Protection of Dentate Hilar Cells from Prolonged Stimulation by Intracellular Calcium Chelation

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Prolonged afferent stimulation of the rat dentate gyrus in vivo leads to degeneration only of those cells that lack immunoreactivity for the calcium binding proteins parvalbumin and calbindin. In order to test the hypothesis that calcium binding proteins protect against the effects of prolonged stimulation, intracellular recordings were made in hippocampal slices from cells that lack immunoreactivity for calcium binding proteins. Calcium binding protein-negative cells showed electrophysiological signs of deterioration during prolonged stimulation; cells containing calcium binding protein did not. When neurons without calcium binding proteins were impaled with microelectrodes containing the calcium chelator BAPTA, and BAPTA was allowed to diffuse into the cells, these cells showed no deterioration. These results indicate that, in a complex tissue of the central nervous system, an activity-induced increase in intracellular calcium can trigger processes leading to cell deterioration, and that increasing the calcium binding capacity of a cell decreases its vulnerability to damage.

CALCIUM-MEDIATED PROCESSES inside neurons are critical to a variety of cell functions (1). Intracellular free Ca\(^{2+}\) is normally maintained at a low level, but the level may increase significantly by events that release intracellular stores of bound Ca\(^{2+}\) or that allow Ca\(^{2+}\) flux across the membrane. Large increases of intracellular Ca\(^{2+}\) are thought to occur during periods of excessive neuronal excitation and trigger processes that lead to cell death (2). Specific cell types appear to be especially vulnerable to damage under conditions that facilitate Ca\(^{2+}\) influx via voltage- or neurotransmitter-gated channels. Intracellular Ca\(^{2+}\) binding proteins are important in regulating free Ca\(^{2+}\) (3), and their capacity to buffer intracellular Ca\(^{2+}\) may be important in determining cell vulnerability under conditions associated with Ca\(^{2+}\) influx.

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In vivo experiments on rat dentate gyrus, prolonged stimulation of the major afferent pathway to dentate granule cells (the perforant path) was shown to lead to the histological degeneration of specific cell populations in the adjacent hilar region, without damage to the granule cells (4). The hilar cells that degenerated were the large spiny cells called mossy cells and a subpopulation of aspiny local circuit neurons (interneurons) that are somatostatin immunoreactive (4, 5). The mechanisms underlying the selective vulnerability of the mossy cells and the somatostatin-immunoreactive interneurons are unknown. However, immunocytochemical studies have shown that the dentate neurons that survive the period of stimulation (granule cells and some interneurons) contain significant levels of a Ca²⁺-binding protein (CaBP)—either parvalbumin or calbindin (D₂₈k) (5). In contrast, the vulnerable hilar cells—the mossy cells and other interneurons—contain neither CaBP (5). Does low Ca²⁺ binding capacity underlie the vulnerability of these hilar cells, and can vulnerable neurons be rendered resistant by increasing their Ca²⁺ binding capacity experimentally? These questions were examined by means of a model of stimulation-induced cell damage (4, 5) modified for the hippocampal slice preparation. We assessed the effects of stimulation on CaBP-negative hilar cells impaled with microelectrodes filled with or without the Ca²⁺ chelator BAPTA [1,2 bis-(2-amino phenoxymethyl)-N,N,N',N'-tetra acetic acid] (6). BAPTA was chosen in preference to other chelators, such as EGTA, since BAPTA is faster acting, is highly specific for Ca²⁺, as opposed to other cations, and has little nonspecific effect on cell function over the short periods of our experiments (7).

Hippocampal slices were prepared and maintained as described (8). Either granule cells, interneurons, or mossy cells were recorded intracellularly. Granule cells, interneurons, and mossy cells were easily distinguished on the basis of their locations and different physiological properties, properties that had been previously correlated with intracellular staining to define cell morphology (9). In addition to intracellular recording, an extracellular recording electrode was used to simultaneously monitor the granule cell population response to stimulation of the perforant path (10). The pattern of sustained perforant path stimulation was similar to the "intermittent" pattern of stimulation used in similar studies of cell death performed in vivo (4, 11).

Simultaneous extracellular and intracellular recordings from granule cells demonstrated that, after 10 to 90 min of intermittent stimulation, there were no electrophysiological signs of deterioration in granule cells (n = 16; Table 1). Electrophysiological indications of deterioration were defined as (i) over a 20-mV depolarization from pre-stimulation resting membrane potential (RMP) that persisted after stimulation ceased (until the end of the experiment, from 30 min to 2 hours after stimulation stopped), (ii) a 50% or more loss of input resistance (R cohorts) (12), and (iii) a 30-mV or more decrease in action potential (AP) amplitude. In contrast to granule cells, mossy cells were extremely sensitive to stimulation and had a much lower threshold for stimulation-evoked APs than granule cells. When tested with intermittent stimulation, all mossy cells depolarized dramatically (up to 60 mV) within seconds of the first 20-Hz stimulus train; in addition, AP amplitude decreased (Table 1) and AP duration increased. Of eight cells tested, only two were able to repolarize and recover healthy AP amplitude after a brief period (less than 5 min) of intermittent stimulation. These two mossy cells were located extremely far (over 1.5 mm) from the stimulating electrode relative to the other, more sensitive mossy cells, and this proximity may have contributed to their lesser sensitivities. However, further intermittent stimulation of these two mossy cells (18 and 19 min, respectively) led to their depolarization without subsequent recovery; AP amplitude and cell R cohorts decreased concomitantly. In these eight experiments, there was no change in the amplitude or waveform of the granule cell population spike at the time when mossy cells showed electrophysiological deterioration. Thus, cells that had been shown, histologically, to degenerate after sustained stimulation in vivo experiments (11) were also less sensitive to intermittent stimulation (Table 1 and Fig. 1) and were also less sensitive to single stimuli (Fig. 1). During an initial 20 to 30 min of cell impalement with BAPTA-containing electrodes (before intermittent stimulation), mossy cell and granule cell properties were monitored; there were no changes in RMP, AP amplitude or waveform, or R cohorts. Recording with BAPTA electrodes in granule cells (n = 3) and CA3 pyramidal cells (n = 10), however, showed that processes known to involve intracellular Ca²⁺ increases (for example, spike frequency adaptation, or the afterhyperpolarization af-

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Before or after stimulation</th>
<th>RMP (mV)</th>
<th>R cohorts (MO)</th>
<th>AP amplitude (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granule cells (no BAPTA)</td>
<td>Before</td>
<td>−76.4 ± 1.2</td>
<td>67.5 ± 4.6</td>
<td>85.1 ± 2.8</td>
</tr>
<tr>
<td>(n = 16)</td>
<td>After</td>
<td>−83.8 ± 1.0*</td>
<td>90.6 ± 6.0*</td>
<td>90.3 ± 2.7</td>
</tr>
<tr>
<td>Insensitive interneurons (no BAPTA)</td>
<td>Before</td>
<td>−62.7 ± 3.8</td>
<td>138.2 ± 7.1</td>
<td>68.0 ± 4.3</td>
</tr>
<tr>
<td>(n = 10)</td>
<td>After</td>
<td>−70.3 ± 3.8</td>
<td>158.0 ± 6.9</td>
<td>68.2 ± 4.1</td>
</tr>
<tr>
<td>Mossy cells (no BAPTA)</td>
<td>Before</td>
<td>−64.8 ± 1.9</td>
<td>88.8 ± 6.7</td>
<td>79.0 ± 5.2</td>
</tr>
<tr>
<td>(n = 8)</td>
<td>After</td>
<td>−32.5 ± 6.2*</td>
<td>13.3 ± 20.0*</td>
<td>18.5 ± 9.4*</td>
</tr>
<tr>
<td>Mossy cells (BAPTA)</td>
<td>Before</td>
<td>−64.3 ± 3.0</td>
<td>94.0 ± 8.3</td>
<td>79.7 ± 0.9</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>After</td>
<td>−66.5 ± 6.1</td>
<td>93.3 ± 6.0</td>
<td>81.8 ± 1.2</td>
</tr>
<tr>
<td>Sensitive interneurons (no BAPTA)</td>
<td>Before</td>
<td>−66.8 ± 2.6</td>
<td>101.2 ± 7.2</td>
<td>59.8 ± 4.6</td>
</tr>
<tr>
<td>(n = 4)</td>
<td>After</td>
<td>−21.4 ± 6.0*</td>
<td>45.2 ± 20.2*</td>
<td>18.2 ± 9.4*</td>
</tr>
</tbody>
</table>

*Difference between before and after stimulation measures statistically significant, P < 0.05; t test.
ter a burst of APs) decreased 20 to 30 min after impairment, suggesting that 20 to 30 min were sufficient to allow for diffusion of BAPTA into the impaired cell. Therefore, mossy cells were activated via intermittent perforant path stimulation 20 to 30 min after impairment. These mossy cells impaired with BAPTA electrodes did not depolarize (or depolarized only slightly) during the initial segment of 20-Hz stimulus trains, and actually hyperpolarized during 2-Hz stimulus trains (Fig. 1B). After 10 min of intermittent stimulation, these cells showed no deterioration by electrophysiological criteria. This response to intermittent stimulation of CaBP-negative cells that were impaired with BAPTA-containing electrodes was in contrast to the response obtained from comparable cells impaired with microelectrodes containing no Ca²⁺ chelator and was similar to the response of CaBP-positive cells such as granule cells.

Two mossy cells that were recorded with BAPTA-containing electrodes were stimulated intermittently soon after impairment (0-10 min, before substantial BAPTA diffusion occurred) and 30 min after impairment (Fig. 1). Their responses to the first period of intermittent stimulation were similar to the responses of mossy cells impaired without BAPTA-containing microelectrodes (Fig. 1A). However, when the same stimulus was delivered 30 min after impairment, the same mossy cells were relatively resistant to stimulus-induced depolarization, decreases in AP amplitude, broadening of APs, and loss of Rm (Fig. 1B).

Other neurons of the dentate hilus that were sensitive to sustained intermittent stimulation had electrophysiological characteristics of interneurons. They were infrequently encountered and difficult to hold in stable penetrations. Like the mossy cells, these sensitive interneurons depolarized, lost Rm, and deteriorated during sustained intermittent stimulation (n = 4; Table 1). Some interneurons in the hilus responded to intermittent stimulation like the granule cells; these insensitive interneurons did not depolarize, lose Rm, or deteriorate during stimulation (n = 10; Table 1). No immunocytochemistry was performed in these experiments, so it was not possible to determine whether the sensitive interneurons lacked Ca²⁺ binding proteins, or whether they contained somatostatin, as experiments performed in vivo would suggest (4, 5).

However, it was possible to differentiate the two populations of interneurons in another way. The interneurons that were sensitive to sustained intermittent stimulation had a very low threshold for AP generation evoked by perforant path stimulation, whereas the insensitive interneurons had a threshold that was similar to granule cells. Two sensitive interneurons with low thresholds were impaled with microelectrodes containing BAPTA, and intermittent stimulation was delivered 30 min after impairment. Intermittent stimulation led to transient, minimal depolarizations (up to 4 mV) and cells completely repolarized after intermittent stimulation; there were no significant changes in Rm (Table 1).

The mechanism of the protective effect of BAPTA is most likely the chelation of intracellular free Ca²⁺, which would limit the rise in Ca²⁺ during stimulation. Calcium levels may rise during stimulation because of the release of free Ca²⁺ from intracellular stores, or as a result of Ca²⁺ influx through voltage-dependent or receptor-linked Ca²⁺ channels. To test whether the deterioration was mediated by N-methyl-D-aspartate (NMDA) channels, as suggested by other studies (2), we carried out two manipulations. In ten experiments, extracellular Mg²⁺ concentration was raised to 2 mM, a level more effective in blocking NMDA-gated channels than our baseline level of 1 mM (13). In two other experiments, the specific NMDA receptor antagonist Ni₃-amino-phosphonovaleate (APV) (100 µM) (14) was added to our standard medium. Both manipulations blocked the damaging effects of intermittent stimulation on electrophysiologically characterized mossy cells, demonstrating that the Ca²⁺ induced cell deterioration was due to influx via NMDA-gated channels, which were likely opened by the high level of excitatory input produced by perforant path stimulation (15).

In summary, we have used the Ca²⁺ chelator BAPTA to reduce the damaging effects of sustained stimulation on cells found in the hilus of the fascia dentata that appear to lack Ca²⁺ binding proteins. Since we have no way of assessing long-term survival of neurons studied in our in vitro experiments, it is not possible to state unequivocally that electrophysiological deterioration is equivalent to cell death. However, the marked and long-lasting electrophysiological changes we encountered have long been associated with dying neurons and correlate well with morphological studies that show death of the same neuron populations (4, 5). Thus, we interpret our results to suggest that effective buffering of intracellular free Ca²⁺ during periods of neuronal excitation is crucial to cell survival. Our data indicate that in complex organized central nervous system tissue, high levels of activation of normal synaptic connections can lead to a rise in Ca²⁺ that causes irreversible degenerative changes in neuronal physiology. Supplementing the Ca²⁺ binding capacity of vulnerable cells can prevent cell damage.

REFERENCES AND NOTES

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6. Intracellular electrodes were pooled from borosilicate glass (80 to 120 megohms). Electrodes were filled with either 4M potassium acetate or 200 mM HAPTA (hexapotassium salt; Molecular Probes) in 4M potassium acetate.


8. Adult female Sprague-Dawley rats were decapitated and a section of brain containing the hippocampus was quickly removed. Longitudinal, 400 µm thick slices were cut in 4°C buffer with a Vibratome (Frederick Haer). Slices were placed immediately after dissection in a warmed (35°C), humidified (95% O₂, 5% CO₂) recording chamber at an interface of air and modified Krebs-Ringer buffer (120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, and 10 mM dextrose, pH 7.4). [H. E. Scharff and P. A. Schwartzkroin, *J. Neurosci.* 8, 3812 (1988)].

9. Granule cells were distinguished from hilar cells by their cell body location in the granule cell layer, as well as by several morphological properties, such as a negative RMP (compared to pyramidal cells, inter-neurons, and mossy cells), a low level of spontaneous activity, and marked spike frequency adaptation [M. A. Frack and D. A. Price, *J. Neurophysiol.* 51, 195 (1984)]. Mossy cells were impaled only in the hilus proper (D. A. Price, *J. Comp. Neurol.* 182, 851 (1978)).

10. R mp was determined from the amplitudes of responses at steady state to hyperpolarizing subthreshold current pulses (0.1 to 1.0 nA for 100 ms) and defined as the slope of linear portion of the current-voltage (I–V) curve. AP amplitude was measured from RMP to peak.


12. APV (Sigma) was added to the perfusate before and during stimulation. APV had no detectable nonspecific effects on cell properties or the population spike.

13. Although receptor binding studies have not indicated that the dentate hilus has a particularly high concentration of NMDA receptors, studies to date have not had sufficient resolution to detect differences among individual hilar cells [D. T. Monaghan and C. W. Cotman, *J. Neurosci.* 5, 2909 (1985); E. W. Harris et al., *Brain Res.* 352, 174 (1983); W. F. Maragos, J. R. Pessoney, A. B. Young, *J. Neurosci.* 8, 493 (1988)].

14. These studies were supported by National Institute of Neurological and Communicative Disorders and Stroke grants NS-01744, NS-15317, and NS-18895. P.A.S. is an affiliate of the Child Development and Mental Retardation Center, University of Washington.

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