Intact neurons       |                         |                         |
| 0.005 mM Gln         | 0.012                   | 0.038 ± 0.05 (8)        |
| 2 mM Gln             | Increases exponentially  | 0.030±0.006 (8)        |
| Damaged neurons      |                         |                         |
| Freeze-thaw          |                         |                         |
| 0.15 mM Gln          | Increases linearily      | 0.035 ± 0.006 (8)      |
| 2 mM Gln             | Increases linearily      | 0.79 ± 0.08 (8)        |
| NMDA Treated         |                         |                         |
| 0.15 mM Gln          | Increases linearily      | 0.020 ± 0.013 (8)      |
| 2 mM Gln             | Increases linearily      | 0.54 ± 0.09 (8)        |

TABLE II
Kinetic constants for glutamate uptake and production in pure glial and mixed glial-neuronal cultures

Rate constants for clearance of glutamate from cell culture medium were determined as described in the legend to Fig. 1. The addition of 2 mM glutamine had, within error, no effect on the measured rate of glutamate uptake from mixed glial-neuronal cultures (n = 4 cultures wells). Likewise, 5 h of hypoxia had no effect on glutamate uptake in mixed cultures (n = 8 cultures wells). Steady state glutamate concentrations were measured at 5 h (mixed cultures) or 7 h (glial cultures) after medium change and were constant, within error, for at least 9 h. Rates of glutamate production were calculated from the steady state equation (see "Experimental Procedures"). n values are given in parentheses.

| Culture          | Glutamate uptake (k) | Glutamate concentration | Glutamate production (S) |
|------------------|----------------------|-------------------------|-------------------------|
| Glial            |                      |                         |                         |
| 0.15 mM Gln      | 0.083                | 0.14 ± 0.04 (10)        | 0.012                   |
| 2 mM Gln         | 0.36 ± 0.06 (10)     | 0.03                    |
| Glial-neuronal   |                      |                         |                         |
| 0.15 mM Gln      | 0.072                | 0.37 ± 0.06 (25)        | 0.027                   |
| 2 mM Gln         | 0.83 ± 0.47 (20)     | 0.06                    |

Glutamate Production by Mixed Glial-Neuronal Cultures—Added glutamate (15 μM) was rapidly cleared from the medium of the mixed glial-neuronal cultures (Fig. 1). The uptake again fit to a first order process and occurred with a rate constant of 0.072 min⁻¹. The observed rate constant suggests that the glial cells constitute the primary site of glutamate uptake in the mixed cultures. When the intact mixed cultures were maintained in the absence or presence of added 2 mM glutamine, they also maintained steady state concentrations of glutamate below 1 μM (0.37 ± 0.06 μM and 0.83 ± 0.47 μM, respectively). Thus, from the steady state equation, the rates of glutamate production were estimated to be 0.027 μmol/min (11 pmol/min) in the absence of added glutamine and 0.060 μmol/min (24 pmol/min) in the presence of 2 mM glutamine. The various kinetic constants measured for intact glial-neuronal cultures are summarized in Table II.

Glutamate production by intact neurons exposed to glutamine can be estimated from the difference in the steady state rates of glutamate production by glial and mixed glial-neuronal cultures exposed to glutamine. This value of 0.030 μmol/min (12 pmol/min) agrees well with the initial rate of production of glutamate by pure neuronal cultures exposed to glutamine (above).
The effects of the thiol-reactive and membrane-impermeant mercurial compound p-chloromercuriphenylsulfonate were next determined. This reagent inhibits the glutaminase in permeabilized but not intact renal mitochondria (17). Fig. 4 illustrates results of an experiment in which pure neuronal cultures were first either incubated under control conditions or were damaged by exposure to NMDA. Following this, 2 mM glutamine was added to both sets of cultures, and the effects of 30 μM pCMPS on the resulting generation of glutamate were then assayed. As illustrated for a shorter time period in Fig. 2, the exposure of healthy neurons to 2 mM glutamine resulted in steadily increasing rates of glutamate generation (Fig. 4, upper panel, curve 1). This delayed hydrolysis of glutamine was prevented by the inclusion of 30 μM pCMPS in the culture medium (curve 2). NMDA treatment of neurons resulted in rapid hydrolysis of added glutamine, and this was almost completely inhibited by 30 μM pCMPS (curves 3 and 4, respectively). When added at 600 μM, pCMPS completely abolished glutamate generation by NMDA-damaged neurons exposed to cell culture medium and glutamine (not shown).

Evidence for Involvement of the Glutaminase in Neurotoxicity—In addition to blocking the gradual increase in glutamate hydrolysis in neuronal cultures exposed to glutamine (Fig. 4, upper panel), 30 μM pCMPS also prevented the release of LDH that is caused by glutamine exposure (Fig. 4, lower panel). The neuroprotection by pCMPS in this protocol was verified by morphological assessment, which showed that neurons retained their normal appearance after 24 h in the presence of 30 μM pCMPS and 2 mM glutamine but were severely damaged if exposed to 2 mM glutamine alone (data not shown).

The neuroprotection observed with pCMPS, together with its inhibition of glutamate generation, strongly suggests that the glutaminase contributes directly to neuronal death in neuronal cultures exposed to glutamine. Problems with toxicity prevented a direct examination of the effects of DON and pCMPS in the standard model of hypoxic neuronal injury in mixed neuronal-glial cultures (26, 27). However, it was possible to investigate the relationship between extracellular glutamate concentrations, glutamine exposure, and neuronal death in mixed neuronal-glial cultures exposed to combinations of hypoxia and glutamine. Data on glutamate and glutamine concentrations in the medium of mixed cultures exposed to combinations of glutamine and hypoxia are summarized in Table IV. Data on glial cultures are presented as a control to confirm the neuronal origin of increased glutamate production caused by combined glutamine and hypoxia.

The exposure of mixed neuronal-glial cultures to the combination of glutamine and hypoxia produced a medium glutamate concentration of 3 μM (Table IV), which was significantly greater than that produced by hypoxia alone (0.3 μM, Table IV) or glutamine alone (0.8 μM, Table II) (p < 4 × 10⁻⁴ for both comparisons). This increase in medium glutamate was associated with 42.2 ± 3.1% (n = 23) of total LDH release 24 h after the beginning of hypoxia, or 16% over that produced with hypoxia alone and 25% over that produced by glutamate alone (p < 2.5 × 10⁻⁴ for both comparisons). Exposure to 500 μM NMDA for 5 h followed by 2 mM glutamine resulted in a glutamate concentration of 7.0 ± 0.6 μM (n = 16) in the medium at 4 h after glutamine addition and 84 ± 12% (n = 24) of total LDH release at 24 h. Values for LDH release were also measured immediately after hypoxia or NMDA treatment and were uniformly 60–70% of those measured at 24 h (data not shown).

We conclude that increased glutamate production in this model

| Condition | Glu μM/min |
|-----------|------------|
| Saline    | 0.011 ± 0.004 (7) |
| 20 mM phosphate | 0.011 ± 0.004 (8) |
| 2 mM Glu, 2 mM Gln | 0.49 ± 0.04 (8) |
| 20 mM phosphate, 2 mM Gln | 3.0 ± 0.81 (8) |
| 2 mM Glu, 2 mM DON | 0.16 ± 0.02 (7) |
| 2 mM Glu, 20 mM phosphate, 2 mM DON | 0.30 ± 0.02 (8) |

Table III

Glutamate generation by neuronal cultures after 500 μM NMDA: effects of phosphate and DON

Glutamate generation by neuronal cultures after 500 μM NMDA, followed by replacement of medium with saline (116 mM sodium chloride, 5.4 mM potassium chloride, 0.8 mM magnesium sulfate, 1.8 mM calcium chloride, 14.7 mM sodium bicarbonate, 10 mM Hepes, pH 7.4), containing sodium phosphate (pH 7.4), glutamine, and DON as indicated. Glutamate production was linear with time for the various conditions, and the data are the means and S.E. for the rates in individual culture wells (two independent experiments, with four culture wells for each condition). n values are given in parentheses.
of hypoxic neuronal injury is proportional to the amount of neuronal death and dependent on the presence of glutamine, properties that implicate the glutaminase.

Consistent with previous reports (38, 39) hypoxia (5 h, n = 8 culture wells) and glutamine (2 mM, n = 4 culture wells) had no effect on the clearance of glutamate from mixed cultures (data not shown). These results indicate that altered uptake is not responsible for glutamine toxicity in hypoxic mixed cultures.

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The experimental condition and time of sampling are given in the left column. Zero time is the time immediately after medium change and the beginning of hypoxia (5 h for mixed cultures and 7 h for glial cultures).

| Condition     | Time | Glu | Gln  |
|---------------|------|-----|------|
| Mixed Cultures|      |     |      |
| Hypoxia       | 5 h  | 0.3 ± 0.04 (22) | 0.06 ± 0.005 (4) |
| Hypoxia, 2 mM Gln | 5 h  | 3.1 ± 0.28 (21) | 2.3 ± 0.1 (4) |
| Glial Cultures |      |     |      |
| Hypoxia       | 7 h  | 0.11 ± 0.09 (10) | 0.06 ± 0.007 (6) |
| Hypoxia, 2 mM Gln | 7 h  | 0.39 ± 0.09 (10) | 2.0 ± 0.16 (6) |

**FIG. 5. Effect of NMDA on the localization of glutamine hydrolysis and glutaminase immunoreactivity: comparison to lactate dehydrogenase.** Pure neuronal cultures were incubated in culture medium with or without 500 μM NMDA for 5 h. The upper panel compares the localization of glutaminase and lactate dehydrogenase activity in damaged cells versus cell culture medium after the 5-h NMDA exposure. For localization of the activity of the glutaminase, medium was removed from cells, and fresh medium was then added to the remaining cell layer. Glutamine (2 mM) was added to both cells and the isolated medium, followed by removal of aliquots at various times for determination of medium glutamate. At the end of the experiment, both the cell and medium samples were subjected to freeze-thaw, and LDH activity was measured. Data are for 12 culture wells from one experiment. Essentially identical data were obtained in a duplicate experiment. The lower panel illustrates immunological localization and characterization of the enzyme in control and damaged neuronal cultures. Aliquots of cellular protein and of the particulate material recovered in the culture media were subjected to SDS-gel electrophoresis and Western blot analysis. Lanes 1 and 6 contain 10 μg of a crude rat brain homogenate. Lanes 2 and 3 contain 1/5 of the total protein recovered from the cellular layer of control or NMDA-treated cultures, respectively. Lanes 4 and 5 contain the total particulate protein recovered from the media of control and NMDA-treated cultures, respectively. Results were duplicated in an independent experiment.

**DISCUSSION**

Neuronal death produces high rates of glutamine hydrolysis, which persist unchanged for at least 4 h. This prolonged generation of glutamate from glutamine by damaged neurons is at least 10-fold greater than glutamate generation by undamaged neuronal and glial cultures exposed to glutamine. Although measurable amounts of glutamate are released by neuronal death itself, this amount is equivalent to only 6–8 min of glutamate hydrolysis by damaged neurons in the presence of 2 mM glutamine.

The studies with inhibitors and activators strongly support the hypothesis that a single molecular entity, the mitochondrial glutaminase, is responsible for the great majority of glutamine hydrolysis following neuronal death: Although DON and pCMPS are not selective for the glutaminase, they work by different mechanisms, and it is unlikely that another brain enzyme that hydrolyzes glutamine to glutamate is inhibited by both. Furthermore, the requirement for polyvalent anions (such as phosphate) for activation is unique to the glutaminase.

Western blotting, as well as measurement of glutaminase activity in cells and cell culture medium, shows that the glutaminase remains in cell fragments after a 5-h exposure to 500 μM NMDA. The observation that the electrophoretic mobility of the enzyme remains unchanged suggests that the glutaminase retained in NMDA-damaged cells is not degraded and is con-
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sistent with the continued activity of the enzyme after neuronal death. One possibility is that the association of the enzyme with the mitochondrial membrane (19) may protect it from degradation by proteases. Further study of enzyme stability and activity after neuronal death, particularly in vivo models of stroke, will be useful in further evaluating the role of the enzyme in pathological glutamate production in vivo.

Although the glutaminase remains localized in cell fragments in the initial stages after excitotoxic cell death, it is accessible to inhibition by the membrane-impermeant inhibitor, pCMPS. Since pCMPS does not inhibit glutaminase activity in intact rat renal mitochondria (17), this is probably a direct effect on the enzyme as opposed to an indirect effect on membrane transport of glutamine. This result suggests that the active form of the glutaminase is not membrane-limited and that the net increase in glutamine hydrolysis that is observed after excitotoxic neuronal death is due to membrane breakdown. A consequence of this conclusion is that it should be possible to develop agents that, by virtue of their membrane transport/permeability properties, inhibit only pathological breakdown. A consequence of this conclusion is that it should be possible to develop agents that, by virtue of their membrane transport/permeability properties, inhibit only pathological glutamate production by exposed glutaminase but do not inhibit normal glutaminase activity in intact mitochondria. Such agents would potentially block pathological glutamate production without interfering with normal glutamaticer transmission.

Concentrations of glutamate sufficient to cause toxicity (i.e., significantly above 1 µM; Refs. 21 and 27) were found in the medium of cultures where there is both neuronal damage and 2 mM added glutamine but were not found with undamaged cultures. The 3 µM concentration of glutamate that was observed in hypoxic mixed cultures exposed to glutamine is consistent with the IC₅₀ for toxicity of glutamate observed in neuronal cultures (2–10 µM) and the increased amount of neuronal death observed in these cultures. The addition of 2 mM glutamate does not cause accumulation of concentrations of glutamate of over 1 µM in mixed cultures not subjected to added stress (Table II). Likewise, the combination of hypoxia and glutamine does not cause glutamate to exceed 0.5 µM in the medium of glial cultures. The 2 mM concentration of glutamine is similar to that found in brain (0.3–0.5 mM in cerebrospinal fluid and 2–4 mM in bulk nervous tissue; Refs. 11 and 40) and is probably relevant to ischemic injury in vivo.

The experiments on glutamine neurotoxicity suggest that the contribution of the glutaminase to neuronal damage depends strongly on the glial clearance of glutamate, which ultimately regulates the concentration of glutamate achieved at any given rate of generation. In the neuronal cultures, removal of medium glutamate is slow. Here, glutamine exposure eventually results in steadily increasing concentrations of glutamate and rates of glutamine hydrolysis, leading to complete neuronal death. In the mixed cultures, glial uptake maintains low concentrations of glutamate, even in the presence of 2 mM glutamine, and glutamine toxicity is observed only if combined with an additional stress, such as hypoxia.

In these experiments, glucose was present during hypoxia, and glial uptake of glutamate remained unchanged in hypoxic mixed cultures exposed to glutamine. However, glial uptake is reduced by hypoglycemia and acidosis in ischemia in vivo (38).

Further studies are required to define precisely how decreased glial uptake will interact with glutamine hydrolysis by the glutaminase following neuronal damage in vivo. Selective inhibitors of the glutaminase should provide the most direct answers about the role of the glutaminase in neuronal injury in vivo and may prove to be clinically useful.

In summary, mechanical or excitotoxic neuronal death activates the prolonged hydrolysis of extracellular glutamine by the mitochondrial glutaminase. This elevated activity is a feature of damaged neurons, since intact neurons or glia exposed to glutamine did not generate glutamate at comparable rates. The neuroprotection observed with the membrane-impermeant pCMPS indicates that it may be possible to produce agents that selectively inhibit the elevated glutaminase activity by damaged neurons while leaving the activity of the glutaminase in undamaged cells relatively intact. Such an approach could be a useful complement or alternative to existing strategies aimed at producing neuroprotection following stroke and other disorders by blockade of the postsynaptic receptors for glutamate.