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N-methyl-D-aspartate Induces a Rapid, Reversible, and Calcium-Dependent Intracellular Acidosis in Cultured Fetal Rat Hippocampal Neurons

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The ability of NMDA to alter intracellular pH (pH_i) was studied in fetal rat hippocampal neurons and glia using the pH-sensitive fluorescent indicator 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF). Brief exposure (60 sec) of hippocampal neurons to NMDA (2.5–250 μM) results in a rapid, and in most cells reversible, reduction in pH_i, with full recovery to baseline pH_i values taking several minutes following removal of NMDA. In contrast, little or no change in pH_i was observed in glial cells exposed to these same concentrations of NMDA. The NMDA-induced acidification of neurons was concentration and time dependent, with an EC_{50} of 39 μM and E_{max} (ΔpH) of −0.53. More prolonged exposure to NMDA (≥10 min) resulted in a more prolonged reduction in pH_i values over the ensuing 20 min observation period. The intracellular acidification resulting from NMDA exposure of hippocampal neurons was blocked by the NMDA receptor antagonist 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP). Moreover, removal of extracellular Ca^{2+} eliminated both the selective NMDA-induced elevation in $[Ca^{2+}]_i$ and the reduction in pH_i, indicating that Ca^{2+} influx may be required for the decrease in pH_i induced by NMDA receptor activation. Finally, the NMDA-induced reduction in pH_i was not significantly attenuated when extracellular $[H^+]$ was decreased by increasing extracellular pH to 8.0. The latter suggests that an intracellular source of H^+ is responsible for the NMDA-induced reduction in neuronal pH_i. The reduction in neuronal pH_i induced by NMDA receptor activation may mediate some of the physiological and (or) pathophysiological actions of glutamate.

[Key words: NMDA, receptor, intracellular pH, acidosis, calcium, neurotoxicity]

Glutamic acid, the major excitatory neurotransmitter in the CNS, is involved in many physiologically important CNS functions, including learning and memory and neuronal plasticity (Mayer and Westbrook, 1987; Cotman et al., 1988). Glutamate and its various receptors may also play a role in the pathogenesis of neurodegenerative disorders such as Alzheimer's disease, amyotrophic lateral sclerosis, and Huntington's disease (Beal, 1992) as well as the delayed neuronal death that occurs as a result of trauma or cerebral ischemia (Choi, 1992). In fact, prolonged or excessive glutamate exposure can be toxic to many populations of neurons and it appears that certain neurons (e.g., CA1 neurons of the hippocampus) are more vulnerable to both glutamate toxicity (excitotoxicity) (Mattson et al., 1989) and ischemic insult (Benveniste, 1991). The "excitotoxic" action of glutamate, at least in primary cultures of fetal rat or mouse neurons, is mediated primarily by the NMDA subtype of glutamate receptor, although other glutamate receptors may play a role (Choi, 1992). Although the exact cellular mechanism(s) underlying the neurotoxicity induced by excessive NMDA receptor activation is unknown, there is considerable evidence that NMDA-induced excitotoxicity is mediated, in part, by a receptor-mediated rise in intracellular calcium ($[Ca^{2+}]_i$) (Mayer and Westbrook, 1987; Mattson et al., 1989; Benveniste, 1991; Choi, 1992). The rise in $[Ca^{2+}]_i$ induced by NMDA/glutamate is believed to initiate a cascade of as yet unknown intracellular events ultimately leading to neuronal death.

While there appear to be multiple homeostatic mechanisms that strictly regulate intracellular pH (pH_i) in most cells (Frelin et al., 1988), including neurons (Chesler, 1990), recent reports suggest that significant changes in pH_i can result from alterations in neuronal activity (Chesler and Kaila, 1992). Relatively small deviations in pH_i could potentially have profound effects on a variety of cellular functions, including the rate of protein synthesis (Chambard and Pouyssegur, 1986), formation of second and third messengers (Harwood and Hawthorne, 1969), as well as by altering the activity of certain metabolic enzymes (Trivedi and Danforth, 1966). Moreover, since the pH optima of many intracellular enzymes are not necessarily the same as those of the intracellular milieu, changes in pH_i may be involved in regulating enzyme activity.

Several recent reports (Tang et al., 1990; Traynelis and Cull-Candy, 1990; Vyklický et al., 1990) have demonstrated that changes in extracellular pH (pH_e) can markedly modulate NMDA receptor–mediated responses. In these experiments, increases in extracellular $[H^+]_i$ were shown to reduce NMDA receptor–mediated cation conductance dramatically in cultured fetal hippocampal neurons and cerebellar granule cells measured with patch-clamp techniques. Since changes in pH_e can effect NMDA receptor responses, we have now examined the actions of NMDA in altering pH_i in cultured fetal hippocampal neurons using the pH-sensitive fluorescent indicator 2′,7′-bis 2-(carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF). Our findings demonstrate that exposure of fetal rat hippocampal neurons to either subtoxic

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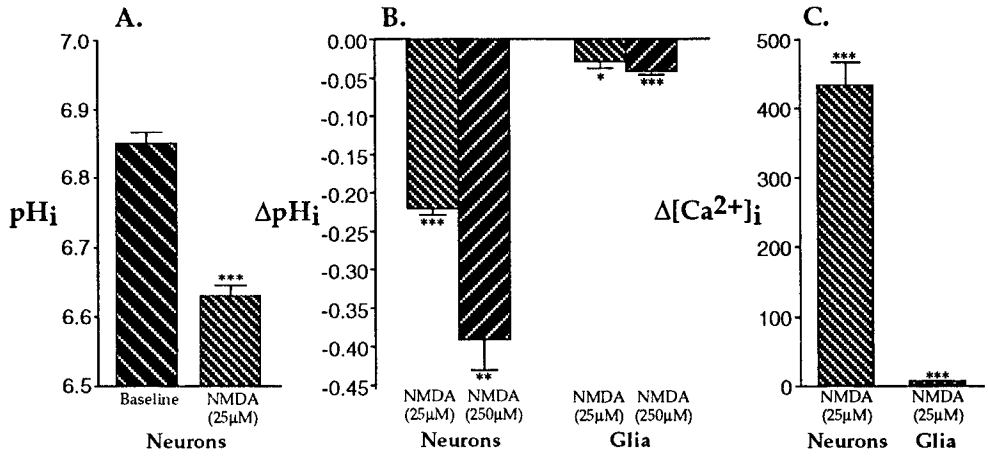

Figure 1. Brief exposure of fetal rat hippocampal neurons and glia to NMDA induces a marked elevation of $[Ca^{2+}]_i$ and reduction in pH_i of neurons with only minimal changes in $[Ca^{2+}]_i$ and pH_i of glia. Intracellular pH was measured using BCECF (see Materials and Methods). Intracellular Ca^{2+} was measured as described in Grynkiewicz et al. (1985) (see Materials and Methods for details). Data are from one to seven independent experiments, each with 5–17 cells, and represent the mean ± SEM for pH_i of neurons at baseline and following NMDA exposure (*A*), and the maximal change in $[Ca^{2+}]_i$ and pH_i from individual neurons and glia following NMDA exposure (*B and C*). *A,* Cultured fetal rat hippocampal neurons were exposed to NMDA (25 μM, $n = 73$) for 60 sec as described in Materials and Methods and Results. *B,* The pH_i of neurons exposed (60 sec) to NMDA (25 μM, $n = 73$; 250 μM, $n = 5$) and glia exposed (60 sec) to NMDA (25 μM, $n = 18$; 250 μM, $n = 15$). *C,* NMDA (25 μM) exposure (60 sec) results in a marked rise in $[Ca^{2+}]_i$ in neurons ($n = 48$) with only minimal elevations of $[Ca^{2+}]_i$ in glia ($n = 23$). *, $p \leq 0.005$; **, $p \leq 0.001$; ***, $p \leq 0.0001$.

or toxic concentrations of NMDA results in a time- and concentration-dependent intracellular acidosis.

These data have been presented in preliminary form (Irwin and Paul, 1992).

Materials and Methods

Tissue culture. Hippocampal neurons from 18–19-d-old Sprague–Dawley rat embryos were maintained in primary culture as described by Segal (1983). Briefly, hippocampal tissue was rapidly dissected and mechanically disrupted, and the cell suspension plated onto poly-L-lysine (Sigma, St. Louis, MO)–coated glass-bottom 35 mm culture dishes (MatTek, Ashland, MA) containing Modified Eagle's Medium with Earle's salts (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum, 10% horse serum, 6000 gm/liter glucose, and 2 mM glutamine. Cells were incubated in a humidified atmosphere containing 5% CO_2 and 95% air. Culture media lacking fetal calf serum but containing 5% horse serum were added 7 d after plating. All experiments were carried out using cells maintained 7–14 d *in vitro.*

Perfusion solutions. Cultures were washed three times with buffer containing (in mM) NaCl, 143; KCl, 2.5; HEPES, 10; $CaCl_2$, 1; $NaHCO_3$, 2.4; and glucose, 10 (adjusted to pH 7.4 with NaOH). The small concentration of HCO_3^- was added to the buffer to provide cells an external source of bicarbonate and to avoid the wide fluctuations of pH_e associated with high HCO_3^- buffers. Air containing 1% CO_2 was gently blown over the cells during experiments to prevent CO_2 loss from the buffer. When monitored, pH_e did not change significantly during the course of the experiment; that is, any changes were generally <0.05 pH units. Cells were incubated with BCECF acetoxymethyl ester (BCECF/AM) (1 μM) or fura-2/AM (2.5 μM) for 30–45 min in the dark at 37°C. Following the incubation period, cells were washed three times with buffer and allowed ≥15 min to complete hydrolysis of the ester. In each experiment, a culture dish containing neurons/glia was placed on the stage of an inverted microscope (Nikon Diaphot) and the cells continuously perfused with buffer at a rate of approximately 200 μl/min at 37°C. The perfusion device consisted of a 12-barrel array of tubes emptying into a common glass tip positioned approximately 1 mm from the cells being imaged. All solutions contained 2 μM glycine to saturate the strychnine-insensitive glycine site on the NMDA receptor–channel complex. In experiments carried out under calcium-free conditions, the perfusion media/solutions contained EGTA (20μM) to chelate trace amounts of residual calcium.

Quantitative microfluorimetric imaging. Intracellular pH (pH_i) and calcium ($[Ca^{2+}]_i$) were measured by fluorescence ratio imaging using BCECF as an indicator for pH (Rink et al., 1982) and fura-2 for Ca^{2+} (Grynkiewicz et al., 1985). The fluorescent indicator was excited using a xenon arc lamp with excitation wavelengths alternating with a filter wheel (Sutter Instrument Co., Novato, CA). Excitation of BCECF was at 495 and 440 nm, with emitted light monitored at 535 nm. Excitation of fura-2 was at 340 and 380 nm, with emitted light monitored at 510 nm. Neurons were visualized using phase-contrast microscopy and were easily identified by their characteristic morphology including prominent neurites and processes. Similarly, glial cells were also identified by their characteristic size, refractivity, and morphology. Cells that could not be unambiguously identified were excluded from analysis. Cell-derived fluorescent images were visualized using a 40×, 1.30 NA oil-immersion objective and intensified with a Videoscope KS1381 intensifier (Videoscope International, LTD, Washington, DC) before entering the camera (Dage-MTI, Michigan City, IN). Images were digitized and stored for subsequent analysis (Universal Imaging Co, West Chester, PA). Back-

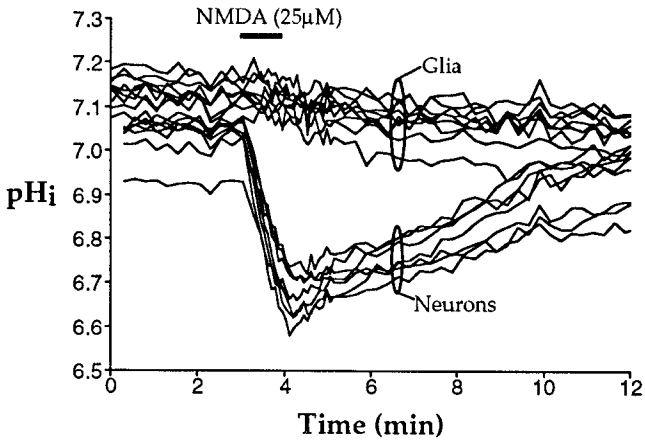

Figure 2. NMDA induces a rapid and reversible reduction in pH_i of neurons with little change in the pH_i of glia: representative experiment illustrating NMDA-induced changes in pH_i of fetal rat hippocampal neurons and glia. Neurons and glia were identified by phase-contrast microscopy on the basis of morphological criteria and exposed to NMDA as described in Materials and Methods. Each line represents the calculated pH_i of one cell. Note that, under these conditions following relatively brief (60 sec) exposure to NMDA, most neurons recover their baseline pH_i over approximately 10 min.

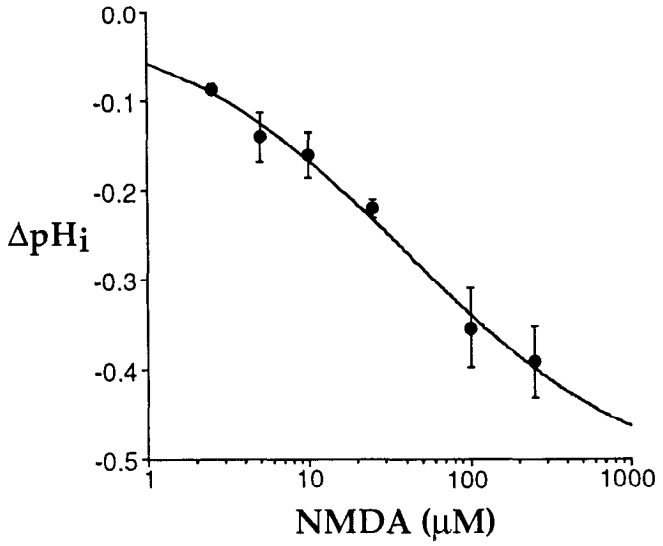

Figure 3. Concentration–response curve for NMDA-induced reduction in pH_i in fetal rat hippocampal neurons. Neurons were exposed to NMDA at various concentrations for 60 sec. Each point represents the mean ± SEM of the maximal change in pH_i from one to seven independent experiments with 5–17 neurons measured in each experiment. Intracellular pH was calculated as described in Materials and Methods and Figure 1. The data were fit according to the equation $\Delta pH_i = (E_{max}[NMDA]^n)/(EC_{50}{}^n + [NMDA]^n)$, where $E_{max} = -0.53$ pH units, $EC_{50} = 39\mu M$ and $n = 0.58$.

ground intensity was determined for each wavelength and gain setting, and subtracted from the signal for each cell. The background intensity never exceeded 10% of the signal.

In situ calibration of pH_i measured with BCECF was performed by exposing cells to nigericin (10 μM) along with high K^+ buffers (Thomas et al., 1979) ranging from pH 6.4 to 7.8 at 37°C. Following subtraction of background intensities at both excitation wavelengths, the ratio of intensities at 495 and 440 nm at each pH standard was fit by linear regression analysis. This equation was then used to calculate apparent pH_i. Calcium concentration was determined by the ratio method (Grynkiewicz et al., 1985). R_{max} and R_{min} were determined *in situ* (Irwin et al., 1992) using either Ca^{2+} (2 mM) or EGTA (3 mM) plus EGTA tetraacetoxymethyl ester (40 μM) in a high-K^+ buffer at 37°C and pH 7.2 with ionomycin (15 μM) and carbonyl cyanide *m*-chlorophenyl-hydrazone (10 μM). An apparent dissociation constant of 285 nM (Groden et al., 1991) was used for $[Ca^{2+}]_i$ calculations.

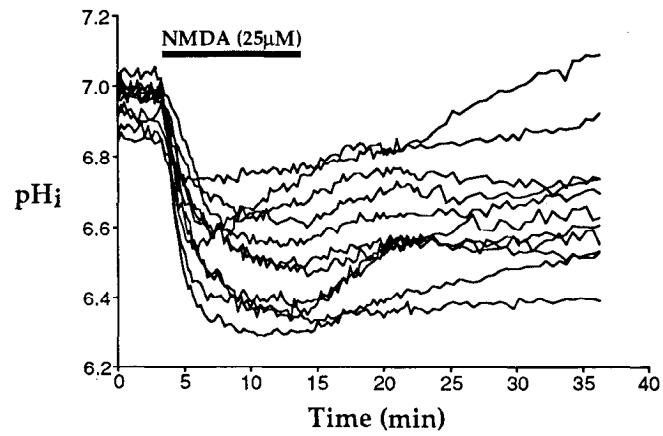

Figure 4. Longer exposure of hippocampal neurons to NMDA results in a marked reduction in pH_i with a more variable and delayed recovery. Fetal rat hippocampal neurons were exposed to NMDA (25 μM) for 10 min and pH_i measured as described in Figure 1 and Results. In the experiment illustrated, baseline pH_i was 6.95 ± 0.02, which was reduced to 6.51 ± 0.05 following NMDA exposure. NMDA exposure for 20 min gave similar results (see Results for mean value of experiments). Note the robust reduction in pH_i in some cells as compared to others. Also note the variable recovery over the ensuing 20 min observation period, with many cells never fully returning to their baseline pH_i values.

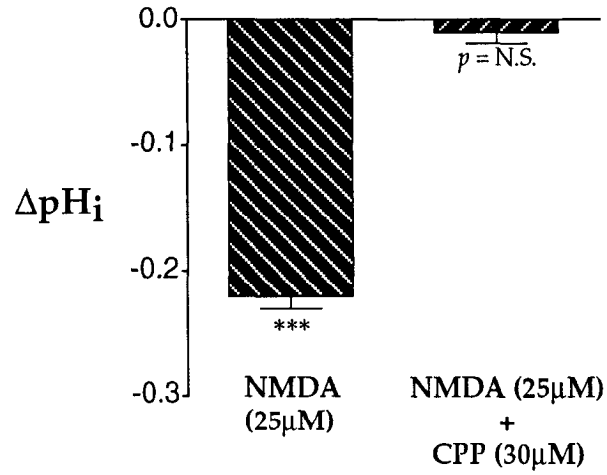

Figure 5. NMDA-induced reduction in pH_i is blocked by CPP. CPP was applied to cells 1–2 min prior to the application of NMDA + CPP. Exposure of CPP alone over this period of time had no significant effect on pH_i. See Results and Figure 1 for other details. Data represents the mean ± SEM of the change in pH_i of hippocampal neurons following exposure (60 sec) to NMDA (25μM, $n = 73$) or with NMDA (25 μM) plus CPP (30 μM; $n = 19$). *N.S.*, not significant; ***, $p \leq 0.0001$.

Materials and statistics. BCECF/AM, fura-2/AM, and EGTA/AM were from Molecular Probes (Eugene, OR). NMDA and 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) were from Research Biochemicals Incorporated (Natick, MA). Ionomycin was from Calbiochem (La Jolla, CA). All other chemicals were obtained from Sigma (St. Louis, MO). Data are presented as the mean ± SEM. Unless otherwise stated, statistical comparisons were made using the Student's *t* test with a significance level of <0.05. Curve fitting was performed using nonlinear regression analysis.

Results

Exposure to NMDA rapidly and reversibly reduces pH_i in hippocampal neurons

The "resting" pH_i of hippocampal neurons and glia was found to be 6.95 ± 0.01 (range, 6.52–7.52) ($n = 259$) and 7.10 ± 0.01 (range, 6.92–7.24) ($n = 33$), respectively. In a subset of hippocampal neurons, brief exposure (60 sec) to NMDA (25 μM) resulted in a reduction of 0.22 ± 0.01 pH units ($n = 73$, $p \leq 0.0001$ by paired *t* test) (Fig. 1*A*). Some cells had little or no response to NMDA while in others there was a rather robust (>0.4 pH units) reduction in pH_i. Following brief (≤60 sec) exposure to NMDA we observed recovery to near baseline pH_i values for most cells over a time interval of several minutes (Fig. 2). To determine whether the NMDA-induced reduction in pH_i occurs in non-neuronal cells, we measured pH_i of co-cultured glial cells that were identified on the basis of their characteristic morphology. In contrast to neurons, the exposure of glia to NMDA (25 and 250 μM) resulted in little or no change of pH_i (Figs. 1*B*, 2). As expected, glial cells showed little to no rise in $[Ca^{2+}]_i$ following NMDA exposure (Fig. 1*C*), while for neurons NMDA produced a more than fivefold increase in $[Ca^{2+}]_i$. The effect of NMDA in reducing pH_i of hippocampal neurons was dependent on NMDA concentration (2.5 μM–250 μM) (Fig. 3) and length of exposure (Fig. 4). Exposure of hippocampal neurons to NMDA (25 μM) for longer periods of time (10–20

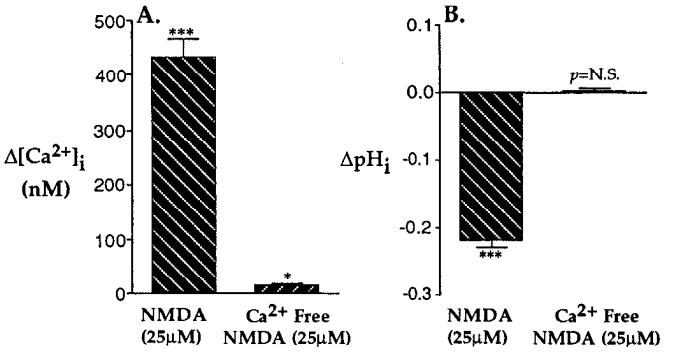

Figure 6. Removal of extracellular Ca^{2+} blocks the NMDA-induced rise in $[Ca^{2+}]_i$ and the reduction in pH_i in hippocampal neurons. See Materials and Methods for experimental details. *A*, In the presence of extracellular Ca^{2+}, NMDA (25 μM) induces a marked rise of $[Ca^{2+}]_i$ from a baseline value of 78 ± 7 nM to 511 ± 37 nM ($n = 48$). However, when external Ca^{2+} is removed, NMDA induces little change in $[Ca^{2+}]_i$ ($n = 25$). *B*, Similarly, NMDA induces a decrease in pH_i when external Ca^{2+} is present ($n = 73$), but fails to alter pH_i when external Ca^{2+} is removed ($n = 37$). *N.S., not significant*; *, $p \leq 0.005$; ***, $p \leq 0.0001$.

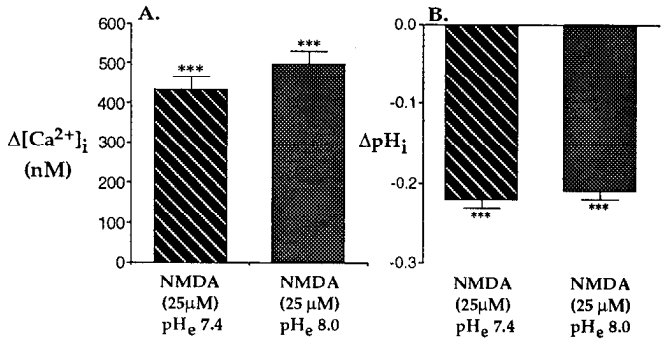

Figure 7. Increasing extracellular pH fails to alter the NMDA-induced rise in $[Ca^{2+}]_i$ and reduction in pH_i. Data are from several independent experiments and represent the mean ± SEM. *A*, Intracellular $[Ca^{2+}]$ was measured with fura-2 as described in Materials and Methods. Hippocampal neurons were exposed (60 sec) to NMDA (25 μM) at pH_e 7.4 ($n = 48$) or at pH_e 8.0 (buffer pH adjusted with NaOH, $n = 63$). The $\Delta[Ca^{2+}]_i$ between groups was not significantly different by ANOVA. *B*, As in *A* but showing change in pH_i, measured with BCECF, for pH_e 7.4 ($n = 73$) and 8.0 ($n = 43$). ΔpH_i between groups was not significantly different by ANOVA; ***, $p \leq 0.0001$.

min) resulted in a reduction in pH_i from 6.91 ± 0.03 to 6.50 ± 0.04 (10 and 20 min experiments combined), $n = 24$. While the pH_i of some neurons (≤25%) started to recover during NMDA exposure, the pH_i of most neurons did not begin recovery until the application of NMDA was terminated (Fig. 4). Additionally, following longer exposure to NMDA (10 or 20 min), we observed little or no recovery of pH_i in many neurons for the duration of the 20 min observation period (Fig. 4, data not shown). The effect of NMDA in reducing pH_i was blocked by the selective competitive NMDA receptor antagonist CPP (Fig. 5). These data confirm that activation of the NMDA subtype of glutamate receptor leads to a rapid concentration-dependent intracellular acidosis in hippocampal neurons.

The NMDA receptor–mediated reduction in pH_i requires extracellular Ca^{2+}

Since NMDA receptor–gated ion channels are permeable to Ca^{2+}, we examined whether Ca^{2+} conductance is involved in the NMDA receptor–mediated intracellular acidification response. Cells were exposed to Ca^{2+}-free buffer with a low concentration of EGTA (20 μM) for 1–2 min prior to the addition of NMDA. In the absence of external Ca^{2+}, the application of NMDA (25 μM) resulted in little or no change in pH_i in virtually all neurons examined (Fig. 6*B*). Additionally, we observed that in many neurons and glia, the removal of Ca^{2+} from solutions bathing the cells resulted in a gradual alkalosis (data not shown). Using the Ca^{2+} fluorescent probe fura-2 to measure $[Ca^{2+}]_i$, we observed that under these same experimental conditions removal of extracellular Ca^{2+} nearly eliminated the rise in $[Ca^{2+}]_i$ observed following NMDA exposure (Fig. 6*A*).

Reducing extracellular $[H^+]$ does not attenuate the NMDA-induced rise of intracellular $[H^+]$

To see if lowering extracellular $[H^+]$ attenuates the NMDA-induced reduction in pH_i we examined pH_i responses following NMDA exposure at a pH_e of 8.0 (which corresponds to a reduction in extracellular $[H^+]$ of ~75%) and compared the ΔpH_i to that observed at a pH_e of 7.4. The reduction in pH_i observed following NMDA exposure at pH_e of 8.0 was not significantly different from that observed at pH 7.4 (Fig. 7*B*). Moreover, the rise in $[Ca^{2+}]_i$ induced by NMDA at pH 8.0 was almost identical to that observed at pH 7.4 (Fig. 7*A*).

Discussion

In the present study, we measured both pH_i and $[Ca^{2+}]_i$ of fetal rat hippocampal neurons and glia maintained *in vitro* using microfluorimetric imaging with the pH-sensitive indicator BCECF (Rink et al., 1982) and the Ca^{2+}-sensitive indicator fura-2 (Grynkiewicz et al., 1985). The "resting" pH_i, and variability in pH_i, of fetal rat hippocampal neurons and glia observed in our experiments was similar to that previously reported by other investigators (Chesler, 1990; Raley-Susman et al., 1991). We have observed that exposure of cultured fetal rat hippocampal neurons to both subtoxic or toxic concentrations of NMDA induces a relatively rapid and concentration-dependent intracellular acidification. By contrast, in cocultured glial cells (which lack NMDA receptors) we observed a small, although statistically significant, change in pH_i following exposure to NMDA (25 and 250 μM). The pharmacologic implications of this minor effect of NMDA on glia pH_i remain unclear at the present time. The NMDA-induced reduction in neuronal pH_i is blocked by the NMDA receptor antagonist CPP. Removal of extracellular Ca^{2+} during exposure to NMDA blocks both the cytosolic rise in Ca^{2+} and H^+, suggesting an important role for Ca^{2+} in the neuronal acidification response. Finally, decreasing external $[H^+]$ by ~75% did not significantly attenuate the NMDA-induced reduction in neuronal pH_i. Although pH_e has been shown to alter the sensitivity of NMDA receptors, the decrease in $[H^+]_e$ resulting from an increase in pH_e to 8.0 would correspond to only a small augmentation (~10%) in NMDA receptor–mediated responses (Tang et al., 1990; Vyklický et al., 1990). We should emphasize that our experiments were carried out using HEPES buffer (10 mM) containing bicarbonate (2.4 mM). Experiments are currently underway to determine the effects of NMDA (and other excitatory amino acids) on neuronal pH_i using different buffering conditions and higher bicarbonate concentrations (e.g., 24 mM).

The reduction of pH_i we observed in cultured hippocampal neurons following NMDA exposure is similar to that previously observed by Endres et al. (1986) in frog spinal cord motoneurons

using pH-sensitive microelectrodes. The decrease in pH_i reported by these investigators corresponds to a triphasic response of pH_e, with a small acidification during the first minute of NMDA exposure followed by an alkaline pH transient and eventually a long-lasting acidification. Similarly, Jarolimek et al. (1989) reported that pH_e is transiently increased following glutamate application to hippocampal slices, which is associated with an increase in extracellular $[K^+]$. The latter suggests that extracellular alkaline transients may be caused by proton influx through cationic channels. If the NMDA-induced intracellular acidification observed in our experiments, however, was due to H^+ entering neurons via NMDA receptor–associated cation channels, then the removal of external Ca^{2+} should fail to affect the reduction in pH_i induced by NMDA. Similarly, the influx of Na^+ following application of NMDA, in the absence of external Ca^{2+}, suggests that Na^+ influx per se is not likely to be critical to the reduction in pH_i. Protons, however, could conceivably enter the cell as a consequence of other mechanisms involved with the removal of intracellular Ca^{2+} from the cell during or following glutamate exposure (i.e., via Na^+/Ca^{2+} exchange). Alternatively, Ca^{2+} could also compete for H^+ binding sites or exchange for H^+ in the mitochondria or other intracellular organelles (Meech and Thomas, 1977). Since lowering extracellular H^+ by ~75% did not significantly attenuate the NMDA-induced reduction in pH_i, an intracellular source of protons seems most likely. However, we would caution that additional interpretations of these data are possible, including effects of pH_e on possible outer membrane H^+ transport mechanisms. It is also plausible that the rise in $[Ca^{2+}]_i$ induced by NMDA exposure results in an increase in neuronal energy metabolism/utilization resulting in ATP breakdown or depletion and thus an intracellular acidosis. However, the rather rapid (<30 sec) reduction in pH_i following NMDA exposure would argue against this. Nonetheless, the exact source of H^+ responsible for the reduction in pH_i induced by NMDA exposure is unknown and currently being investigated.

Electrical stimulation of neurons has previously been reported to result in a reduction in pH_i. The electrically induced acidification of Molluscan neurons was also found to follow the rise in Ca^{2+} influx by ~200–400 msec. Further, the removal of extracellular Ca^{2+} eliminated the electrically induced acidification (Ahmed and Connor, 1980). Direct intracellular injection of Ca^{2+} into snail neurons has also been reported to cause a rapid decrease in pH_i that was directly proportional to the amount of Ca^{2+} injected (Meech and Thomas, 1977). In this regard, Thomas (1989) found that in snail neurons H^+ currents are unidirectional (i.e., only flowing out of the cell). The latter again suggests that the electrically induced reduction in pH_i is not a consequence of H^+ entering the neuron, but rather that Ca^{2+} (entering the cell following depolarization) displaces H^+ from internal binding or sequestration sites.

Hypoxia and/or glucose depletion in hippocampal slices has also been shown to result in a decrease in pH_i within 5 min of exposure (Fujiwara et al., 1992). Furthermore, brain ischemia has been reported to lower brain pH_e and pH_i (Nedergaard et al., 1991). Taken together with our data and others, it is possible that the reduction in pH_i induced by hypoxia/ischemia may be mediated, in part, by glutamate released during ischemia (Benveniste et al., 1989).

What cellular effects, if any, would result from the reduction in pH_i of the magnitude we observed (~0.5 pH units) following exposure of hippocampal neurons to NMDA? Although the exact consequences of the NMDA receptor–induced reduction in pH_i we have observed are unknown, the activity of certain important metabolic enzymes show a marked sensitivity to changes in pH. For example, muscle phosphofructokinase (PFK) (a key regulatory enzyme in glycolysis) is extremely sensitive to changes in pH (Trivedi and Danforth, 1966). Whereas brain PFK is somewhat less sensitive to changes in pH than the muscle enzyme (Bazaes and Kemp, 1990), brain PFK would, nonetheless, be profoundly inhibited by the reduction in pH_i of the magnitude we observed following NMDA exposure. At physiologically relevant substrate concentrations, for example, brain PFK was reported to be inhibited by >80% at a pH 6.7 (Bazaes and Kemp, 1990). The marked pH dependence of muscle and brain PFK suggests that changes in pH_i may inhibit glycolysis. We postulate that a sustained reduction in neuronal pH_i induced by NMDA (or other excitotoxins) might therefore result in ATP depletion and cell death due to loss of ATP-dependent functions (i.e., Na^+ K^+ ATPase activity). Thus, the reduction in pH_i observed in cultured hippocampal neurons following NMDA exposure, which appears to be mediated by a receptor-mediated rise in $[Ca^{2+}]_i$, may be an important cellular "event" in the intracellular cascade leading to "excitotoxic" death of these neurons. In fact, exposure of neurons to NMDA for more prolonged periods of time (≥5 min) results in widespread neuronal death (Choi, 1992). Further, removal of extracellular Ca^{2+} blocks both the reduction in pH_i induced by NMDA and the excitotoxicity induced by glutamate (Choi, 1988). Additionally, it is conceivable that, following NMDA receptor–induced acidification, H^+ could move out of the neuron and alter NMDA receptor activity via the proposed external H^+ site on the NMDA receptor complex. The latter may constitute a negative feedback loop to reduce receptor activation. The ability (or inability) of neurons to restore normal pH_i following exposure to excitatory amino acids may therefore determine their susceptibility to excitotoxins. This hypothesis is currently being tested.

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