Cumulative Effects of Glutamate Microstimulation on Ca\textsuperscript{2+} Responses of CA1 Hippocampal Pyramidal Neurons in Slice

JOHN A. CONNOR AND ROBERT J. CORMIER
Department of Neuroscience, University of New Mexico School of Medicine, Albuquerque, New Mexico 87131

Connor, John A. and Robert J. Cormier. Cumulative effects of glutamate microstimulation on Ca\textsuperscript{2+} responses of CA1 hippocampal pyramidal neurons in slice. J. Neurophysiol. 83: 90–98, 2000. Glutamate stimulation of hippocampal CA1 neurons in slice was delivered via iontophoresis from a microelectrode. Five pulses (approximately 5 \(\mu\)A, 10 s duration, repeated at 1 min intervals) were applied with the electrode tip positioned in the stratum radiatum near the dendrites of a neuron filled with the Ca\textsuperscript{2+} indicator fura-2. A single stimulus set produced Ca\textsuperscript{2+} elevations that ranged from several hundred nM to several \(\mu\)M and that, in all but a few neurons, recovered within 1 min of stimulus termination. Subsequent identical stimulation produced Ca\textsuperscript{2+} elevations that outlasted the local glutamate elevations by several minutes as judged by response recoveries in neighboring cells or in other parts of the same neuron. These long responses ultimately recovered but persisted for up to 10 min and were most prominent in the mid and distal dendrites. Recovery was not observed for responses that spread to the soma. The elevated Ca\textsuperscript{2+} levels were accompanied by membrane depolarization but did not appear to depend on the depolarization. High-resolution images demonstrated responsive areas that involved only a few \(\mu\)m of dendrite. Our results confirm the previous general findings from isolated and cell culture neurons that glutamate stimulation, if carried beyond a certain range, results in long-lasting Ca\textsuperscript{2+} elevation. The response characterized here in mature in situ neurons was significantly different in terms of time course and reversibility. We suggest that the extended Ca\textsuperscript{2+} elevations might serve not only as a trigger for delayed neuron death but, where more spatially restricted, as a signal for local remodeling in dendrites.

INTRODUCTION

When brain tissue is subjected to ischemic insult or mechanical trauma, there is a large release of glutamate from damaged or otherwise directly affected cells that results in widespread exposure of all neurons in the affected region to high levels of glutamate and other neurotransmitters (Benveniste et al. 1984; Globus et al. 1991; Mitani et al. 1990, 1994). In these catastrophic models, the glutamate exposure has long been thought to trigger excitotoxic reactions involving intracellular Ca\textsuperscript{2+} in neurons, resulting in cell death that extends far beyond the initially damaged region (Choi 1988; Manev et al. 1989; Rothman and Olney 1986). Glutamate-Ca\textsuperscript{2+}–initiated processes have also been proposed in models of more subtle neuron destruction such as amyotrophic lateral sclerosis and Alzheimer’s, among others (Loopuis and Schmidt 1998; Mattson 1994; Olney 1990; Schousboe et al. 1997).

The effects of excessive exposure to glutamate and the resulting toxicity have been extensively studied in neurons isolated from embryonic or neonatal brains maintained in tissue culture (Choi et al. 1988; Dubinsky and Rothman 1991; Glaum et al. 1990; Randall and Thayer 1992; Vornov et al. 1991) and to a lesser extent, in completely isolated neurons from adult tissue (Chen et al. 1997; Connor et al. 1988; Wadman et al. 1993). However, there are often difficulties in drawing conclusions from these types of preparation about the behavior of mature neurons in situ. Because of the powerful ability of glia to remove glutamate from the extracellular space (reviewed in Barbour and Haussert 1997), it has proved more difficult to examine the effects of glutamate in vivo or in brain slices in a controlled fashion. In the present study, we exploited microiontophoresis of glutamate to deliver locally high concentrations of this agonist to only a few cells in the slice, thereby stimulating cells with no compromise of oxygenation and no widespread excitation in the slice as with global superfusion. Using this method, we investigated whether there are overt, cumulative effects on Ca\textsuperscript{2+} homeostasis of local stimulation by glutamate, and we explored the cellular regions in which these effects are expressed in adult in situ neurons. We show that local applications of glutamate not only cause the expected rapid increases in intracellular Ca\textsuperscript{2+} that recover within seconds after the application but, if applications are sufficiently large and repeated, that they trigger disproportionately long-lasting increases in the dendrites. These increases persisted up to 10 min before recovery was attained. The long-persisting increases were little affected by applying hyperpolarizing current during the extended response.

METHODS

Coronal brain slices (350–400 \(\mu\)m thick) were prepared from adult Sprague Dawley rats (Harlan; 5–10 wk old) by standard methods that optimize in situ visualization of neurons near the cut surface of the slice (Aghajanian and Rasmussen 1989). Animals were deeply anesthetized (0.85 mg/kg ketamine and 0.15 mg/kg xylazine) and perfused through the right cardiac ventricle. The perfusion solution was ice-cold and consisted of, in mM: 250 sucrose, 3 KCl, 1.25 NaH\textsubscript{2}PO\textsubscript{4}, 26 NaHCO\textsubscript{3}, 6 MgCl\textsubscript{2}, 0.4 CaCl\textsubscript{2}, 10 dextrose, and 0.1 kynurenate. The perfusion solution was bubbled with 95% O\textsubscript{2}, 5% CO\textsubscript{2}, as were all solutions in the present study. After the perfusate was clear of blood (\(\leq 1\) min), decapitation of the animal and dissection of the brain began. Brain slices were made under the cold saline on a vibrotome (Ted Pella). After preparation, the slices were warmed to 28°C over the course of 30 min in artificial cerebrospinal fluid (ACSF) that contained (in mM): 126 NaCl, 3 KCl, 1.25 NaH\textsubscript{2}PO\textsubscript{4}, 26 NaHCO\textsubscript{3}, 1 MgSO\textsubscript{4}, 2 CaCl\textsubscript{2}, and 10 dextrose. The slices remained under these conditions until use, at least 1.5 h. ACSF (31 ± 0.5°C, gassed with a mixture of 95% O\textsubscript{2}/5% CO\textsubscript{2} flowed at 1–2 ml/min through the submersion chamber used to study individual slices.
Injection/recording microelectrodes were filled at the tip with 12 μM fura-2 in 0.5 mM K-acetate. They were then back-filled with 3 M KCl/1 M K-acetate. After cell impalement, filling to an adequate level of fura-2 (100–200 μM; Molecular Probes, Eugene, OR) required 15–20 min, during which time a small negative current (200–400 nA) was applied. After this filling time, enough KCl/K-acetate had diffused to the tip to lower electrode resistance to \( \leq 100 \) MΩ. Only cells with input resistance \( \geq 50 \) MΩ and steady resting membrane potentials more negative than \(-60\) mV after dye injection were used in the experimental population. Holding current was adjusted to maintain the potential between \(-65\) and \(-75\) mV under nonstimulated conditions. 

An Axoclamp 2A amplifier in bridge mode was used to record voltage signals. Orthodromic electrical stimulation was delivered via monopolar electrodes and positioned in the stratum radiatum (50–100 μm, 100 ms applied current).

Ester loading of fura-2 followed the basic procedure of Regehr and Tank (1991). To fill CA1 neurons, a large bore micropipette (tip \( \sim 15 \) μm) filled with fura-2/AM:DMSO (10 μM:0.3%) was positioned in the alveus and pressure pulses (1 Hz, 0.5 duty cycle) were applied for 15 or more minutes. Basal dendrites of CA1 neurons steadily accumulated fura-2 that diffused to cell bodies and apical dendrites. This positioning of the pipette allowed measurements to be made in the cell body layer, distant from the loading site. Calcium measurements were made by ratio imaging of fura-2 (Grynkiewicz et al. 1985) using 350/380 nm excitation, an upright microscope, and a cooled frame-transfer charge coupled device camera system (Petrozzino et al. 1995). For injected neurons, fura-2 ratios were converted to \([\text{Ca}^{2+}]\) using in vitro standards. Measurements with ester-loaded fura-2 were left as ratios because of the following factors. First, nonspecific loading leads to background from out-of-focus cells, which may include damaged high-Ca²⁺ cells near the slice surface. Second, the fluorescence signal includes a nonquantifiable component from the unconverted ester form of fura-2/AM, which does not bind Ca²⁺.

Iontophoresis pipettes (1 M Na-glutamate, \( \sim 10 \) ΜΩ) were positioned 20–50 μm from the primary apical dendrite of the fura-2-filled neuron. Glutamate was ejected by five iontophoretic pulses (10 s duration at intervals of 60 s), and the pipette was then withdrawn from the slice. Control experiments demonstrated that iontophoresis current itself had no effect on synaptic transmission, membrane potential, or intracellular \([\text{Ca}^{2+}]\). Recovery times for Ca²⁺ transients are expressed as elapsed time between termination of iontophoretic delivery of glutamate and 90% recovery (0.9 × Δ[Ca²⁺] peak to prestimulus levels).

**RESULTS**

**Conditions for extended Ca²⁺ response**

Glutamate was administered via microelectrode iontophoresis using a protocol that has been used for long-term potentiation (LTP) induction (Cormier et al. 1993); 10-s pulses re-
peated five times at a frequency of 1/min. The iontophoresis electrode was positioned in the mid region of the stratum radiatum. Although previously used for LTP induction, the purpose here was to administer a stimulation protocol that, administered singly, had no known deleterious effects. We then explored the cumulative effects of multiple applications of this stimulus.

To assess the effective spread of glutamate away from the electrode, we examined Ca^2+ responses in a number of neighboring cells filled noninvasively by fura-2/AM (see METHODS). Figure 1 shows responses in a field of cells to glutamate iontophoresis from an electrode positioned in the lower right hand region of the field. The amplitude of the iontophoretic current was increased with each pulse in the train (0.83, 2.5, 3.3, 4.2, and 8.7 μA). The first (smallest) pulse elicited a small response only in cell 5, but as the pulse amplitude was increased, responses were recruited in neurons at greater and greater distances from the electrode tip. The most distant cell (cell 1) showed no appreciable response until the fifth pulse and then gave a large, rapidly recovering response. Effective concentrations of other transmitters such as dopamine are achieved at lower current intensities (Nicholson 1995) as expected from a lower density of uptake sites. It would appear, however, that the larger amplitude ejection currents are necessary to stimulate neurons outside of a radius of about 40–70 μm and that iontophoretic currents in >3 μA are required to overcome the rapid glutamate uptake capacity. Presumably the large ejection currents (glutamate quantities) are necessary to

FIG. 2. Comparison of Ca^2+ responses for 2 sequential stimulus episodes in a single neuron microinjected with fura-2. Left: Ca^2+ transients recorded at 5 locations (inset) in a previously unstimulated neuron. Small horizontal bars mark iontophoretic current applications. Proximal locations (1, 2, and 3) show more rapid recovery than distal regions (4 and 5). Recovery was complete (>90%) in all regions 1 min after stimulus termination. Right: responses to a 2nd, identical set of stimuli show a greatly extended time course as compared with the 1st. Ca^2+ levels in the most distal dendrite (5) now remain elevated for 6–7 min after stimulus termination. Recovery in the soma is also slowed, possibly reflecting diffusion of Ca^2+ from the dendrite and persisting membrane depolarization.
overcome the glutamate uptake capacity of glia in the vicinity of the ejection site. Because of the branching of dendritic trees, it is not possible to place a more exact radius on the effective diffusion of glutamate.

After the fifth (largest) glutamate ejection cells 2 and 4 showed a much slower recovery rate than cells 1, 3, and 5. The positioning of cell 2 between the rapidly recovering cells 1 and 3 argues against the slow recovery reflecting merely the restoration of extracellular glutamate to its normal levels and for the possibility that factors intrinsic to the individual neurons are responsible. Heterogeneity of Ca\(^{2+}\) recovery within single neurons and on a much smaller spatial scale is shown in Figs. 5 and 6. At this time we cannot say whether the very different recovery rates are due to preexisting differences in the state of the cells or if they result from differences in mechanical or chemical manipulations during and after slicing. Response heterogeneity was also seen in fura-2–injected neurons, forcing our observations to be descriptive in nature.

A total of 27 neurons microinjected with fura-2 met the electrophysiological acceptance criteria outlined in METHODS. To assess the likelihood of delayed effects of the stimulation, 12 of these neurons were given one set of stimuli and then observed for at least 25 min during which time no further exogenous glutamate was applied. Ca\(^{2+}\) levels in nine of these neurons recovered to prestimulus levels within 1 min after termination of the fifth iontophoretic pulse and remained there for the duration of the observation period. The remaining three neurons failed to restore low Ca\(^{2+}\) levels, with two of these losing the fura-2 during the observation period, indicating membrane breakdown. We concluded that if a cell recovered from the initial stimulation it was unlikely that there would be a delayed change in Ca\(^{2+}\). Fifteen additional neurons recovered from the initial stimulus set and were given a subsequent stimulation set after a recovery period of at least 10 min.

Figure 2 illustrates cellular responses to two stimulus sets tracked at five locations, one somatic and four dendritic. Figure 2, left, shows the rapidly recovering response typically evoked by the initial stimulus set. Although in many cases the recovery progressively slowed with the course of the stimuli, there was no clear discrepancy between these recovery times and the times that might be needed to clear the extracellular space of glutamate and to buffer/sequester and pump out the intracellular Ca\(^{2+}\) increase. Both glutamate uptake and Ca\(^{2+}\) regulation might be expected to slow somewhat with the cumulative demand. Note that recovery in the main portion of the dendrite is somewhat more rapid than in the soma, but that recovery in the distal dendrites requires the greatest time.

Behavior different from this occurred when the subsequent stimulus set was applied. A recovery period of 15 min was given between the stimulus episodes shown. Figure 2, right, shows responses of this same neuron to a second set of glutamate pulses delivered at approximately the same location as the first set. Although there are significant differences in the time course of Ca\(^{2+}\)responses in the soma and proximal apical dendrite between the two episodes (increased maximum amplitude and slowing of recovery, especially after the fifth pulse), the greatest difference is seen in the more distal apical dendrites. Instead of mirroring the soma/proximal dendrite response, the more distal dendrite Ca\(^{2+}\) levels plateau or

---

**FIG. 3.** Distribution of cell responses in neurons given at least two sets of glutamate stimulation. Response of one of the neurons in the no recovery bin is shown in detail in Fig. 5.
“hang” for several minutes after termination of the last stimulus pulse before recovering to their prestimulus levels in a relatively rapid fashion. This plateau-rapid recovery waveform was typical of the cells studied. A vestige of the long dendritic response appears as a shoulder on the recovery phase of the soma/proximal dendrite response.

The range of recovery times observed in these 15 neurons is summarized in Fig. 3. One of the neurons failed to recover after the second stimulus set whereas three of the neurons showed no increase in response duration with the second pulse (recovery, 1 min). Of these latter cells, one produced a prolonged response after four stimulus sets and a second cell failed to recover after a third stimulus set.

Figure 4 shows electrophysiological data that demonstrate that the extended Ca elevations and the induction of LTP were not related; that is, potentiation could be induced without an extended response and the ultimate formation of an extended response did not depend on the prior induction of potentiation. Excitatory post synaptic potentials (EPSPs) in response to stimulation of the stratum radiatum, before and after a single set of glutamate pulses, are shown superimposed for each cell. For both examples, Ca$^{2+}$ levels had returned to prestimulation levels at the time the “Post” records were made. A subsequent glutamate stimulation set gave rise to an extended response in both cases. In Fig. 4A, paired pulse stimulation was delivered to a single pathway. The response shows clear potentiation of the initial and paired (facilitated) EPSPs. In Fig. 4B, two-pathway stimulation was delivered to a second cell via two electrodes positioned in stratum radiatum. No potentiation or significant depression was seen for either pathway. Analysis of EPSPs during the maintained responses was not attempted because of complications raised by maintained depolarization.

**Extended response results from intracellular not extracellular signalling**

A primary concern in experiments such as these is whether the extended Ca$^{2+}$ elevations result from interesting changes in the properties of the neurons or from unexpectedly long persisting glutamate elevations in the extracellular space, even though the long duration of the responses would make this seem unlikely. We judged that the clearest way of separating the two possibilities was to demonstrate that large differences in intracellular Ca$^{2+}$ could exist in different dendritic branches that were very close together. Diffusion times should be on the order of a few seconds for distances of several μm in the extracellular space (Nicholson 1995) and, more importantly, the local region would have been subject to the same initial concentrations of glutamate during the stimuli. Figure 5 shows data from branching tertiary dendrites arising from a common stalk. Morphology of the dendrites and recording locations are shown using fura-2 fluorescence (top). There were no detectable changes such as beading as a result of stimulation. Two stimulus sets were given to the cell; the first was followed by rapid recovery of Ca$^{2+}$ levels whereas recovery from the second was much more extended and is shown in the plots of Fig. 5. All regions started at approximately the same levels and gave robust Ca$^{2+}$ increases during the five iontophoretic stimuli. Regions 1 and 2 showed partial recovery after the first stimuli; however region 3 showed no appreciable recovery during the interpulse intervals and maintained the longest Ca$^{2+}$ elevation in the several minutes after removal of the iontophoresis electrode. Even the most rapidly recovering region (region 1) required 5 min before baseline Ca$^{2+}$ levels were reset. Ca$^{2+}$ levels at region 3 remained elevated for at least 3 min after levels in the parent (region 1) and parallel (region 2) branches had reset to prestimulus values. The physical separation of these regions across the extracellular space is <30 μm. It is extremely unlikely that appreciable gradients of glutamate could exist for such a long time over so small a distance unless some very unusual and undemonstrated properties of glial uptake and release of glutamate are invoked. Ca$^{2+}$ profiles along the branched dendrite at three different time points of the response are shown in Fig. 5, right. It can be seen from these that all regions of the dendrite are responsive to glutamate during the applications, and that only a part of the lower branch shows the long-lasting Ca$^{2+}$ elevation.

Although both branches involved in the extended response shown in Fig. 5 were tertiary dendrites, such differences in the time course of recovery were not limited to dendrites of the same size. Figure 6 shows data taken from a primary dendrite
and a small side branch during the recovery phase following a second stimulus epoch. Fig. 6, right, shows a picture of the neuron (fura-2 fluorescence, 380 nm excitation) and the regions from which Ca\textsuperscript{2+} measurements were taken. The small dendrite branch reached much higher levels than the main dendrite during stimuli, but after the plateau period shown in the initial portion of the plot abruptly recovered. Levels in the main dendrite and another branch increased and remained at higher than normal Ca\textsuperscript{2+} levels for the duration of the measurements. Again there were only \sim 10 \mu m separating the different measurement regions, and all regions showed robust responses to the glutamate application. Thus the difference in time course of the response was present in dendrites that were widely different in size as well as in small spine-bearing dendrites. The responses observed were indicative of secondary processes set off within the dendrites and not of persistent significant differences in extracellular glutamate concentration.

**Voltage dependence**

Microelectrode penetration was sometimes lost during the course of these experiments. In general this did not affect the condition of the cell as judged from Ca\textsuperscript{2+} levels and the ability to respond to glutamate pulses. Electrical recordings were maintained in 16 of the neurons analyzed. During the first set of iontophoretic pulses, cells depolarized to approximately \sim 10 mV and recovered, between pulses, more rapidly than the Ca\textsuperscript{2+} levels illustrated in Fig. 1. During the prolonged Ca\textsuperscript{2+} response, membrane voltage remained depolarized to levels between \sim 50 to \sim 30 mV. Recovery of Vm to normal resting levels always preceded the restoration of Ca\textsuperscript{2+}. Depolarization
to −10 to 0 mV by current injections lasting many minutes have been analyzed previously. Such depolarizations generate large Ca\(^{2+}\) increases in CA1 and CA3 pyramidal neurons, exceeding 5 μM in many cases (Perkel et al. 1993, Pozzoli-Miller et al. 1996). These increases show partial recovery during the depolarization, and in the absence of synaptic stimulation or glutamate application, concurrent with the period of high Ca\(^{2+}\), resting levels of Ca\(^{2+}\) are restored within 1–2 min after return to resting potential. The depolarization-repolarization cycle can be repeated a number of times. Therefore, simple depolarization and the associated Ca\(^{2+}\) increase are insufficient to generate the type of extended responses reported here.

In four experiments, a negative holding current was applied to bring the neuron soma voltage to −80 mV. In the example shown in Fig. 7, hyperpolarization brought a partial recovery to the soma Ca\(^{2+}\) level but caused relatively little change in dendrites remote from the soma. While this behavior might seem unexpected, it would follow from a model in which a persisting current (carried partly or totally by Ca\(^{2+}\)) was being generated in the dendrites because of the glutamate exposure. This current would depolarize the soma as well as the dendritic tree and activate voltage-gated Ca channels (VGCCs) in the soma. Hyperpolarizing the soma would shut VGCCs there but might have little or no effect on the dendritic influx, given observations from other laboratories (see DISCUSSION).

DISCUSSION

The data demonstrate glutamate-stimulated Ca\(^{2+}\) elevations in mature CA1 neurons that outlast focally applied glutamate stimulus by considerable periods. This effect was rarely seen after a single LTP-inducing stimulation that we have here termed “moderate stimulation,” but generally was expressed only for repeated stimuli of this type. It would therefore appear that LTP and the maintained Ca elevations are separate or at least only partially intersecting phenomena. There were very significant differences in the response time course observed among neighboring cells responding to the same focal glutamate application. In some cases, as in Fig. 1, the time course differences are difficult to ascribe to amount or distribution of extracellular glutamate, because a neuron showing a prolonged Ca\(^{2+}\) elevation was sandwiched between two rapidly recovering neurons. Large time course differences were pursued on a much finer spatial scale by showing that different dendrites on the same neuron, separated by only a few μm, showed responses of significantly different time course. These findings indicate that the extended responses are not caused by unexpectedly long persistence of glutamate in the extracellular space. The extended Ca\(^{2+}\) elevations occurred in the presence of normal in vitro oxygenation and glucose. Therefore, we ascribe the cause of the response to glutamate activation and not to compromised metabolic capacity of the neurons involved.

Ca\(^{2+}\) increases that were transient and not immediately lethal, but unexpectedly long, were our main concern because this response may recapitulate, in a limited way, what occurs in situ during an ischemic insult or other events that release significant quantities of glutamate in the important CA1 region. During the ischemic insult itself, CA1 neurons experience intracellular Ca increases in the micromolar range (Silver and Erecinska 1990, 1992), but the great bulk of them do not die until 2 to 4 days later. In fact, using the gerbil 5 min occlusion model (Kirino 1982), our laboratory has shown that resting Ca\(^{2+}\) levels on the day after an ischemic insult were the same as in control neurons (Connor et al. 1999). This behavior would indicate that high intracellular Ca\(^{2+}\) is a trigger for the following degenerative events, but high ambient Ca\(^{2+}\) is not an enduring factor in the times nearer to cell death (see also Dubinsky 1993). This in situ behavior points up a shortcoming of in vitro models. When put under conditions of anoxia with glucose deprivation for periods of 5–10 min, neurons in hippocampal slice do undergo large intracellular Ca\(^{2+}\) increases (Lobner and Lipton 1993; Mitani et al. 1993; Tanaka et al. 1999), but these changes seldom reverse and the cells show signs of rapid deterioration. We also observed irreversible changes in the experiments in the present study, but this population was not emphasized because of the difficulty in establishing the actual cause of death in the acute slice preparation.

The initial study showing prolonged Ca\(^{2+}\) responses to exogenous glutamate in CA1 neurons (Connor et al. 1988) was carried out in acutely isolated neurons. This and a subsequent study demonstrated that in neurons pretreated with the protein kinase inhibitor sphingosine, the prolonged response, but not transient Ca increases, was blocked (Wadman and Connor 1992). This finding suggested that the response was not the result of simple rundown of Ca\(^{2+}\) pumping or sequestration caused by the repeated Ca loads, but of kinase-mediated processes triggered by the glutamate receptor excitation and the resulting Ca\(^{2+}\) influx. In the acutely isolated neurons, the response, once established, did not subside, but high Ca\(^{2+}\) levels persisted until the neuron died, usually within 15–20 min. Persisting elevations of Ca\(^{2+}\) were also shown to occur in small dendrites and spines of CA3 neurons after repeated orthodromic tetani to the associational-commisssural pathways (Müller and Connor 1991). Although less well studied than the CA1 neurons, delayed death also occurs in CA3 pyramidal neurons after more prolonged ischemic insults (Hatakeyama et al. 1988; Yanagihara et al. 1985). Subsequently, studies done on neurons in tissue culture also demonstrated that prolonged Ca\(^{2+}\) elevations followed repeated exposures to glutamate (Randall and Thayer 1992; Weiss et al. 1993). Sphingosine preexposure also blocked the extended response in culture (Weiss et al. 1993). The occurrence of a secondary response in tissue culture neurons was shown to be an early marker of cells that would die after glutamate exposure (Tymianski et al. 1993).

Unlike the acutely isolated or cultured neurons, the fully developed neurons in slice studied here did not show appreciable Ca\(^{2+}\) recovery before the long plateau response started. The plateau was either already developed after the final stimulus or it did not occur. It is not clear whether this represents a physiological difference in neurons of the different preparations (slice vs. culture or acutely isolated) or differences in the stimulus protocols. In the rat ischemia model, Silver and Erecinska (1990) reported the development of a delayed or secondary Ca\(^{2+}\) increase after reperfusion. This ischemia-induced increase was irreversible although the observation required long implanation with ion sensing electrodes, which may contribute to cell deterioration. Future work will investigate different glutamate application protocols in the slice preparation.
Recent electrophysiology studies employing whole-cell current measurements in acutely isolated CA1 neurons have demonstrated the developments of a persisting inward current after prolonged stimulation by glutamate, N-methyl-D-aspartate, or high intracellular Ca\(^{2+}\) (Chen et al. 1997, 1998). Ca\(^{2+}\) influx is a significant component of this current, and continuing Ca\(^{2+}\) influx feeds further increase of the current. A point of particular interest is that the persisting current showed only a weak dependence on membrane voltage. Thus there are similarities in the responses but further experiments will be required to make better comparison. The use of zinc as a blocker (Chen et al. 1998) presents problems for Ca\(^{2+}\) indicator experiments in that there is a finite membrane permeability to zinc, and this ion binds with high affinity to bis-(o-aminophenoxy)-N,N',N'-tetraacetic acid family indicators (fura-2, etc.) as with other blocker ions, e.g., cadmium. Interpretation of fluorescence signals is not straightforward (Connor et al. 1987). Whereas the membrane current in the acutely isolated neurons, once established, persisted until obvious cell death (Chen et al. 1988), the response analyzed here, if not pushed too far, recovered. Assuming that the underlying membrane current is the same, this difference may be one degree of stimulation or may reflect differences in the physiological state of acutely isolated neurons and those in slice. Isolated neurons on which Ca\(^{2+}\) measurements were made (Connor et al. 1988; Wadam and Connor 1992) also did not recover from multiple stimuli once a secondary response was established. We note that the distal dendritic tree, which in the experiments here was most sensitive to the response development, is generally lost in the acute isolation of neurons.

There is no shortage of potential factors leading to cell death that might be activated by prolonged Ca\(^{2+}\) elevations. Among them are certain members of the caspase family, (Fink et al. 1998; Namura et al. 1998), phosphatases such as calcineurin (Rao et al. 1997, Wang et al. 1999), or more complex cascades involving Ca-activated factors such as Ca-calmodulin-kinases (Aronowski et al. 1992; Churn et al. 1992; Picone et al. 1989) and mitogen-activated protein kinase (Murray et al. 1998).

The appreciable differences in the Ca\(^{2+}\) responses of different dendrites of the same neuron also illustrate the fact that secondary processes activated by glutamate need not involve the whole cell but can occur rather independently in different parts of the same neuron. This might imply that the responses illustrated in the present study could be used in, among other things, dendritic “pruning.” That is, the long Ca\(^{2+}\) responses might selectively activate degradative enzymes in these selected dendrites for a sufficient period to promote their destruction whereas in uninvolved dendrites there would be no such activation. The extended duration of the Ca\(^{2+}\) response provides a second dimension for the Ca\(^{2+}\) signaling. That is, in addition to the absolute magnitude of the signal, which can be quite large in nondestructive activity, reaching into the 30–50 \(\mu\text{M}\) range during LTP induction (Petrozzino et al. 1995), there is the second dimension of time; the signal may be several minutes rather than several seconds. If the cascades triggered by an initial Ca\(^{2+}\) increase have downstream steps that are also biased by ambient Ca\(^{2+}\) levels, then the ultimate outcomes could be very different depending on whether Ca\(^{2+}\) is high or low when subsequent steps in a cascade are reached.


