The Journal of Neuroscience, February 1995, 15(2): 1318–1328

Mitochondria and Na^+/Ca^{2+} Exchange Buffer Glutamate-induced Calcium Loads in Cultured Cortical Neurons

R. James White and Ian J. Reynolds

Center for Neuroscience and Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

Utilizing Indo-1 microfluorimetry, we have investigated the role of mitochondria and Na^+/Ca^{2+} exchange in buffering calcium loads induced by glutamate stimulation or depolarization of cultured rat forebrain neurons. A 15 sec pulse of 3 μM glutamate or 50 mM potassium with veratridine was followed by a 2 min wash with a solution containing either Na^+-free buffer or the mitochondrial uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), or both. For glutamate-induced Ca^{2+} loads, a Na^+-free wash delayed recovery to baseline by twofold, mitochondrial uncoupling delayed recovery by greater than fourfold, and the combined treatment essentially prevented recovery of $[Ca^{2+}]_i$ for the duration of the wash. Although the depolarization stimulus was able to elicit a larger peak $[Ca^{2+}]_i$, the neurons required significantly less time to recover from depolarization-induced Ca^{2+} loads after identical wash manipulations, indicating a fundamental difference between calcium loads induced by glutamate as opposed to those induced by depolarization. We show evidence that the delayed recovery is not primarily the result of perturbations in intracellular pH regulation and have also demonstrated that a substantial portion of the delayed recovery is independent of Ca^{2+} entry during the washout phase. We conclude that glutamate and depolarization both induce Ca^{2+} loads whose buffering is critically dependent on functional mitochondria and secondarily reliant on Na^+/Ca^{2+} exchange. The two systems overlap and seem to be responsible for buffering most of the glutamate-induced Ca^{2+} load, because manipulations that compromised both systems completely disabled the neurons' ability to recover $[Ca^{2+}]_i$ to baseline.

[Key words: intracellular calcium, calcium homeostasis, glutamate, depolarization, cortical neurons, mitochondria, sodium/calcium exchange]

Free intracellular calcium, $[Ca^{2+}]_i$, plays a central role in the regulation of neuronal signaling. The importance of Ca^{2+} in neuronal function is underscored by the numerous mechanisms by which $[Ca^{2+}]_i$ is increased, including voltage-gated calcium channels and glutamate receptors (Miller, 1987; Mayer and Miller, 1990; Bertolino and Llinas, 1992) as well as both IP_3 and ryanodine receptors (Brorson et al., 1991; Friel and Tsien, 1992b; Henzi and MacDermott, 1992). The $[Ca^{2+}]_i$ transients produced by activation of these various proteins have been well described, but until recently, very little was known about the mechanisms used by vertebrate neurons to buffer the resulting calcium loads. Components of the homeostatic system include Ca^{2+}-binding proteins, the endoplasmic reticulum, and mitochondria, as well as two plasmalemmal extrusion molecules, the Na^+/Ca^{2+} exchanger and Ca^{2+}-ATPase (for review, see Miller, 1991). A detailed understanding of the qualitative and quantitative contributions of each buffering component under various Ca^{2+} loading circumstances might shed light on the ways in which Ca^{2+} performs its physiologic as well as pathologic roles.

Several investigators have described the mechanisms by which mammalian dorsal root ganglion (DRG) neurons buffer physiologic calcium loads (Duchen et al., 1990; Thayer and Miller, 1990; Benham et al., 1992; Mironov et al., 1993; Werth and Thayer, 1994), and other groups have examined in detail the important role of the endoplasmic reticulum in bullfrog sympathetic neurons (Friel and Tsien, 1992a; Hua et al., 1993). These studies have focused on non-neurotoxic stimuli, and indeed, some of them have used physiologic stimulation to induce $[Ca^{2+}]_i$ transients. Although the results are not uniform, these studies of peripheral neurons collectively show evidence indicating a Ca^{2+} buffering role for the endoplasmic reticulum, mitochondria, Na^+/Ca^{2+} exchange, and Ca^{2+}-ATPase.

In contrast, much less is known about how central mammalian neurons buffer calcium loads. Two recent reports utilized subcortical neurons to examine the role of mitochondria, endoplasmic reticulum stores, Na^+/Ca^{2+} exchangers, and Ca^{2+}-ATPases in buffering action potential–induced calcium loads (Bleakman et al., 1993; Tatsumi and Katayama, 1993). Although Bleakman et al. (1993) did not find evidence supporting a primary role for any of the systems, Tatsumi and Katayama (1993) identified buffering contributions from mitochondria, Ca^{2+}-ATPases, and Na^+/Ca^{2+} exchange. Bleakman et al. (1993) also measured $[Ca^{2+}]_i$ responses after stimulation of the three ionotropic glutamate receptor subtypes, but they did not investigate the systems involved in buffering the agonist-induced Ca^{2+} loads. Thus, while these reports added to what is known about Ca^{2+} homeostasis in central neurons, several important issues remained to be clarified.

In this study of pyramidally shaped neurons cultured from embryonic rat forebrains, we used Indo-1 microfluorimetry to measure $[Ca^{2+}]_i$ transients in single cells. We chose rapidly acting

Received May 5, 1994; revised July 21, 1994; accepted Aug. 8, 1994.

We gratefully acknowledge Kristi Rothermund for the preparation of cell cultures, Dr. Stan Thayer for advice concerning the experimental equipment, and Drs. Jon Johnson and Elias Aizenman for critical review of the manuscript. This work was supported by the NIH (DA 07409) and a Grant-in-Aid from the American Heart Association. I.J.R. is an Established Investigator of the American Heart Association, and R.J.W. is supported by an NIH Medical Scientist Training Program Grant (5T32GM08208).

Correspondence should be addressed to Ian J. Reynolds, Ph.D., Department of Pharmacology, W1313 Biomedical Science Tower, Pittsburgh, PA 15261.

inhibitors of Na^+/Ca^{2+} exchange and mitochondria, which allowed us to identify the role of these mechanisms during *recovery* from a Ca^{2+} load rather than during the application of the stimulus. We found that mitochondria and Na^+/Ca^{2+} exchange were individually important in buffering these calcium loads, but that a dramatic effect was seen only when both systems were compromised. Finally, the data indicate that a glutamate-induced Ca^{2+} load imposes a greater burden on the two systems we studied than does a depolarization-induced $[Ca^{2+}]_i$ change of similar magnitude.

Materials and Methods

Cell culture. Forebrains were obtained from embryonic day 17 Sprague-Dawley rats and dissociated according to a method modified from Snodgrass (1980). Briefly, the lobes were incubated in 0.005–0.01% trypsin in 2 ml of Ca^{2+}/Mg^{2+}-free medium (in mM: 116 NaCl, 5.4 KCl, 26.2 $NaHCO_3$, 11.7 NaH_2PO_4, 5 glucose, 0.001% phenol red, and minimum essential medium amino acids; pH adjusted to 7.4 with NaOH) for 30–35 min at 37°C. The tissue was then triturated 8–10 times before the volume was brought to 10 ml and viability determinations were made with trypan blue (0.08%) exclusion.

The plating suspension was diluted to 150–400,000 cells/ml using plating medium (v/v solution of 90% Dulbecco's modified Eagle's medium, 10% heat-inactivated fetal bovine serum, 24 U/ml penicillin, 24 μg/ml streptomycin; final glutamine concentration, 3.1 mM). Cells were plated onto poly-L-lysine–coated (40 μg/ml) 35 mm glass coverslips that were inverted 1 d later in a maintenance medium (horse serum substituted for fetal calf serum, all other constituents identical). Inversion of the coverslips prevented glial proliferation, and no further medium changes were made until the neurons were utilized for Ca^{2+} recordings or ATP measurements 12–18 d later. Each type of experiment described in this article was generally performed on cells from four different culture dates but never less than two.

Our study was limited to pyramidally shaped neurons to reduce potential cell type variability. We only studied cells whose soma could be described as triangular or diamond-shaped with three or more visible processes. Photographs were made of all neurons and double checked for these features before being used in the analysis. Figure 1*A* shows a prototypical pyramidal neuron. Having passed the morphological criteria, neurons were monitored for baseline ratios reflective of $[Ca^{2+}]_i$ in the range of 50–150 nM and dye loading sufficient to have a 490 nm emission signal at least 20-fold greater than background.

Calcium measurements. Neurons were loaded for 55–65 min at 37°C in a solution of 5 μM Indo-1AM in HEPES buffered salt solution (HBSS; containing, in mM, 137 NaCl, 5 KCl, 0.9 $MgSO_4$, 1.4 $CaCl_2$, 10 $NaHCO_3$, 0.6 Na_2HPO_4, 0.4 KH_2PO_4, 5.6 glucose, and 20 HEPES; pH adjusted to 7.4 with NaOH) supplemented with 5 mg/ml bovine serum albumin (BSA) to enhance dye penetration into the cells. The dye was washed and the cells were reincubated at 37°C for an additional 20–30 min to allow for uniform cleavage of the acetyl-methoxyester moiety on the dye.

The coverslips were placed in a laminar-flow recording chamber (1 ml vol) fitted to a Nikon microscope, and the cells were constantly perfused at 20 ml/min except when exposed to ω-conotoxin GVIA. Excitation light from a Nikon 100 W Hg lamp was directed in series through two neutral density filters (ND32 and ND8), filtered (peak transmittance, 340 ± 7.5 nm), and reflected off of a dichroic mirror onto the cell. Emitted light transmitted through the dichroic (390 nm cutoff) was reflected sequentially off of two additional dichroic mirrors, first a 515 nm and then a 440 nm. The neurons were continuously visualized with long-wavelength red light through a video camera placed after the 515 nm dichroic, which had been coated to maximize reflectance of the shorter wavelengths (Omega 515DRLPEXT02). The light after the 440 nm dichroic was filtered at 405 ± 17 nm and 490 ± 10 nm for the reflected and transmitted beams, respectively. Two Thorn EMI photomultiplier tubes (PMTs) were used to measure the emitted light in parallel, and the currents from the PMTs were converted to voltage signals that were subsequently filtered at 10 Hz. Each of the two raw fluorescence signals was digitized by an A/D converter with DMA (Omega DAS-16) at 200 Hz and averaged on line by an 80386-based computer to generate a fluorescence ratio at 1 Hz; the raw data were saved for later off-line analysis. Single cells were spatially defined by a rectangular diaphragm placed before the video camera and PMTs, but gradients within single cells were not resolved. Much of the electronics hardware was obtained from the University of Pennsylvania Biomedical Instrumentation Group.

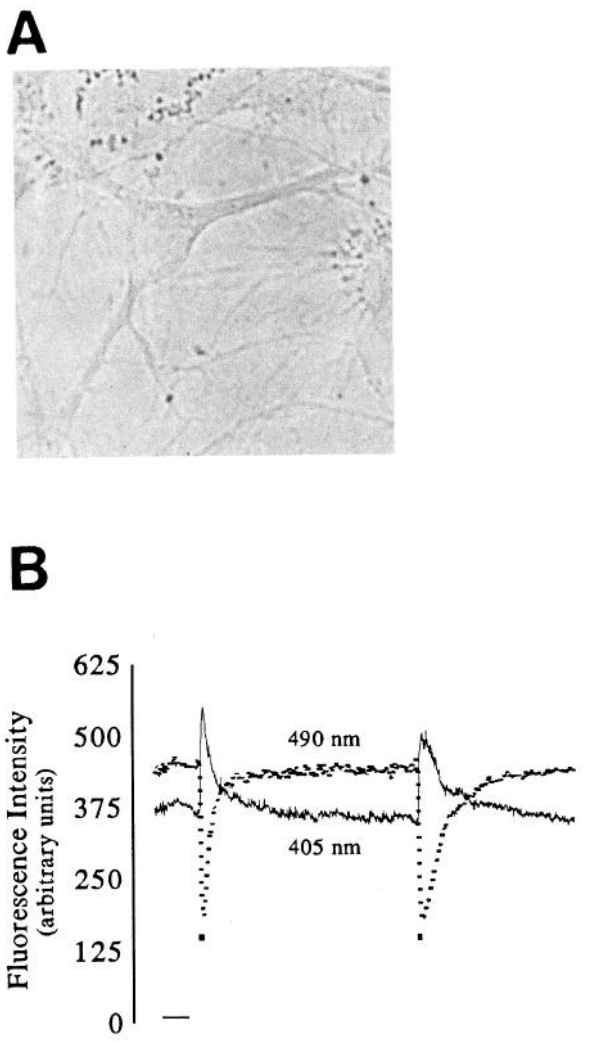

Figure 1. Indo-1 microfluorimetry in cultured rat forebrain neurons. *A*, A prototypical pyramidal neuron meeting our criteria of diamond or triangular shape with three or more processes. This cell was used to obtain the $[Ca^{2+}]_i$ recording shown in Figure 3*A*. *B*, Raw fluorescence data from this neuron showing less than 15% rundown in the absolute fluorescence signal over the course of a 25 min recording. This cell was stimulated for 15 sec with an iso-osmolar 50 mM KCl HBSS supplemented with 2.5 μM veratridine (*bars under the lower trace* indicate agonist application). Y-axis maximum value, 4096. Time calibration (*lower left*), 100 sec.

We used coverslips for only one complete experiment prior to discarding them. As indicated in the results, a complete experiment involved a 3 min collection of baseline $[Ca^{2+}]_i$, a 15 sec pulse of glutamate or depolarizing potassium as an internal control, a 15 min recovery period, and finally, a second identical stimulus that was immediately followed by a 2 min wash with the solution indicated.

Calibration. In situ calibration of the dye in cortical neurons was performed as a modification of the method of Wahl et al. (1990) At the same time that the dye was being loaded, neurons were energy depleted for 40 min in glucose-free cytoplasmic buffer (in mM: 120 KCl, 10 NaCl, 1 2-deoxyglucose, 10 $NaHCO_3$, 10 HEPES, 1 EGTA, 0.05 $MgSO_4$, 1 KH_2PO_4, and 1 μM antimycin-A) supplemented with 5 μM Indo-1AM and 5 mg/ml BSA. The dye was washed in cytoplasmic buffer and the cells were returned to the incubator for another 20 min. This procedure largely removed the ATP required for calcium pumping activity, and

the cells looked swollen but well loaded with dye; apparently, the treatment allowed for quicker loading. After measuring baseline $[Ca^{2+}]_i$, the chamber fluid was exchanged for cytoplasmic buffer containing 20 μM 4Br-A23187 and 5 μM nigericin. After a new equilibrium was established, the fluid was exchanged for cytoplasmic buffer that contained no EGTA and 10 mM $CaCl_2$ as well as the 4Br-A23187 and nigericin. Calibration data were collected from eight different cells.

The fluorescence ratios at the two extreme extracellular $[Ca^{2+}]$ were used as constants in the equation described by Grynkiewicz et al. (1985): $[Ca^{2+}]_i = K_D \cdot \beta \cdot (R - R_{min})/(R_{max} - R)$, to convert observed fluorescence ratios (R) to $[Ca^{2+}]_i$. In our system, β, the ratio of the fluorescence measured at 490 nm in the presence and absence of saturating $[Ca^{2+}]_i$, was 4.82; R_{min}, the fluorescence ratio in the nominal absence of $[Ca^{2+}]_i$, was 0.47; R_{max}, the ratio with saturating $[Ca^{2+}]_i$, was 4.79. K_D is the dissociation constant for the dye, 250 nM (Grynkiewicz et al., 1985). Background fluorescence was collected after each experiment and subtracted from the observed raw data prior to calcium value calculations; typically, background represented between 2% and 5% of the total signal.

As with any fluorometric measurement of intracellular ion concentrations, the actual $[Ca^{2+}]_i$ values reported here are estimates. Although we have gone to great lengths to provide an accurate calibration, we are almost certainly overestimating $[Ca^{2+}]_i$ largely because of difficulty in saturating the dye during calibration. Furthermore, to avoid bleaching over the relatively long time course of some of the experiments, we have reduced the excitation intensity to a minimum. This system specific problem, coupled with a fundamental decrease in the excitation efficiency for eliciting the 490 nm emission as one approaches Ca^{2+} saturation of the dye (Grynkiewicz et al., 1985), caused our denominator in the ratio measurements to become small and subject to substantial noise. However, we were mainly interested in time to recovery, and thus our most critical measurements were made well within the range of reasonable $[Ca^{2+}]_i$ values. We have assured that there is no substantial relationship between the peak values and the recovery times. Additionally, regardless of the actual value of $[Ca^{2+}]_i$, the relative relationships between the different peak values are reliable since they were acquired under identical conditions.

ATP measurements. Following the appropriate treatment, neurons were washed with ice-cold HBSS and then with ice-cold phosphate-buffered saline (PBS; in mM: 128 NaCl, 1.2 KCl, 8.1 Na_2HPO_4, 2.7 KH_2PO_4, pH adjusted to 7.4 with NaOH). The PBS was aspirated and 100 μl of 10% trichloroacetic acid (TCA) was added to the center of the coverslips. Cells were scraped and the extract collected; the TCA extraction was repeated and the two extracts were combined. The extracts were sonicated (3 × 5 sec) and centrifuged at 10,000–15,000 × g for 5 min. The protein pellet was retained for assay using the BCA reagent (Pierce Chemical). The supernatant was removed, measured by weight on an analytical balance, and then combined with 180 μl of an organic solvent (500 mM tri-*n*-octylamine in Freon). The samples were vortexed (2 × 5 sec) and centrifuged again at 10,000–15,000 × g for 1 min. The lipids in the organic phase on the bottom were discarded and the nucleotides in the top aqueous phase were retained; 150 μl of 100 mM K_2HPO_4 was added to each tube prior to storage at −70°C. Reverse-phase HPLC on a C_8 column was used to separate the samples prior to absorption measurements at 254 nm with an array of photodiodes (Waters System, Millipore). The absorption peak was integrated and converted to an ATP amount by linear regression from a standard curve generated using pure ATP; finally, the ATP content of each sample was normalized to protein.

Reagents. Nominally calcium-free solutions contained no added calcium and no chelating agents. Unless noted otherwise, nominally sodium-free solutions were made with 137 mM *N*-methyl-D-glucamine (NMG) as the sodium substitute with no added bicarbonate ion; the pH was adjusted to 7.4 with concentrated HCl. Where indicated, NMG solutions contained 5 mM $KHCO_3$ in place of 5 mM KCl. Lithium-substituted solutions contained 137 mM LiCl and 5 mM $KHCO_3$, and the pH was adjusted to 7.4 with Tris base. Potassium solutions (50 mM KCl) contained an iso-osmolar substitution of KCl for NaCl. Stock 1 mM nimodipine (a gift from Dr. Alexander Scriabine, Miles, Inc., West Haven, CT) and stock 5 mM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) solutions were made in methanol giving a final MeOH concentration of 0.1%. Veratridine stock solutions (2.5 mM) were made in 10% HCl. ω-Conotoxin GVIA (Bachem) was dissolved to 1 mM in distilled H_2O, aliquoted, and removed only once from the freezer before being discarded. Nigericin was made as a 10 mM stock in ethanol. Indo-1AM and 4Br-A23187 were obtained from Molecular Probes, aliquoted in anhydrous dimethyl sulfoxide, and frozen. Cell culture supplies were purchased from GIBCO, and, unless otherwise noted, all other reagents were of the highest grade available from Sigma.

Analysis and statistics. Peak $[Ca^{2+}]_i$ data were obtained and a $\delta[Ca^{2+}]_i$ calculated by subtracting the baseline $[Ca^{2+}]_i$. The 25% recovery time was defined as the time at which the cell *last* crossed the $[Ca^{2+}]_i$ value corresponding to (peak $[Ca^{2+}]_i - (0.25 \cdot \delta[Ca^{2+}]_i)$). A similar calculation was made for the 90% recovery time. For the main body of data, statistical significance was measured using a one-way ANOVA as calculated by INSTAT v2.0 (Graph Pad Software, San Diego, CA); post hoc analyses were performed with a Newman-Keuls test. Variability in our sample sizes precluded the use of multifactorial ANOVA where such use might have been appropriate (Fig. 4); instead, we used multiple t tests and a Bonferroni correction to compensate for repeated tests.

Results

Because cortical neuronal cultures are inherently heterogeneous, we sought to decrease the potential cell-type variability by restricting our study to neurons whose morphology was distinctly pyramidal (see Materials and Methods). A prototypical neuron is shown in Figure 1*A*, and the $[Ca^{2+}]_i$ recording of that cell is shown in Figure 3*A*. Figure 1*B* illustrates the raw fluorescence values from the same experiment showing less than 15% run-down over the course of a 25 min recording. In 158 neurons, the basal $[Ca^{2+}]_i$ was 85 ± 2 nM.

Mitochondria and Na^+/Ca^{2+} exchange buffer Ca^{2+} loads

The importance of mitochondria and Na^+/Ca^{2+} exchange in buffering Ca^{2+} loads elicited by two different stimuli (glutamate and depolarization) is demonstrated in Figure 2. In Figure 2*A*, a 15 sec control pulse of 3 μM glutamate and 1 μM glycine was followed 15 min later by an identical glutamate pulse that was washed out with the various reagents shown. The majority of cells had buffered their $[Ca^{2+}]_i$ to baseline values within 2 min of the first 15 sec glutamate pulse. Thus, we hypothesized that if a particular buffering system were important in the normal homeostatic response to our glutamate-induced Ca^{2+} load, disruption of that system during the first 2 min would delay the neuron's recovery to baseline $[Ca^{2+}]_i$. We chose to disrupt mitochondria and Na^+/Ca^{2+} exchange during that first 2 min period in which most cells would normally have recovered. We assumed that after 15 min, the cell would be in essentially the same homeostatic state for the second glutamate pulse as it had been for the first. In about half of our control neurons, the recovery times for the two transients were nearly identical, but recovery times in the remaining controls were longer for the second transient than for the first. To account for this variability in making comparisons between cells, we classified the cell as having "increased recovery time" when the time to 90% recovery for the second $[Ca^{2+}]_i$ transient was 2.5-fold greater than the first transient. By this criterion, only 1 of 12 control neurons had an "increased recovery time."

In all of our experiments, the second peak $[Ca^{2+}]_i$ was substantially smaller (e.g., see Fig. 2*A*, trace i). This apparent run-down was dependent on UV light and occurred for Ca^{2+} loads induced by both glutamate and depolarizing potassium solutions; other investigators have also found that the dynamic range of Indo-1 decays over time (Randall and Thayer, 1992). The stimuli were identical and the similar recovery times for the two pulses under control conditions suggested that our problem was one of measurement as opposed to a true difference in $[Ca^{2+}]_i$. In 49 neurons stimulated with glutamate, the average peak $[Ca^{2+}]_i$ following the first stimulation was 3.37 ± 0.30 μM (mean ± SEM).

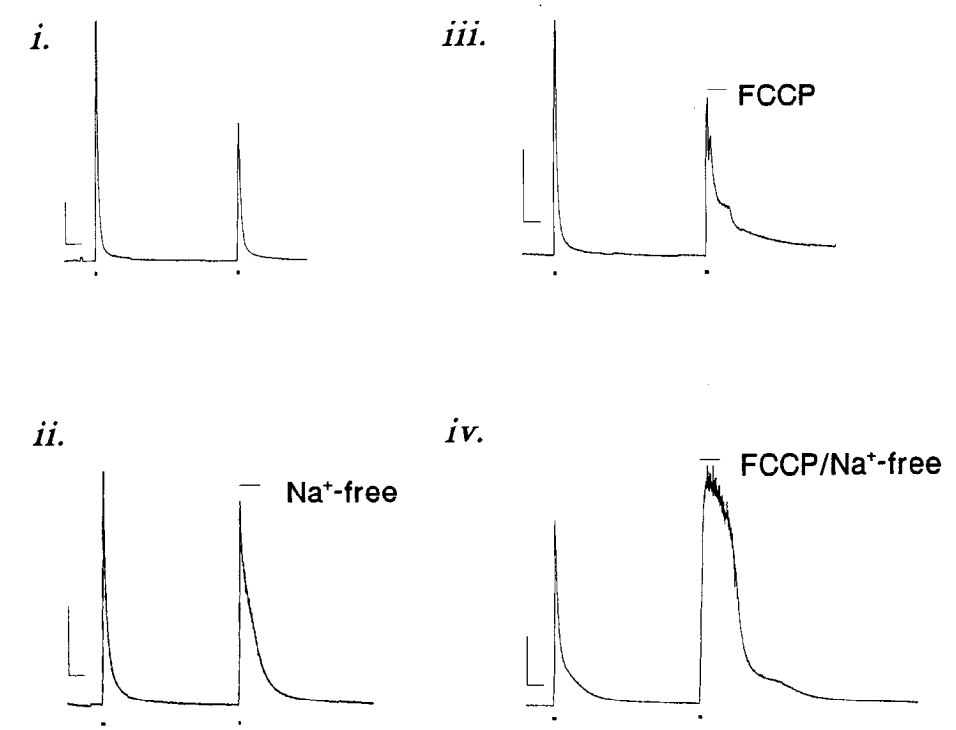

Figure 2. Washing with FCCP or Na^+-free buffer delays recovery from glutamate and depolarization induced calcium loads. *A,* Neurons were stimulated for 15 sec with 3 μM glutamate and 1 μM glycine (*bars under each trace* indicate agonist application). After a fixed 15 min interval, a second glutamate/glycine pulse was delivered and washed for 2 min with the solutions indicated: *i,* control HBSS; *ii,* Na^+-free, *N*-methyl-D-glucamine (NMG)-substituted HBSS; *iii,* 5 μM FCCP; *iv,* 5 μM FCCP in NMG-substituted buffer. The traces are representative of the mean recovery times for the respective conditions (n = 12, 15, 10, and 13). Note in *iii* that the $[Ca^{2+}]_i$ recovers to a plateau prior to FCCP washout; this was a common finding. In *iv,* the neuron is unable to buffer $[Ca^{2+}]_i$ even halfway to baseline in the presence of the modified buffer; this occurred in 11 of 13 cells. Calibration: 500 nM $[Ca^{2+}]_i$, 100 sec. *B,* Neurons were depolarized for 15 sec with an iso-osmolar 50 mM KCl HBSS supplemented with 2.5 μM veratridine (*bars under each trace* indicate agonist application). As in *A,* the second pulse was washed for 2 min with the solutions indicated: *i,* control HBSS; *ii,* Na^+-free, NMG-substituted HBSS; *iii,* 5 μM FCCP; *iv,* 5 μM FCCP in NMG-substituted buffer. Again, the traces were chosen to represent the mean recovery times (n = 6, 11, 8, 14, respectively). Note in particular that the peak $[Ca^{2+}]_i$ level achieved with the depolarization stimulus was almost always greater than the peak with a glutamate stimulus. Yet in *iv,* unlike recovery from the glutamate Ca^{2+} load, the neuron stimulated by depolarization was able to buffer partially the Ca^{2+} load while exposed to the modified wash solution. Calibration: 500 nM $[Ca^{2+}]_i$, 100 sec.

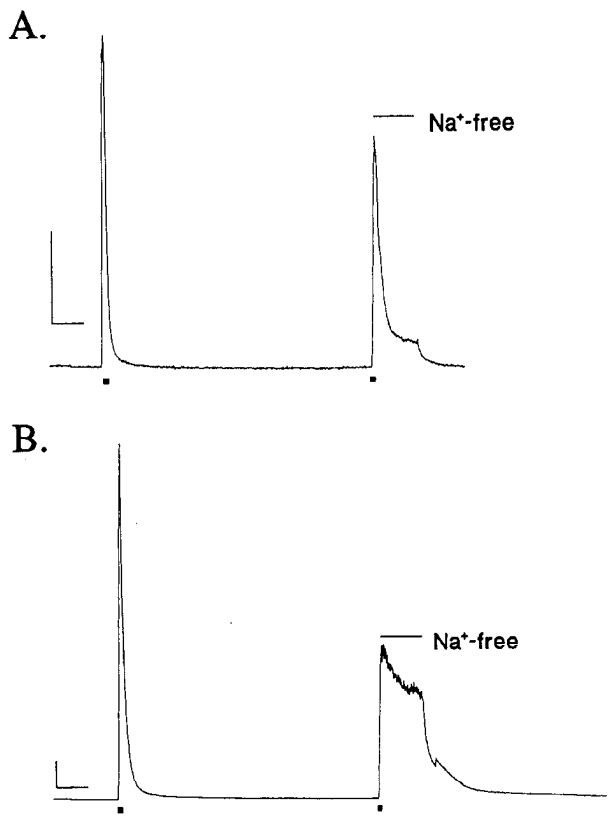

Figure 3. Na^+-free buffer reveals a variable degree of dependence on Na^+/Ca^{2+} exchange. *A*, This neuron was depolarized for 15 sec with an iso-osmolar 50 mM KCl HBSS supplemented with 2.5 μM veratridine. The trace illustrates a phenomena that we saw in 6 of 24 neurons from the depolarization group and 10 of 27 neurons from the glutamate group. In this significant minority of neurons, the cell cannot fully recover without Na^+. *B*, This neuron was stimulated for 15 sec with 3 μM glutamate and 1 μM glycine. The trace illustrates a remarkable level of dependence on Na^+/Ca^{2+} exchange as this neuron recovers little in the first 2 min, but rapidly buffers $[Ca^{2+}]_i$ when Na^+ is reintroduced. We observed this behavior only in cells stimulated with glutamate ($n = 4$ of 27 observations, 0 of 24 with depolarization). Na^+-free buffer did not change the baseline $[Ca^{2+}]_i$ when applied without prior glutamate or depolarization stimuli (not shown). *Bars under each trace* indicate agonist application. Calibration: 500 nM $[Ca^{2+}]_i$, 100 sec.

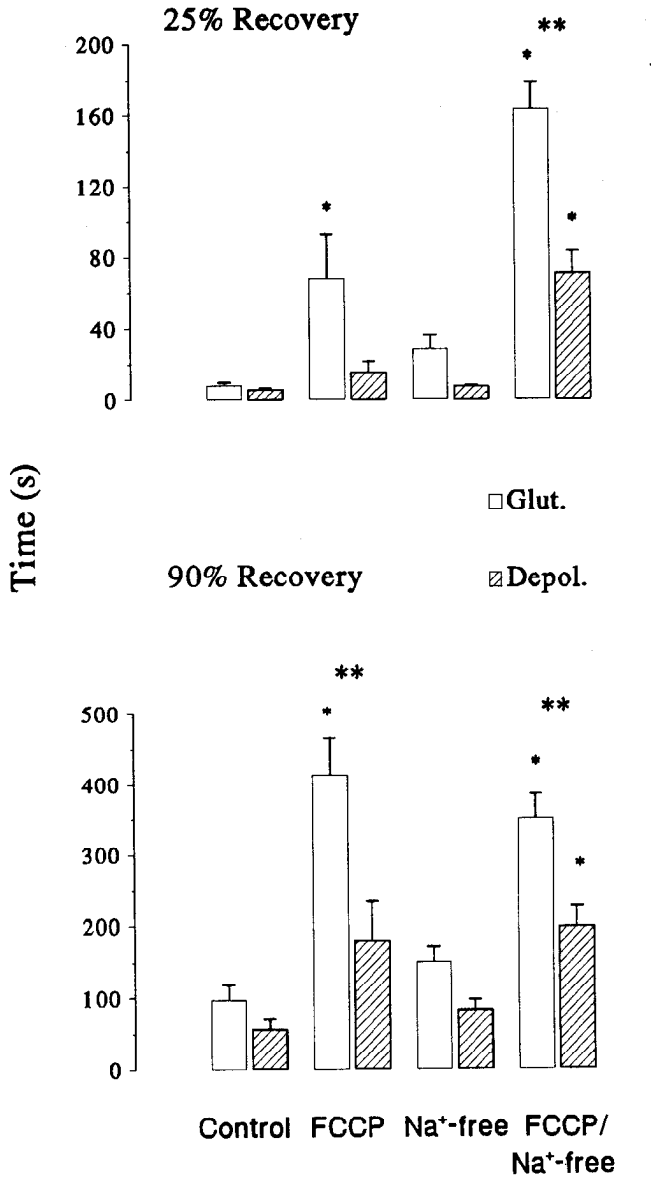

Figure 4. Buffering of glutamate-induced calcium loads is more sensitive to disruption of mitochondria and Na^+/Ca^{2+} exchange. This figure summarizes the recovery time analysis for 89 separate recordings. Neurons were stimulated according to the protocols described in Figure 2, and the time to 25% and 90% recovery was measured in each trace as described in Materials and Methods. Each bar represents the mean ± SEM of 6–15 individual experiments performed in three or four different culture preparations, and reflects the mean of the data for the experiments shown in Figure 2. *, condition was significantly different from its control ($p < 0.05$, Newman-Keuls post hoc following one-way ANOVA); ** (above *pairs of bars*), recovery times were significantly different between the glutamate and depolarization stimuli ($p < 0.01$, Student's t test; we made a Bonferroni correction to account for multiple t tests).

Washing with Na^+-free buffer revealed a dependence on Na^+/Ca^{2+} exchange (Fig. 2*A*, ii) with a correspondingly increased recovery time in 10 of the 15 cells tested. However, the extent to which the neurons were affected by Na^+-free buffer was variable. Of the 10 cells that were affected, five could not recover to baseline $[Ca^{2+}]_i$ until Na^+ was reintroduced into the buffer (Fig. 3*A*). Three of the 10 neurons seemed to be very dependent on the Na^+/Ca^{2+} exchanger and recovered only slightly without Na^+, rapidly returning to baseline when Na^+ was provided in the bath (Fig. 3*B*; we did not leave the Na^+-free buffer on longer to explore whether this population of cells would have ever recovered in the absence of Na^+). Na^+-free buffer did not change the baseline $[Ca^{2+}]_i$ when applied without prior glutamate or depolarization stimuli (not shown). Washing with Na^+-free buffer did not significantly increase the mean recovery time largely because of the variability in the degree of dependence on the Na^+/Ca^{2+} exchange. However, by the criteria of "increased recovery time" 10 of 15 cells were affected by a Na^+-free wash, in contrast to 1 of 12 cells that had an increased recovery time following control HBSS wash.

The mitochondrial uncoupler FCCP is a rapidly acting and reversible agent that collapses the proton gradient across the mitochondrial membrane and ultimately inhibits mitochondrial Ca^{2+} uptake (Heytler, 1981; Miller, 1991). Neurons stimulated with glutamate and washed with 5 μM FCCP ($n = 11$; Fig. 2*A*, iii; see also Fig. 4) uniformly showed an increased recovery time before $[Ca^{2+}]_i$ returned to baseline values. The trace also shows

Table 1. Effect of different Na^+-free buffer composition on 90% recovery time

	Recovery time (sec)			
		FCCP		
Stimulus	Control	Li^+	NMG	NMG (no HCO_3^-)
Glutamate	96 ± 23 (12)	373 ± 88 (6)	274 ± 13 (7)	350 ± 35 (13)
Depolarization	57 ± 15 (6)	191 ± 39 (6)	206 ± 41 (6)	199 ± 29 (14)

Neurons were stimulated for 15 sec with either 3 μM glutamate and 1 μM glycine or 50 mM K^+ and 2.5 μM veratridine. The stimuli were washed out for 2 min with either a Li^+-substituted, Na^+-free solution containing $KHCO_3$, or an NMG-substituted buffer containing $KHCO_3$, or our standard NMG-substituted buffer without HCO_3^-; each Na^+-free buffer solution also contained 5 μM FCCP. The Li^+-substituted solution blocks Na^+/Ca^{2+} exchange but leaves both Na^+/H^+ and HCO_3/Cl^- exchange intact; the NMG-substituted solution blocks both Na^+/Ca^{2+} exchange and Na^+/H^+ exchange but provides HCO_3. We continued to find increased recovery times in cells without Na^+/Ca^{2+} exchange even when the transporters that help to regulate pH were supported. Values represent mean ± SEM (number of cells).

the interesting ability of FCCP to generate a Ca^{2+} plateau (seen in every cell).

A Na^+-free wash solution containing 5 μM FCCP completely disabled the neurons' ability to buffer the glutamate-induced Ca^{2+} load, resulting in an increased recovery time in every cell (Fig. 2*A*, iv; $n = 13$; see also Fig. 4). As the wash solution entered the chamber, the initial $[Ca^{2+}]_i$ peak was followed by a sustained $[Ca^{2+}]_i$ elevation ("hump") in every neuron tested, and frequently (11 of 13), the cell was unable to recover $[Ca^{2+}]_i$ even 50% until ordinary buffer was reintroduced. It appears that by short-circuiting the mitochondrial Ca^{2+} uniporter and inhibiting (or reversing) the plasmalemmal Na^+/Ca^{2+} exchanger, we effectively disrupted the neurons' ability to handle a glutamate-induced Ca^{2+} load.

In our neurons, a depolarizing solution containing 50 mM K^+ induced a peak $[Ca^{2+}]_i$ less than the glutamate stimulus. In order to better match both the peak and the recovery time to our glutamate-induced load, we added 2.5 μM veratridine, a compound known to block rapid sodium channel inactivation (Catterall, 1980). The combined depolarizing stimulus induced a $[Ca^{2+}]_i$ transient whose peak $[Ca^{2+}]_i$ was somewhat larger with the depolarizing stimulus ($n = 39$, 5.83 ± 0.54 μM $[Ca^{2+}]_i$, mean ± SEM). Analogous traces to those in Figure 2*A* are shown for the depolarizing stimulus in Figure 2*B*. Again, inhibition of either mitochondrial Ca^{2+} uptake or the plasma membrane exchanger delayed the recovery of $[Ca^{2+}]_i$ to baseline, but blocking both buffering mechanisms induced a similar "hump" in most cells and significantly delayed recovery. As with the glutamate-induced Ca^{2+} load, washing the depolarization stimulus with Na^+-free buffer did not increase the mean recovery time in a statistically significant way. In our experiments, neurons were variably dependent on Na^+/Ca^{2+} exchange in that some absolutely required a functional exchange and others appeared to be indifferent to its activity.

To buffer glutamate-induced Ca^{2+} loads, central neurons rely heavily on mitochondria and Na^+/Ca^{2+} exchange

Despite the lower peak $[Ca^{2+}]_i$ levels induced by glutamate, Figure 4 illustrates a key finding: the glutamate-induced Ca^{2+} loads were significantly more sensitive to our manipulations than were the corresponding depolarization-induced loads. We chose to measure time to 25% recovery to reflect the early part of the recovery process and time to 90% recovery to indicate the time required to return to near baseline values. We will address this issue more thoroughly in the Discussion, but our experiments reveal an important difference between the Ca^{2+} loads induced by these two different stimuli. It is possible that the other buffering systems (which we left intact) were more effective in handling the depolarization-induced load during the early phase of recovery. It is also possible that the absolute amount of Ca^{2+} influx was greater for the glutamate responses, even though peak $[Ca^{2+}]_i$ values elicited by depolarization were consistently larger than for those induced by glutamate.

Changes in intracellular pH regulation are not responsible for changes in $[Ca^{2+}]_i$ homeostasis

Recent work has indicated that pathologic concentrations of glutamate can disrupt H^+ homeostasis and that calcium loading can acidify neuronal cytoplasm (Hartley and Dubinsky, 1993; Koch and Barish, 1994; Werth and Thayer, 1994). Because our standard Na^+-free solution disrupted both the HCO_3^-/Cl^- transporter and the Na^+/H^+ transporter, we felt it important to rule out alteration of H^+ homeostasis as the primary mechanism for our ability to disturb the neurons' Ca^{2+} buffering. To dissect the contribution made by each of the transport systems whose activity affects pH, we repeated the experiments previously described utilizing two additional Na^+-free solutions, one Li^+ substituted containing bicarbonate ion and one NMG substituted also with bicarbonate. The Li^+-substituted solution supported Na^+/H^+ transport while removing Na^+/Ca^{2+} exchange; the NMG solution removed both of those transporters but, unlike our standard Na^+-free buffer, left HCO_3^-/Cl^- exchange intact by providing extracellular bicarbonate. The data are summarized in Table 1, and Figure 5 illustrates the results of these control experiments: when we utilized Na^+-free buffers in our FCCP/Na^+-free washes, disruption of Na^+/Ca^{2+} exchange was the effect of primary importance, not disruption of the transporters important in pH regulation. The cells showed similar responses in all three Na^+-free solutions, and there were no substantial differences among the three types of Na^+-free buffers when they were used in the FCCP, Na^+-free protocol (Table 1). Thus, the traces in Figures 2, 3, and 5 are representative of data obtained in all three types of Na^+-free solutions, and the total cell counts given in the legend to Figure 3 reflect a pooling of all Na^+-free experiments. We concluded that the effects of Na^+-free buffer were not primarily the result of a change in intracellular pH regulation.

Many of the available pharmacologic tools for blocking both directions of the Na^+/Ca^{2+} exchange have a relatively large effect on voltage-gated Na^+- and Ca^{2+} channels, making them unattractive for our experiments. We tried to use one of the more specific, commercially available agents, dichlorobenzamil, but found that it was fluorescent in the 405 nm range; thus, it was not useful in our setup.

With respect to pH, we had an additional concern. It was possible that a 5 μM concentration of FCCP might have an effect

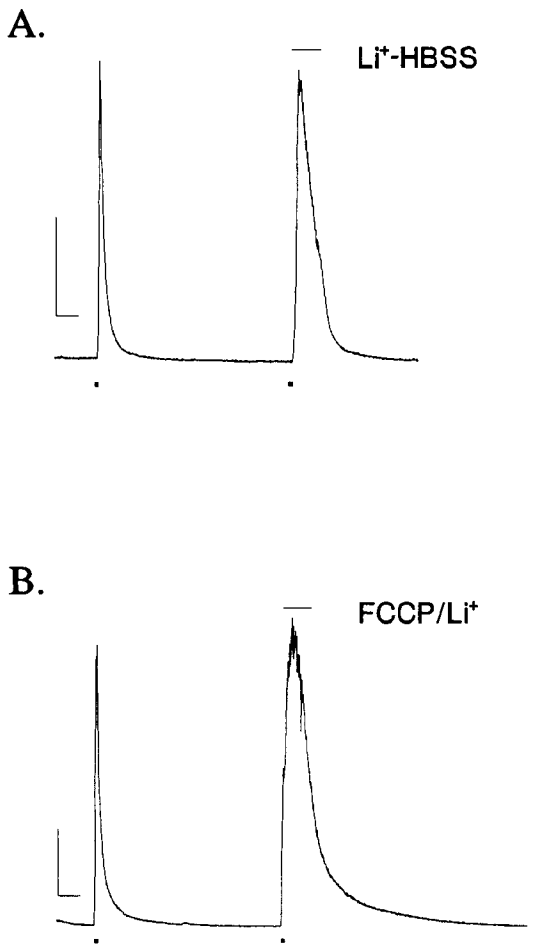

Figure 5. Effect of Na^+-free buffer on recovery from $[Ca^{2+}]_i$ increases is not the result of perturbed intracellular pH regulation. *A,* This neuron was stimulated for 15 sec with 3 μM glutamate and 1 μM glycine at the time indicated by the *bars below the trace.* Following the second stimulation, the cell was washed for 2 min with an Li^+-substituted, Na^+-free solution containing $KHCO_3$. This solution blocked Na^+/Ca^{2+} exchange but supported Na^+/H^+ and HCO_3^-/Cl^- exchange; the cell's recovery to baseline $[Ca^{2+}]_i$ levels remained retarded. *B,* This neuron was stimulated for 15 sec with 3 μM glutamate and 1 μM glycine at the time indicated by the *bars.* The 2 min washout solution contained 5 μM FCCP in an Li^+-substituted, Na^+-free buffer containing $KHCO_3$, thus blocking mitochondria and Na^+/Ca^{2+} exchange but leaving transporters that regulate pH intact. The two traces are reflective of the mean recovery time data obtained in a total of 24 cells using Li^+-substituted and NMG-substituted solutions, both containing HCO_3^-. An additional 25 cells (not shown) confirmed these findings for the depolarization-induced Ca^{2+} load. Calibration: 500 nM $[Ca^{2+}]_i$, 100 sec.

on the plasma membrane in addition to its desired effect of dissipating the mitochondrial pH gradient. Specifically, the neurons could depolarize if 5 μM FCCP allowed H^+ ions to move across the plasma membrane toward their Nernst electrochemical equilibrium potential. If large enough, such a depolarization would result in a Ca^{2+} influx through voltage-gated calcium channels (VGCC); other investigators may have observed this phenomena (Duchen, 1990). Figure 6 shows that 5 μM FCCP caused a Ca^{2+} influx (trace i) that could be largely attenuated with the L- and N-type VGCC blockers nimodipine and ω-conotoxin (trace ii); a reduction in the peak $[Ca^{2+}]_i$ transient was seen in all cells tested (8 of 8). There was a great deal of variability in the duration and size of the response to 5 μM FCCP, and although the calcium channel antagonists always substantially reduced the peak response, $[Ca^{2+}]_i$ continued to fluctuate for 5-15 min after washout of all drugs.

The effects of 750 nM FCCP were more consistent with respect to $[Ca^{2+}]_i$ transients, in that the transients were slower and smaller (Fig. 6, trace iii). More importantly, $[Ca^{2+}]_i$ rapidly returned to baseline following washout of FCCP. In addition, almost all of the rise in $[Ca^{2+}]_i$ could be blocked with the combined calcium channel antagonists (Fig. 6, trace iv) at the lower FCCP concentration (n = 3 of 3).

We sought to establish that the lower FCCP concentration disrupted neuronal $[Ca^{2+}]_i$ homeostasis equally effectively. In particular, we were concerned that our effect might be completely dependent upon a large calcium influx through VGCCs during the 2 min wash phase. However, there were no qualitative differences between 750 nM FCCP and the higher concentration used for the majority of our experiments. Glutamate-induced Ca^{2+} loads washed with 750 nM FCCP (n = 5) had increased recovery times, and washing the glutamate with 750 nM FCCP in a Na^+-free buffer severely disturbed the neuron's ability to recover from a glutamate-induced Ca^{2+} load (n = 5). Qualitatively, therefore, the two FCCP concentrations produced identical results. It is perhaps noteworthy, however, that a quantitative difference remained between the magnitude of the recovery time increases for the two FCCP concentrations [glutamate-induced Ca^{2+} load washed with 5 μM FCCP in Na^+-free buffer (n = 13): time to 25% recovery = 163 ± 16 sec, 90% recovery = 350 ± 35 sec; 750 nM FCCP in Na^+-free buffer (n = 5): time to 25% recovery = 130 ± 19 sec, 90% recovery = 253 ± 45 sec; all values mean ± SEM].

ATP depletion and extracellular Ca^{2+} are not the principal determinants of the $[Ca^{2+}]_i$ hump

Two key questions remained at this stage. The first issue concerned ATP depletion, and while other work in this laboratory has suggested the relative resilience of these neurons to ATP loss (Rajdev and Reynolds, unpublished observations), it seemed important to investigate the possibility that some of our effects reflected defeat of the Ca^{2+}-ATPase pumps. Coverslips resting in individual culture dishes were washed with HBSS, 15 sec of 3 μM glutamate, or 15 sec of glutamate followed by 2 min of 5 μM FCCP in Na^+-free buffer; a positive control in which cells were ATP-depleted using 1 mM deoxyglucose and 1 μM antimycin-A (30 min) was performed in parallel. The treatment was terminated on ice and cell extracts were processed as described in Materials and Methods. Reverse-phase HPLC was used to separate the samples and a photodiode array determined the ATP concentration. This value was normalized to proteins. The numbers given are the average of two or three dishes for each condition, and similar results were found three times in experiments lacking the positive control. We saw no ATP depletion as a result of our Na^+-free/FCCP treatment (mean in nmol/mg protein ± SEM): control, 292 ± 231; glutamate, 359 ± 41; glutamate washed with FCCP in Na^+-free buffer, 164 ± 77; ATP-depleted positive control, −22 ± 8. The negative value indicates ATP levels below the detection level. These data demonstrate that brief periods of mitochondrial inhibition did not cause ATP depletion in our neurons and may indicate the presence of well-developed glycolytic pathways (McConnell et al., 1992).

The second issue was related to the source of the Ca^{2+} "hump." Although our data suggested that the effects were based upon

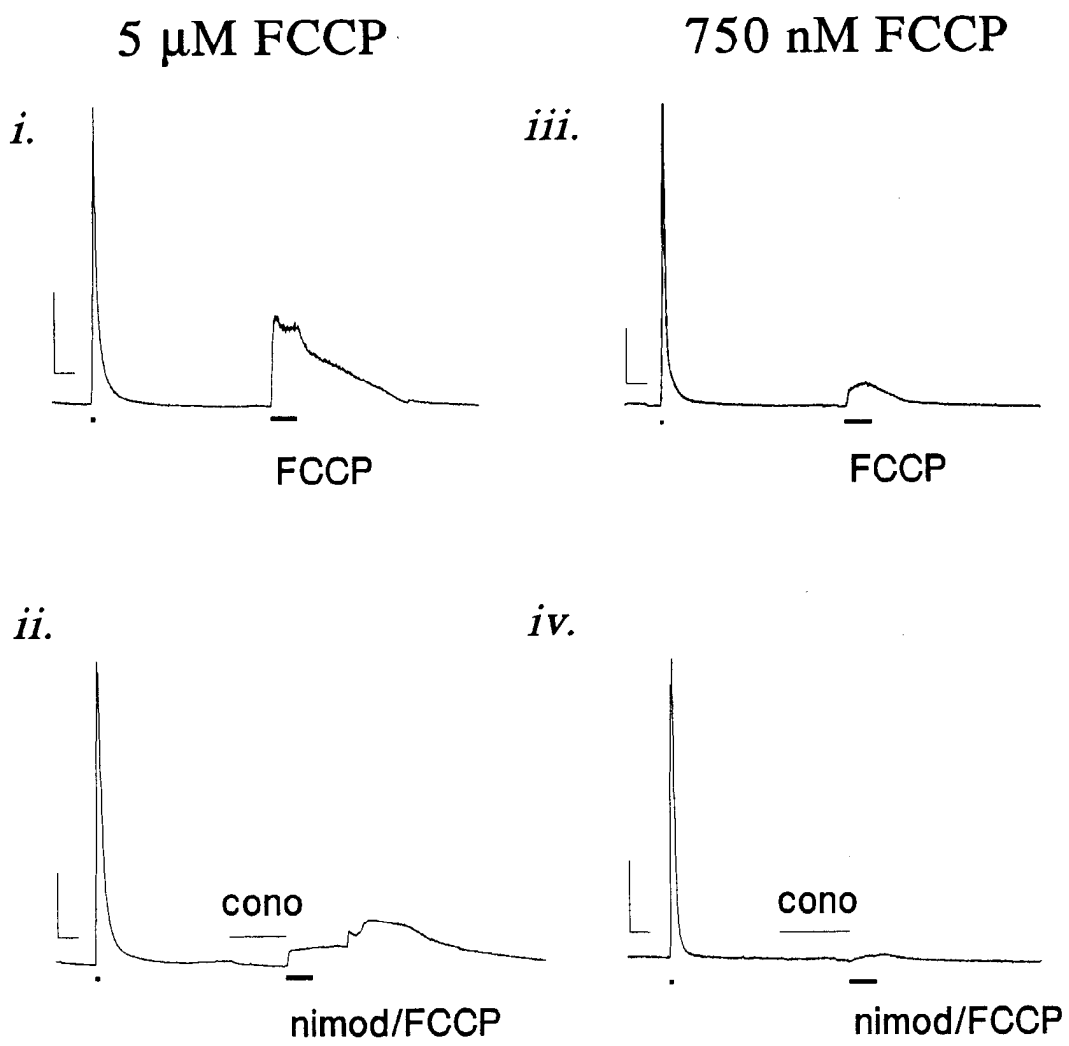

Figure 6. FCCP causes $[Ca^{2+}]_i$ elevations blockable by L- and N-type calcium channel antagonists. Neurons exposed to 2 min of 5 μM FCCP (*trace i*) rapidly responded with $[Ca^{2+}]_i$ transients of variable size. The cells required several minutes to buffer $[Ca^{2+}]_i$ levels back to baseline following washout of FCCP, and these recovery times were also highly variable. A control glutamate stimulus (15 sec, 3 μM) confirmed the neuron's excitability, but reversing the experimental arrangement had no effect (not shown). Following a 5 min pretreatment with 1 μM ω-conotoxin GVIA, application of 5 μM FCCP and 1 μM nimodipine (*trace ii*) resulted in a substantial reduction of the peak $[Ca^{2+}]_i$ seen in *trace i. Traces iii and iv* illustrate similar experiments performed with 750 nM FCCP. At the lower FCCP concentration, the resulting transients were more consistent and had much smaller peak $[Ca^{2+}]_i$ values. Note that the cells recover rapidly following washout of 750 nM FCCP and that almost all of the transient is blocked by calcium channel antagonists. The traces reflect the mean peak $[Ca^{2+}]_i$ and recovery times for the respective conditions (n = 9, 8, 2, 3). *Bars under each trace* indicate glutamate (15 sec, 3 μM) and then FCCP application. Calibration: 500 nM $[Ca^{2+}]_i$, 100 sec.

the perturbation of intracellular events, determining the source of the rising $[Ca^{2+}]_i$ *after* glutamate washout was a critical issue. To examine the potential role of Ca^{2+} entry during the recovery phase, we washed the glutamate pulse with Na^+-free, Ca^{2+}-free HBSS containing FCCP. Under these conditions, the $[Ca^{2+}]_i$ rise during washout (the "hump") persisted, indicating an intracellular source for the pool of Ca^{2+} involved. There was also a significant increase in recovery time as compared to controls. However, compared to the recovery time in the presence of FCCP in Ca^{2+}-containing, Na^+-free HBSS, neuronal $[Ca^{2+}]_i$ recovered more quickly in the absence of extracellular Ca^{2+} [glutamate-induced Ca^{2+} load washed with 5 μM FCCP in Na^+-free buffer (n = 13): time to 25% recovery = 163 ± 16 sec, 90% recovery = 350 ± 35 sec; FCCP in Na^+-free, Ca^{2+}-free buffer (n = 9): time to 25% recovery = 70 ± 20 sec, 90% recovery = 164 ± 35 sec]. The data are pooled from experiments using both concentrations of FCCP, and interestingly, there was now no quantitative difference between the mean recovery latencies for the different FCCP concentrations. This lack of difference between the two concentrations may be reflective of the Ca^{2+} influx that occurred during the 2 min of 5 μM FCCP application; the influx was clearly less with 750 nM FCCP (see above and Fig. 6). There appears to be a component of influx in our experiments performed in Ca^{2+}-containing buffer; further experiments are necessary to quantitate the contributions made by continued activation of Ca^{2+}-permeable ion channels and reversal of Na^+/Ca^{2+} exchange.

Discussion

In this study, we have demonstrated that neuronal buffering of Ca^{2+} loads induced by both depolarization and glutamate is critically dependent on mitochondria and secondarily reliant on Na^+/Ca^{2+} exchange. In most of the cells, the buffering capacity of each system is apparently sufficient to compensate partially

for a deficiency in the other; however, when both systems are incapacitated, it seems that the Ca^{2+} load cannot be effectively managed by the neurons' other potential Ca^{2+} buffering resources. It is also likely that glutamate imposes a greater Ca^{2+} burden on neurons than does a depolarizing stimulus as inferred from the different recovery time characteristics.

Two issues regarding our experimental paradigm are noteworthy. First, by manipulating only the wash out solution, we specifically avoided the potential problem of comparing calcium loads induced under different conditions. Other investigators (Bleakman et al., 1993; Tatsumi and Katayama, 1993) have introduced Na^+-free buffer or FCCP analogs into the experiment before the second stimulus, thus potentially changing the nature of the calcium load and complicating the interpretation of the results. Especially with respect to experiments conducted in Na^+-free buffer, two important complications are avoided by removing Na^+ only after the stimulus delivery. Hartley and Dubinsky (1993) showed that a 5 min exposure to NMG-substituted buffer is sufficient to induce substantial intracellular acidification, probably reflecting the tonic activity of the Na^+/H^+ exchanger. It is possible that such an acidification might alter a host of intracellular functions, and in any case, it is theoretically unattractive to compare Ca^{2+} loads delivered to cells with different states of intracellular pH. A stronger argument against removing Na^+ prior to the stimulus exists: in the presence of Na^+, both depolarization and glutamate will result in increases in $[Na^+]_i$. Glutamate will open ligand-gated channels permeable to Na^+, and both stimuli will open voltage-gated Na^+ channels. Removing the Na^+ prior to the stimulus thus fundamentally changes the stimulus itself. Particularly with respect to finding a role for Na^+/Ca^{2+} exchange in buffering Ca^{2+} loads, the $[Na^+]_i$ is critical (Kiedrowski et al., 1994), and again, identical stimuli are theoretically more attractive.

Thayer and Miller (1990) have shown that in DRG neurons, the mitochondria buffer Ca^{2+} loads induced by depolarization. In their experiments, addition of an uncoupler to stimulated neurons caused net efflux of mitochondrial Ca^{2+} that had been stored during and immediately following the stimulus. Matrix Ca^{2+} is moved into the cytoplasm through the mitochondrial Na^+/Ca^{2+} exchange, whose activity is normally concealed by the mitochondrial Ca^{2+} uniporter (Miller, 1991). Our experimental protocol prevented mitochondrial buffering after the stimulus but would have allowed the mitochondria to store a significant amount of Ca^{2+} during the stimulus period, in contrast to other investigators who have applied FCCP throughout the stimulus. Once mitochondrial Ca^{2+} uptake was prevented, the tonic activity of the mitochondrial Na^+/Ca^{2+} exchange could release a great deal of stored Ca^{2+}. When $[Na^+]_i$ increased following glutamate stimulation or our veratridine-supplemented depolarization, two distinct processes were influenced: (1) there was a smaller gradient for Ca^{2+} extrusion (Kiedrowski et al., 1994), and (2) there was a larger gradient for movement of matrix Ca^{2+} into the cytosol by the mitochondrial exchange. Thus, mitochondria were probably the source of the Ca^{2+} hump that we observed during simultaneous inhibition of mitochondrial Ca^{2+} uptake and plasmalemmal Na^+/Ca^{2+} exchange.

The second issue regarding the paradigm is our choice of glutamate concentration and stimulus duration. Our 15 sec 3 μM glutamate application is probably not an accurate reflection of normal synaptic physiology. However, such a stimulus has not been neurotoxic in any of the studies with which we are familiar. In our laboratory, 3 μM glutamate produces a peak $[Ca^{2+}]_i$ transient that is almost at the top of the dose–response curve but is also readily buffered by the cell. At this glutamate concentration, most of the $[Ca^{2+}]_i$ response is mediated by NMDA receptors (Rajdev and Reynolds, 1994). Thus, it was a suitable stimulus to give reliable calcium loads whose buffering could be consistently manipulated. In that our paradigm produced maximal Ca^{2+} responses without any toxicity, we would consider this a modest glutamate-induced Ca^{2+} load.

The mechanisms by which central neurons buffer modest glutamate-induced Ca^{2+} loads has received little attention, yet the cells' homeostatic response is a subject of tremendous import. Because Ca^{2+} influx is clearly implicated in the signal transduction that leads to excitotoxic cell death (Choi, 1992), there has been a significant interest in studying $[Ca^{2+}]_i$ in neurons following excitotoxic glutamate stimulation. Many recent studies (Manev et al., 1989; Frandsen and Schousboe, 1991; Milani et al., 1991; Randall and Thayer, 1992; Dubinsky, 1993; Elliott and Sapolsky, 1993; Frandsen and Schousboe, 1993; Mattson et al., 1993) have implicated delayed increases of $[Ca^{2+}]_i$ in the toxicity produced by pathologic glutamate stimulation. Most of those reports cite alterations in $[Ca^{2+}]_i$ homeostasis as a fundamental part of the process leading to neuronal cell death, and yet it seems difficult to make statements about alterations in $[Ca^{2+}]_i$ homeostasis when so little is known about the way central neurons handle Ca^{2+} loads induced by non-neurotoxic glutamate stimulation. Our study represents an important first step toward understanding how central neurons handle modest glutamate-induced Ca^{2+} loads. Thus, it may now be easier to generate testable hypotheses concerning the alterations in $[Ca^{2+}]_i$ homeostasis resulting from pathologic glutamate stimulation.

A delayed or long-lasting elevation of $[Ca^{2+}]_i$ may indicate that the normal Ca^{2+} buffering mechanisms have failed, but, especially with respect to $[Ca^{2+}]_i$, a "black box" exists between the initial $[Ca^{2+}]_i$ transient and subsequent neurodegeneration. It is possible that either (1) the size of the Ca^{2+} load or (2) the way in which that load is handled, or both, may be primary factors in determining the toxicity of a stimulus. Two recent studies have evaluated different Ca^{2+} loading stimuli with respect to their ability to cause cell death *in vitro* (Hartley et al., 1993; Tymianski et al., 1993). Both studies concluded that glutamate was the most neurotoxic Ca^{2+} loading stimulus; in keeping with earlier reports, the toxicity was closely related to NMDA receptor activity but could not be correlated with $[Ca^{2+}]_i$ (Choi, 1992).

Hartley et al. (1993) produced data strongly supporting the conclusion that the total Ca^{2+} load was a major determinant of subsequent cell death. Although they did not discuss the issue, data from their study illustrated the remarkable capacity that neurons have to buffer greater than 99% of large Ca^{2+} loads and thus maintain $[Ca^{2+}]_i$ in the 0.5–5 μM range despite Ca^{2+} influx that should result in millimolar $[Ca^{2+}]_i$. Since their investigation indicates that the absolute Ca^{2+} load is critical, it is possible that the Ca^{2+} fluxes generated by pathologic stimulation of the NMDA receptor subtype are much greater than the fluxes induced by equipotent activation of other ligand- or voltage-gated channels (Brocard et al., 1993; Eimerl and Schramm, 1994). While our experiments do not specifically address the issues of total Ca^{2+} load, our recovery time data indicate that the cell is better able to buffer a depolarization-induced Ca^{2+} load without the normal contributions from mitochondria and Na^+/Ca^{2+} exchange as compared to a glutamate-induced load, which produces an equivalent (or smaller) increment in $[Ca^{2+}]_i$. One po-

tential explanation for this finding is that despite the larger $[Ca^{2+}]_i$ observed following a depolarization stimulus, the absolute Ca^{2+} load induced by glutamate may be substantially larger. Although our stimuli are different from those used by Hartley et al. (1993), both sets of data are consistent with the idea that glutamate Ca^{2+} loads are larger than depolarization loads even when the measured $[Ca^{2+}]_i$ transients are comparable. If glutamate does induce larger Ca^{2+} loads, then we could conclude that glutamate Ca^{2+} influx either occurs more slowly or is buffered more efficiently than depolarization influx, because $[Ca^{2+}]_i$ is greater in the latter. It is difficult to tease these two hypotheses apart, but Reichling and MacDermott (1993) have reported that depolarization induces faster rates of $[Ca^{2+}]_i$ rise in spinal neurons than does glutamate. If cortical neurons handle glutamate Ca^{2+} loads more quickly, then these intermediate buffering mechanisms appear to rely on mitochondria and Na^+/Ca^{2+} exchange for more complete Ca^{2+} removal, as our results clearly show that simultaneous inhibition of mitochondria and Na^+/Ca^{2+} exchange almost completely inhibits the final recovery of $[Ca^{2+}]_i$. Thus, while not direct proof for a greater Ca^{2+} load resulting from glutamate stimulation (as opposed to depolarization), our data are at least consistent with the hypothesis.

Tymianski et al. (1993) emphasized the "source specificity" of the Ca^{2+} load, indicating that Ca^{2+} entering through the NMDA channel was more toxic than Ca^{2+} entering through other sources. This special toxicity of NMDA Ca^{2+}, they suggest, may be the result of the NMDA channel's unique access to proteins or compartments that makes NMDA Ca^{2+} more efficacious in producing neuronal cell death. In our study, $[Ca^{2+}]_i$ transients produced by glutamate were smaller than those produced by depolarization yet the recovery from the glutamate loads was more sensitive to inhibition of mitochondria and Na^+/Ca^{2+} exchange. These data may be further indirect evidence for compartmentalization of glutamate receptors with special proteins and organelles, if one infers that the neurons' greater reliance on those two buffering systems for glutamate-induced Ca^{2+} loads is a function of the proximity between the source of the load and the buffers. Direct experiments measuring absolute Ca^{2+} fluxes and mitochondrial Ca^{2+} uptake will be required to distinguish between the hypotheses of total Ca^{2+} load and special access to compartments, explanations that are certainly not mutually exclusive.

A thorough understanding of $[Ca^{2+}]_i$ homeostasis is absolutely critical if we are to make further progress dissecting, and ultimately treating, excitotoxic neuronal injury. Since $[Na^+]_i$ increases during neuronal tissue injury (Young, 1992), it is possible that Na^+/Ca^{2+} exchange is inhibited or even reversed in light of the depolarized membrane and the electrogenic nature of the exchange (Stys et al., 1992). Our experiments provide two different pieces of evidence suggesting that the exchange may be reversed in intact cells. Figure 3 illustrates that one-third of our cells stimulated with glutamate were unable to recover until Na^+ was reintroduced into the wash; a comparison of Figure 7 and Figure 2*A*, trace iv, reveals that there is a substantial influx of Ca^{2+} that follows the stimulus if Na^+ is removed from the wash solution. There is substantial evidence for an allosteric Ca^{2+}-binding regulatory site in isolated preparations of the Na^+/Ca^{2+} exchanger (Philipson and Nicoll, 1993), and our experiments suggest the existence of such regulation in intact cells. Under resting conditions the exchanger's reverse operation did not play a prominent role, but once stimulated by high $[Ca^{2+}]_i$,

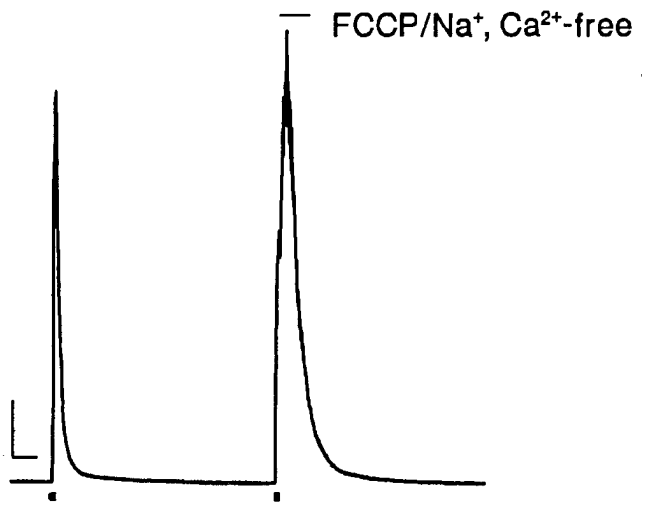

Figure 7. Alterations in $[Ca^{2+}]_i$ during the wash phase are not primarily the result of Ca^{2+} entry. A glutamate stimulus (applied at the *bars under the trace,* 15 sec, 3 μM) washed with 5 μM FCCP in Na^+/Ca^{2+}-free HBSS demonstrates that Ca^{2+} influx during the wash treatment was not the sole mediator of the cell's inability to recover. This trace reflects the mean recovery time data from a total of nine experiments. Calibration: 500 nM $[Ca^{2+}]_i$, 100 sec.

the exchanger placed an additional Ca^{2+} burden on the neuron when the cells were washed in Na^+-free buffer. In some of the cells (Fig. 3*B*), the additional Ca^{2+} burden seems to be more than the mitochondrial Ca^{2+} uniporter can handle.

It is probable that the importance of Na^+/Ca^{2+} exchange increases for the clearance of Ca^{2+} loads induced by more toxic concentrations of glutamate (Kiedrowski et al., 1994). Thus, during ischemia, the inhibition (or reversal) of this transport protein's normal operation may be an important piece of the disruption in $[Ca^{2+}]_i$ homeostasis that leads to excitotoxicity (Mattson et al., 1989; Andreeva et al., 1991). Similar arguments can be made for the importance of understanding the mitochondrial contribution to $[Ca^{2+}]_i$ homeostasis. In addition to the ATP depletion that will ultimately affect Ca^{2+}-ATPases, a decrease in mitochondrial respiration will cause early impairment of the mitochondrial Ca^{2+} uniporter and significantly hinder the neurons' ability to buffer glutamate-induced Ca^{2+} loads. Other investigators have already begun to uncover the important role of mitochondria in neuronal cell death (Beal, 1992; Beal et al., 1993; Mattson et al., 1993), although no one has experimentally targeted the mitochondria's Ca^{2+} buffering.

References

Andreeva N, Khodorov B, Stelmashook E, Cragoe E Jr, Victorov I (1991) Inhibition of Na^+/Ca^{2+} exchange enhances delayed neuronal death elicited by glutamate in cerebellar granule cell cultures. Brain Res 548:322–325.

Beal MF (1992) Does impairment of energy metabolism result in excitotoxic neuronal death in neurodegenerative illnesses? Ann Neurol 31:119–130.

Beal MF, Brouillet E, Jenkins BG, Ferrante RJ, Kowall NW, Miller JM, Storey E, Srivastava R, Rosen BR, Hyman BT (1993) Neurochemical and histologic characterization of striatal excitotoxic lesions produced by the mitochondrial toxin 3-nitropropionic acid. J Neurosci 13:4181–4192.

Benham CD, Evans ML, McBain CJ (1992) Ca^{2+} efflux mechanisms following depolarization evoked calcium transients in cultured rat sensory neurones. J Physiol (Lond) 455:567–583.

Bertolino M, Llinas RR (1992) The central role of voltage-activated and receptor-operated calcium channels in neuronal cells. Annu Rev Pharmacol Toxicol 32:399–421.

Bleakman D, Roback JD, Wainer BH, Miller RJ, Harrison NL (1993) Calcium homeostasis in rat septal neurons in tissue culture. Brain Res 600:257–267.

Brocard JB, Rajdev S, Reynolds IJ (1993) Glutamate-induced increases in intracellular free Mg^{2+} in cultured cortical neurons. Neuron 11: 751–757.

Brorson JR, Bleakman D, Gibbons SJ, Miller RJ (1991) The properties of intracellular calcium stores in cultured rat cerebellar neurons. J Neurosci 11:4024–4043.

Catterall WA (1980) Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. Annu Rev Pharmacol Toxicol 20: 15–43.

Choi DW (1992) Excitotoxic cell death. J Neurobiol 23:1261–1276.

Dubinsky JM (1993) Intracellular calcium levels during the period of delayed excitotoxicity. J Neurosci 13:623–631.

Duchen MR (1990) Effects of metabolic inhibition on the membrane properties of isolated mouse primary sensory neurones. J Physiol (Lond) 424:387–409.

Duchen MR, Valdeolmillos M, O'Neill SC, Eisner DA (1990) Effects of metabolic blockade on the regulation of intracellular calcium in dissociated mouse sensory neurones. J Physiol (Lond) 424:411–426.

Eimerl S, Schramm M (1994) The quantity of calcium that appears to induce neuronal death. J Neurochem 62:1223–1226.

Elliott EM, Sapolsky RM (1993) Corticosterone impairs hippocampal neuronal calcium regulation—possible mediating mechanisms. Brain Res 602:84–90.

Frandsen A, Schousboe A (1991) Dantrolene prevents glutamate cytotoxicity and Ca^{2+} release from intracellular stores in cultured cerebral cortical neurons. J Neurochem 56:1075–1078.

Frandsen A, Schousboe A (1993) Excitatory amino acid-mediated cytotoxicity and calcium homeostasis in cultured neurons. J Neurochem 60:1202–1211.

Friel DD, Tsien RW (1992a) A caffeine- and ryanodine-sensitive Ca^{2+} store in bullfrog sympathetic neurones modulates effects of Ca^{2+} entry on $[Ca^{2+}]_i$. J Physiol (Lond) 450:217–246.

Friel DD, Tsien RW (1992b) Phase-dependent contributions from Ca^{2+} entry and Ca^{2+} release to caffeine-induced $[Ca^{2+}]_i$ oscillations in bullfrog sympathetic neurons. Neuron 8:1109–1125.

Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450.

Hartley DM, Kurth MC, Bjerkness L, Weiss JH, Choi DW (1993) Glutamate receptor-induced $^{45}Ca^{2+}$ accumulation in cortical cell culture correlates with subsequent neuronal degeneration. J Neurosci 13: 1993–2000.

Hartley Z, Dubinsky JM (1993) Changes in intracellular pH associated with glutamate excitotoxicity. J Neurosci 13:4690–4699.

Henzi V, MacDermott AB (1992) Characteristics and function of Ca^{2+}- and inositol 1,4,5-trisphosphate-releasable stores of Ca^{2+} in neurons. Neuroscience 46:251–273.

Heytler PG (1981) Uncouplers of oxidative phosphorylation. In: Inhibitors of mitochondrial function (Erecinska M, Wilson DF, eds), pp 199–210. Oxford: Pergamon.

Hua SY, Nohmi M, Kuba K (1993) Characteristics of Ca^{2+} release induced by Ca^{2+} influx in cultured bullfrog sympathetic neurones. J Physiol (Lond) 464:245–272.

Kiedrowski L, Brooker G, Costa E, Wroblewski JT (1994) Glutamate impairs neuronal calcium extrusion while reducing sodium gradient. Neuron 12:295–300.

Koch RA, Barish ME (1994) Perturbation of intracellular calcium and hydrogen ion regulation in cultured mouse hippocampal neurons by reduction of the sodium ion concentration gradient. J Neurosci 14: 2585–2593.

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.

Mattson MP, Guthrie PB, Kater SB (1989) A role for Na^+-dependent Ca^{2+} extrusion in protection against neuronal excitotoxicity. FASEB J 3:2519–2526.

Mattson MP, Zhang Y, Bose S (1993) Growth factors prevent mitochondrial dysfunction, loss of calcium homeostasis, and cell injury, but not ATP depletion in hippocampal neurons deprived of glucose. Exp Neurol 121:1–13.

Mayer ML, Miller RJ (1990) Excitatory amino acid receptors, second messengers and regulation of intracellular Ca^{2+} in mammalian neurons. Trends Pharmacol Sci 11:254–260.

McConnell HM, Owicki JC, Parce JW, Miller DL, Baxter GT, Wada HG, Pitchford S (1992) The cytosensor microphysiometer: biological applications of silicon technology. Science 257:1906–1912.

Milani D, Guidolin D, Facci L, Pozzan T, Buso M, Leon A, Skaper SD (1991) Excitatory amino acid-induced alterations of cytoplasmic free Ca^{2+} in individual cerebellar granule neurons: role in neurotoxicity. J Neurosci Res 28:434–441.

Miller RJ (1987) Multiple calcium channels and neuronal function. Science 235:46–52.

Miller RJ (1991) The control of neuronal Ca^{2+} homeostasis. Prog Neurobiol 37:255–285.

Mironov SL, Usachev Y, Lux HD (1993) Spatial and temporal control of intracellular free Ca^{2+} in chick sensory neurons. Pfluegers Arch 424:183–191.

Philipson KD, Nicoll DA (1993) Molecular and kinetic aspects of sodium-calcium exchange. Int Rev Cytol 1993:199–227.

Rajdev S, Reynolds IJ (1994) Glutamate-induced intracellular calcium changes and neurotoxicity in cortical neurons *in vitro*: effect of chemical ischemia. Neuroscience, in press.

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

Reichling DB, MacDermott AB (1993) Brief calcium transients evoked by glutamate receptor agonists in rat dorsal horn neurons: fast kinetics and mechanisms. J Physiol (Lond) 469:67–88.

Snodgrass S, White W, Biales B, Dichter M (1980) Biochemical correlates of GABA function in rat cortical neurons in culture. Brain Res 190:123–138.

Stys PK, Waxman SG, Ransom BR (1992) Ionic mechanisms of anoxic injury in mammalian CNS white matter: role of Na^+ channels and Na^+-Ca^{2+} exchanger. J Neurosci 12:430–439.

Tatsumi H, Katayama Y (1993) Regulation of the intracellular free calcium concentration in acutely dissociated neurones from rat nucleus basalis. J Physiol (Lond) 464:165–181.

Thayer SA, Miller RJ (1990) Regulation of the intracellular free calcium concentration in single rat dorsal root ganglion neurones *in vitro*. J Physiol (Lond) 425:85–115.

Tymianski M, Charlton MP, Carlen PL, Tator CH (1993) Source specificity of early calcium neurotoxicity in cultured embryonic spinal neurons. J Neurosci 13:2085–2104.

Wahl M, Lucherini MJ, Gruenstein E (1990) Intracellular Ca^{2+} measurement with Indo-1 in substrate-attached cells: advantages and special considerations. Cell Calcium 11:487–500.

Werth JL, Thayer SA (1994) Mitochondria buffer physiological calcium loads in cultured rat dorsal root ganglion neurons. J Neurosci 14:346–356.

Young W (1992) Role of calcium in central nervous system injuries. J Neurotrauma 9:S9–S25.