Calcium Dynamics in Thorny Excrescences of CA3 Pyramidal Neurons

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Jaffe, David B. and Thomas H. Brown. Calcium dynamics in thorny excrescences of CA3 pyramidal neurons. J. Neurophysiol. 78: 10–18, 1997. Confocal laser scanning microscopy was used to visualize Ca$^{2+}$ transients in a particular type of dendritic spine, known as a thorny excrescence, in hippocampal CA3 pyramidal neurons. These large excrescences or thorns, which serve as the postsynaptic target for the mossy-fiber synaptic inputs, were identified on the basis of their location, frequency, and size. Whole cell recordings were made from superficial CA3 pyramidal neurons in thick hippocampal slices with the use of infrared video microscopy; cells with proximal apical dendrites close to the surface of the slice were selected. Changes in intracellular Ca$^{2+}$ levels were monitored by imaging changes in fluorescence of the dyes Calcium Green-1 and Fluo-3. Dual-emission fluorescence imaging was also employed with the use of a combination of Fluo-3 and the Ca$^{2+}$-insensitive dye seminaphthorhodafluor-1. This method was used to decrease the potential influence of background fluorescence on the calculated changes in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]). Somatic depolarization produced increases in [Ca$^{2+}$], in both the thorn and the immediately adjacent dendrite. Changes in [Ca$^{2+}$], were time locked with the onset of depolarization and the decay began immediately after the termination of depolarization. The peak increase in the Ca$^{2+}$ signal was significantly greater in the thorns than in the adjacent dendritic shafts. With the use of high-temporal-resolution methods (line scans), differences were also seen in the time course of Ca$^{2+}$ signals in these two regions. The decay time constants of the Ca$^{2+}$ signal were faster in thorns than in the adjacent dendritic shafts. These observations suggest that voltage-gated Ca$^{2+}$ channels are localized directly on the dendritic spines receiving mossy-fiber input. Furthermore, Ca$^{2+}$ homeostasis within thorny excrescences is distinct from Ca$^{2+}$ regulation in the dendritic shaft, at least over brief time periods, a finding that could have important implications for synaptic plasticity and signaling.

INTRODUCTION

Most excitatory synapses in the mammalian CNS make contacts onto specialized structures called dendritic spines (Harris and Kater 1992). The exact role of spines in synaptic transmission and plasticity has remained a mystery. We and others have been especially interested in the questions of whether these spines contain voltage-gated Ca$^{2+}$ channels, which could be relevant both to synaptic signaling and plasticity (Holmes and Levy 1990; Segev and Rall 1988; Zador et al. 1990).

Recent experiments have demonstrated that subthreshold synaptic stimulation is sufficient to trigger voltage-gated Ca$^{2+}$ channels in dendrites (Eilers et al. 1995; Magee and Johnston 1995; Miyakawa et al. 1992), and we previously demonstrated that voltage-gated Ca$^{2+}$ channels are located on the dendritic spines of CA1 pyramidal neurons (Jaffe et al. 1994a; see also Yuste and Denk 1995). Here we examine Ca$^{2+}$ dynamics in a very different kind of spine, called a thorn or thorny excrescence (Amaral and Dent 1981; Blackstad and Kjaerheim 1961; Chicurel and Harris 1992), that is located on the proximal dendrites of CA3 pyramidal neurons. Thorns are the postsynaptic targets of the mossy-fiber synaptic inputs to the hippocampus from the dentate gyrus.

The size and morphology of thorns, as determined by three-dimensional reconstructions from serial electron micrographs, can vary significantly in size and shape, from relatively small (1–2 μm in length) and unbranched protuberances with a single head to large (5–6 μm in length) and complex branched structures with as many as 12 heads (Chicurel and Harris 1992). Thorns are generally found in clusters (DeLeon et al. 1994), in contrast to the more uniform distribution of typical spines on hippocampal pyramidal neurons (Harris and Stevens 1989).

Thorny excrescences are fascinating in their own right. Owing to their large size and distinguishing characteristics, they can be readily identified with the use of light microscopic imaging methods (Brown and Zador 1990; Jaffe and Brown 1992), offering certain experimental advantages for imaging Ca$^{2+}$ dynamics.

METHODS

Whole cell recording in thick hippocampal slices

Hippocampal slices (300 μm) from 14- to 25-day-old Sprague-Dawley rats were sliced with the use of a vibratome in “cutting” artificial cerebrospinal fluid (aCSF) containing (in mM) 124 choline chloride, 2.5 KCl, 26 NaHCO$_3$, 2 MgCl$_2$, 2 CaCl$_2$, 1.25 NaH$_2$PO$_4$, and 10 dextrose. Slices were incubated at room temperature (~22°C) in a holding chamber with normal aCSF (124 mM NaCl replaced the choline) oxygenated with 95% O$_2$-5% CO$_2$. Slices were transferred as needed to a submersion-type recording chamber perfused with oxygenated aCSF, also at room temperature.

Cell bodies and dendrites of CA3 neurons close to the surface (within 50 μm) were identified with the use of infrared video microscopy and differential interference contrast optics (Dodt 1993; Keenan et al. 1988; MacVicar 1984) beneath a water immersion objective (Zeiss, ×100, 0.75 numerical aperture). An example of CA3 pyramidal neuron cell bodies and dendrites visualized with infrared video microscopy is shown in Fig. 1A. Whole cell patch recordings from cell somata or dendrites were made under visual guidance as previously described (Jaffe and Brown 1994b; Stuart and Sakmann 1994; Stuart et al. 1993). Pipette tips contained (in mM) 150 potassium gluconate, 20 KCl, 0.1–1 ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 2 MgCl$_2$, 2 Na$_2$ATP, and 10 N-2-hydroxyethylpiperazine-N′-2-eth-
anesulfonic acid, pH 7.3. Electrodes were backfilled with the same solution with 100–200 μM of either Fluo-3 or Calcium Green-1 (CG-1, Molecular Probes). Some Fluo-3 experiments included 200 μM of the Ca$^{2+}$-insensitive dye seminaphthorhodafluor-1 (SNARF-1), a pH-sensitive dye.

Electrical signals (Fig. 1B) were monitored with an Axoclamp 2a amplifier under the control of a Master-8 stimulus generator. Electrodes had access resistances, measured with a bridge balance, of <20 MΩ. Signals were digitized on-line with custom software and analyzed with the use of PV-WAVE (Precision Numerics). Mossy fibers were stimulated (50-μs monophasic pulses) with a bipolar stimulating electrode placed in the dentate granule cell layer or a patch pipette containing aCSF placed in stratum lucidum 100–200 μm from a selected CA3 neuron.

Confocal microscopy

HIGH-SPATIAL-RESOLUTION IMAGING. An upright BioRad MRC-600 confocal laser scanning system was employed in these experiments. The 40× water immersion objective, mentioned above, was also used to visualize dye-filled neurons. The 488-nm line of a krypton-argon laser excited fluorescent dye (CG-1, Fluo-3, or SNARF-1) injected into single CA3 neurons. We detected fluorescence with the use of one of two photomultiplier tubes at wavelengths >515 nm with a long-pass barrier filter. A dichroic mirror (band-pass wavelengths 500–550 nm) was used in some experiments to direct wavelengths >600 nm to the second photomultiplier tube. The confocal aperture was maximally open for a depth of field (optical section thickness) of ~6 μm (measured as the half-width of reflected in-focus light). An electronic zoom of 6–8× was used for the visualization of thorns. In some preliminary experiments, thorns and simple spines were visualized on an inverted confocal system with a 63× oil immersion objective (1.25 numerical aperture).

High-spatial-resolution fluorescent images (128 × 128 pixels) were taken at 500-ms intervals as previously described (Jaffe and Brown 1994a,b). Changes in fluorescence (ΔF) were normalized to resting fluorescence levels (ΔF/F) and reported as a percentage. Assuming that changes in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]i) were within the linear range of the dye, uncalibrated ΔF/F values should be proportional to changes in [Ca$^{2+}$]i, (Jaffe et al. 1992; Lev-Ram et al. 1992)

$$\Delta F/F = \frac{F - F_{auto}}{F_{rest} - F_{auto}}$$

 Autofluorescence (F$_{auto}$), measured from an averaged pixel region (50–100 pixels) not including the dye-filled neuron, was subtracted from resting fluorescence levels (F$_{rest}$). Autofluorescence was determined from pixels within the same optical section as the dendrite taken during each protocol. All resting signals, including Fluo-3 signals, were higher than autofluorescence.

It is important to point out that the photomultiplier detectors were equipped with offset and gain controls. These values were initially adjusted to achieve maximum contrast at rest between thorns and background. No further adjustments were made during the course of an experiment. Because of the offset to the output signal, the absolute ΔF/F and autofluorescence fluorescence levels that we measured represent the sum of the actual fluorescence and the offset. All results are therefore presented with the autofluorescence correction to subtract this offset from measured ΔF/F values.

In some Fluo-3 experiments ΔF was measured at 500–550 nm by photomultiplier 1. This was normalized to the ΔF/F of SNARF-1 measured at wavelengths >600 nm by photomultiplier 2. This ratio was termed ΔF/S. SNARF-1 was chosen as a Ca$^{2+}$-independent indicator on the basis of its excitation at 488 nm and large Stokes shift (emission >600 nm). Assuming that SNARF-1 concentrations and pH ratios were uniform throughout the neuron, and that changes in [Ca$^{2+}$]i, from rest were within the linear range of Fluo-3, ΔF/S, like ΔF/F, should be proportional to changes in [Ca$^{2+}$]i. The time course of ΔF in both cases was measured as the average of 25–50 pixels and filtered at 7–10 Hz.

HIGH-TEMPORAL-RESOLUTION MEASUREMENTS. A full frame image (768 × 512 pixels) of each neuron was taken during depolarization (Fluo-3 experiments) or at rest (CG-1 or SNARF-1 experiments) to identify the location of thorns on the apical dendrites. Fluorescence measurements were made over a single horizontal position consisting of a line of 768 pixels. These data are therefore referred to as line scans. Up to 512 line scans at 4-ms intervals were made across thorns and their corresponding dendritic shafts.
An average of five pixels over a selected horizontal location, thorn or dendrite, was used for calculating percent ΔF/F. The time course of \( \Delta F/F \) was then filtered at 20 Hz. All scanning, including both high-spatial-resolution and high-temporal-resolution imaging, was under the control of the Master-8 stimulus generator to coordinate electrical stimulation with confocal imaging. Finally, time constants for the recovery of \( [Ca^{2+}] \) were determined with the use of the exponential fitting program DISCRETE (Provincher 1976).

**Z SERIES PROJECTIONS.** After most experiments, neurons were depolarized, allowing a large influx of \( Ca^{2+} \). This resulted in large fluorescence increases throughout the soma and dendrites. A series of 10–40 high-resolution images (768 × 512 pixels), referred to as a Z series, was taken at 0.5- to 1-μm intervals along the Z-axis with the use of Kalman averaging (Kalman 1960) or photon counting. The confocal aperture was adjusted for ~1-μm optical sections. The maximum projection of the Z series or a three-dimensional volume rendering (with the use of VoxelView) was employed to distinguish thorns from secondary dendrites.

**Statistics**

All comparisons between thorns and dendrites were assessed with the use of two-tailed Student’s t-tests for paired samples unless otherwise noted. All values are presented as means ± SE.

**Results**

**Visualization of thorns in living tissue with the use of confocal microscopy**

We first assessed the use of confocal microscopy for visualizing thorns in living, unclared acute hippocampal slices. Although thorns are relatively larger than most other types of dendritic spines, the detection of emitted fluorescence from thorns is substantially degraded by the opacity of the slice. Signal averaging (Kalman averaging) or photon counting was used to increase the fluorescence signal-to-noise ratio. Examples of thorns identified on proximal apical dendrites are shown in Fig. 2. Groups of smaller thorns (2–3 μm in length) were normally observed, although longer thorns (up to 6 μm) were also seen (see Fig. 3). The location and morphology of thorns were distinguished from those of the simple spines seen on more distal apical or basal dendrites (compare Fig. 2, B and C, with Fig. 2D).

Imaging dynamic \( \Delta F \) with signal averaging, to the extent used above, was not feasible because of its limited temporal resolution. The potential effects of phototoxicity also had to be considered. Two conditions were required for us to image dynamic \( \Delta F \) within thorns. First, we used hippocampal slices from younger animals (14–20 days old); these slices were more translucent than slices from older animals. Second, we specifically selected neurons with dendrites within 50 μm of the surface of the slice (Fig. 1). In some experiments recordings were made directly from proximal apical dendrites that extended close to the surface of the slice.

At the beginning of each experiment thorns were identified on the basis of size, location, and distribution (Amaral and Dent 1981; Brown and Johnston 1983; Chicurel and Harris 1992; DeLeon et al. 1994) with the \( \Delta F/F \) of CG-1, SNARF-1 fluorescence, or the fluorescence of Fluo-3 during repetitive cell firing (from an applied depolarizing current). Included within our sample were just those structures proximal (within 50–100 μm) to the cell body layer, consistent with the distribution of the mossy-fiber synapses. Thorns were also identifiable by virtue of occurring in clusters of short thorns (2–3 μm) (DeLeon et al. 1994) or as individual long thorns (up to 6 μm) (Figs. 3A and 4A), in contrast to the generally uniform density of the ordinary spines (Fig. 2D). Finally, thorns could be differentiated on the basis of length (2–6 vs. 1–2 μm long for ordinary spines).

**Voltage-stimulated \( Ca^{2+} \) influx into thorns**

We triggered trains of action potentials by 0.5- to 1-s depolarizing current pulses or voltage steps to elicit voltage-gated \( Ca^{2+} \) entry. Increases in \( [Ca^{2+}] \), were detected in both thorns and dendritic shafts (Fig. 3, B and C). The time course of these \( Ca^{2+} \) transients, measured at 500-ms intervals, was consistent with previous dendritic measurements of voltage-gated \( Ca^{2+} \) accumulation (Jaffe et al. 1992); increases in \( [Ca^{2+}] \), were time locked with the onset of depolarization and recovery of \( Ca^{2+} \) immediately followed the end of the depolarizing pulse.

The overall magnitude of changes in \( [Ca^{2+}] \), in most thorns was frequently larger than that measured in corresponding dendritic shafts (Fig. 3B, Table 1). There was no clear evidence that the choice of dye (Fluo-3, Fluo-3/SNARF-1, or CG-1) was a critical determinant of the overall tendency, which was that most often the \( Ca^{2+} \) signal in the thorn was greater than the signal in the adjacent dendritic shaft. In making this comparison, we considered there to be no difference between the shaft and thorn if the signals were within 5% of each other. From 47 thorns identified on 18 CA3 pyramidal neurons we compared peak \( \Delta F/F \) or \( \Delta F/\Delta S \) values between thorns and dendrites (Table 2). The mean ratio of the change in \( Ca^{2+} \) in thorns compared with their dendritic shafts (T/D) was >1 for all three dye combinations.

An important concern was the issue of light scattering. Small structures adjacent to larger ones with significantly higher fluorescence may be contaminated by the scattering of light. Although the “flare” produced by light scattering is reduced in confocal microscopy, scattering could result in an edge effect of higher \( \Delta F/F \) values. To control for this we analyzed pixels adjacent to the dendritic shaft but lacking thorns. Signals were only detected from pixel areas that contained thorns (Fig. 3B).

**High-temporal-resolution measurements of \( Ca^{2+} \) transients in thorns**

Although the time course of measured \( Ca^{2+} \) transients can be on the order of seconds (see Fig. 3B), components of spike-mediated \( Ca^{2+} \) accumulation may be more rapid (Jaffe and Brown 1994c). To achieve greater temporal resolution, a single laser line was scanned at 4-ms intervals over both identified thorns and the corresponding dendritic shaft. In Fig. 4A, the position of the scanned line over thorns and the primary apical dendrite of a CA3 pyramidal neuron is illustrated. In response to a brief depolarizing current pulse (0.5 s), changes in Fluo-3 fluorescence along the scanned line were observed in both thorn and dendrite (measured at arrows labeled T and D, respectively, in Fig. 4B).

The time courses for both the thorn and dendrite \( Ca^{2+} \)
signals are plotted in Fig. 4C. As observed with the lower-temporal-resolution method, changes in \([\text{Ca}^{2+}]\), in thorns were larger than in the dendrite (Fig. 4C, Table 2). The T/D ratio of peak \(\Delta F/F\) was significantly larger for the line-scanned measurements compared with values from lower-temporal-resolution imaging (Table 1; \(t = 3.46\), \(df = 36\), \(P < 0.05\), 2-tailed Student’s \(t\)-test for unpaired samples). However, the effects of averaging boxed regions (Fig. 3A) of pixels over both neuronal and nonneuronal regions may have contributed overall to smaller spinous signals.

The time course of Ca\(^{2+}\) accumulation in thorns was only \(~10\%\) faster than that of the parent dendrite (Table 3). For a 500-ms depolarizing voltage step, the mean time to peaks in the thorn and dendrite were 233 and 273 ms, respectively. Variations in these times were due to the inactivation of voltage-gated Ca\(^{2+}\) channels and/or poor voltage clamping of the Ca\(^{2+}\) currents. In contrast, the recovery of \([\text{Ca}^{2+}]\), was significantly faster in thorns compared with the parent dendrite. In 9 of 11 thorns examined, the decay of Ca\(^{2+}\) in thorns and dendrites was best fit by two exponentials. This is similar to the decay of dendritic \([\text{Ca}^{2+}]\), which is also best fit by two exponentials (Jaffe and Brown 1994c; Regehr and Tank 1992). The time constant of the slowest fitted exponential was \(~50\%\) faster in thorns than in the corresponding dendritic shafts (Table 3). This observation further indicates that we were resolving differences in fluorescence changes between thorn and dendrite.

**Synaptically mediated Ca\(^{2+}\) signals**

We attempted to examine the effects of mossy-fiber synaptic stimulation on Ca\(^{2+}\) transients in the proximal dendrites and thorns of CA3 neurons. Stimulating electrodes were placed either along the hilar side of the dentate granule cell layer or in stratum lucidum within 100–200 \(\mu m\) of an identified CA3 cell. This latter location was chosen in some experiments to maximize stimulation of fibers innervating a specific neuron. We surmised that as the stimulating electrode is placed farther from the recording site, the probability that the stimulated mossy fibers leave the plane of the slice might increase. This would limit the number of activated synapses onto the particular cell of interest. Although placing a stimulating electrode in stratum lucidum could increase the likeli-
FIG. 3. Membrane depolarization produces larger changes in Ca$$^{2+}$$ in thorns than accompanying dendrites. Trains of action potentials were produced by 1-s depolarizing current pulse applied through whole cell patch pipette. A: micrograph of a proximal apical dendrite of a CA3 pyramidal neuron illustrating a long thorn (T1), with possibly 2 heads in succession, and a cluster of apparently 2 smaller thorns (T2). B: time course of changes in Ca$$^{2+}$$ in response to a 1-s current pulse (horizontal black bar). Dashed trace: average change in fluorescence for thorn. Solid trace: corresponding dendrite (corresponding to dashed and solid boxes in A). Bottom trace: lack of signal in an area adjacent to dendrite. C: 3-dimensional volume rendering of CA3 pyramidal neuron from A and B. Rendering was produced from a Z series (see METHODS) of 22 optical sections taken at 1-μm intervals. Again, apparently 2 heads in succession can be seen for thorn T1 and cluster of 2 thorns is identified by T2. ΔF/F, resting fluorescence level; T, thorn; D, dendrite.

Discussion

Visualization of spines in living brain slices

Visualizing living spines within a thick, acute brain slice presents a major technical problem. The optical properties of living brain tissue are far from ideal because of its high opacity. Emitted fluorescence rapidly scatters with distance through the slice, reducing the efficiency of photon detection. In this and other studies, visualizing dendrites closest to the surface is the most obvious solution (Guthrie et al. 1991; Hosakawa et al. 1992; Jaffe and Brown 1992; Muller and Connor 1991). Alternatively, spines on living neurons have also be visualized in culture (Murphy et al. 1994; Papa et al. 1995; Pozzo-Miller et al. 1993; Segal 1995). Recently, two-photon fluorescence imaging has been used to visualize and record changes in Ca$$^{2+}$$ within CA1 pyramidal neuron dendritic spines in acute brain slices and to confirm the presence of voltage-gated Ca$$^{2+}$$ channels in their dendritic spines (Svoboda et al. 1996; Yuste and Denk 1995). Tissue
from younger animals (<25 days old) is preferred for optical methods because it is more translucent (Keenan et al. 1988) and therefore reduces loss of emitted fluorescence due to scattering. An important concern with young tissue, however, is the possibility that there may be significant anatomic and physiological differences between immature and adult neurons (Amaral and Dent 1981).

**Ca\(^{2+}\) dynamics in thorns**

The work presented here is the first high-time-resolution study of Ca\(^{2+}\) dynamics within thorns of CA3 pyramidal neurons (but see Pozzo-Miller et al. 1993; Segal 1995). We found that membrane depolarization produced increases in [Ca\(^{2+}\)], within both the proximal apical dendrites and thorns. However, Ca\(^{2+}\) signals in most thorns were larger than the signals in the corresponding dendritic shaft (Figs. 3 and 4, Table 1). This conclusion was drawn from three different analyses: 1) changes in [Ca\(^{2+}\)], as determined by the percent \(\Delta F/F\) for cells filled with Fluo-3 or CG-1; 2) \(\Delta F\) normalized to the fluorescence of a Ca\(^{2+}\)-independent dye (\(\Delta F/S\)); and 3) high-time-resolution measurements of \(\Delta F/F\).

### Table 1. Comparison of thorn and dendrite peak fluorescence ratios

<table>
<thead>
<tr>
<th>Dye</th>
<th>T &gt; D</th>
<th>T &lt; D</th>
<th>T = D</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(^{2+}) Green</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Fluo-3</td>
<td>19</td>
<td>3</td>
<td>5</td>
<td>27</td>
</tr>
<tr>
<td>Fluo-3/SNARF-1</td>
<td>11</td>
<td>1</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>5</td>
<td>6</td>
<td>47</td>
</tr>
</tbody>
</table>

T, thorn; D, dendrite; SNARF-1, seminaphthorhodfluor-1. n, number of comparisons.

The simplest explanation for larger signals in thorns is entry of Ca\(^{2+}\) through voltage-gated channels located on thorns and differences in surface-to-volume ratios. The magnitude of Ca\(^{2+}\) accumulation produced by entry through the plasma membrane is dependent on the Ca\(^{2+}\) current density and inversely related to the volume of the compartment. If one assumes an equivalent density of channels on the shaft and the thorn, and therefore an equivalent Ca\(^{2+}\) current density, Ca\(^{2+}\) accumulation will be larger in the thorn because of its smaller volume. Similar results have been observed in spines on hippocampal CA1 pyramidal neurons and hippocampal pyramidal neurons in culture (Jaffe et al. 1994a; Segal 1995; Yuste and Denk 1995). This hypothesis is further supported by our high-time-resolution measurements. If the source of calcium were solely from the dendritic shaft, we would expect a delay in the onset as well as the time-to-peak of the thorn signal. One recent set of experiments suggests that diffusion from the dendrite into spines may be on the order of 50–100 ms, depending on the size of the spine (Svoboda et al. 1996).

### Table 2. Peak changes in fluorescence compared between thorns and dendrite

<table>
<thead>
<tr>
<th>Dye</th>
<th>n</th>
<th>T/D Ratio</th>
<th>Student’s t-Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(^{2+}) Green</td>
<td>7</td>
<td>1.7 ± 0.3</td>
<td>(t = 1.97, P &gt; 0.05)</td>
</tr>
<tr>
<td>Fluo-3</td>
<td>27</td>
<td>1.4 ± 0.1</td>
<td>(t = 5.57, P &lt; 0.05)</td>
</tr>
<tr>
<td>Fluo-3/SNARF-1</td>
<td>13</td>
<td>1.5 ± 0.1</td>
<td>(t = 2.93, P &lt; 0.05)</td>
</tr>
<tr>
<td>Line scan Fluo-3</td>
<td>11</td>
<td>2.5 ± 0.5</td>
<td>(t = 3.09, P &lt; 0.05)</td>
</tr>
</tbody>
</table>

Values in column 2 are means ± SE. n, number of comparisons. For abbreviations, see Table 1.
Alternative hypothesis: calcium-induced calcium release

A more complex hypothesis to explain our results is that rapid diffusion of calcium from the dendritic shaft into thorns triggers calcium-induced calcium release (CICR). Local amplification due to CICR results in higher levels of calcium relative to the dendritic shaft. Recent work suggests that there are potent Ca\(^{2+}\) release mechanisms in hippocampal pyramidal neurons (Alford et al. 1993; Jaffe and Brown 1994a) and voltage-gated Ca\(^{2+}\) entry has been reported to trigger CICR in cerebellar Purkinje neurons as well (Llano et al. 1994). Generally, CICR channels are activated by micromolar levels of free Ca\(^{2+}\) (Bezprozvanny et al. 1991), but there may be significant variation in their sensitivities depending on the cell type and experimental conditions.

The CICR hypothesis, however, is not supported by our observation that the time course of Ca\(^{2+}\) accumulation in thorns is not slower than in the dendritic shaft (see also Segal 1995). Given that there should be some threshold level of calcium needed to trigger CICR, and if there were only dendritic voltage-gated Ca\(^{2+}\) channels, we would expect some delay of the signal for Ca\(^{2+}\) diffusion from the dendrite into the thorn (Svoboda et al. 1996). Some amplification of Ca\(^{2+}\) entry into the thorn, from the dendrite or directly into the thorn, by CICR cannot be ruled out by these experiments and remains an interesting possibility.

Diffusion of Ca\(^{2+}\) from the thorn into the dendrite might, however, account for the more rapid recovery of Ca\(^{2+}\) in thorns. The dendritic shaft could serve as a large sink for Ca\(^{2+}\) to rapidly diffuse from the dendrite into the thorn.

Another possibility is that there may be differences in Ca\(^{2+}\) homeostatic mechanisms, such as Ca\(^{2+}\) sequestration into endoplasmic reticulum, between thorn and dendrite. A simpler explanation, however, is that the large surface-to-volume ratio of thorns (compared with dendrites) allows for greater Ca\(^{2+}\) extrusion or sequestration, via Na\(^+\)/Ca\(^{2+}\) exchange or Ca\(^{2+}\)-ATPase, and therefore a faster decay.

Single-wavelength Ca\(^{2+}\) imaging

Single-wavelength imaging, in contrast to dual-wavelength ratiometric imaging, depends on normalizing \(\Delta F\) to \(\Delta F/F\) (Jaffe and Brown 1994b; Jaffe et al. 1992; Lev-Ram et al. 1992). There are two primary assumptions associated with this method: that changes in [Ca\(^{2+}\)] are within the linear range of the dye if there is no calibration, and that resting levels of Ca\(^{2+}\) are uniform throughout the cell. If these assumptions are satisfied, then measured \(\Delta F/F\) values should be proportional to changes in [Ca\(^{2+}\)]. Saturation of dye might limit our resolution of significant differences in Ca\(^{2+}\) transients between thorn and dendrite. Thus failure to see significant differences with a high-affinity dye might reflect a saturation effect. However, we were able to detect differences between thorn and dendrite with the use of both of the indicator dyes (Table 1).

Synaptically mediated Ca\(^{2+}\) transients in thorns

Subthreshold synaptic stimulation can activate voltage-gated Ca\(^{2+}\) channels in hippocampal, cortical, and cerebellar neurons (Eilers et al. 1995; Magee and Johnston 1995, 1997;
Markram and Sakmann 1994; Miyakawa et al. 1992; Papa et al. 1995). It is therefore possible that voltage-gated channels on thorns are also activated by subthreshold synaptic stimulation. Activation of these channels may serve to raise local Ca$^{2+}$ levels and stimulate Ca$^{2+}$-dependent biochemical mechanisms. Alternatively, subthreshold activation of voltage-gated channels in spines might be important for amplifying synaptic potentials (Segev and Rall 1988).

There are, of course, potential sources of Ca$^{2+}$ in hippocampal neurons other than voltage-gated Ca$^{2+}$ channels. The localized Ca$^{2+}$ transients observed in response to mossy-fiber stimulation may be generated by the low density of N-methyl-D-aspartate activated by mossy-fiber stimulation (Jonas et al. 1994; Spruston et al. 1995). Release of Ca$^{2+}$ from intracellular stores by metabotropic glutamate receptor activation (Jaffe and Brown 1994a) could also account for localized increases in Ca$^{2+}$ at mossy-fiber synapses.

Conclusions

We have shown, with the use of both high- and low-temporal-resolution imaging methods, that membrane depolarization resulted in larger Ca$^{2+}$ increases in thorns than in the adjacent dendritic shafts. Our results are consistent with the hypothesis that voltage-gated Ca$^{2+}$ channels are located on the thorny excrescences of CA3 pyramidal neurons. In principle, rapid diffusion of calcium from the dendrite into the spine may trigger CICR and lead to changes in calcium as well. Regardless of the mechanism, the data suggest that there are differences in Ca$^{2+}$ homeostatic mechanisms between thorns and dendrites. The activation of voltage-gated Ca$^{2+}$ channels on thorns may therefore be important for mediating Ca$^{2+}$-dependent processes at this synapse where there is a low density of N-methyl-D-aspartate receptors.

This work was supported by a National Institute of Neurological Disorders and Stroke postdoctoral fellowship award to D. B. Jaffe and grants from the Office of Naval Research and the National Institutes of Health to T. H. Brown.

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Received 28 December 1995; accepted in final form 25 February 1997.

REFERENCES


