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Perturbation of Intracellular Calcium and Hydrogen Ion Regulation in Cultured Mouse Hippocampal Neurons by Reduction of the Sodium Ion Concentration Gradient

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Na^+–Ca^{2+} exchange has been identified as a mechanism for regulation of intracellular Ca ion concentration ($[Ca^{2+}]_i$) in neurons of invertebrates and vertebrates, but for mammalian central neurons its role in restoration of resting $[Ca^{2+}]_i$ after transient increases induced by stimulation has been less clear. We have examined the recovery of $[Ca^{2+}]_i$ following K^+ depolarization and glutamate receptor activation of cultured mouse hippocampal neurons using the Ca^{2+}-sensitive dye Fura-2. Reduction of the transmembrane Na^+ gradient by removal of external Na^+ slowed the recovery of neurons from imposed Ca^{2+} loads. We observed that $[Ca^{2+}]_i$ regulation was disrupted more severely when *N*-methyl-D-glucamine (*N*-MG), Tris, or choline rather than Li^+ replaced external Na^+. Additional disruption of intracellular pH regulation by substitutes other than Li^+ may account for this difference. Measurement of $[Ca^{2+}]_i$ and $[H^+]_i$ (using the H^+-sensitive dye BCECF) during glutamate receptor activation indicated that Ca^{2+} influx resulted in production of intracellular H^+, and that Li^+ but not *N*-MG could prevent cytoplasmic acidification on removal of external Na^+. We also observed that intracellular acidification alone was sufficient to slow recovery from Ca^{2+} load. We conclude, therefore, that Na^+–Ca^{2+} exchange contributes to recovery of $[Ca^{2+}]_i$ after stimulation leading to Ca^{2+} entry into hippocampal neurons, and that Na^+–H^+ exchange limits the acidification (and secondary increase in $[Ca^{2+}]_i$) that accompanies Ca^{2+} influx. We suggest that because both Na^+–Ca^{2+} and Na^+–H^+ exchangers will be compromised during ischemia and hypoglycemia, increased intracellular H^+ may synergize with cytoplasmic Ca^{2+} to potentiate excitotoxic neuronal death.

[Key words: sodium–calcium exchange, sodium–hydrogen exchange, Fura-2, BCECF, intracellular Ca^{2+} regulation, intracellular H^+ regulation, hippocampal neurons, mouse]

The processes responsible for restoring intracellular calcium ion concentrations ($[Ca^{2+}]_i$) of neurons to resting levels after stimulation have been divided into three general classes (Blaustein, 1978, 1988): those involving (1) binding to intracellular sites, (2) sequestration into intracellular organelles, and (3) extrusion into the extracellular space. Mechanisms of Ca^{2+} extrusion include an ATP-driven Ca^{2+} pump, and an electrogenic exchange of external Na^+ for internal Ca^{2+} (Na^+–Ca^{2+} exchange). The relative contributions of these processes to overall Ca^{2+} homeostasis vary. This report focuses on the Na^+–Ca^{2+} exchanger in hippocampal neurons and the consequences of its inhibition by reduction of the transmembrane Na^+ gradient. Because of their shared dependence on external Na^+, intracellular H^+ and activity of the Na^+–H^+ exchanger are also considered.

The fundamental properties of the Na^+–Ca^{2+} exchangers in a variety of cells have been extensively investigated and recently reviewed (Allen et al., 1989; Blaustein et al., 1991a). Less well understood is how the activities of these Na^+–Ca^{2+} exchangers are integrated into cellular functioning. In neurons, a contribution of Na^+–Ca^{2+} exchange to regulation of $[Ca^{2+}]_i$ has been established from investigations of large axons in invertebrates (Baker et al., 1969; Blaustein and Hodgkin, 1969) and of synaptosomes derived from mammalian brain (Blaustein and Wiesmann, 1970).

Here, we report on the possible role of Na^+–Ca^{2+} exchange in rapid restoration of $[Ca^{2+}]_i$ to resting levels following increases resulting from depolarization or glutamate receptor activation. Na^+–Ca^{2+} exchange was inhibited by decrease in $[Na^+]_o$ by substitution with Li^+, *N*-methyl-D-glucamine (*N*-MG), choline, or Tris. Concentrations of intracellular Ca^{2+} and H^+ were measured using the dyes Fura-2 and BCECF. Results of $[Ca^{2+}]_i$ measurements indicate that in cultured hippocampal neurons the activity of the Na^+–Ca^{2+} exchanger is important in determining the time course of return of $[Ca^{2+}]_i$ to resting levels after stimulation. Further, measurements of $[H^+]_i$ suggest that Ca^{2+} influx imposes an acid load normally buffered by Na^+–H^+ exchange, and that inhibition of Na^+–H^+ exchange results in loss of intracellular pH regulation during periods of Ca^{2+} influx. We suggest that under conditions in which both Na^+–Ca^{2+} exchange and Na^+–H^+ exchange are compromised, as occurs during ischemia and hypoglycemia, cytoplasmic acidification may exacerbate the deleterious effects of excess intracellular Ca^{2+}.

A preliminary report of some of these findings has appeared previously (Koch and Barish, 1991).

Materials and Methods

Cultures of mouse hippocampal neurons. Timed pregnant female Swiss–Webster mice were obtained from Simonsen. Cell cultures were prepared using cells dissociated from hippocampi of day 15–16 (E15–E16) em-

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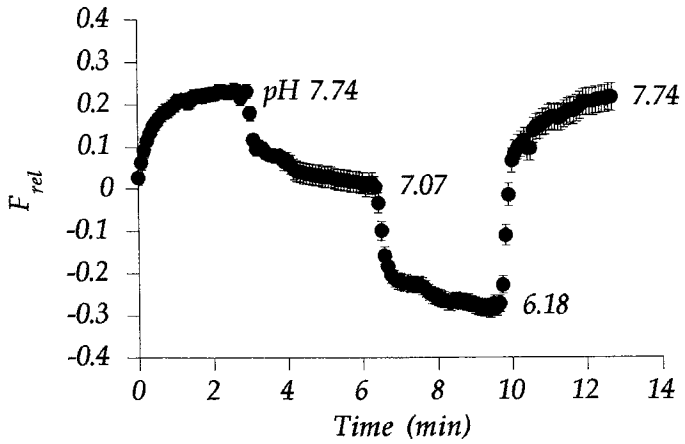

Figure 1. Calibration of BCECF fluorescence and measurement of intracellular pH. Shown is the relative change in fluorescence (F_{rel}; mean ± SEM, $n = 36$ cells), where $F_{rel} = (F - F_0)/F_0$. Here, F is the fluorescence at a given time point and F_0 is the fluorescence at time 0. Neurons were permeabilized with nigericin (10 μM) and superfused with KCl-based external solution buffered with HEPES and PIPES at pH_o of 7.74, 7.07, and 6.18 as indicated. Despite large differences in absolute values of fluorescence (not shown), values of F_{rel} equilibrated with external pH within 3 min, and were consistent across many cells.

bryos using procedures described previously (Wu and Barish, 1992). Cells were grown in coverslip chambers constructed by gluing 12-mm-diameter Teflon O-rings onto 25-mm-diameter circular glass coverslips with Sylgard 182 or 184 (Dow Corning) cured by heating on a hot plate. The cover glass surface was coated with 100 μg/ml poly-D-lysine for 1 hr, rinsed three times with sterile water, and then overlaid with 25 μl of 12.5 μg/ml laminin in Hank's Balanced Salt Solution (HBSS) for 2–3 hr. Aliquots of cells were plated into this solution at concentrations giving final cell densities of 25 or 50 × 10^3 cells/cm^2. Cultures were treated with antimitotic agents (10 μM fluorodeoxyuridine or cytosine arabinoside) after 1–2 d. Cells were used for experiments after 3–7 d in culture.

Measurement of intracellular Ca^{2+} and H^+ ion concentrations. Procedures for determination of intracellular ion concentrations were similar to those described previously (Barish, 1991). Measurements were made using an inverted fluorescence microscope (Zeiss ICM-405) and an Olympus 40× UV APO (1.4 NA) objective. Illumination was by a 75 W xenon bulb and a computer-controlled filter changer in the excitation path (Lambda-10; Sutter Instruments). The microscope was coupled to an intensified CCD camera (Hamamatsu). Images were acquired, analyzed, and intracellular ion concentrations calculated using VideoProbe hardware and software (ETM Systems).

Cells were loaded with Fura-2/AM or BCECF/AM (both from Molecular Probes) in an Mg^{2+}-free HBSS-based loading solution containing (in mM) 1.8 CaCl and 25 HEPES. For both Fura-2/AM and BCECF/AM, stocks (1 mM from 50 μg aliquots) were dissolved in dry dimethyl sulfoxide (DMSO). For Fura-2, 10 μl of Fura-2/AM stock, 10 μl of a 10% Pluronic F-127 aqueous solution, and 20 μl of DMSO were added to each milliliter of loading solution. For BCECF, 5 μl of BCECF/AM stock was added to each milliliter of loading solution. For both dyes, cells were incubated in loading solution for 1 hr at 37°C, rinsed with Mg-free HBSS, and then held for a minimum of 2 hr at room temperature to allow for cleavage of the acetoxymethyl (AM) ester.

Fluorescence of the Ca^{2+}-sensitive dye Fura-2 was measured using excitation filters of 350 and 380 nm (bandwidths of 10 and 13 nm), a dichroic mirror at 400 nm, and an emission filter at 510 nm (bandwidth of 40 nm). Intracellular Ca^{2+} was calculated from the ratio (R) of fluorescence emissions (F) at excitation wavelengths of 350 and 380 nm (F_{350}/F_{380}) according to the relation (Grynkiewicz et al., 1985)

$$[Ca^{2+}]_i = K_d\,(F_{380,min}/F_{380,max})\,[(R - R_{min})/(R_{max} - R)],$$

where the K_d for Fura-2 at room temperature was 135 nM and the subscripts min and max denote values obtained at minimal and saturating concentrations of internal Ca^{2+}. Minimal $[Ca^{2+}]_i$ was attained after incubating neurons in a Ca^{2+}-free saline containing 5 μg/ml ionomycin and 10 mM EGTA for 30 min. Saturating $[Ca^{2+}]_i$ was then obtained by superfusing the same cells with a similar saline containing 5 μg/ml ionomycin and 10 mM Ca^{2+}.

Fluorescence of the H^+-sensitive dye BCECF was measured using a standard FITC filter set. Intracellular H^+ was calculated from the relative change in BCECF fluorescence, F_{rel}, from its initial value (F_0), where

$$F_{rel} = (F - F_0)/F_0$$

using the relation

$$[H^+]_i = K_d\,(F_{rel} - F_{rel,min})/(F_{rel,max} - F_{rel}).$$

Here, K_d for BCECF was 105 nM (calculated from $pK_a = 6.98$; Molecular Probes), and $F_{rel,min}$ and $F_{rel,max}$ were determined for nigericin-permeabilized neurons (10 μM nigericin in KCl-based solution) by varying the external pH. We confirmed (Fig. 1) that F_{rel} remained consistent across many cells and varying absolute intensities of fluorescence as the internal pH of nigericin-permeabilized neurons was shifted to values spanning the dynamic range of BCECF. When quantitative measurements of pH_i were made, BCECF fluorescence was calibrated by determining $F_{rel,min}$ and $F_{rel,max}$ for each field of cells. Cells were permeabilized with nigericin (10 μM) and exposed to KCl-based external solutions (containing, in mM, 130 KCl, 10 NaCl, 1 $MgSO_4$, 10 HEPES, 10 PIPES) with pH adjusted to values between 6.0 and 8.0. Nigericin was dissolved in methanol to make a 10 mM stock, and this stock was diluted 1:1000 into the KCl-based buffer.

Fluorescence images were acquired and stored at 512 × 512 pixels. Measurements of fluorescence in individual cells were made by outlining areas of interest over cells identified as neurons based on the presence of high rounded somas and tapering dendrites. Measurements of $[Ca^{2+}]_i$ were made for neuron somas only, and thus include values for cytoplasm and nucleus and exclude those for dendrites and axons. $[Ca^{2+}]_i$ in neuronal processes showed qualitatively similar behavior in all experiments, but was not analyzed in detail. Astroglial cells form a thin, flat monolayer under the serum-containing growth conditions employed here, and are easily distinguished from neurons. Synaptic transmission between neurons was not evident in these cultures, probably because cell density was relatively low and the cultures were not allowed to develop for extended periods.

Experimental procedures. The normal external solution was based on HBSS and contained (in mM) 137 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 4.2 $NaHCO_3$, 10 HEPES; pH 7.3–7.4; 300–310 mOsm/kg (measured with a Wescor vapor pressure osmometer). In Na^+-free external solutions, NaCl was replaced on an equiosmolar basis with (in mM) 144 LiCl, 168 *N*-methyl-D-glucamine, 146 choline-Cl, or 144 Tris-Cl (pH 7.3–7.4; 290–310 mOsm/kg in all cases). All solutions contained 0.3 μM tetrodotoxin to prevent action potential generation during stimulation. All glutamate-containing solutions also contained 10 μM glycine to maximize activation of NMDA-type glutamate receptors (Johnson and Ascher, 1987).

Solutions were applied using a puffer pipette threaded with five PE-10 polyethylene tubes, each connected to a slightly pressurized syringe reservoir. The pipette (orifice diameter of ~100 μm) was positioned approximately 50 μm from the cell under study. Normal external solution flowed continuously. To apply test solutions, the valve metering the flow of normal solution was closed at the same time that the valve for the test solution was opened. Solution exchange at the cell required approximately 250 msec as judged by the change in junction potential of a 0.1 M KCl-filled pipette placed at the cell position as the puffed solution was changed from 0.1 M KCl to 0.1 M NaCl.

All experiments were performed at room temperature (20–22°C).

We observed quantitative differences in the amplitudes and recovery times of $[Ca^{2+}]_i$ responses between batches of cultures, and accommodated to this variability within individual experiments by comparing control and test responses from the same neurons, and replicating experiments across batches of cultures. Statistical significance within experiments was thus evaluated by paired two-tailed t test on data from individually identified cells. Statistical significance between experiments was evaluated as described in the notes to Tables 1 and 2. Differences were considered significant at $p < 0.05$.

Results

The stimuli used in this study, either K^+ or glutamate, were chosen to maximize activation of Ca^{2+}-permeant ion channels. Potassium at 30 or 50 mM (the usual concentrations employed) would be expected to depolarize the (K^+-selective) membrane

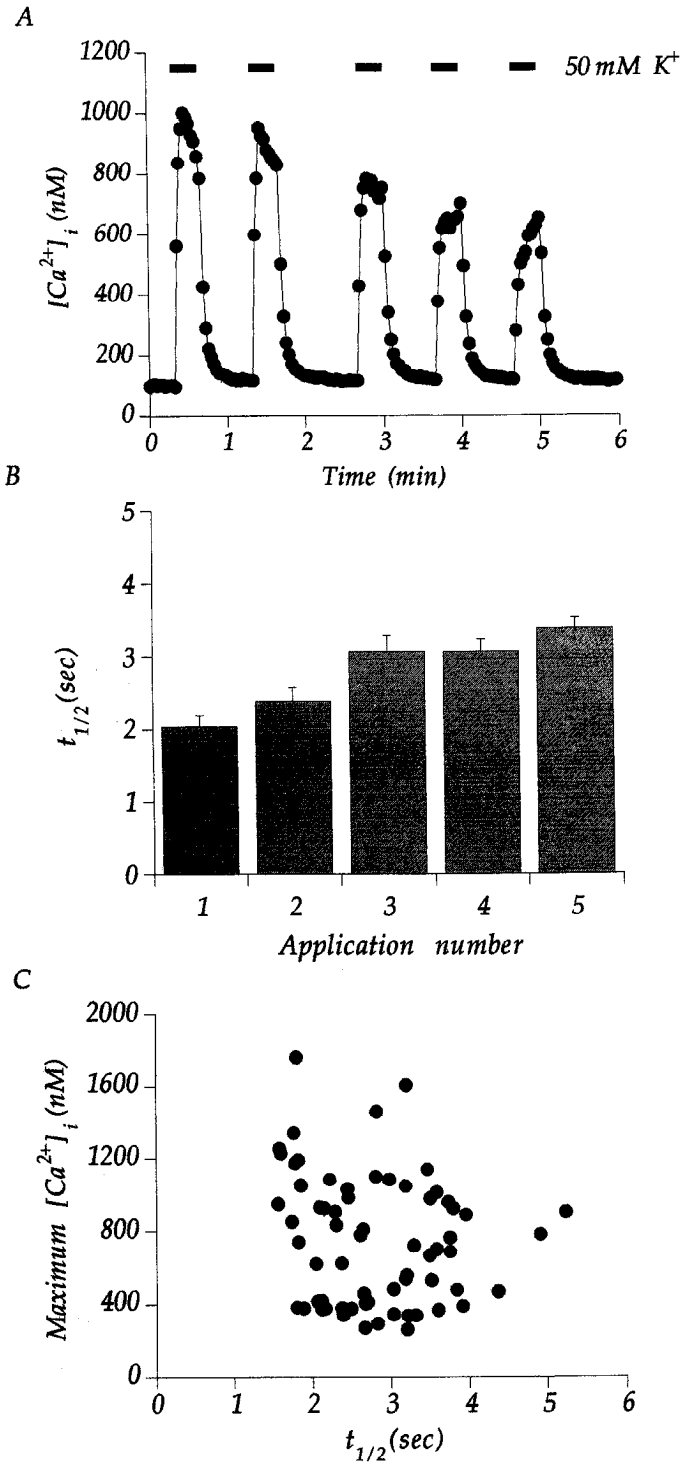

Figure 2. *A*, $[Ca^{2+}]_i$ with successive K^+ depolarizations. *Circles* represent means (n = 14 cells). SEMs were generally smaller than the symbols, and in this and other, similar figures that follow were omitted for clarity. *Horizontal bars* indicate the period of 50 mM K^+ application. The decrease in response amplitude was statistically significant ($p < 0.0001$, peak 5 vs peak 1). *B*, Half-decay times ($t_{1/2}$) for $[Ca^{2+}]_i$ following removal of 50 mM K^+ (mean ± SEM). Tine for half-decay rather than time constant was used as an index of $[Ca^{2+}]_i$ relaxation time because the return of Ca^{2+} to baseline was not always exponential (see following figures). For comparison, these control values of $t_{1/2}$ are presented using the same vertical scale as used for the experimental data (see Figs. 5, 6). The increase in $t_{1/2}$ with successive depolarizations was statistically significant when values for each depolarization were compared to those for the first depolarization on a cell-by-cell basis ($p < 0.03$, peak 2 vs peak 1; $p < 0.0001$, peaks 3–5 vs peak 1). *C*, Comparison of maximum $[Ca^{2+}]_i$ attained during K^+ depolarization versus $t_{1/2}$ for its decline. There was little correlation over this series of stimuli between the quantity of Ca^{2+} that accumulated internally and the time course for its decline (a line of the form $y = -76.32x + 967.85$ could be fit by linear regression, with $r = 0.18$). Data in *A–C* were taken from the same cells.

to approximately −33 or −15 mV (Barish, 1991) and activate all classes of voltage-gated Ca currents. Glutamate at 20 or 50 μM would be expected to activate both NMDA- and non-NMDA–classes of glutamate receptors (Sands and Barish, 1989; Patneau and Mayer, 1990), and is within the range of extracellular concentrations expected during ischemia (Benveniste, 1991).

The results of control experiments (Fig. 2) established that time courses of recovery of $[Ca^{2+}]_i$ following repetitive transient stimulations with K^+ or glutamate were sufficiently stable for the analysis undertaken here, and that the Ca^{2+} loads imposed by these stimuli could be comfortably buffered by available $[Ca^{2+}]_i$-regulating mechanisms. After five applications of 50 mM K^+–containing HBSS, the responses of 14 neurons showed a decrease in amplitude of 33 ± 3% (mean ± SEM; Fig. 2*A*), while the mean time for $[Ca^{2+}]_i$ to decline to one-half of the difference between resting and peak concentrations ($t_{1/2}$) showed a progressive increase over five depolarizations (from 2.04 ± 0.16 sec for the first depolarization to 3.39 ± 0.13 sec for the fifth; Fig. 2*B*). While these gradual increases were seen consistently and were statistically significant, they were "well behaved" (i.e., monotonic) and small compared to those induced by substitution for extracellular Na^+ (compare with Figs. 3, 4). Further, for a given stimulus there was little correlation within a population of neurons between peak response amplitude and $t_{1/2}$ recovery (Fig. 2*C*). The results presented in Figure 2 were obtained with 50 mM K^+ as a stimulus; similar results were obtained with other concentrations of K^+, and with glutamate stimulation.

Transient stimulation and substitution for external Na^+

Na^+–Ca^{2+} exchange was blocked by substitution of Li^+, *N*-MG, choline, or Tris for external Na^+. Application of *N*-MG caused a transient elevation of $[Ca^{2+}]_i$ in 16 of 18 cells. Similar $[Ca^{2+}]_i$ transients were observed in 8 of 18 cells with choline and 24 of 24 cells with Tris, but were never observed on application of Li^+ (0 of 16 cells).

As illustrated in Figure 3, removal of external Na^+ often reduced the amplitude of the $[Ca^{2+}]_i$ increase induced by 50 mM K^+, especially for the second application (16 of 16 for Li^+ on first application; 8 of 8 for *N*-MG on first application; 7 of 12 and 12 of 12 for Tris on first and second applications, respectively; 2 of 18 and 18 of 18 for choline on first and second applications, respectively). As K^+ depolarization would be expected to activate voltage-gated Ca channels, the smaller responses could be due to reduction of voltage-gated Ca current by Na^+ substitution as suggested by Korn and Horn (1989).

Most importantly, substitution for extracellular Na^+ consistently and reversibly slowed recovery of $[Ca^{2+}]_i$ after brief K^+ depolarizations (Fig. 3, Table 1; $p < 0.0003$–0.0001 for all substitutions). This effect of Na^+ removal was seen with Li^+, Tris, *N*-MG, and choline. Since none of these Na^+ substitutes participate in exchange for internal Ca^{2+}, they might be expected to have similar actions. However, we observed that the magnitudes of the effects seen with Li^+ substitution (Fig. 3*A*) were not as large as those seen with *N*-MG, Tris, or choline (Fig. 3*B*–

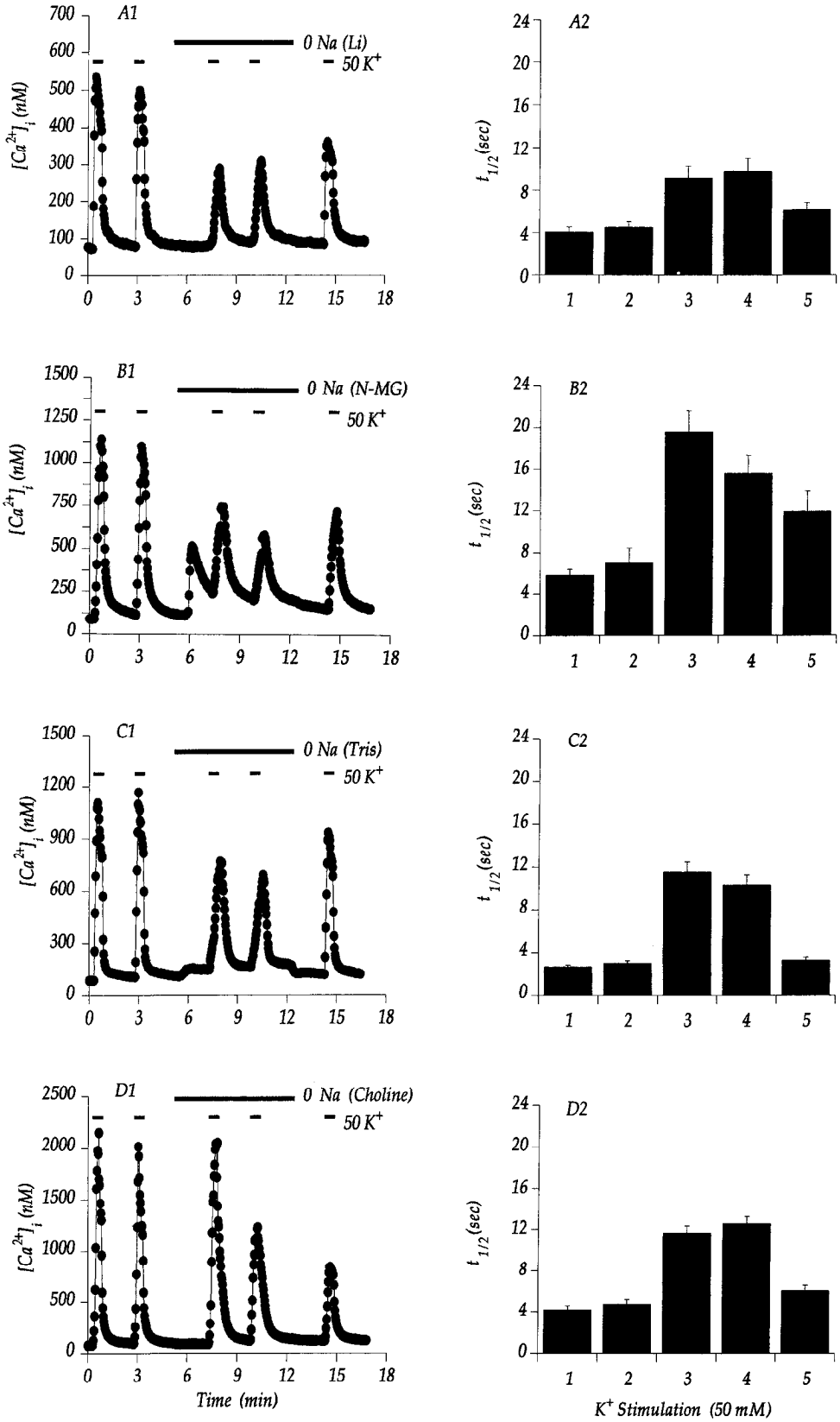

Figure 3. $[Ca^{2+}]_i$ and time courses of return to resting levels during and after K^+ depolarizations (50 mM), in the presence and absence of external Na^+. In each portion of the figure, *left panels* show plots of $[Ca^{2+}]_i$ versus time and *right panels* indicate the times required for one-half recovery of resting Ca^{2+} levels ($t_{1/2}$) during each period following K^+ depolarization (the applications of K^+-containing solution are sequentially numbered). *A*, Na^+ substitution by Li^+; *B*, substitution by *N*-MG; *C*, substitution by Tris; *D*, substitution by choline. In each plot of $[Ca^{2+}]_i$ versus time, *bars* mark the duration of K^+ stimulation (30 sec) and the period of Na^+ substitution (7 min), and *data points* represent means (SEMs were omitted). In bar graphs, the *columns* show means ± SEM. Data presented in *A–D* were taken from cells on sister coverslips derived from the same plating. This procedure was adopted to ensure neurons were not previously exposed to Na^+-free solutions. In *B*, values for $t_{1/2}$ during Na^+ substitution were measured relative to resting $[Ca^{2+}]_i$ levels before application of *N*-MG. Numbers of cells were 16 for Li^+, 8 for *N*-MG, 24 for Tris, and 18 for choline.

D). While substitution of Li^+ for Na^+ prolonged the recovery of $[Ca^{2+}]_i$ after K^+ depolarization by more than twofold, increases in $t_{1/2}$ were three- to fourfold when *N*-MG, Tris, or choline was substituted for Na^+ (although only for Tris was the enhanced effect statistically significant; Table 1).

Li^+ differs from the other Na^+ substitutes in that it can substitute for Na^+ in the Na^+–H^+ exchanger (see Aronson, 1985). When data derived from the three non-Li^+ substitutes were combined (a grouping based on the inability of these substitutes to support Na^+–H^+ exchange) and then compared with data for

Table 1. Time for one-half decay of intracellular Ca^{2+} ($t_{1/2}$) following K^+ depolarization or glutamate stimulation before and during substitution for external Na^+

Stimulus	Na^+ substitute	Control $t_{1/2}$ (sec)	$t_{1/2}$ with substitute (sec)	Mean $t_{1/2}$ ratio (vs control) (sec)	p vs control*	p vs Li^+†	Mean $t_{1/2}$ ratio (all non-Li^+) (sec)	p vs Li^+‡
K^+ depolarization	Li^+ (n = 16)	3.7 ± 0.3	8.2 ± 0.7	2.4 ± 0.2	<0.0001			
	N-MG (n = 8)	5.8 ± 0.6	19.6 ± 1.9	3.6 ± 0.5	<0.0003	<0.057		
	Tris (n = 24)	2.7 ± 0.2	11.5 ± 1.0	4.3 ± 0.3	<0.0001	<0.0003	3.8 ± 0.2	<0.0005
	Choline (n = 17)	4.2 ± 0.4	11.6 ± 0.7	3.0 ± 0.2	<0.0001	<0.4314		
Glutamate	Li^+ (n = 16)	6.8 ± 0.5	63.1 ± 8.6	10.4 ± 1.7	<0.0001			
	N-MG (n = 15)	8.3 ± 0.5	114.4 ± 6.2	13.9 ± 0.6	<0.0001	<0.062		

Neurons were depolarized by K^+ (50 mM) for 30 sec twice before and twice during Na^+ substitution (see Fig. 3). Data are calculated for the first stimulation before and during the test, and are presented as mean ± SEM.

* Significance evaluated by paired two-tailed t test.

† Significance for K^+ depolarization data evaluated by Bonferroni multiple-comparison t test for each substitute versus Li^+. Significance for glutamate stimulation data evaluated by unpaired t test for N-MG versus Li^+.

‡ Significance evaluated by unpaired two-tailed t test for all non-Li^+ substitutes versus Li^+.

Li^+ substitution, the enhanced increase in $t_{1/2}$ by non-Li^+ substitutes was statistically significant (Table 1; $p < 0.0005$).

When glutamate receptor activation was used as a stimulus, $t_{1/2}$ for recovery of $[Ca^{2+}]_i$ after stimulation also showed large reversible increases during substitution for external Na^+ (Fig. 4, Table 1). Because a secondary increase in $[Ca^{2+}]_i$ was observed following removal of glutamate under Na^+-substituted conditions, the relative increases in $t_{1/2}$ were larger than seen with K^+ depolarization (10–14-fold). As was seen for K^+ depolarization, $t_{1/2}$ recovery was consistently longer with N-MG rather than Li^+ substitution (although it did not quite reach statistical significance, $p < 0.062$ vs Li^+). The secondary increase was also more pronounced in the presence of the non-Li^+ substitute.

The data presented in Figures 3 and 4 are representative of 20 experiments on neurons in culture for 3–10 d from E15–E16 embryos in which Li^+, N-MG, choline, or Tris was used as substitute for external Na^+ during stimulation by 50 mM K^+ or 20 μM glutamate. Removal of external Na^+ resulted in delayed recovery of $[Ca^{2+}]_i$ in every instance, and in cases in which the effects of Na^+ substitution with Li^+ were compared to those of substitution with N-MG, Tris, or choline, the effects of large cations were always more pronounced.

Intracellular Ca^{2+} and H^+ concentrations during continuous glutamate receptor activation

We compared the effects of substitution of external Na^+ with Li^+ or N-MG on both $[Ca^{2+}]_i$ and $[H^+]_i$ during continuous glutamate receptor activation. $[Ca^{2+}]_i$ and $[H^+]_i$ were measured

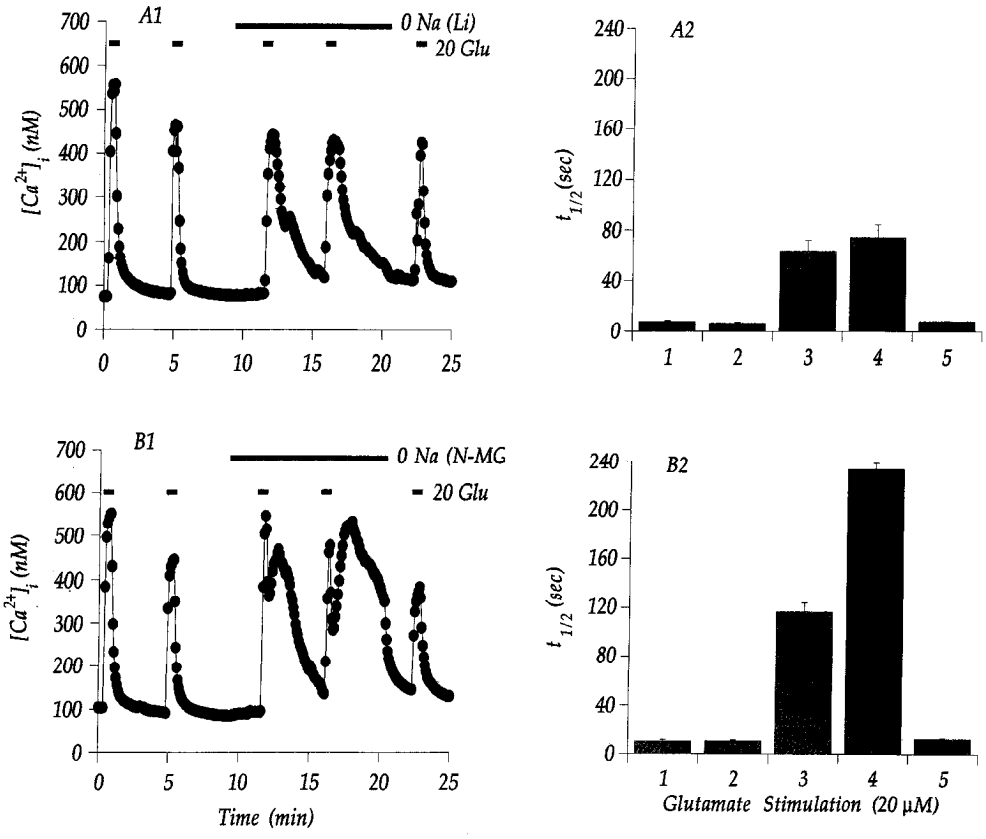

Figure 4. $[Ca^{2+}]_i$ and time courses of its recovery during and after glutamate receptor activation (20 μM), in the presence and absence of external Na^+. The data are presented as in Figure 3. *A*, Na^+ substitution by Li^+; *B*, substitution by N-MG. Sister coverslips were used. In the *left panels* data points represent means; in the *right panels* the *columns* show mean ± SEM. Numbers of cells were 17 for Li^+ and 22 for N-MG.

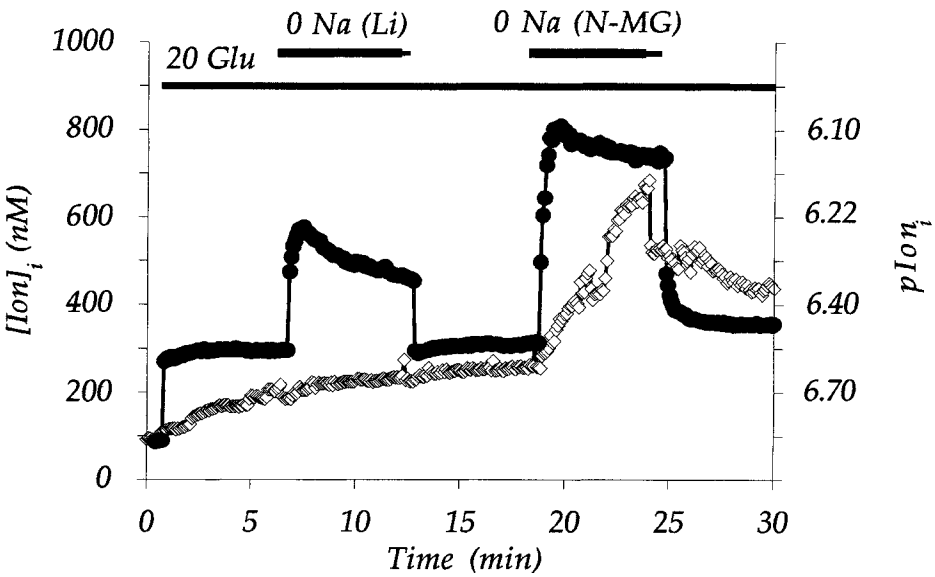

Figure 5. $[Ca^{2+}]_i$ and $[H^+]_i$ during glutamate stimulation in the presence and absence of external Na^+. These data were collected in sequential experiments from neurons on sister coverslips from the same plating. *Solid circles* represent $[Ca^{2+}]_i$ and *open diamonds* represent $[H^+]_i$ as measured in nM against the *left vertical scale* and as pCa and pH on the *right vertical scale.* The *lower horizontal bar* indicates the duration of glutamate stimulation (20 μM) and the *upper horizontal bars* mark the 6 min intervals of substitution for Na^+. Data points represent means; means ± SEM are noted in the Results for resting and peak values. The times of transitions from one solution to another differed by a few seconds in the two experiments; for accuracy in presentation of the data, the *thick portions of the horizontal bars* apply to the $[H^+]_i$ data, and the *narrow portions* represent the additional time during which Na^+-free conditions existed in the $[Ca^{2+}]_i$ experiment. Numbers of cells analyzed were 24 for $[Ca^{2+}]_i$ and 36 for $[H^+]_i$.

from sister coverslips under identical ionic conditions (Fig. 5). Data are shown plotted on vertical scales indexed in both linear (molar concentration) and negative logarithmic (pIon) units.

While substitution of Li^+ or *N*-MG for external Na^+ had qualitatively similar effects on $[Ca^{2+}]_i$, the effects of *N*-MG were more severe. Resting $[Ca^{2+}]_i$ was 88.9 ± 0.4 nM, and this was rapidly increased to a stable value of 292 ± 0.8 nM by application of 20 μM glutamate (n = 24 cells). Substitution of Li^+ for Na^+ (in the continued presence of glutamate) resulted in an increase to a peak value of 578 ± 49 nM that relaxed toward a steady state (504 ± 4.0 nM) and that returned to its resting value on return of external Na^+. Subsequent replacement of Na^+ by *N*-MG resulted in a larger increase to a peak of 810 ± 22 nM that relaxed more slowly (steady state value, 756 ± 3.7 nM) and that reversed less completely on return to Na^+-containing solution.

Li^+ and *N*-MG had markedly different effects on the slow acidification induced by application of 20 μM glutamate. Resting pH_i was 7.03 ± 0.01 (~93 nM; mean ± SEM, n = 36 cells). The initial application of glutamate (in Na^+-containing solution) triggered a slow fall in pH_i to 6.71 ± 0.28 (~195 nM) after 6 min. Substitution of Li^+ for Na^+ and reintroduction of Na^+ had minimal if any effect on this slow acidification. In contrast, substitution with *N*-MG elicited a rapid fall in pH_i to 6.16 ± 0.27 (~692 nM) by the conclusion of the 6 min period of exposure to Na^+-free solution. Subsequently, $[H^+]_i$ only partially recovered during 6 min in Na^+-containing solution.

A similar pattern of effects on intracellular Ca^{2+} and H^+ regulation was seen in 8 of 8 experiments comparing Li^+ with *N*-MG as Na^+ substitutes.

Intracellular acidification and recovery from Ca^{2+} load

Because our data suggested that some substitutions for external Na^+ could result in enhanced accumulation of H^+ during Ca^{2+} influx, we examined whether intracellular acidification would itself slow the return of $[Ca^{2+}]_i$ to resting levels after glutamate stimulation. In this experiment, recovery of $[Ca^{2+}]_i$ was compared before and during exposure to acidic external solution containing 10 mM lactate. Exploratory experiments established that application of this solution caused intracellular pH to fall to approximately 6.6 after 16 min. We observed that intracellular acidification resulted in a greater than twofold increase in $t_{1/2}$ after exposure to 20 μM glutamate (Table 2).

Discussion

Reduction in the inward driving force on Na^+ will affect at least two mechanisms acting in parallel, Na^+–Ca^{2+} exchange and Na^+–H^+ exchange. Our data indicate that inhibition of the Na^+–Ca^{2+} exchange alone will compromise intracellular Ca^{2+} regulation, and that intracellular acidification also retards recovery of $[Ca^{2+}]_i$ after stimulation. Thus simultaneous inhibition of Na^+–Ca^{2+} and Na^+–H^+ exchangers will disrupt Ca^{2+} homeostasis both directly and indirectly.

That the effects of substitution for external Na^+ involved the Na^+–Ca^{2+} exchanger rather than some other mechanism (such as alteration of second messenger systems by Li^+; Berridge and Irvine, 1989) is indicated by the rapid onset and reversibility of the delay in recovery of $[Ca^{2+}]_i$ by Na^+ substitutes, and the qualitative similarity of the effects of Li^+, *N*-MG, choline, and Tris. The additional influence of pH_i on $[Ca^{2+}]_i$ regulation was indicated by comparison of the effects of substitution with Li^+ versus *N*-MG. Cytoplasmic acidification accompanied Na^+ substitution by *N*-MG but not by Li^+ during glutamate receptor activation, and we observed in other experiments that acidification of the cytoplasm (using external lactate) resulted in delays in recovery of $[Ca^{2+}]_i$ after stimulation.

Thus, we suggest that the effects of *N*-MG, Tris, or choline substitution on $[Ca^{2+}]_i$ regulation were always more severe than

those seen with Li^+ because, in addition to blockade of Na^+–Ca^{2+} exchanger, the acid load accompanying Ca^{2+} influx could not be dissipated by the Na^+–H^+ exchanger. The additional intracellular Ca^{2+} seen with *N*-MG substitution, for example (Fig. 5), may reflect the contribution of Ca^{2+} displaced from cytoplasmic binding sites by excess H^+ (Rose and Rick, 1978; Dickens et al., 1989), or partial inhibition of remaining Ca^{2+}-regulating mechanisms.

Viewed from another perspective, the results reported here emphasize the care with which substitutes for external Na^+ must be chosen. Choline and *N*-MG are commonly employed in investigations of putative activities of Na^+–Ca^{2+} exchange. As they also profoundly altered intracellular pH regulation in the face of increase in $[Ca^{2+}]_i$, a role of H^+ in effects commonly attributed to cytoplasmic Ca^{2+} and inhibition of Na^+–Ca^{2+} exchange should also be considered.

Table 2. Time for one-half decay of intracellular Ca^{2+} ($t_{1/2}$) following glutamate stimulation before and during intracellular acidification

	Lactate ($n = 9$)
Control $t_{1/2}$ before acidification (sec)	83 ± 0.7
$t_{1/2}$ during acidification (sec)	19.2 ± 4.0
Mean $t_{1/2}$ ratio (vs control) (sec)	2.3 ± 0.4
p vs control	<0.02

Neurons were stimulated by glutamate (20 μM) for 30 sec before and during intracellular acidification imposed by application of HBSS containing 10 mM lactate. Data are presented as mean ± SEM. Significance was evaluated using paired two-tailed t test as for Table 1. Results of identical parallel experiments in which intracellular pH was measured using BCECF indicate that lactate shifted pH_i from 7.22 ± 0.10 to 6.62 ± 0.08 after 16 min of equilibration (mean ± SEM, $n = 12$).

Na^+–Ca^{2+} exchange in neuronal Ca^{2+} regulation

A role of the Na^+–Ca^{2+} exchanger in regulation of neuronal $[Ca^{2+}]_i$ was first appreciated for large invertebrate axons and synaptosomes (see introductory remarks). Na^+–Ca^{2+} exchange has been characterized as a low-affinity and high-capacity mechanism for extrusion of cytoplasmic Ca^{2+}. In general, the present results are consistent with the conclusion of other studies that Na^+–Ca^{2+} exchange is less important for regulation of basal $[Ca^{2+}]_i$ levels but more significant at elevated Ca^{2+} concentrations.

The results of several recent experimental efforts support Na^+-dependent Ca^{2+} efflux as a mechanism of recovery from transient intracellular Ca^{2+} load in *Aplysia* neurons (Levy and Tillotson, 1988), rat brain synaptosomes (Nachshen, 1985; Nachshen et al., 1986; Taglialatela et al., 1990), pituitary cell lines (Korn and Weight, 1987; Korn and Horn, 1989), neuroblastoma and pheochromocytoma cells (Dickens et al., 1989), and presynaptic terminals at crayfish and squid synapses (Zipser et al., 1991; Mulkey and Zucker, 1992), as well as in cultured rat hippocampal neurons (Blaustein et al., 1991b; Segal and Manor, 1992). In these studies, removal of external Na^+ resulted in delayed restoration of resting levels of $[Ca^{2+}]_i$ in the seconds immediately following stimulation when this was determined by using Ca^{2+}-sensitive dyes, Ca^{2+}-activated Cl^- current, or $^{45}Ca^{2+}$ flux. Taken together with the results reported here, these studies made using different detection methodologies indicate that Na^+–Ca^{2+} exchange can participate in dynamic regulation of neuronal intracellular Ca^{2+} over periods of seconds to minutes during and immediately following stimulation.

The contribution of Na^+–Ca^{2+} exchange to regulation of $[Ca^{2+}]_i$ transients may not be universal. In several investigations of peripheral sensory neurons (cultured rat dorsal root ganglion neurons: Benham et al., 1989; Thayer and Miller, 1990; acutely dissociated mouse dorsal root ganglion neurons: Duchen et al., 1990), little evidence was obtained for a contribution of Na^+–Ca^{2+} exchange to restoration of $[Ca^{2+}]_i$ to resting levels following stimulation. This difference may reflect variation in the contribution of Na^+–Ca^{2+} exchange to overall $[Ca^{2+}]_i$ regulation in different cells, and/or a consistent difference in mechanisms of intracellular Ca^{2+} regulation between central and peripheral neurons.

Na^+–H^+ exchange and neuronal H^+ regulation

We measured pH_i in unstimulated hippocampal neurons to be approximately 7.03, a value similar to that obtained in other studies of hippocampal and other neurons (see Chesler, 1990, for review). Our data are consistent with the results of studies indicating the importance of Na^+–H^+ exchange in regulation of intracellular pH in cultured rat hippocampal neurons (Raley-Susman et al., 1991), rat brain synaptosomes (Nachshen and Drapeau, 1988), cultured sympathetic neurons (Tolkovsky and Richards, 1987), and identified leech neurons (Deitmer and Schlue, 1988).

Our results extend this previous work by emphasizing the role of the Na^+–H^+ exchanger in extrusion of the acid generated by glutamate receptor activation. In the experiment illustrated in Figure 5, $[H^+]_i$ increased steadily during a period of Na^+–H^+ exchange inhibition in the face of steady glutamate-induced Ca^{2+} influx. In Na^+-free external solution, $[Ca^{2+}]_i$ and $[H^+]_i$ reached almost identical levels (approximately 810 nM for Ca^{2+} and 692 for H^+), and $[H^+]_i$ continued to increase even after 6 min. Ca^{2+} accumulation at the inner surface of the cell membrane will displace H^+ from intracellular sites and induce local acidification (Meech and Thomas, 1977, 1980; Ahmed and Connor, 1980), and the Na^+–H^+ exchanger may provide a rapid and localized mechanism for extrusion of this H^+. If Na^+–H^+ exchange was impaired during a period of prolonged Ca^{2+} influx (as during ischemia), our results suggest the H^+ concentration of the cytosol could exceed that of Ca^{2+}.

Relevance to the physiology of hippocampal neurons

Electrophysiology. In principle, the activity of the Na^+–Ca^{2+} exchanger may influence the firing properties of neurons by two different mechanisms: (1) by regulating the Ca^{2+} concentration at the inner surface cell membrane in the vicinity of Ca^{2+}-dependent ion channels, and (2) by electrogenic production of depolarizing current. While neither of these possibilities has been investigated in hippocampal neurons, the rate of Ca^{2+} clearance by Na^+–Ca^{2+} exchange influences the time course of a slow afterhyperpolarization in bullfrog sympathetic ganglion (Goh et al., 1992; see also Korn and Horn, 1989), and electrogenic Na^+–Ca^{2+} exchange current is responsible for a slow depolarizing afterpotential in neocortical neurons (Friedman et al., 1992). Similar processes may operate in hippocampal neurons.

Further, Na^+–Ca^{2+} exchange may participate in activity-dependent modulation of transmitter release by influencing $[Ca^{2+}]_i$ in presynaptic terminals. In presynaptic terminals at crayfish neuromuscular junction, inhibition of Na^+–Ca^{2+} exchange by increase in internal Na^+ or by substitution of Li^+ for external

Na^+ prolonged the decay of both $[Ca^{2+}]_i$ and postsynaptic potential amplitude following tetanic stimulation (posttetanic potentiation; Mulkey and Zucker, 1992).

Excitotoxicity. We noted a striking similarity in the concentrations of Ca^{2+} and H^+ during glutamate stimulation (approaching 1 μM) if Na^+-dependent mechanisms of Ca^{2+} and H^+ extrusion were blocked (Fig. 5). Episodes of cerebral ischemia and hypoglycemia are accompanied by a decrease in intracellular ATP, increase in intracellular Na^+, and increase in extracellular H^+ (Nedergaard et al., 1990). Inhibition of Ca^{2+} and H^+ transport may result, as (1) both the Na^+–Ca^{2+} (see DiPolo and Beaugé, 1983, 1991; Collins et al., 1992) and Na^+–H^+ (Cassel et al., 1986; Weissberg et al., 1989) exchangers are positively regulated by internal ATP, (2) Na^+–H^+ exchange is inhibited by external H^+ (see Aronson, 1985), and (3) both exchangers are inhibited by a rise in cytoplasmic Na^+. Thus, during the initial period of ischemia and hypoglycemia characterized by high concentrations of glutamate in extracellular space, both Na^+–Ca^{2+} and Na^+–H^+ exchangers may be compromised.

An increase in $[H^+]_i$ in parallel with $[Ca^{2+}]_i$ during ischemia and hypoglycemia could result in significant cellular damage. Our data indicate that exposure to a moderate concentration of glutamate (20 μM) can lower pH_i to below 6.15 in only a few minutes if Na^+–H^+ exchange is impaired. This value of pH_i is well within the toxic range for neurons (Nedergaard et al., 1991). While lactic acid accumulation is often considered the mechanism of intracellular acidosis in ischemia (see Plum, 1983), H^+ accumulation may also occur as a consequence of Ca^{2+}-dependent displacement of H^+ from shared binding sites and reduced Na^+–H^+ exchange. Evidence consistent with this possibility was presented by Pirttilä and Kauppinen (1992), who observed in brain slice that complexing extracellular Ca^{2+} in Na^+-free medium reduced acidification during anoxia. In addition to being itself toxic (Nedergaard et al., 1991), intracellular H^+ could also synergize with Ca^{2+} in causing neuronal damage and death by several concurrently activated pathways (see Tombaugh and Sapolsky, 1990a), including displacement of Ca^{2+} from intracellular binding sites and blockade of several classes of potassium channels that normally act to stabilize membrane potential (see Moody, 1984).

While excitotoxicity is often considered dependent on Ca^{2+} entry (Choi, 1987), the magnitude of glutamate-induced increase in $[Ca^{2+}]_i$ is often not precisely correlated with cell death when these are tallied for individual cells (Michaels and Rothman, 1990; Dubinsky and Rothman, 1991; but see Ogura et al., 1988; de Erausquin et al., 1990). Thus, an additional process consequent on glutamate receptor activation and Ca^{2+} influx may be involved (Choi, 1990; Choi and Rothman, 1990), and increased intracellular H^+ could be such an additional factor. As described, regulation of both $[Ca^{2+}]_i$ and $[H^+]_i$ will be affected during ischemia and hypoglycemia, and glutamate receptor activation will impose strong H^+ and Ca^{2+} loads. This notion is particularly attractive because studies proposing a role of Na^+–Ca^{2+} exchange in protection from excitotoxic insult (Mattson et al., 1989a; Mattson, 1990) have been performed using Na^+ substitutions (e.g., choline) that will also block Na^+–H^+ exchange. It has been further proposed that growth factors may increase the ability of hippocampal neurons to tolerate a glutamate- or hypoglycemia-induced Ca^{2+} load (Mattson et al., 1989b; Mattson and Rychlik, 1990; Cheng and Mattson, 1991) by increasing the activity of Na^+-dependent extrusion of internal Ca^{2+} (Mattson, 1990). As stimulating actions of growth factors on Na^+–H^+ exchange are well known for non-neuronal cells (for reviews, see Moolenaar et al., 1986; Grinstein et al., 1989), it may be that growth factor–induced potentiation of Na^+–H^+ exchange (possibly compromised by a fall in $[ATP]_i$) enhances the ability of cells to buffer H^+ displaced by Ca^{2+} during accumulation to excitotoxic concentrations.

These potentially toxic actions of strong *intracellular* acidification exist in contrast to the neuroprotective effects of mild *extracellular* acidification (Giffard et al., 1990; Tombaugh and Sapolsky, 1990b; Takadera et al., 1992), which appear to be a consequence of block of the NMDA class of glutamate receptor by external H^+ (Giffard et al., 1990; Tang et al., 1990; Traynelis and Cull-Candy, 1990; Vyklický et al., 1990).

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