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Changes in Intracellular pH Associated with Glutamate Excitotoxicity

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Excitotoxic neuronal injury is known to be associated with increases in cytosolic calcium ion concentrations. However, it is not known if perturbations in other intracellular ions are also associated with glutamate (GLU)-induced neuronal death. Accordingly, intracellular hydrogen ion concentrations were measured in cultured hippocampal neurons with the fluorescent dye BCECF during and after toxic exposures. Five minute GLU applications produced an initial cytosolic acidification. During the hour after GLU removal, intracellular pH (pH_i) recovered steadily, resulting in a rebound cytosolic alkalinization. Lowering extracellular calcium depressed the initial GLU-induced acidification, suggesting that the rapid acidification may result partly as a consequence of calcium entry. An acidification-induced rebound alkalinization appeared to be activated by GLU exposure. Inhibitors of intracellular pH regulation, harmaline, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), and replacement of external Na^+ with *N*-methyl-glucamine$^+$ (NMG^+), retarded the rate of recovery from GLU-induced acidification. The rapid acidification and rebound alkalinization could be mimicked by challenging neurons with elevated external K^+ or replacement of external Na^+ with NMG^+. Two or more hours following toxic GLU exposure, hydrogen ion concentration did not stabilize at initial levels but progressively increased. High K^+ or Na^+ removal did not produce this long-term acidification and were not toxic. The cumulative increase in intracellular hydrogen ion may reflect the declining health of injured neurons and could contribute directly to neuronal death. Therefore, cytosolic acidification may act synergistically with increases in calcium concentration in mediating excitotoxicity.

[Key words: excitotoxicity, neurotoxicity, intracellular pH, glutamate, excitatory amino acids, hydrogen ion concentration, BCECF]

Delayed glutamate (GLU) excitotoxicity is thought to occur through a number of calcium-mediated events. Entry of calcium through both NMDA and non-NMDA ionotropic GLU channels (MacDermott and Dale, 1987; Murphy et al., 1987; Glaum et al., 1990; Iino et al., 1990; Gilbertson et al., 1991) has been postulated to activate calcium-dependent kinases, phosphatases, phospholipases, and endonucleases that eventually destabilize intracellular homeostasis and lead to neuronal damage and death (Orrenius et al., 1988; Choi, 1990). While GLU application was originally reported to produce sustained rises in $[Ca^{2+}]_i$ (Connor et al., 1988; Ogura et al., 1988; Manev et al., 1989; Wahl et al., 1989; De Erausquin et al., 1990; Glaum et al., 1990; Ciardo and Meldolesi, 1991; Dubinsky and Rothman, 1991), recent experiments have described complete recovery of basal $[Ca^{2+}]_i$ for clearly toxic GLU exposures (Randall and Thayer, 1992; Dubinsky, 1993b). Thus, calcium-mediated processes associated with neuronal death must be activated in the hour or so following GLU overstimulation. This view is consistent with the observation that removal of extracellular calcium is protective against GLU-induced toxicity (Choi et al., 1987; Rothman et al., 1987).

However, other experiments have questioned the sole involvement of calcium, since neuronal death can occur without large deviations in $[Ca^{2+}]_i$ and high potassium or cyanide induced increases in $[Ca^{2+}]_i$ do not produce toxicity (Michaels and Rothman, 1990; Dubinsky and Rothman, 1991). Intracellular acidification has been postulated to contribute to ischemic neuronal death (Tombaugh and Sapolsky, 1990; Nedergaard et al., 1991; see Siesjo, 1992, for review). Previous calculations suggested that ischemia-induced shifts in whole brain pH could be accounted for by the changes within glial cells (Kraig et al., 1986). Neurotransmitter-induced variations in astroglial pH_i and brain extracellular pH have been documented extensively (Chesler and Chan, 1988; Jarolimek et al., 1989; Kraig and Chesler, 1990; Chesler and Rice, 1991; Chen and Chesler, 1992a; Chesler and Kaila, 1992). Reexamination of the relationship between changes in lactate accumulation and extracellular pH during ischemia has challenged the notion that the hydrogen ion distribution differs between glial and neuronal compartments (Katsura et al., 1991). Thus, internal neuronal pH disturbances could possible accompany excitotoxicity. Indeed, measurements with H^+-sensitive microelectrodes have demonstrated that GLU and GABA application can directly acidify frog motoneurons and crayfish stretch receptor neurons, respectively (Enders et al., 1986; Kaila et al., 1992). Moreover, physiological levels of GLU produced parallel increases in $[H^+]_i$ and $[Ca^{2+}]_i$ among hippocampal neurons, suggestive of a synergistic contribution to excitotoxic neuronal death (Koch and Barish, 1991).

Since extended periods of elevated $[H^+]_i$ have been demonstrated to be neurotoxic *in vitro* (Nedergaard et al., 1991), we have examined GLU-induced changes in pH_i among cultured hippocampal neurons with the hydrogen ion–sensitive dye 2′,7′-bis-(2-carboxyethyl)-5-(and-6)carboxyfluorescein (BCECF). These experiments were designed to parallel those previously

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reported for $[Ca^{2+}]_i$ on both short and long time scales (Dubinsky, 1993b).

Materials and Methods

Tissue culture. Hippocampal cultures from postnatal day 1 rat pups (Sprague–Dawley, Harlan) were prepared according to established procedures (Dubinsky, 1989, 1993b; Yamada et al., 1989). Neurons were plated onto a preplated astroglial feeder layer on polylysine- and collagen-coated, glass-bottomed, 35 mm petri dishes (pH measurements) or plastic petri dishes (toxicity measurements) at a density of 500,000 cells per dish. Cultures were maintained in minimum essential medium without glutamine containing 27.75 mM glucose, 10% NuSerum (Collaborative Research), 50 U/ml penicillin, and 50 μg/ml streptomycin, 335 mOsm at 37°C in a humidified atmosphere containing 5% CO_2 for 12–18 d before use.

Intracellular pH measurements. Intracellular hydrogen ion concentration ($[H^+]_i$) was assessed with ratio measurements of the hydrogen ion–sensitive dye BCECF. Cultures were loaded with the dye by incubation of a 4 μM concentration of the acetoxymethyl ester form (BCECF-AM; lot 1114, Molecular Probes) for 5 min in the growing medium at 37°C. BCECF loading was terminated by subsequent rinsing with a basic salt solution containing (mM) 139 NaCl, 3 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 1.0 $NaHCO_3$, 27.75 glucose, 15 sucrose, 10 Na-HEPES, and 0.01 glycine, 329 mOsm, pH 7.3 at 37°C, and cultures were placed on the heated stage of an Olympus IMT-2 inverted microscope for 15–20 min prior to data acquisition. In some experiments, $CaCl_2$ was increased to 10 mM or omitted altogether. In experiments assessing pH_i many hours after GLU exposure, cultures were initially rinsed with Earle's Balanced Salt Solution (EBSS+) containing (mM) 116 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.1 $MgSO_4$, 0.9 NaH_2PO_4, 26.2 $NaHCO_3$, 27.75 glucose, 35 sucrose, 0.01 glycine, and phenol red, 328 mOsm, and exposed to 500 μM GLU for 5 min at 37°C in 95% air, 5% CO_2. Cultures were rinsed in fresh EBSS+ and returned to the incubator for variable periods of time; 4 μM BCECF-AM was subsequently added to the EBSS+ for 5 min prior to fluorescence measurements.

Responses of individual neurons at 35°C to 500 μM GLU application were monitored by intermittent ratio imaging following delivery of a concentrated stock solution of GLU to the edge of the dish accompanied by gentle mixing. After 5 min of GLU exposure, the dishes were manually rinsed three times with warmed balanced salt solution. This method of application mimics the procedures used in previously published calcium measurement and toxicity experiments (Dubinsky, 1993b). 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS; Sigma) and harmaline (Sigma) were prepared as 100× stock solutions in basic salt solution and added similarly for final concentrations of 100 μM. The pH of the external solution at the beginning and end of the experiments remained consistent at 7.3.

Ratio measurements were calculated from digitized images of emitted BCECF fluorescence (520–555 nm; dichroic cutoff, 515 nm) for excitation at 495 and 440 nm from a 75 W xenon source through a 5% transmittance neutral density filter. A Nikon CF Fluor 40× oil 1.3 NA objective was used to capture the fluorescence in conjunction with a DAGE-MTI GenIIsis Image Intensifier and CCD-72 camera. Fields for imaging were selected under bright-field illumination prior to any fluorescence measurements and contained nonclumped, healthy, intact neurons.

Calibrations were performed on individual neurons from separate cultures incubated in nigericin containing solutions of known pH (Thomas et al., 1979). Specifically, these solutions contained (mM) 150 KCl, 1.8 $CaCl_2$, 0.8 $MgCl_2$, 10 μg/ml nigericin (Molecular Probes), and 10 mM concentrations of 2-[*N*-morpholino]ethanesulfonic acid (pH 5.6, 6.2), (3-[*N*-morpholino]propanesulfonic acid (pH 6.6, 7.1), or HEPES (pH 7.4, 7.85, 8.2). The resulting relationship between ratio values and pH_i was fitted with the sigmoidal curve; ratio = min + [max/(1 + $(10^{pH}/10^{mid})^{slope}$)], where min = 1.72, max = 9.71, mid = 6.97, and slope = 0.958. The midpoint of the curve (mid) corresponded to the pH value for half-maximal BCECF sensitivity. The calculated value agreed well with the pK'_a value published for BCECF of 6.97 determined by more rigorous calibration procedures (James-Kracke, 1992). Harmaline contributed an additional fluorescence signal at 440 nm that sometimes affected measurements obtained at very high gains. Accordingly, calibration values in harmaline were probably different and hence no pH_i equivalences are provided. All changes reported for ratio values in harmaline were observed in the 495 nm values and were not attributable to harmaline autofluorescence. Calibration values were not changed by the addition of DIDS.

To prevent both bleaching of the dye and phototoxic damage to the neurons, measurements were made intermittently at intervals greater than or equal to 60 sec. In addition, no attempt was made to calculate the internal neuronal buffering capacity for H^+. Therefore, we were unable to calculate H^+ flux rates. In order to compare the rates of recovery from internal acidification under different experimental conditions, an empirical measure of the rate of rise of the ratio values (in ratio units/min) was tabulated from all data points collected following removal of the acid-loading agent.

All statistics were performed on ratio values. For the GLU response curves, distinctions between significance levels below $p < 0.05$ have been omitted for graphical clarity.

Toxicity experiments. Neuronal survival was assessed by counting neurons containing and excluding trypan blue 24 hr following exposure to various test solutions as previously described (Dubinsky, 1993b). Briefly, growing medium was replaced with EBSS+ and 500 μM GLU was added from a concentrated stock solution. After a 5 min incubation at 37°C in 5% CO_2, cultures were rinsed in fresh EBSS+ and incubated overnight. For the ion substitution experiments, EBSS-like solutions containing 50 mM K^+ substituted for Na^+ or 145 mM *N*-methyl-glucamine$^+$ (NMG^+) substituted for Na^+ were added instead of EBSS+ during the 5 min exposure. Similar ion substitutions were made in the balanced salt solution used for the pH_i experiments.

Results

Short-term GLU-induced changes in intracellular pH

Initial BCECF ratio measurements in untreated cultures bathed in HEPES-buffered basic salt solution were 5.77 ± 0.24 (mean ± SEM, $N = 28$ neurons from four experiments), corresponding to a pH_i of 7.00 ± 0.05 or an intracellular hydrogen ion concentration ($[H^+]_i$) of 100 nM. Similar values for neuronal pH_i in HEPES-buffered media have been reported previously (Nachshen and Drapeau, 1988; Koch and Barish, 1991; Raley-Susman et al., 1991). Following the establishment of a stable resting pH_i, ratios were obtained intermittently during a 5 min bath application of 500 μM GLU (Fig. 1*A*). Immediately upon introduction of GLU, fluorescence ratios fell rapidly, indicating an intracellular acidification. In four experiments, the average minimum ratio value attained in the presence of GLU was 3.68 ± 0.07, corresponding to a pH_i of 6.48 ± 0.02, or 331 nM $[H^+]_i$. After several minutes in the continued presence of GLU, ratios began to creep upward, suggesting the onset of a recovery process.

Upon removal of the GLU, ratio values recovered initial levels and continued to climb, indicating an intracellular alkalinization. The rate of recovery was slow compared to the initial drop and varied between culture dishes. Thirty to sixty minutes were generally required to regain and surpass initial pH_i levels. A secondary alkalinization was observed at 60 min in 27 out of 28 neurons from four experiments. Since the ratios were continuing to increase at the end of the measurement period, maximum levels for the alkalosis could not be established. No changes in basal pH_i were observed when control solution changes were performed with basic salt solution (Fig. 1*B*).

GLU toxicity has principally been associated with an influx of extracellular calcium. Therefore, GLU induced changes in pH_i were monitored in cultures incubated in extracellular solutions with either no added calcium or 10 mM calcium, conditions that should alter the influx of calcium. Nominally "calcium-free" solution should minimize and the high external calcium solution should augment the GLU-induced increase in intracellular calcium concentration ($[Ca^{2+}]_i$), respectively. If GLU-induced $[H^+]_i$ changes were linked to $[Ca^{2+}]_i$ changes, then these solutions might be expected similarly to minimize or aug-

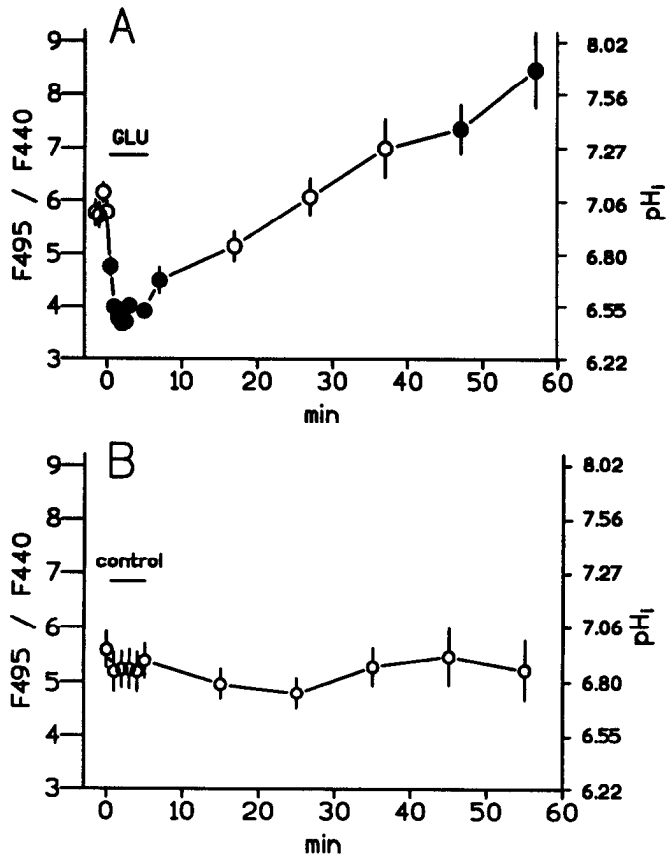

Figure 1. Hippocampal neurons responded to 500 μM GLU exposure (*A*) with an initial acidification followed by a rebound alkalinization. Data points represent mean ratio values (±SEM) for 27 neurons from three separate experiments. *B*, Intracellular pH remained unaltered during control solution changes. In each curve, *solid symbols* represent data points significantly different from the initial value at 0 min (Dunnett's test, $p < 0.05$). Data are from four neurons in one of two replicate experiments.

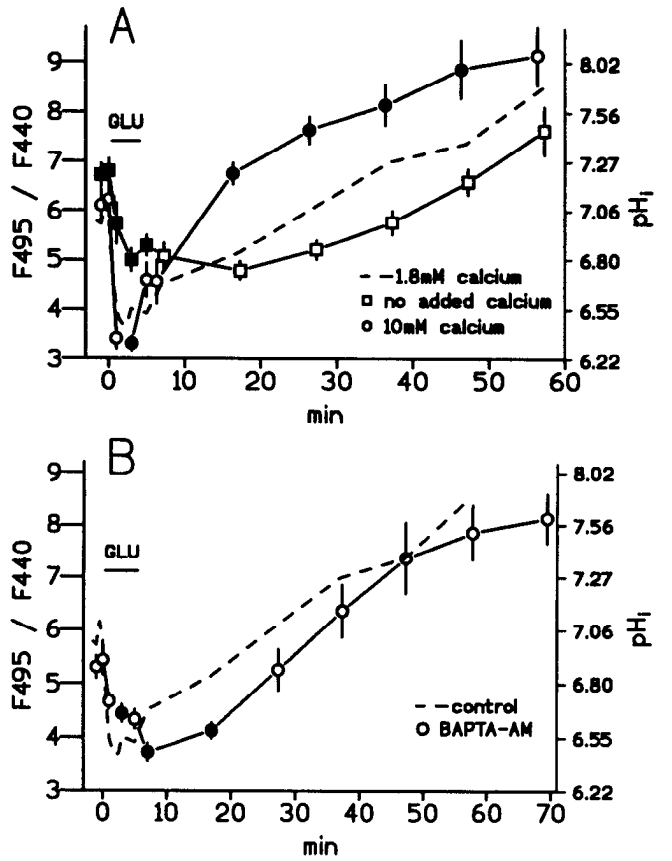

Figure 2. Manipulations of calcium availability altered the extent of pH_i changes in response to GLU. *A*, Lowering extracellular calcium muted the initial acidification and delayed the rebound alkalinization. Raising extracellular calcium speeded the onset of alkalinization. *B*, Introduction of the cell-permeant calcium chelator BAPTA-AM slowed the initial acidification and delayed the rebound alkalinization. *Solid symbols* in *A* and *B* represent data points differing from the coincident time points in the GLU response in normal calcium (Fig. 1*A*; represented by *dashed line*; $p < 0.05$, ANOVA followed by Student-Newman-Keuls test). In all curves, data points during the GLU-induced acidification were significantly lower than ratios at 0 min ($p < 0.05$, Dunnett's test). Ratios were significantly more alkaline after 30 min in 10 mM calcium and after 40 min in BAPTA ($p < 0.05$, Dunnett's test). Data were combined from 16 or 17 neurons in three experiments in each condition.

ment the observed changes in BCECF fluorescence. In lowered external calcium solution the basal pH_i was higher and the initial fall in pH_i occurred more slowly and was not as prominent (Fig. 2*A*). Subsequently, the recovery of basal levels and secondary increase in pH_i appeared delayed in onset and overall time course. Elevated external calcium did not alter the initial pH_i decrease appreciably, but the recovery phase occurred more rapidly. The steady state rate of recovery from acidification was only slightly retarded by the nominally "calcium-free" condition (Table 1). Other manipulations of external calcium failed to produce any changes in the rates of recovery (Table 1).

To manipulate $[Ca^{2+}]_i$ further, cultures were preincubated for an hour in 4 μM BAPTA-AM, a cell-permeant calcium chelator. BAPTA-AM was expected to blunt and prolong the initial increase in $[Ca^{2+}]_i$ but not to prevent it (Dubinsky, 1993a). Incubation with BAPTA-AM did not significantly alter the basal ratio values (5.49 ± 0.21, $N = 22$ from three experiments, corresponding to pH_i of 6.94 ± 0.04, $[H^+] = 115$ nM). As with the lowered external calcium condition, BAPTA-AM–treated neurons responded to GLU with a slower, less prominent initial acidification and a delayed onset of recovery and secondary alkalinization (Fig. 2*B*). In general, though, BAPTA-AM treatment was not as effective as removal of external calcium in altering the changes in pH_i. Thus, GLU-induced changes in $[H^+]_i$ appeared to be influenced by the concomitant alterations in $[Ca^{2+}]_i$.

A prominent influx of Na^+ was also expected following GLU receptor activation. Since the Na^+-H^+ antiporter has been implicated in the regulation of pH_i in hippocampal neurons (Raley-Susman et al., 1991), Na^+ fluxes or alterations in the Na gradient may have contributed to the observed pH_i changes. Slowed operation of the Na^+-H^+ antiporter following Na^+ influx through GLU channels could have contributed to the GLU-induced fall in pH_i, or activation of the antiporter could be responsible for the subsequent increase in pH_i. Two different treatments were applied to test the involvement of the Na^+-H^+ antiporter. Harmaline, a specific inhibitor of this pump in hippocampal tissue, human placental brush border membranes, and renal microvillus membranes (Aronson and Bounds, 1980; Balkovetz et al., 1986; Raley-Susman et al., 1991), was employed both during and after GLU exposure. Introduction of 100 μM harmaline without rinsing produced an initial fall in BCECF fluorescence to a stable plateau (data not shown), indicating that the Na^+-

Table 1. Steady state rates of recovery from the initial GLU-induced acidification

Experiment	Recovery rate
GLU	0.0900 ± 0.0128 (28)
No added calcium	0.0505 ± 0.0037 (16)*
10 mM External calcium	0.0695 ± 0.0109 (20)
BAPTA-AM	0.1005 ± 0.0156 (16)
Harmaline	0.0245 ± 0.0042 (20)***
DIDS	0.0395 ± 0.0047 (17)**
NMG^+ in rinse	0.0482 ± 0.0028 (23)**
Delay after pretreatments	
0.5 hr	0.0267 ± 0.0106 (18)***
1 hr	−0.0040 ± 0.0104 (16)***
2 hr	0.0416 ± 0.0049 (21)***
4 hr	0.0395 ± 0.0111 (12)**
6 hr	0.0546 ± 0.0067 (19)*
8 hr	0.0359 ± 0.0141 (25)***
12 hr	0.0404 ± 0.0036 (21)***

For each neuron the rate of recovery was calculated as the slope of the linear regression line fit to all time points following GLU removal. Values are the mean ± SEM of calculated slopes from all neurons (N), in ratio units/min.

*$p < 0.05$, ANOVA followed by two-tailed t test with Bonferroni correction compared to GLU.

**$p < 0.01$.

***$p < 0.001$.

H^+ antiporter was active at rest to remove H^+. In the continued presence of harmaline, GLU still produced a further rapid fall in ratio values (Fig. 3*A*). The recovery from this additional acidification was significantly slowed in comparison to control solutions (Table 1) and recovery beyond initial levels was not observed within 60 min. When Na^+-H^+ antiporter activity was blocked by NMG^+ substitution for external Na^+ either before or after GLU treatment, the recovery from acidification was also retarded (Fig. 3*B*, Table 1). Therefore, Na^+-H^+ antiporter activity appeared to be involved in the recovery from the initial acidification and the rebound alkalinization. In addition, the Na-dependent Cl^-/HCO_3^- exchanger, also present within hippocampal neurons (Raley-Susman et al., 1991), contributed to the recovery from acidification since its inhibitor, DIDS (Boron et al., 1981), also slowed the recovery process when present throughout the 60 min observation period (Fig. 3*C*, Table 1). In the continuous presence of DIDS alone, neuronal pH_i declined and then recovered to a slightly elevated level (data not shown).

Other conditions producing short-term acidification

Similar cellular acid-loading followed by a slow recovery and rebound alkalinization could be observed when hippocampal cultures were bathed in 50 mM K^+ for 5 min. In the presence of elevated potassium, ratios in three dishes dropped to 4.39 ± 0.15 or a pH_i of 6.68 ± 0.04 or 209 nM $[H^+]_i$ ($N = 26$ neurons from three experiments; Fig. 4*A*). Removal of extracellular sodium and substitution of NMG^+, a cation that is not a substrate for the Na^+-H^+ antiporter, also produced an acidification among hippocampal neurons (Koch and Barish, 1991; Raley-Susman et al., 1991). In our experiments, pH_i declined during Na^+ removal and NMG^+ exposure to comparable levels (ratio of 4.43 ± 0.16, $N = 34$ neurons from three experiments, corresponding to 6.69 ± 0.04 pH_i, 204 nM $[H^+]_i$) followed by a slow recovery and subsequent rebound increase in pH_i (Fig. 4*B*).

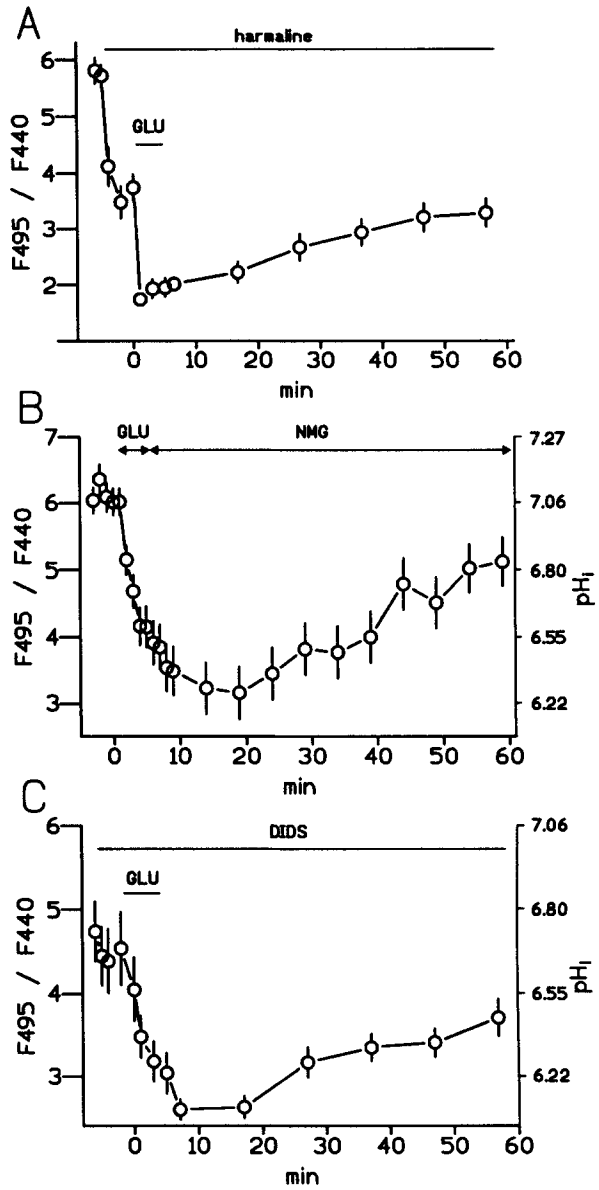

Figure 3. Inhibitors of hydrogen extrusion depressed the recovery from GLU-induced initial acidification. In the continuous presence of 100 μM harmaline (*A*) or DIDS (*C*), GLU produced an initial acidification with a slowed recovery. *B*, NMG^+ substitution for external Na^+ in the rinse produced a further acidification and retarded the subsequent recovery. Data represent 17–23 neurons from single experiments selected from two to four replicates.

Long-term GLU-induced acidification

The approximately 1 hr observation period following BCECF-AM loading was not sufficient to determine if the rebound alkalinization was severe enough to contribute to the eventual toxicity expected from this dose of GLU. To monitor pH_i changes during the hours following GLU exposure, cultures were pretreated with an equivalent exposure to GLU, rinsed, and incubated for variable periods of time prior to loading with BCECF-AM. Initial ratio measurements in morphologically intact neurons at various times after GLU pretreatment revealed that pH_i remained elevated for about an hour, returning to basal levels by 1.5 hr (Fig. 5). pH_i did not remain stable, however, but continued to fall over the next 10 hr. This progressive increase

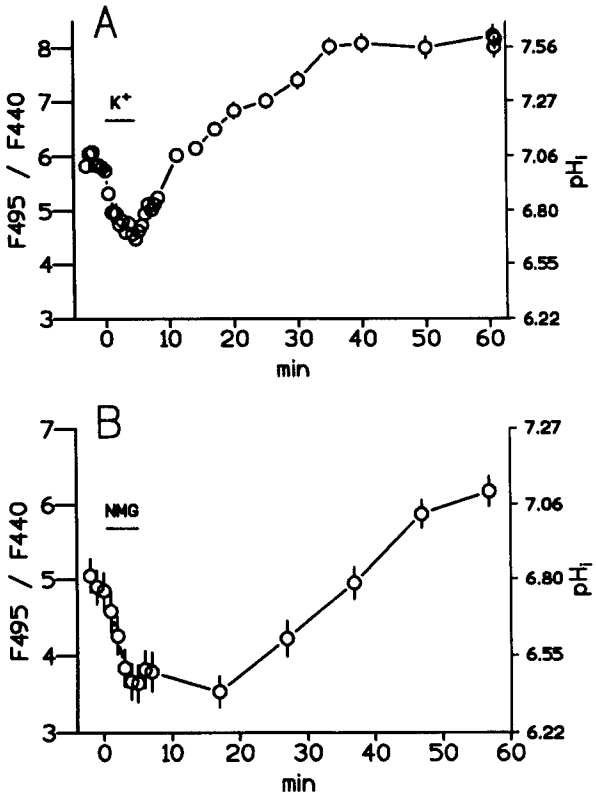

Figure 4. Short-term acidification and rebound alkalinization produced by 50 mM external potassium (*A*) and NMG^+ substitution for external sodium (*B*). Single representative experiments of seven or eight neurons each are illustrated from among three replicates.

Figure 5. BCECF ratio measurements taken at long intervals after 5 min acidifying pretreatments. Neurons were pretreated with GLU and returned to the incubator for the indicated time prior to loading with BCECF (*circles*). *Solid circles* indicate ratio values significantly different from the initial ratios in naive cultures (0 hr, no pretreatment; $p < 0.05$, ANOVA followed by Dunnett's test). Other pretreatments failing to produce the decline in pH_i at 12 hr were 500 μM GLU plus 20 μM CNQX and 20 μM MK-801 (*square*), 50 mM external K^+ (*diamond*), and NMG^+ substitution for external Na^+ (*cross*), and solution change controls (*triangle*). At 12 hr, only the GLU pretreatment was significantly different from the solution change controls ($p < 0.05$, ANOVA followed by Dunnett's test). Data are combined initial measurements from three to five experiments (12–27 neurons) at each time point.

in $[H^+]_i$ constituted a third, long-term effect of GLU that reflected a clear loss of hydrogen ion homeostasis.

Other treatments (high external potassium and NMG substitution for sodium) that produced immediate acidification and the secondary rebound alkalinization did not result in a long-term acidification 12 hr after exposure (Fig. 5). Hippocampal cultures exposed to elevated potassium concentration or NMG^+ replacement of external Na^+ exhibited normal ratio values (pH_i levels) after this prolonged interval. Similarly, control cultures receiving simple solution changes failed to show any deterioration in basal ratio values after 12 hr.

During this extended period after a toxic GLU exposure, hippocampal neurons retained their ability to respond to GLU both electrophysiologically and with an increase in $[Ca^{2+}]_i$ (Dubinsky, 1993b). Therefore, cultures were tested to determine if the GLU-induced $[H^+]$ effects were also preserved despite the progressive decline in basal pH_i. BCECF responses of hippocampal cultures to application of GLU at various times after a toxic pretreatment varied depending upon the pH_i level attained during the interval following pretreatment (Fig. 6). At short intervals of 0.5–1 hr, the rapid GLU-induced acid response was greatly attenuated or nonexistent (Fig. 6*A*). The absence of a response was notable during this period of rebound alkalinization following the pretreatment. A new, secondary increase in pH_i was difficult to distinguish from the continuously rising values produced by the GLU pretreatment. In the 2–6 hr interval following pretreatment, when pH_i was slightly depressed, GLU induced both the rapid acidification and subsequent recovery and alkalosis (Fig. 6*B*). Once pH_i had declined substantially, at 8 and 12 hr after pretreatment, the rapid acidification was again muted but the secondary rise in pH_i was observed (Fig. 6*C*). At all of these extended times, the steady state rate of recovery was substantially retarded compared to the rate observed upon single GLU exposure (Table 1). Deterioration of somal morphology could not account for the variability among responses since all imaged neurons appeared healthy. Thus, after toxic pretreatment, the GLU-induced initial decline in pH_i and the ensuing rate of recovery appeared labile and possibly dependent upon the existing $[H^+]_i$ or $[Ca^{2+}]_i$.

GLU receptor antagonists block long-term acidification

Antagonists of ionotropic GLU receptors, 6-cyano-7-dinitroquinoxaline-2,3-dione (CNQX) and MK-801, failed to block totally the GLU-induced initial acidification and secondary rebound alkalinization in the majority of neurons (Fig. 7*A*). The minimum ratio value attained in the presence of antagonists (4.22 ± 0.13, $N = 25$, corresponding to a pH_i of 6.63 ± 0.03 or 234 nM $[H^+]_i$) reflected a significantly reduced accumulation of $[H^+]_i$ compared to GLU alone ($pH_i = 6.48$; see above; $p < 0.001$ with two-tailed t test). In only 3 out of 28 neurons tested in five experiments, CNQX and MK-801 were effective at preventing the initial decrease in pH_i (Fig. 7*B*). In these three neurons, in the absence of the initial acid transient, no subsequent alkalosis was observed.

CNQX and MK-801 did, however, prevent the long-term acidification induced by toxic GLU exposure (Fig. 5). Twelve hours after a combined treatment with GLU, CNQX, and MK-801, ratio values remained at levels comparable to initial baseline ratios, even though an initial acidification and rebound alkalinization probably occurred at the time of treatment.

Toxicity associated with treatments producing acidification

Hippocampal neuronal survival was assessed 24 hr following the various treatments found to manipulate pH_i (Fig. 8). A 5 min treatment with 500 μM GLU proved lethal, in agreement

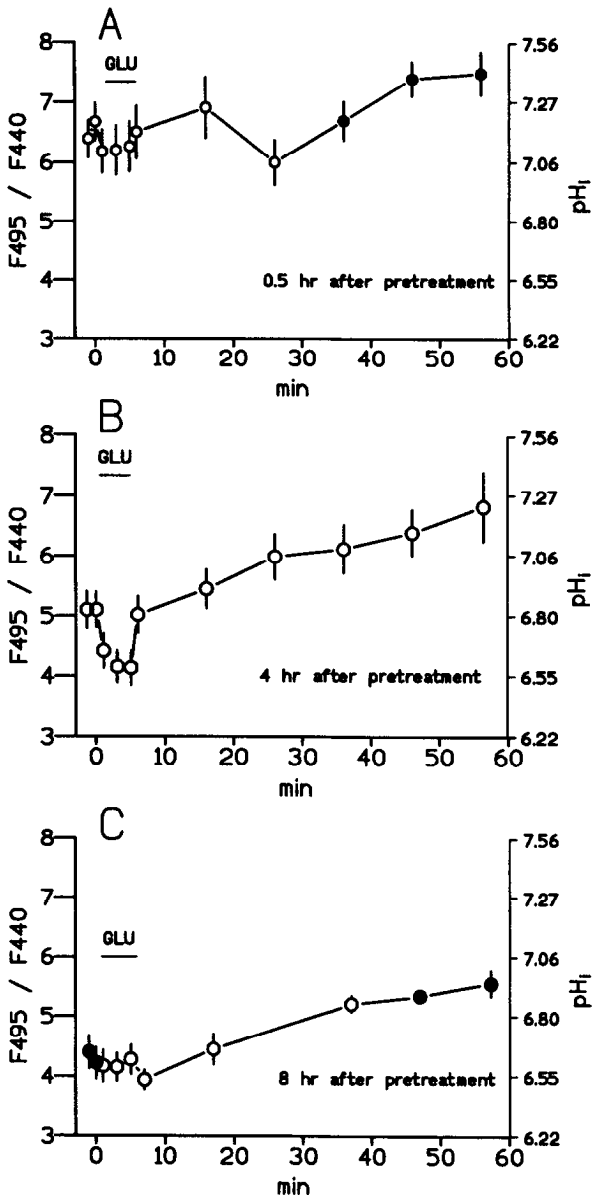

Figure 6. Response of hippocampal neurons to GLU at various times after a previous GLU pretreatment. *A*, Cultures were pretreated for 5 min with 500 μM GLU, rinsed and incubated for 30 min prior to BCECF-AM loading, and monitored during and after the 5 min test GLU exposure. Ratio values are not significantly different from initial levels over the course of an hour (Dunnett's test). *B*, Four hour interval between pretreatment and test exposure. Only the ratio at 57 min is significantly different from the initial value at 0 min ($p < 0.05$, Dunnett's test). *C*, Eight hour interval between pretreatment and test exposure. Ratios beyond 30 min are significantly different from that at 0 min ($p < 0.05$, Dunnett's test). In all curves, *solid symbols* represent data points significantly different from corresponding points in the GLU response in normal calcium (*dashed line* from Fig. 1*A*; $p < 0.05$, ANOVA followed by Student-Newman-Keuls test). Traces represent averages of three experiments each (15–21 neurons).

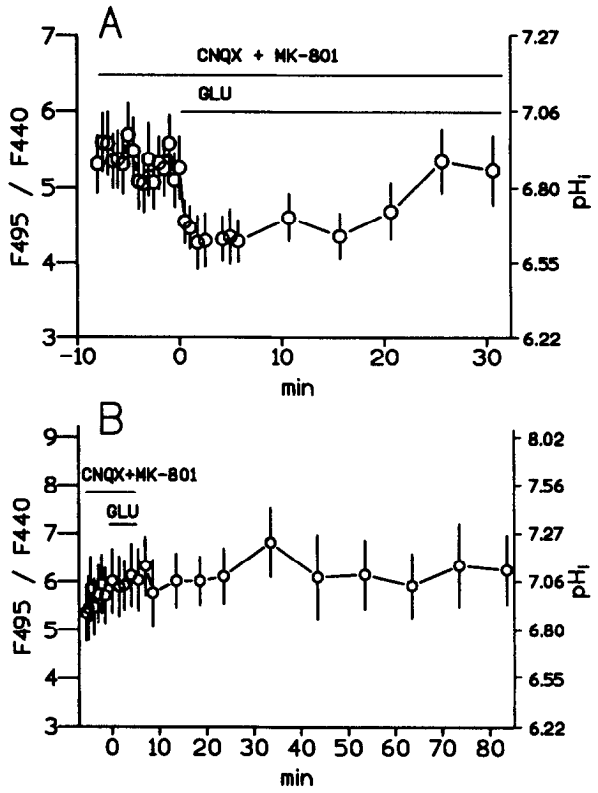

Figure 7. Short-term GLU-induced $[H^+]_i$ responses were not prevented by the combination of 20 μM CNQX and 20 μM MK-801 in the majority of neurons tested (*A*). In a minority of neurons, ionotropic GLU antagonists prevented both the initial acidification and the subsequent rebound alkalinization (*B*). Two out of six experiments (three or four neurons each) are illustrated.

with previous experiments (Michaels and Rothman, 1990; Dubinsky and Rothman, 1991). Ionotropic GLU receptor antagonists protected against excitotoxicity, as expected (Michaels and Rothman, 1990), despite their failure to prevent the initial decrease in pH_i. Similarly, hippocampal neurons survived short-term elevations in external K^+ or removal of external Na^+. Thus, all treatments producing the initial acidification and rebound alkalinization, but not the long-term decline in pH_i, were *not* toxic. GLU exposure was notably the only treatment resulting in both the progressive, long-term decline in pH_i and cell death.

Discussion

Mechanisms of GLU-induced alterations in pH_i

Toxic exposure to GLU produced a continually varying pattern of changes in $[H^+]_i$ among hippocampal neurons. Initially, during the GLU overstimulation, $[H^+]_i$ increased rapidly to a peak value and began to recover. Secondarily, after removal of GLU, $[H^+]_i$ steadily decreased, attaining and undershooting basal levels over the course of the next hour. Subsequently, $[H^+]_i$ did not ever appear to remain stable at initial homeostatic levels but gradually and continuously rose over the course of many hours. Only the long-term changes in pH_i appeared to be associated with the delayed neuronal death observed during this time period. Each of these changes in $[H^+]_i$ may reflect different intracellular events consequent to GLU receptor activation.

Intracellular acidification following GLU exposure was expected from hydrogen ion–sensitive microelectrode recordings of frog motoneurons (Enders et al., 1986) and from recently reported fluorescence measurements in hippocampal neurons (Koch and Barish, 1991; Irwin and Paul, 1992; Raley-Susman et al., 1992a). The postulated H^+ fluxes would be consistent with extracellularly recorded alkaline transients in other preparations. Stimulus-evoked external alkaline shifts, recorded in the molecular layer of the turtle cerebellum, are antagonized by

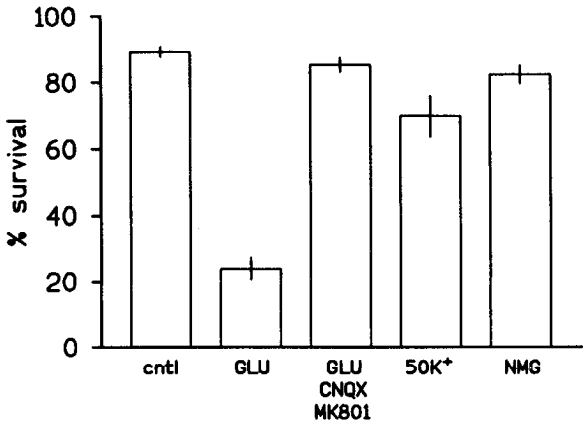

Figure 8. Survival of hippocampal neurons 24 hr following a 5 min exposure to various treatments that altered pH_i. Bars represent control solution changes (*cntl*), 500 μM GLU (*GLU*), 500 μM GLU plus 20 μM CNQX and 20 μM MK-801 (*GLU CNQX MK801*), 50 mM external K^+ (*50K*$^+$), and NMG^+ substitution for external Na^+ (*NMG*). Data are combined from 12 fields in two experiments.

kynurenate and mimicked by GLU iontophoresis (Chesler and Chan, 1988; Chen and Chesler, 1991; Chesler and Kaila, 1992). Similarly in hippocampal slices, stimulation of the Schaffer collaterals produces an alkaline shift in CA1 extracellular space, sensitive to GLU antagonists (Chen and Chesler, 1992a,b). These external alkalinizations appear associated with postsynaptic receptor activation rather than neuronal firing (Chen and Chesler, 1992b; Chesler and Kaila, 1992).

Displacement of H^+ from internal binding sites by the GLU-induced increase in cytosolic Ca^{2+} constitutes a likely explanation for the initial GLU-induced accumulation of H^+. Direct injection of Ca^{2+} into snail neurons and *Myxicola* axoplasm leads to an increased $[H^+]_i$ (Meech and Thomas, 1977; Abercrombie and Hart, 1986). Conversely, acidification of molluscan neurons, *Myxicola* axoplasm, neuroblastoma cells, and PC12 cells causes a concomitant increase in $[Ca^{2+}]_i$ (Ahmed and Connor, 1980; Abercrombie and Hart, 1986; Dickens et al., 1989). Under experimental conditions comparable to those producing the increased $[H^+]_i$ reported here, GLU produced rapid elevations in $[Ca^{2+}]_i$, persisting for about an hour (Dubinsky, 1993b). The abundance of free calcium ions would have maintained the hydrogen ion displacement and prevented repeated GLU-induced acidifications. Two hours after GLU exposure, with calcium homeostasis reestablished and $[H^+]_i$ near the normal range though not stable, a second GLU exposure could again produce increases in both $[Ca^{2+}]_i$ and $[H^+]_i$. Many hours after GLU exposure, the elevated $[H^+]_i$ would largely prevent GLU-induced Ca^{2+} influx from further displacing bound H^+. Elevated external K^+, which should depolarize neurons to approximately −27 mV, activate voltage-gated calcium channels, and cause an increase in $[Ca^{2+}]_i$, produced a similar acidification, consistent with this displacement mechanism.

An initial acidification produced by Ca^{2+} displacement of H^+ from internal binding sites could also possibly explain the inability of ionotropic GLU antagonists to prevent the increase in $[H^+]_i$. Activation of unblocked metabotropic receptors causes a transient increase in $[Ca^{2+}]_i$ that recovers to a plateau level (Furuya et al., 1989; Murphy and Miller, 1989; Dubinsky, 1993b). The release of calcium from internal stores could similarly displace bound hydrogen and transiently acidify hippocampal neurons. However, MK-801 alone can prevent GLU-induced increases in $[Ca^{2+}]_i$ measured with fura-2 (Dubinsky and Rothman, 1991) and combined CNQX and MK-801 together prevented rises in $[Ca^{2+}]_i$ with GLU applications identical to those employed here (Z. Hartley and J. M. Dubinsky, unpublished observations). Therefore, an additional GLU-induced process may contribute to the rapid acidification, independent of ionotropic receptor activation or changes in intracellular calcium.

Other explanations for the initial alterations in pH_i appear less satisfactory. Involvement of the Na^+-H^+ antiporter can be ruled out since GLU persisted in acidifying neurons even when the antiporter was blocked by harmaline. The increased ATP hydrolysis required to reestablish calcium homeostasis might produce an overabundance of H^+. However, this would not account for the absence of a second response at short intervals or the secondary alkalinization.

One often suggested possibility involves hydrogen ions entering neurons directly either through GLU channels, hydrogen ion channels, or other voltage-activated channels (Thomas and Meech, 1982; Mozhayeva and Naumov, 1983; Barish and Baud, 1984; Byerly et al., 1984; Chesler and Chan, 1988). Yet very little inward current has been observed through H^+ channels (Barish and Baud, 1984; Byerly et al., 1984; Decoursey, 1991). H^+ influx through other channel types may also be insufficient for the following reasons. At rest the H^+ equilibrium potential is −18 mV. If an ohmic conductance pathway were opened by GLU or GLU-induced depolarization, H^+ flux would initially be inward and could contribute to intracellular acidification. As V_m approached −10 mV, as demonstrated in hippocampal cultures (Rothman et al., 1987), the hydrogen influx would decrease rapidly and reverse direction. If enough H^+ entered in the initial milliseconds of GLU exposure to increase $[H^+]_i$ measurably, then the H^+ equilibrium potential would become more negative and the hydrogen flux would reverse direction sooner. Thus, only a very rapid initial acid transient might accompany GLU application. Constant depolarization in the continued presence of GLU would promote H^+ efflux rather than the observed continual acidification over the course of several minutes. Therefore, H^+ influx via channel activation may not be sufficient to explain the observed initial acidification. The slow time course of acidification itself argues against a channel-mediated process. Additionally, the absence of a second GLU-induced increase in $[H^+]_i$ 0.5 hr following GLU pretreatment makes a channel-specific route of entry less probable.

Subsequent to the initial acidification, pH_i began to recover, in many neurons even before removal of the GLU. The recovery process probably reflected operation of exchange mechanisms for removal of the excess free cytoplasmic H^+. Two different transport processes have been identified in hippocampal neurons recovering from an NH_4Cl-induced acid load: a DIDS-sensitive HCO_3^--dependent acid extruder, and an amiloride-insensitive, harmaline-sensitive Na^+-H^+ antiporter (Raley-Susman et al., 1991). Similar exchange mechanisms have been characterized in sympathetic neurons, synaptosomes, and leech neurons (Deitmer and Schlue, 1987; Tolkovsky and Richards, 1987; Nachshen and Drapeau, 1988). In agreement with this previous work, DIDS, harmaline, and removal of external sodium reduced the rate of recovery from GLU-induced acid loads among hippocampal cells. DIDS had no apparent effect upon resting pH_i levels while harmaline produced a decrease in pH_i. Thus, as previously reported, the Na^+-H^+ antiporter was active at rest and in response to perturbations in pH_i, while the HCO_3^--

dependent extrusion mechanism was only activated after intracellular acidification (Raley-Susman et al., 1991). The recovery processes were activated irrespective of the method of acid loading: GLU, high K^+, or external Na^+ removal.

Accompanying the recovery from acid loading was the rebound alkalinization and overshooting of the initial basal pH_i levels. Increasing $[H^+]_i$ shifts the pH dependence of Na^+-H^+ antiporter operation to higher pH levels (Aronson et al., 1982; Grinstein et al., 1984). Activation of an internal allosteric modifier site on the Na^+-H^+ antiporter by $[H^+]_i$ increases the level to which pH_i must rise before the exchanger turns off (Moolenaar, 1986). Phosphorylation of a serine residue on the cytoplasmic domain of the transporter may also produce an alkaline shift in pH_i dependence of transporter function (Grinstein et al., 1992; Wakabayashi et al., 1992). The average alkaline pH_i attained in the experiments reported here ranged from 7.77 (17 nM $[H^+]_i$) at the end of the 60 min initial observation period (Fig. 1*A*) to 7.15 (71 nM $[H^+]_i$) in freshly loaded neurons 0.5 hr after GLU exposure (Fig. 5). These values are in the range of previously reported pH levels from other tissues (7.4–7.5, Grinstein and Rothstein, 1986). Manipulations effecting $[Ca^{2+}]_i$ did not consistently alter the steady state rate of recovery from acidification (Table 1), in agreement with the absence of direct regulation of the Na^+-H^+ antiporter by internal calcium (Grinstein et al., 1985b).

After attaining the slightly alkaline pH_i levels, $[H^+]_i$ in GLU-exposed neurons began to return to initial basal levels. The gradual removal of H^+ from the internal regulatory site on the Na^+-H^+ antiporter should restore the original $[H^+]_i$. However, $[H^+]_i$ homeostasis was not restored and $[H^+]_i$ continued to climb gradually over the course of hours. The cumulative increase in $[H^+]_i$ may reflect an increase in glycolytic metabolism and lactate production or a decline in cellular ability to buffer H^+. The Na^+-H^+ antiporter remained functional in this time period since an additional GLU challenge produced the characteristic secondary rebound recovery. The rate at which the transporter was able to extrude H^+ was, however, reduced in the hours following GLU pretreatment, since recovery from an additional GLU-induced acid load was slower than in nonpretreated cultures (Table 1). Since hydrogen effluxes increase with increasing acidity (Grinstein and Rothstein, 1986), the decline in recovery rate cannot be attributable to the lowered pH_i at these extended times.

Operation of the Na^+-H^+ antiporter depends upon the Na gradient but metabolic energy may contribute to its pumping ability and modulation by $[H^+]_i$ (Cassel et al., 1986; Grinstein and Rothstein, 1986; Weissberg et al., 1989; Wakabayashi et al., 1992). With reduction of cellular ATP, the exchanger decreases its affinity for hydrogen at the internal regulatory site (Cassel et al., 1986; Wakabayashi et al., 1992). Therefore, the decline in overall neuronal metabolism several hours after GLU exposure (Raley-Susman et al., 1992b) would indirectly contribute to the observed reduction of antiporter activity. If Na/K-ATPase activity were compromised, a decline in the Na gradient, and hence rate of hydrogen removal, might be expected. Indeed, following anoxic exposure in hippocampal slices, intracellular potassium concentrations fall and sodium concentrations rise, consistent with a metabolic decrease in Na/K-ATPase activity (Kass and Lipton, 1982).

Alternatively, the decline in Na^+-H^+ antiporter activity may be attributable to dephosphorylation of an internal regulatory site or regulatory protein. Indirect evidence suggests that protein kinase C may stimulate the antiporter via a phosphorylation-dependent regulatory site in neuroblastoma cells and lymphocytes (Moolenaar et al., 1984; Grinstein et al., 1985a). Regulatory phosphorylation sites have also been implicated by antiporter sensitivity to calmodulin antagonists in cardiac ventriculocytes (Weissberg et al., 1989). With the loss of high-energy phosphate sources that accompanies excitotoxic damage (Kass and Lipton, 1982; Rothman et al., 1987), this regulatory site may become dephosphorylated, resulting in a slower rate of hydrogen extrusion. If the antiporter became unable to keep up with the normal hydrogen influx, a gradual intracellular acidification could ensue.

pH_i and excitotoxicity

The observed changes in $[H^+]_i$ could contribute to excitotoxic injury in several ways. The initial increase in $[H^+]_i$ and/or the hour or so of elevated $[OH^-]$ could initiate intracellular production of free radical species, leading to eventual neuronal death (Kogure et al., 1985; Monyer et al., 1990; Agardh et al., 1991). Since internal acidification of synaptosomes results in calcium-independent release of neurotransmitter (Drapeau and Nachshen, 1988), it is possible that GLU could be released from synaptic pools during this transient decrease in pH_i, further compounding excitotoxic damage.

However, the acidification was not prolonged nor was the secondary alkalinization extensive. Moreover, similar initial perturbations produced by high potassium or sodium replacement were not toxic. During ischemia, brain pH_i falls rapidly and is followed by a secondary alkalosis during reperfusion (von Hanwehr et al., 1986; Silver and Erecinska, 1992). Anoxic brain slices similarly exhibited immediate acidification followed by rebound alkalinization (Pirttila and Kauppinen, 1992). In both *in vivo* and *in vitro* ischemic models, recovery of pH_i was notably slow (Pirttila and Kauppinen, 1992; Silver and Erecinska, 1992). Prolonged intracellular acidification has been demonstrated to cause neuronal death, in the absence of GLU exposure (Tombaugh and Sapolsky, 1990; Nedergaard et al., 1991). While the absolute levels of H^+ accumulation reported here were less than those previously reported to be toxic, they were recorded from neurons still surviving at prolonged times after GLU exposure. The $[H^+]_i$ achieved in hippocampal neurons at the time of death may be even greater.

Excitotoxic neuronal death is generally thought to be mediated by the influx of calcium during excitatory amino acid overstimulation (Choi, 1990). The rise in $[Ca^{2+}]_i$ persists for about an hour after an insult comparable to that used in the present experiments. Yet calcium homeostasis is restored and remains stable in neurons surviving hours after the insult (Randall and Thayer, 1992; Dubinsky, 1993b). In contrast, $[H^+]_i$ homeostasis appears to be permanently disrupted following toxic insult. Cellular damage or death could result at any time from abnormal pH_i regulation. Indeed, the progressive increase in $[H^+]_i$ in the hours following an excitotoxic insult may act synergistically with other calcium-mediated processes to potentiate neuronal death.

References

Abercrombie RF, Hart CE (1986) Calcium and proton buffering and diffusion in isolated cytoplasm from *Myxicola* axons. Am J Physiol 250:C391–C405.

Agardh CD, Zhang H, Smith M-L, Siesjo BK (1991) Free radical production and ischemic brain damage influence of postischemic oxygen tension. Int J Dev Neurosci 9:127–138.

Ahmed Z, Connor JA (1980) Intracellular pH changes induced by

calcium influx during electrical activity in molluscan neurons. J Gen Physiol 75:403–426.
Aronson PS, Bounds SE (1980) Harmaline inhibition of Na-dependent transport in renal microvillus membrane vesicles. Am J Physiol 238: F210–F217.
Aronson PS, Nee J, Suhm MA (1982) Modifier role of internal H^+ in activating the Na^+/H^+ exchanger in renal microvillus membrane vesicles. Nature 299:161–163.
Balkovetz DF, Leibach FH, Mahesh VB, Devoe LD, Cragoe EJ Jr, Ganapathy V (1986) Na^+-H^+ exchanger of human placental brush border membrane: identification and characterization. Am J Physiol 251:C852–C860.
Barish ME, Baud C (1984) A voltage-gated hydrogen ion current in the oocyte membrane of the axolotl, *Ambystoma*. J Physiol (Lond) 352:243–263.
Boron WF, McCormick WC, Roos A (1981) pH regulation in barnacle muscle fibers: dependence on extracellular sodium and bicarbonate. Am J Physiol 240:C80–C89.
Byerly L, Meech R, Moody W Jr (1984) Rapidly activating hydrogen ion currents in perfused neurones of the snail, *Lymnaea stagnalis*. J Physiol (Lond) 351:199–216.
Cassel D, Katz M, Rotman M (1986) Depletion of cellular ATP inhibits Na^+/H^+ antiport in cultured human cells. J Biol Chem 261: 5460–5466.
Chen JCT, Chesler M (1991) Extracellular alkalinization evoked by GABA and its relationship to activity-dependent pH shifts in turtle cerebellum. J Physiol (Lond) 442:431–446.
Chen JCT, Chesler M (1992a) Modulation of extracellular pH by glutamate and GABA in rat hippocampal slices. J Neurophysiol 67: 29–36.
Chen JCT, Chesler M (1992b) Extracellular alkaline shifts in rat hippocampal slice are mediated by NMDA and non-NMDA receptors. J Neurophysiol 68:342–344.
Chesler M, Chan CY (1988) Stimulus-induced extracellular pH transients in the *in vitro* turtle cerebellum. Neuroscience 27:941–948.
Chesler M, Kaila K (1992) Modulation of pH by neuronal activity. Trends Neurosci 15:396–402.
Chesler M, Rice ME (1991) Extracellular alkaline-acid pH shifts evoked by iontophoresis of glutamate and aspartate in turtle cerebellum. Neuroscience 41:257–267.
Choi DW (1990) Cerebral hypoxia: some new approaches and unanswered questions. J Neurosci 10:2493–2501.
Choi DW, Maulucci-Gedde M, Kriegstein AR (1987) Glutamate neurotoxicity in cortical cell culture. J Neurosci 7:357–368.
Ciardo A, Meldolesi J (1991) Regulation of intracellular calcium in cerebellar granule neurons: effects of depolarization and of glutamatergic and cholinergic stimulation. J Neurochem 56:184–191.
Connor JA, Wadman WJ, Hockberger E, Wong RKS (1988) Sustained dendritic gradients of Ca^{2+} induced by excitatory amino acids in CA1 hippocampal neurons. Science 240:649–653.
Decoursey TE (1991) Hydrogen ion currents in rat alveolar epithelial cells. Biophys J 60:1243–1253.
De Erausquin GA, Manev H, Guidotti A, Costa E, Brooker G (1990) Gangliosides normalize distorted single-cell intracellular free Ca^{2+} dynamics after toxic doses of glutamate in cerebellar granule cells. Proc Natl Acad Sci USA 87:8017–8021.
Deitmer JW, Schlue WR (1987) The regulation of intracellular pH by identified glial cells and neurons in the central nervous system of the leech. J Physiol (Lond) 388:261–283.
Dickens CJ, Gillespie JI, Greenwell JR (1989) Interactions between intracellular pH and calcium in single mouse neuroblastoma (N2A) and rat pheochromocytoma cells (PC12). Q J Exp Physiol 74:671–679.
Drapeau P, Nachshen DA (1988) Effects of lowering extracellular and cytosolic pH on calcium fluxes, cytosolic calcium levels, and transmitter release in presynaptic nerve terminals isolated from rat brain. J Gen Physiol 91:305–315.
Dubinsky JM (1989) Development of inhibitory synapses among striatal neurons *in vitro*. J Neurosci 9:3955–3965.
Dubinsky JM (1993a) Effects of calcium chelators on intracellular calcium and excitotoxicity. Neurosci Lett 150:129–132.
Dubinsky JM (1993b) Intracellular calcium levels during the period of delayed excitotoxicity. J Neurosci 13:623–631.
Dubinsky JM, Rothman SM (1991) Intracellular calcium concentrations during "chemical hypoxia" and excitotoxic neuronal injury. J Neurosci 11:2545–2551.
Enders W, Ballanyi K, Serve G, Grafe P (1986) Excitatory amino acids and intracellular pH in motoneurons of the isolated frog spinal cord. Neurosci Lett 72:54–58.
Furuya S, Ohmori H, Shigemoto T, Sugiyama H (1989) Intracellular calcium mobilization triggered by a glutamate receptor in rat cultured hippocampal cells. J Physiol (Lond) 414:539–548.
Gilbertson TA, Scobey R, Wilson M (1991) Permeation of calcium ions through non-NMDA glutamate channels in retinal bipolar cells. Science 251:1613–1615.
Glaum SR, Scholz WK, Miller RJ (1990) Acute- and long-term glutamate-mediated regulation of $[Ca^{++}]_i$ in rat hippocampal pyramidal neurons *in vitro*. J Pharmacol Exp Ther 253:1293–1302.
Grinstein S, Rothstein A (1986) Mechanism of regulation of the Na^+/H^+ exchanger. J Membr Biol 90:1–12.
Grinstein S, Goetz JD, Rothstein A (1984) 22Na fluxes in thymic lymphocytes. II. Amiloride-sensitive Na/H exchange pathway; reversibility of transport and asymmetry of the modifier site. J Gen Physiol 84:585–600.
Grinstein S, Cohen S, Goetz JD, Rothstein A (1985a) Osmotic and phorbol ester-induced activation of Na^+/H^+ exchange: possible role of protein phosphorylation in lymphocyte volume regulation. J Cell Biol 101:269–276.
Grinstein S, Rothstein A, Cohen S (1985b) Mechanism of osmotic activation of Na^+/H^+ exchange in rat thymic lymphocytes. J Gen Physiol 85:765–787.
Grinstein S, Woodside M, Sardet C, Pouyssegur J, Rotin D (1992) Activation of the Na^+/H^+ antiporter during cell volume regulation. Evidence for a phosphorylation-independent mechanism. J Biol Chem 267:23823–23828.
Iino M, Ozawa S, Tsuzuki K (1990) Permeation of calcium through excitatory amino acid receptor channels in cultured rat hippocampal neurones. J Physiol (Lond) 424:151–165.
Irwin RP, Paul SM (1992) Glutamate exposure rapidly decreases intracellular pH in rat hippocampal neurons in culture. Soc Neurosci Abstr 18:257.
James-Kracke MR (1992) Quick and accurate method to convert BCECF fluorescence to pH_i: calibration in three different types of cell preparations. J Cell Physiol 151:596–603.
Jarolimek W, Misgeld U, Lux HD (1989) Activity dependent alkaline and acid transients in guinea pig hippocampal slices. Brain Res 505: 225–232.
Kaila K, Paalasma P, Taira T, Voipio J (1992) pH transients due to monosynaptic activation of $GABA_A$ receptors in rat hippocampal slices. Neuroreport 3:105–108.
Kass IS, Lipton P (1982) Mechanisms involved in irreversible anoxic damage to the *in vitro* rat hippocampal slice. J Physiol (Lond) 332: 459–472.
Katsura K, Ekholm A, Asplund B, Siesjo BK (1991) Extracellular pH in the brain during ischemia: relationship to the severity of lactic acidosis. J Cereb Blood Flow Metab 11:597–599.
Koch RA, Barish ME (1991) Sodium-calcium exchange in cultured embryonic mouse hippocampal neurons. J Cell Biol 115:22a.
Kogure K, Arai H, Abe K, Nakano M (1985) Free radical damage of the brain following ischemia. Prog Brain Res 63:237–259.
Kraig RP, Chesler M (1990) Astrocytic acidosis in hyperglycemic and complete ischemia. J Cereb Blood Flow Metab 10:104–114.
Kraig RP, Pulsinelli WA, Plum F (1986) Carbonic acid buffer changes during complete brain ischemia. Am J Physiol 250:R348–R357.
MacDermott AB, Dale N (1987) Receptors, ion channels and synaptic potentials underlying the integrative actions of excitatory amino acids. Trends Neurosci 10:280–284.
Manev H, Favaron M, Guidotti A, Costa E (1989) Delayed increase of Ca^{2+} influx elicited by glutamate: role in neuronal death. Mol Pharmacol 36:106–112.
Meech RW, Thomas RC (1977) The effect of calcium injection on the intracellular sodium and pH of snail neurones. J Physiol (Lond) 265: 867–879.
Michaels RL, Rothman SM (1990) Glutamate neurotoxicity *in vitro*: antagonist pharmacology and intracellular calcium concentrations. J Neurosci 10:283–292.
Monyer H, Hartley M, Choi DW (1990) 21-Aminosteroids attenuate excitotoxic neuronal injury in cortical cell cultures. Neuron 5:121–126.
Moolenaar WH (1986) Effects of growth factors on intracellular pH regulation. Annu Rev Physiol 48:363–376.
Moolenaar WH, Tsien RY, van der Saag PT, de Laat SW (1984) $Na^+/$

H^+ exchange and cytoplasmic pH in the action of growth factors in human fibroblasts. Nature 304:645–648.

Mozhayeva GN, Naumov AP (1983) The permeability of sodium channels to hydrogen ions in nerve fibres. Pfluegers Arch 396:163–173.

Murphy SN, Miller RJ (1989) Two distinct quisqualate receptors regulate Ca^{2+} homeostasis in hippocampal neurons *in vitro*. Mol Pharmacol 35:671–680.

Murphy SN, Thayer SA, Miller RJ (1987) The effects of excitatory amino acids on intracellular calcium in single mouse striatal neurons *in vitro*. J Neurosci 7:4145–4158.

Nachshen DA, Drapeau P (1988) The regulation of cytosolic pH in isolated presynaptic nerve terminals from rat brain. J Gen Physiol 91:289–303.

Nedergaard M, Goldman SA, Desai S, Pulsinelli WA (1991) Acid-induced death in neurons and glia. J Neurosci 11:2489–2497.

Ogura A, Miyamato M, Kudo Y (1988) Neuronal death *in vitro*: parallelism between survivability of hippocampal neurons and sustained elevation of cytosolic Ca^{2+} after exposure to glutamate receptor agonist. Exp Brain Res 73:447–458.

Orrenius S, McConkey DJ, Jones DP, Nicotera P (1988) Ca^{2+}-activated mechanisms in toxicity and programmed cell death. ISI Atlas Sci 319–324.

Pirttila TRM, Kauppinen RA (1992) Recovery of intracellular pH in cortical brain slices following anoxia studied by nuclear magnetic resonance spectroscopy: role of lactate removal, extracellular sodium and sodium/hydrogen exchange. Neuroscience 47:155–164.

Raley-Susman KM, Cragoe EJ Jr, Sapolsky RM, Kopito RR (1991) Regulation of intracellular pH in cultured hippocampal neurons by an amiloride-insensitive Na^+/H^+ exchanger. J Biol Chem 266:2739–2745.

Raley-Susman KM, Kopito RR, Sapolsky RM (1992a) NMDA exposure reduces intracellular pH in cultured hippocampal neurons. Soc Neurosci Abstr 18:803.

Raley-Susman KM, Miller KR, Owicki JC, Sapolsky RM (1992b) Effects of excitotoxin exposure on metabolic rate of primary hippocampal cultures: application of silicon microphysiometry to neurobiology. J Neurosci 12:773–780.

Randall RD, Thayer SA (1992) Glutamate-induced calcium transient triggers delayed calcium overload and neurotoxicity in rat hippocampal neurons. J Neurosci 12:1882–1895.

Rothman SM, Thurston JH, Hauhart RE (1987) Delayed neurotoxicity of excitatory amino acids *in vitro*. Neuroscience 22:471–480.

Siesjo BK (1992) Pathophysiology and treatment of focal cerebral ischemia. II. Mechanisms of damage and treatment. J Neurosurg 77: 337–354.

Silver IA, Erecinska M (1992) Ion homeostasis in rat brain *in vivo*: intra- and extracellular $[Ca^{2+}]$ and $[H^+]$ in the hippocampus during recovery from short-term, transient ischemia. J Cereb Blood Flow Metab 12:759–772.

Thomas JA, Buchsbaum RN, Zimniak A, Racker E (1979) Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated *in situ*. Biochemistry 18:2210–2218.

Thomas RC, Meech RW (1982) Hydrogen ion currents and intracellular pH in depolarized voltage-clamped snail neurones. Nature 299: 826–828.

Tolkovsky AM, Richards CD (1987) Na^+/Ca^{++} exchange is the major mechanism of pH regulation in cultured sympathetic neurons: measurements in single cell bodies and neurites using a fluorescent pH indicator. Neuroscience 22:1093–1102.

Tombaugh GC, Sapolsky RM (1990) Mechanistic distinctions between excitotoxic and acidotic hippocampal damage in an *in vitro* model of ischemia. J Cereb Blood Flow Metab 10:527–535.

von Hanwehr R, Smith MJ, Siesjo BK (1986) Extra- and intracellular pH during near-complete forebrain ischemia in the rat. J Neurochem 46:331–339.

Wahl P, Schousboe A, Honore T, Drejer J (1989) Glutamate-induced increase in intracellular Ca^{2+} in cerebral cortex neurons is transient in immature cells but permanent in mature cells. J Neurochem 53: 1316–1319.

Wakabayashi S, Sardet C, Fafournoux P, Counillon L, Meloche S, Pages G, Pouyssegur J (1992) Structure function of the growth factor-activatable Na^+/H^+ exchanger (NHE1). Rev Physiol Biochem Pharmacol 119:157–186.

Weissberg PL, Little PJ, Cragoe EJ Jr, Bobik A (1989) The pH of spontaneously beating cultured rat heart cells is regulated by an ATP-calmodulin-dependent Na^+/H^+ antiport. Circ Res 64:676–685.

Yamada KA, Dubinsky JM, Rothman SM (1989) Quantitative physiological characterization of a quinoxalinedione non-NMDA receptor antagonist. J Neurosci 9:3230–3236.