Dendritic Ca\textsuperscript{2+} transients evoked by action potentials in rat dorsal cochlear nucleus pyramidal and cartwheel neurons

Running title: Dendritic Ca\textsuperscript{2+} transients in DCN neurons

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Keywords: dorsal cochlear nucleus, pyramidal cells, cartwheel cells, simple spikes, complex spikes, dendrites
ABSTRACT

Simultaneous fluorescence imaging and electrophysiologic recordings were used to investigate the Ca\(^{2+}\) influx initiated by action potentials (APs) into dorsal cochlear nucleus (DCN) pyramidal cell (PC) and cartwheel cell (CWC) dendrites. Local application of Cd\(^{2+}\) blocked Ca\(^{2+}\) transients in PC and CWC dendrites, demonstrating that the Ca\(^{2+}\) influx was initiated by dendritic Ca\(^{2+}\) channels. In PCs, TTX eliminated the dendritic Ca\(^{2+}\) transients when APs were completely blocked. However, the Ca\(^{2+}\) influx could be partially recovered during an incomplete block of APs, or when a large depolarization was substituted for the blocked APs. In CWCs, dendritic Ca\(^{2+}\) transients evoked by individual APs, or simple spikes, were blocked by TTX and could be recovered during an incomplete block of APs or by a large depolarization. In contrast, dendritic Ca\(^{2+}\) transients evoked by complex spikes, a burst of APs superimposed upon a slow depolarization, were not blocked by TTX, despite eliminating the APs superimposed upon the slow depolarization. These results suggest two different mechanisms for the retrograde activation of dendritic Ca\(^{2+}\) channels: the first requires fast Na\(^+\) channel-mediated APs or a large somatic depolarization, whereas the second is independent of Na\(^+\) channel activation, requiring only the slow depolarization underlying complex spikes.

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INTRODUCTION

The classical view of synaptic integration is that action potentials (APs) are generated by the soma and axon in response to the passive summation of synaptic inputs within the dendritic arbor (Coombs et al. 1957; Fatt 1957). However, intradendritic recordings from mammalian neurons showed that dendrites are not necessarily passive structures (Llinás and Sugimori 1980; Wong et al. 1979). Although it was originally postulated that dendritic voltage-gated conductances are only involved in the anterograde propagation of synaptic responses toward the soma (Llinás and Nicholson 1971), somatic and axonal APs can propagate retrogradely and evoke active dendritic responses. The imaging of ion-sensitive fluorescence indicators has shown that APs evoke a Ca$^{2+}$ influx through dendritic Ca$^{2+}$ channels in cortical (Markram et al. 1995; Schiller et al. 1995) and hippocampal (Jaffe et al. 1992; Spruston et al. 1995) pyramidal neurons, in thalamocortical relay neurons (Zhou et al. 1997), in olfactory bulb mitral cells (Charpak et al. 2001; Margrie et al. 2001), and in spinal motoneurons (Larkum et al. 1996).

Dendritic Ca$^{2+}$ channels may play an important role in the integration of synaptic inputs. Because of the importance of Ca$^{2+}$ as a second messenger (Tsien and Tsien 1990), and because of its limited mobility in cytoplasm (Albritton et al. 1992), dendritic Ca$^{2+}$ channels may also provide an important source of Ca$^{2+}$ at or near synapses. This dendritic Ca$^{2+}$ influx may produce changes in synaptic efficacy when the retrograde propagation of somatic APs into the dendrites is paired with afferent inputs (Bell et al. 1997; Linden 1999; Magee and Johnston 1997; Markram et al. 1997). In addition, the activation of dendritic Ca$^{2+}$ channels by propagating APs can alter the electrophysiologic response properties of neurons. Ca$^{2+}$ channel-mediated burst discharges in hippocampal CA1 and CA3 pyramidal neurons can be induced under conditions that block dendritic voltage-gated K$^{+}$ channels (Golding et al. 1999; Magee and Carruth 1999),
and simultaneous back-propagating APs and dendritic depolarization can also evoke complex spike discharges in neocortical pyramidal neurons (Schwindt and Crill 1999; Williams and Stuart 1999).

Dendritic Ca\textsuperscript{2+} channels may contribute to information processing in at least two neuronal populations within the dorsal cochlear nuclei (DCN). Pyramidal cells (PCs, also known as fusiform cells) receive excitatory afferent input from two spatially and functionally segregated sources. The basal dendrites receive excitatory input from auditory nerve fibers (ANFs) ascending from the cochlea (Hirsch and Oertel 1988b; Kane 1974; Manis and Brownell 1983; Ryugo and May 1993), whereas the apical dendrites receive excitation from parallel fibers (PFs) originating from granule cells scattered throughout the cochlear nucleus (Manis 1989; Osen and Mugnaini 1981). The activation of dendritic Ca\textsuperscript{2+} channels by somatic APs could serve to modulate synaptic efficacy in response to synchronously active ANF and PF inputs. Cartwheel cells (CWCs) can be identified by their characteristic burst discharges or complex spikes (Manis et al. 1994; Zhang and Oertel 1993) that consist of fast Na\textsuperscript{+} channel-mediated APs superimposed upon a slow Ca\textsuperscript{2+} channel-mediated depolarization (Agar et al. 1996; Golding and Oertel 1997). However, complex spiking units also respond with simple spikes or a combination of complex and simple spikes in a variety of preparations (Davis and Young 1997; Ding et al. 1999; Golding and Oertel 1996; Manis et al. 1994; Parham and Kim 1995; Waller and Godfrey 1994; Zhang and Oertel 1993). These distinct electrophysiologic responses could result from differences in the pattern of dendritic Ca\textsuperscript{2+} channel activation initiated by Na\textsuperscript{+}-channel mediated APs.

The experiments in the present study combine whole-cell recordings with the imaging of the fluorescent Ca\textsuperscript{2+} indicator fluo-3 to investigate whether APs evoked by somatic current injection evoke a Ca\textsuperscript{2+} influx into the dendrites of PCs and CWCs. The properties of Ca\textsuperscript{2+}
channels have been investigated in acutely dissociated DCN neurons (Harasztosi et al. 1999; Molitor and Manis 1999); however, no information about dendritic Ca\(^{2+}\) channels could be obtained from this preparation. In the present study, recordings from a brainstem slice preparation show that APs evoke a Ca\(^{2+}\) influx into the apical and basal dendrites of PCs and into the dendrites of CWCs. Na\(^+\) channel-mediated APs are required to evoke a Ca\(^{2+}\) influx into the dendrites of PCs and simple spiking CWCs, whereas the activation of Na\(^+\) channels is not required to evoke a Ca\(^{2+}\) influx into the dendrites of complex spiking CWCs. The activation of dendritic Ca\(^{2+}\) channels by APs may play a significant role in shaping the output of the DCN, both by contributing to the integration of spatially and functionally segregated afferent inputs in PCs, and by shaping the electrophysiologic responses of CWCs.
MATERIALS AND METHODS

Preparation of slices

Rat pups (age P10 - P18) were anesthetized with ketamine (44 mg/kg), decapitated, and the brainstem was quickly removed and placed into an oxygenated HEPES-buffered dissection solution at 30 °C containing (in mM) 138 NaCl, 5 KCl, 1.25 KH$_2$PO$_4$, 10 glucose, 0.2 CaCl$_2$, 4 MgSO$_4$, and 10 HEPES, pH 7.35 with 5 M NaOH. Using an oscillating tissue slicer, the cochlear nuclei were cut into 250 µm thick slices in the trans-strial plane, perpendicular to the parallel fibers, and were placed into an incubation chamber containing an artificial cerebrospinal fluid (ACSF; see composition below) maintained at 34 °C. Slices remained in incubation for at least 1 h before being transferred to the recording chamber. In the recording chamber, slices were superfused with the ACSF (2 - 4 ml/min) and were maintained at 33 °C throughout all recordings. The ACSF solution contained (in mM) 130 NaCl, 3 KCl, 1.25 KH$_2$PO$_4$, 20 NaHCO$_3$, 10 glucose, 2.5 CaCl$_2$, and 1.3 MgSO$_4$, and was continuously perfused with 95% O$_2$ - 5% CO$_2$ to maintain a pH near 7.4. During some experiments, the voltage-gated Na$^+$ channel antagonist TTX was diluted from a concentrated stock solution and added to the ACSF. TTX was purchased from Molecular Probes (Eugene, OR); remaining chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Electrophysiologic Recordings

Whole-cell recording techniques (Edwards et al. 1989; Hamill et al. 1981) were used to provide depolarizing stimuli, to record electrophysiologic responses, and to provide a rapid means of loading individual cells with a fluorescent indicator while minimizing background fluorescence. Individual cells near the slice surface were visualized with infrared video.
microscopy (MacVicar 1984) on a fixed-stage upright microscope (Axioskop FS, Carl Zeiss Inc.) with a 40X, 0.75 N.A. water immersion objective. Whole-cell recordings were obtained using either a List EPC-7 amplifier in current clamp mode, or an Axoprobe 1A current clamp amplifier, filtered at 3 kHz and digitized at 10 kHz with a 12-bit A/D converter (Digidata 1200, Axon Instruments). Although it is known that voltage clamp amplifiers such as the List EPC-7 can distort the measured AP waveform in current clamp mode (Magistretti et al. 1998), no significant differences were observed between the AP-evoked Ca\(^{2+}\) influx in recordings obtained with either amplifier. Recording pipettes were pulled with a horizontal puller (BB-CH-PC, Mecanex Inc.) from borosilicate glass capillaries (KG-33 glass, Garner Glass Co.), fire-polished and coated with Sylgard (Dow Corning). Pipette resistances ranged from 3 - 8 MΩ with a pipette solution containing (in mM) 110 K gluconate, 20 KCl, 4 NaCl, 4 MgATP, 10 HEPES, 10 phosphocreatine, 0.3 GTP, 0.2 EGTA and 0.2 fluo-3 (pentapotassium salt, Molecular Probes), pH 7.20 with 5 M KOH. Although EGTA could reduce fluorescence signals by reducing the available free Ca\(^{2+}\) for binding to fluo-3, we found that the addition of a low concentration of EGTA to the pipette solution improved the detection of fluorescence signals by reducing background and resting fluorescence levels and improved the long-term stability of electrophysiologic recordings. Small amounts of hyperpolarizing holding current (typically < 300 pA for pyramidal cells and < 200 pA for cartwheel cells) were sometimes applied to prevent spontaneous action potentials. Bridge balance errors were corrected on-line for recordings made with the Axoprobe 1A and off-line for recordings made with the List EPC-7. Capacitive transients were blanked and a measured junction potential of 12 mV between the pipette solution and ACSF was subtracted from the voltage traces.
In some experiments, local application of TTX and CdCl₂ via pressure pipette was employed to ascertain the conductance types contributing to the dendritic Ca\(^{2+}\) influx. TTX and CdCl₂ were diluted from concentrated stock solutions into a HEPES-buffered solution containing (in mM) 138 NaCl, 5 KCl, 10 glucose, 2.5 CaCl₂, 1.3 MgCl₂, and 10 HEPES, pH 7.35 with 5 M NaOH. The concentration of TTX (10 µM) and CdCl₂ (1 mM) in the pressure pipette was ten-fold the desired final concentration at the site of application. Pressure pipette solutions were 0.2 µm filtered and centrifuged at 12,000 rpm for 5 minutes to prevent clogging of tips with any insoluble material that may be present. Pressure pipettes were similar to the recording pipettes, although the pressure pipettes generally had smaller tips (resistances 5 – 10 MΩ) than the recording pipettes to limit the mixing of the ACSF with the pipette contents and to limit the spread of the ejected bolus. Under visual control, pressure pipettes were typically placed within 10 µm of the target dendritic process. For each stimulus trial during which a pressure application was given, two or three 10 ms pulses of 3 – 5 PSIG were delivered at 50 Hz by opening a solenoid valve driven by a TTL circuit. Pressure pulses were timed so that the first pulse was delivered prior to the onset of the electrophysiologic stimulus, and the remaining pulses were delivered during the stimulus train. No discernible effects were observed on electrophysiologic or fluorescence recordings from three cells during pressure application of the HEPES-buffered pressure pipette solution without TTX or CdCl₂ (not shown).

**Image Acquisition**

A shuttered mercury arc lamp (HBO 100 W, Carl Zeiss Inc.) was used with 450 - 490 nm bandpass excitation filter to excite dye fluorescence; emissions were filtered with a 520 nm longpass filter. The resulting images were collected with an integrating CCD camera (model
Individual video frames were acquired every 33 ms, and were digitized with a 10 bit frame grabber (Image Lightning 2000, Axon Instruments) controlled by Axon Imaging Workbench (version 2.0 – 2.1, Axon Instruments) in the nonratiometric imaging mode. Digitized images are 640 x 480 pixels, which corresponds to an imaged area of 150 x 115 µm. Both PCs and CWCs possess extensive dendritic arbors; however, the use of a fixed-stage microscope prevented the movement of the objective relative to a cell once recordings began, so the results are limited to the most proximal processes of these neurons. Electrophysiologic stimuli were generated and recordings were obtained by a separate computer that was synchronized to the imaging computer by a common trigger signal. The onset of any electrophysiologic stimuli was delayed by 100 ms relative to the onset of image acquisition to allow for the acquisition of three resting fluorescence images for direct comparison with fluorescence levels during presentation of electrophysiologic stimuli. Repetitive stimuli were presented 10 s apart to allow for a complete return of intracellular Ca\textsuperscript{2+} to resting levels.

Following the termination of electrophysiologic recordings, the recording pipette was withdrawn and a series of integrated (2 – 8 video frames) fluorescence images were obtained from different regions and focal depths to reconstruct the morphology of the neuron from which recordings had been obtained. Montages of these fluorescence images were assembled to simultaneously display all visible anatomic features. PCs were identified as large (long axis of the soma > 25 µm) bipolar neurons with extensively branched apical dendrites extending into the superficial DCN and less-extensively branched basal dendrites extending into deeper layers (Blackstad et al. 1984). CWCs were identified as medium-sized (long axis of soma 15 - 20 µm) multipolar neurons with ovoid cell bodies and spiny dendrites typically limited to the superficial DCN (Wouterlood and Mugnaini 1984). The spines characteristic of CWC (and to a lesser
extent, PC) dendrites were not always visible due to light scatter through the 250 µm thick tissue sections. Therefore, dendritic branching patterns were examined to further distinguish CWCs from other DCN neurons. CWC dendrites typically branch at wider angles, resulting in distal processes that sometimes curve around or toward the soma (Wouterlood and Mugnaini 1984), whereas the dendrites of other neurons in the superficial DCN, such as stellate cells, branch at smaller angles, resulting in dendritic processes that radiate outward from the soma.

*Image Analysis*

Fluorescence images are shown as ΔF/F, which is the fractional change in fluorescence elicited by a stimulus relative to the resting fluorescence. Estimates of the calcium concentration can be computed from ΔF/F if accurate measurements of the dye Kₐ and the resting calcium levels are known (Lev-Ram et al. 1992). Because these parameters have not been measured in our preparation, values are only reported as fractional changes in fluorescence. Given the properties of the fluorescence indicator being used in these experiments, positive ΔF/F values indicate an increase in free Ca²⁺ concentration. Raw fluorescence images were background subtracted and filtered prior to constructing ΔF/F images. Typically, the majority of pixels in a given image measured only background fluorescence, so background subtraction was performed by subtracting the median of a pixel intensity histogram for each image. Following background subtraction, a 3 x 3 filter was used to smooth pixel intensities throughout each image (pixel weights were 4/9 in the center, 1/9 on the sides and 1/36 on the corners). ΔF/F images were then obtained by subtracting the resting fluorescence image obtained immediately before the stimulus presentation from a fluorescence image obtained at the end of the stimulus presentation, and normalizing this difference by the resting fluorescence image.
One difficulty with the calculation of $\Delta F/F$ images is the high level of background fluorescence in a slice preparation (Sandler and Barbara 1999). In these experiments, many dendritic processes at rest were not visible above background and only became visible following a fluorescence increase resulting from a $\text{Ca}^{2+}$ influx. Normalizing fluorescence changes by resting fluorescence values that are not significantly above background levels produces erroneously large $\Delta F/F$ values. To prevent these errors, the value of the background fluorescence was added back to the resting fluorescence image prior to normalizing: $\Delta F/F = (F_{\text{stim}} - F_{\text{rest}})/(F_{\text{rest}} + F_{\text{background}})$. Although this results in an underestimation of the true $\Delta F/F$ value, it does not alter our interpretation of these data, as the focus of this study was to compare relative changes in $\text{Ca}^{2+}$ levels rather than absolute measures of $\text{Ca}^{2+}$ concentration.

Quantitative comparisons of fluorescence changes were obtained using fluorescence profiles. Profiles are curves that follow a visible dendrite along its length; the $\Delta F/F$ value for each pixel that falls along the curve is plotted as a function of path distance from the curve origin, which is typically located where the dendrite originates from the soma. Despite filtering, there was significant variation between neighboring pixels, which produced noisy fluorescence profiles. To reduce this noise, profile data were smoothed by averaging $\Delta F/F$ values from small regions (~1 $\mu\text{m}^2$) centered on each pixel along the profile rather than using $\Delta F/F$ values from individual pixels. Profile data were subsequently averaged across stimulus repetitions; gray shaded regions are used to represent the mean ± S.E.M. on graphs presenting profile averages. In some instances (figures 3 and 4), fluorescence profiles were normalized to compare the spatial distribution of $\text{Ca}^{2+}$ transients evoked under different conditions. Rather than normalizing amplitudes at a fixed point along the profile, scaling factors were calculated to minimize the mean square distance between profiles.
RESULTS

Ca\(^{2+}\) Transients in Pyramidal Cell Dendrites

The ability of subthreshold and suprathreshold stimuli to evoke a Ca\(^{2+}\) influx into the soma and proximal dendrites of PCs was initially examined. PCs were presented with a 100 Hz train of 4 ms current pulses; the magnitude of these pulses was adjusted to produce subthreshold (figure 1A, left) and suprathreshold (figure 1A, middle and right) responses. A subthreshold depolarization alone is not sufficient to elicit a widespread Ca\(^{2+}\) influx; little or no fluorescence increase was evoked in the absence of APs (figure 1B, left image; figure 1C, blue lines). Somatic APs produced fluorescence increases throughout the soma and proximal dendrites of PCs, indicating increased somatic and dendritic Ca\(^{2+}\) levels. A single AP can elicit a widespread dendritic Ca\(^{2+}\) influx; detectable fluorescence increases evoked by an individual AP were observed throughout most visible regions (figure 1B, middle image; figure 1C, green lines). A substantial fluorescence increase in the soma and all visible dendrites was evoked by multiple APs in rapid succession (figure 1B, right image; figure 1C, red lines).

These results demonstrate that the fluorescence increase occurs in both the proximal apical (figure 1B, upper processes; figure 1C, right) and basal (figure 1B, lower right process; figure 1C, left) dendrites of this PC. Although the magnitude of fluorescence changes is greater in the apical dendrite relative to the basal dendrite (figure 1C), this does not necessarily indicate a larger Ca\(^{2+}\) influx into the apical dendrite. Regions where the largest fluorescence changes were observed are usually the same areas where the resting fluorescence was higher compared to neighboring regions. This asymmetric distribution of resting dye fluorescence represents areas of dye accumulation, compartments with decreased surface to volume ratios, or processes closer
to the slice surface, which reduces the amount of overlying tissue that can scatter the emitted fluorescence. Normalizing fluorescence changes by the resting fluorescence should account for a non-uniform resting fluorescence, but our calculations of $\Delta F/F$ include the background fluorescence (see Methods), which reduces the relative magnitudes of spatial differences in the resting fluorescence values. While we can conclude that APs evoke a $Ca^{2+}$ influx into the apical and basal dendrites of PCs, we cannot make any conclusions about the relative magnitudes of the $Ca^{2+}$ changes across different regions of an individual cell.

The involvement of dendritic $Ca^{2+}$ channels in producing the dendritic $Ca^{2+}$ influx was investigated with the $Ca^{2+}$ channel antagonist $Cd^{2+}$. The fluorescence increase in an apical PC dendrite elicited by a train of APs was reduced locally by the first of two consecutive applications of 1 mM $CdCl_2$ via a pressure pipette (figure 2B). No reduction in the AP-evoked fluorescence increase was observed in a basal dendrite farthest from the location of the pressure pipette (figure 2E). $Cd^{2+}$ also reduced the fluorescence of a background region near the pressure pipette in both trials (figure 2C), which presumably results from the binding of $Cd^{2+}$ to residual dye in the extracellular space that accumulated when the pipette approached the cell with positive pressure prior to seal formation. Similar to fluo-3/$Mn^{2+}$ and fluo-3/$Zn^{2+}$ complexes (Kao et al. 1989), the fluorescence of a fluo-3/$Cd^{2+}$ complex may be substantially less than that of a fluo-3/$Ca^{2+}$ complex, resulting in an apparent fluorescence reduction when $Cd^{2+}$ is added to the extracellular environment. Due to light scattering within the tissue section, the background and dendritic fluorescence cannot be independently measured, so that a reduction in background fluorescence could contribute to an apparent reduction in dendritic fluorescence, and vice versa. However, the reduction of fluorescence along the apical dendritic process is more extensive during the first $Cd^{2+}$ application
(figure 2D, blue line and arrows) when compared to the corresponding reduction in background fluorescence (figure 2C, blue line and arrows). In addition, a second Cd\textsuperscript{2+} application 10 s after the first produced a widespread depression in fluorescence levels along the entire apical dendrite (figure 2D, red line) that was not observed in the corresponding background fluorescence profile (figure 2C, red line). Across seven PCs, the dendritic fluorescence reduction around the pressure pipette extended 28.2 ± 3.7 \(\mu\)m during the first and 42.6 ± 6.5 \(\mu\)m during the second of two consecutive Cd\textsuperscript{2+} applications, as measured by the extent of a fluorescence reduction greater than 3 S.E.M. from the control fluorescence profile around the pressure pipette. In contrast, the background fluorescence reduction only extended 14.7 ± 2.0 \(\mu\)m and 18.9 ± 2.9 \(\mu\)m during the same sequence of local Cd\textsuperscript{2+} application. Thus, a reduction of extracellular dye fluorescence by Cd\textsuperscript{2+} cannot account fully for the overall fluorescence decrease, indicating that a reduction of Ca\textsuperscript{2+} influx through dendritic Ca\textsuperscript{2+} channels blocked by Cd\textsuperscript{2+} also contributes to the fluorescence decrease.

A reduction of the AP-mediated Ca\textsuperscript{2+} influx by Cd\textsuperscript{2+} suggests that APs initiate a Ca\textsuperscript{2+} influx through dendritic voltage-gated Ca\textsuperscript{2+} channels. The results of figure 2 and similar results from six other PCs do not imply that a Ca\textsuperscript{2+} influx through Ca\textsuperscript{2+} channels is solely responsible for the increase in intracellular Ca\textsuperscript{2+} levels. It is possible that other sources contribute to the rise in intracellular Ca\textsuperscript{2+} levels, such as Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (Llano et al. 1994; Sandler and Barbara 1999). It has been shown that the majority of the Ca\textsuperscript{2+} influx into acutely isolated PC cell bodies can be attributed to Ca\textsuperscript{2+} channels (Rusznak et al. 2000), although we did not attempt to resolve the relative contributions of separate sources of the dendritic Ca\textsuperscript{2+} influx in the present study. However, it is clear that dendritic Ca\textsuperscript{2+} channels initiate the Ca\textsuperscript{2+} influx; additional
sources may contribute to the overall Ca\(^{2+}\) influx once the initial influx through Ca\(^{2+}\) channels occurs.

It is possible that APs trigger a large somatic Ca\(^{2+}\) increase that diffuses into dendritic processes, accounting for the majority of the observed fluorescence increases. This is not likely, due to the limited diffusion of cytosolic Ca\(^{2+}\) within neurons (Albritton et al. 1992). In addition, a localized reduction of Ca\(^{2+}\) influx due to the application of Cd\(^{2+}\) via a pressure pipette also suggests that the diffusion of somatic Ca\(^{2+}\) is not responsible for the observed dendritic Ca\(^{2+}\) influx. However, if the diffusion of a somatic Ca\(^{2+}\) influx were responsible for the dendritic extent of the fluorescence increase, a larger somatic Ca\(^{2+}\) influx should produce an increased spread of fluorescence, as opposed to a spatially uniform increase with little or no spatial spread. Increasing the number of APs does not produce a more distal spread of fluorescence; instead, larger fluorescence increases were seen in regions that had detectable ∆F/F values elicited by a single AP (figure 3B – D, solid lines). This uniform increase can be also be observed by directly comparing the fluorescence profiles elicited by 1 – 4 APs normalized to the magnitude of the fluorescence profile elicited by 8 APs (figure 3B – D, dashed lines and shaded regions). In the cell and in nine other PCs, the normalized fluorescence magnitudes share a similar profile regardless of the number of APs, suggesting that the dendritic Ca\(^{2+}\) increase results from a Ca\(^{2+}\) influx that is local to the region of the observed fluorescence increase, rather than a diffusion of a somatic Ca\(^{2+}\) influx.

Although the increase in fluorescence changes resulting from additional APs is spatially uniform, the distribution of an AP-mediated Ca\(^{2+}\) influx along a dendritic process may not be spatially homogeneous. Peaks in the fluorescence profiles were observed (figure 3B - D), which may indicate localized regions of high Ca\(^{2+}\) influx, or “hot spots”. Despite examples to the
contrary, a correlation between fluorescence peaks and dendritic branch points appeared to be the case in many PC dendrites, similar to what had been proposed to occur in the dendrites of cerebellar Purkinje neurons (Llinás and Nicholson 1971). However, most hot spots observed here also correspond to regions where the resting fluorescence was higher than in neighboring regions, potentially representing areas of dye accumulation or compartments with lower surface to volume ratios. In addition, continuous dendritic segments sometimes did not reside within a single focal plane, contributing to the appearance of non-uniform fluorescence changes. Therefore, we cannot necessarily conclude that non-uniform fluorescence changes correspond to a non-uniform Ca\textsuperscript{2+} influx along the length of a dendritic process.

The time course of fluorescence changes also supports the idea that the increase in dendritic Ca\textsuperscript{2+} is due to local Ca\textsuperscript{2+} sources rather than the diffusion of Ca\textsuperscript{2+} from the soma or other cellular compartment. If the diffusion of somatic Ca\textsuperscript{2+} were the source of dendritic Ca\textsuperscript{2+} influx, the time course of distal fluorescence transients would be delayed and or prolonged relative to the time course of proximal fluorescence transients. However, the decay of distal fluorescence transients (figure 3E – H, shaded regions) appears to occur at the same rate or faster than the decay of proximal fluorescence transients (figure 3E – H, solid lines). The larger fluorescence magnitudes observed when more APs were elicited (figure 3B - D) reflects an accumulation of Ca\textsuperscript{2+} bound to the fluorophore rather than an increase in Ca\textsuperscript{2+} diffusion. The image acquisition rate (30 Hz) is too slow to resolve the fluorescence increases that occur with each individual AP. However, the high Ca\textsuperscript{2+} binding affinity of the dye (Kd = 400 nM, Minta et al. 1989) and the dye concentration used in these experiments (200 µM) should result in a slow off-rate of Ca\textsuperscript{2+} bound to the dye (Helmchen et al. 1996), as observed by the slow fluorescence decay following a train of APs (figure 3E - H). This slow off-rate produces an accumulation of
dye-bound Ca\(^{2+}\) when APs are elicited in rapid succession, effectively integrating the Ca\(^{2+}\) influx across all APs within the train. This integration of Ca\(^{2+}\) influx can be observed in the rising phase of the fluorescence transients, which continue to increase until the train of APs that initiate the influx has been terminated (figure 3E – H). Although it cannot be determined how the magnitude of the Ca\(^{2+}\) influx varies among individual APs, it does appear that each additional AP elicits additional Ca\(^{2+}\) influx when presented in rapid succession.

**Contribution of Na\(^{+}\) Channels to Dendritic Ca\(^{2+}\) Transients**

The Ca\(^{2+}\) channels that initiate the dendritic Ca\(^{2+}\) influx can also actively propagate a somatic depolarization throughout the dendritic arbor. Other voltage-gated conductances, such as Na\(^{+}\) channels, may also be present in the dendrites and contribute to the spread of a dendritic depolarization. One possibility is that dendritic Na\(^{+}\) channels are responsible for conducting the propagating depolarization into the dendrites, whereas Ca\(^{2+}\) channels respond to this depolarization with a Ca\(^{2+}\) influx but contribute little to the active propagation of APs into the dendritic arbor. To investigate this possibility, the effects of blocking Na\(^{+}\) channels on the dendritic Ca\(^{2+}\) influx were subsequently investigated.

If dendritic Na\(^{+}\) channels contribute to the spread of a somatic depolarization into the dendritic arbor, a local block of Na\(^{+}\) channels could prevent the depolarization from propagating past the site of Na\(^{+}\) channel block, thereby reducing the Ca\(^{2+}\) influx at and distal to that region. Local application of 10 \(\mu\)M TTX slightly reduced the dendritic Ca\(^{2+}\) influx elicited by a train of somatic APs at but not distal to the site of TTX application in an apical PC dendrite (figure 4C). One interpretation of this result is that the TTX applied via pressure pipette did not reach its target; however, three lines of
evidence refute this interpretation. First, application of Cd$^{2+}$ via pressure pipette using similar positioning and pressure pulses resulted in a localized reduction in the Ca$^{2+}$ influx (figure 2). Second, the raw fluorescence images show that the apical dendrite moved slightly in response to the bolus ejected from the pressure pipette (not shown); these raw fluorescence images were reconstructed to align the apical dendrite to its original location prior to the calculation of the $\Delta F/F$ images. Third, a subsequent application of TTX 10 s later blocked all but one AP (figure 4B, bottom), reducing the Ca$^{2+}$ influx into the apical dendrite (figure 4D, solid line). A comparison of the fluorescence profile evoked during the second TTX application normalized to the fluorescence profile evoked under control conditions (figure 4D, dashed line and shaded region) shows that Ca$^{2+}$ transients were still observed throughout the visible apical dendrite, despite a block of Na$^+$ channels whose spatial extent was large enough to affect AP initiation. Similar results were obtained from six other PCs in which the first TTX application did not eliminate APs and in which the pressure pipette was confirmed to be unclogged after recordings had been terminated. Therefore, blocking dendritic Na$^+$ channels does not attenuate the dendritic Ca$^{2+}$ influx, if the extent of the Na$^+$ channel block does not alter the generation of APs evoked by somatic depolarization.

A dendritic Ca$^{2+}$ influx can still be observed when APs are blocked during a block of Na$^+$ channels throughout the entire cell. The Ca$^{2+}$ influx elicited by a train of somatic APs (figure 5C, shaded region) was reduced by the addition of 1 µM TTX to the bathing medium (figure 5C, solid line), which prevented the cell from spiking in response to depolarizing current injection (figure 5B, lower trace). Unlike subthreshold responses (figure 1), a measurable fluorescence increase remains in the presence of TTX. This presumably is due to the larger depolarizing stimulus used to evoke APs under...
control conditions compared with the smaller depolarizing stimulus needed to maintain the cell at subthreshold levels. However, these results did vary, and other PCs showed a more complete block of the original Ca\(^{2+}\) influx by TTX (e.g., figures 6 – 7). The variable amount of Ca\(^{2+}\) influx blocked by TTX likely reflects differences in the stimulus levels used to evoke APs across the different cells. In nine PCs, TTX blocked 76.8% to 96.9% of the AP-evoked fluorescence increase; in seven of these cells, reduced or attenuated APs were observed as TTX washed into the superfusate (figure 5B, middle trace), and these attenuated APs were sufficient to evoke widespread Ca\(^{2+}\) influx (figure 5C, dashed line). The profiles of fluorescence changes evoked by reduced numbers of APs in the presence of TTX closely approximated profiles evoked by similar numbers of APs under control conditions (not shown), suggesting that a dendritic Ca\(^{2+}\) influx can be evoked independent of Na\(^{+}\) channel activation.

If a block of Na\(^{+}\) channels does not eliminate the dendritic Ca\(^{2+}\) influx, then it may also be possible to fully recover the dendritic Ca\(^{2+}\) influx in the absence of any Na\(^{+}\) channel activation. The Ca\(^{2+}\) influx elicited by a train of somatic APs (figure 6C, shaded region) was eliminated by the addition of 1 µM TTX to the bathing medium (figure 6C, solid line), which prevented the cell from spiking in response to depolarizing current injection (figure 6B, lower left). Somatic current pulses of large amplitude and duration elicited an AP that was smaller and broader (figure 6B, lower right) than the original APs evoked prior to the exposure to TTX (figure 6B, upper left). These broad APs were likely to be Ca\(^{2+}\) channel-mediated events that can be evoked in PCs during Na\(^{+}\) channel blockade (Hirsch and Oertel 1988a). When these Ca\(^{2+}\) channel-mediated APs were evoked, a substantial Ca\(^{2+}\) influx was observed (figure 6C, upper dashed line) that was similar to the magnitude of the Ca\(^{2+}\) influx evoked by Na\(^{+}\) channel-mediated APs. In seven PCs tested, 14.8%
to 93.4% of the fluorescence increase blocked by TTX was recovered with increased somatic current injection. However, these fluorescence changes are dependent on the magnitude of the somatic depolarization, rather than the number of Ca\(^{2+}\)-mediated APs: a much smaller fluorescence increase is observed (figure 6C, lower dashed line) when evoked by a smaller somatic depolarization that is still sufficient to evoke a Ca\(^{2+}\)-mediated AP. Therefore, a complete block of Na\(^{+}\) channels does not preclude a dendritic Ca\(^{2+}\) influx, if a sufficient somatic depolarization is provided in the absence of APs.

When Na\(^{+}\) channels are blocked, a local block of Ca\(^{2+}\) channels by Cd\(^{2+}\) produces a region of membrane that is devoid of any depolarizing active conductances, which could prevent the propagation of a Ca\(^{2+}\) spike distal to this region. To test this hypothesis, the effects of applying Cd\(^{2+}\) via a pressure pipette were examined while Na\(^{+}\) channels were blocked by TTX. The Ca\(^{2+}\) influx elicited by a train of APs (figure 7C, shaded region) was reduced (figure 7C, solid line) when the APs were blocked by the addition of 1 µM TTX to the bathing medium (figure 7B, middle). A partial recovery of the original fluorescence increase was obtained (figure 7C, dashed line) by increasing the amplitude and duration of somatic current pulses that elicited small Ca\(^{2+}\) spikes (figure 7B, bottom). A local block of Ca\(^{2+}\) channels via a pressure application of Cd\(^{2+}\) produced a local reduction in the partially recovered fluorescence increase at but not distal to the region of Ca\(^{2+}\) channel block (figure 7D, solid line) without having any discernible effects on the magnitude of the small Ca\(^{2+}\) spikes (not shown). The results from this and a second PC show that a local block of dendritic Ca\(^{2+}\) channels in the absence of any Na\(^{+}\) channel activation does not prevent the propagation of Ca\(^{2+}\) channel-mediated APs, suggesting that any attenuation occurring over a small region of passive membrane is not sufficient to terminate the spread of a dendritic depolarization.
Unlike PCs, CWCs can respond to a suprathreshold stimulus with a complex spike, a burst of fast APs superimposed upon a slow depolarization (Manis et al. 1994; Zhang and Oertel 1993) that is mediated by Ca$^{2+}$ channels (Golding and Oertel 1997). These different electrophysiologic responses may reflect different patterns of dendritic Ca$^{2+}$ channel activation. Complex spikes evoked by small somatic current pulses (figure 8B, upper left) elicited a large dendritic Ca$^{2+}$ influx (figure 8C, shaded regions) in two visible dendrites of a CWC. Addition of 1 µM TTX to the bathing medium blocked the fast APs, but did not block the slow underlying depolarization (figure 8B, upper right) or the dendritic Ca$^{2+}$ influx (figure 8C, black lines). In this CWC, blocking Na$^+$ channels with TTX increased the duration of the slow depolarization (figure 8B, upper right). It is not clear why this occurred; one possibility is a reduction in the activation of K$^+$ channels that normally contribute to the repolarization of fast Na$^+$-mediated APs. A prolonged slow depolarization could augment the dendritic Ca$^{2+}$ influx that would otherwise be reduced in the absence of Na$^+$ channel activation. Although not conclusive, the time course of fluorescence changes suggests that the prolonged slow depolarization extends the duration of the fluorescence changes instead of increasing their amplitudes (figure 8B, lower right). In addition, a dendritic Ca$^{2+}$ influx was observed in other CWCs in which TTX did not produce a similar increase in the duration of the slow depolarization in other CWCs (figure 9). Therefore, a dendritic Ca$^{2+}$ influx into CWC dendrites evoked by a complex spike can occur in the absence of Na$^+$ channel activation.

Unlike PCs, little or no increase in the amplitude and duration of somatic current injection was required to elicit a Ca$^{2+}$ influx into the dendrites of
complex spiking CWCs in the presence of TTX. In five complex spiking CWCs tested, a dendritic Ca$^{2+}$ influx could be elicited in the presence of TTX that was comparable in magnitude (110.0% ± 5.6%, range 95.0% to 121.5%) to the dendritic Ca$^{2+}$ influx elicited under control conditions. In one of these cells, blocking Na$^+$ channels with TTX produced a variable threshold for evoking the slow depolarization underlying the original complex spike (figure 9C), which allowed for a direct comparison of the Ca$^{2+}$ influx evoked in the presence and absence of this slow depolarization (figure 9D). The large depolarization that is directly elicited by the depolarizing current (figure 9C) is an artifact due to an increase in access resistance, and the results of figure 9D clearly indicate that this depolarization is not responsible for the Ca$^{2+}$ influx observed during TTX application. It is possible that this increase in access resistance contributed to a variable threshold for evoking the slow depolarization underlying complex spikes.

However, in two other CWCs, blocking Na$^+$ channels with TTX required a small increase (< 200 pA) in the amplitude of the 4 ms somatic current pulse to recover the slow depolarization and the dendritic Ca$^{2+}$ transients. This is in contrast to the results of similar experiments performed on PCs, in which large increases in the amplitude and/or duration of somatic current pulses were required to recover fluorescence increases in the presence of TTX (figures 6 - 7). Therefore, the slow depolarization underlying complex spikes can occur in the absence of Na$^+$ channel activation, and is responsible for initiating a large Ca$^{2+}$ influx into CWC dendrites.

In addition to complex spikes, CWCs can also respond with individual fast APs (simple spikes) followed by a characteristic afterdepolarization in response to depolarizing somatic current injection (Manis et al. 1994; Zhang and Oertel 1993). The mode of CWC spiking has a dramatic effect on the properties of the AP-mediated Ca$^{2+}$ influx. Simple spikes (figure 10B, left) were blocked by the addition of 1 µM
TTX to the bathing medium (figure 10B, right). Similar to PCs, blocking somatic APs with TTX blocked the dendritic Ca\(^{2+}\) influx (figure 10C, solid line), and a partial release of the Na\(^+\) channel block during the washout of TTX sufficient to evoke 1 – 3 attenuated APs resulted in a partial recovery of the fluorescence increase (figure 10C, dashed lines). In six simple spiking CWCs tested, TTX blocked 66.6\% to 92.2\% of the fluorescence increase elicited under control conditions. In three of these cells, a large increase in the amplitude and duration of the somatic current injection was used to evoke a small Ca\(^{2+}\) channel-mediated AP to recover the fluorescence increase blocked by TTX. These results varied: in two cells, 75\% of the original fluorescence increase was recovered; in the third cell, a large fluorescence increase was observed whose magnitude was 250\% of that obtained under control conditions. Although a Ca\(^{2+}\) influx that was larger in TTX than under control conditions was never observed in PC dendrites, these results suggest that the Ca\(^{2+}\) influx into simple-spiking CWC dendrites is more similar to the Ca\(^{2+}\) influx into PC dendrites than to the Ca\(^{2+}\) influx into complex-spiking CWC dendrites.

As was the case in PCs, the AP-mediated Ca\(^{2+}\) influx into the dendrites of CWCs is initiated by dendritic Ca\(^{2+}\) channels. Consecutive applications of 1 mM CdCl\(_2\) via pressure pipette produced local reductions in the dendritic Ca\(^{2+}\) influx (figure 11C, solid and dashed lines) relative to control levels (figure 11C, shaded region). Similar to the results presented from a PC (figure 2), the negative ΔF/F values in figure 11C may be attributed a reduction of background fluorescence due to the reduced fluorescence of the Cd\(^{2+}\)/fluo-3 complex compared to free fluo-3. However, no apparent reduction in the background fluorescence was observed in this cell (not shown), although the orientation of the pressure pipette along the axis of the dendrite may have obscured any reduction in background fluorescence. These results were obtained from a simple-spiking CWC.
current pulses was increased to elicit a pair of APs, and no slow depolarization underlying the APs characteristic of a complex spike was observed (figure 11B). Similar results were obtained from three other simple-spiking CWCs; fluorescence recordings during a localized application of Cd\(^{2+}\) were not obtained from any complex-spiking CWCs.
DISCUSSION

Ca$^{2+}$ and Na$^+$ Channels in Pyramidal Cell Dendrites

Ca$^{2+}$ channels are present in the apical and basal dendrites of PCs, and initiate a Ca$^{2+}$ influx in response to APs evoked by somatic depolarization. One issue that requires additional consideration is the extent to which the Ca$^{2+}$ influx is observed throughout PC dendrites. The present results were limited to dendritic processes within 100 µm of the soma, and PC dendrites can extend upwards of 500 µm from the cell body (Blackstad et al. 1984). In some cases, measurable Ca$^{2+}$ transients could be observed where dendrites exited the field of view (e.g., figures 4 and 5) indicating that the depolarization continued to propagate toward distal dendritic processes. In other cases, Ca$^{2+}$ transients rapidly tapered off with increasing distance from the soma (e.g., figure 2), even though distal processes were subsequently observed during morphologic reconstruction (not shown). A number of factors could prevent the observation of Ca$^{2+}$ transients in more distal processes, including variations in process depth relative to the focal plane, or fluorescence intensities not significantly above background levels due to small process volumes or inadequate dye spread. Therefore, it is reasonable to assume that the dendritic Ca$^{2+}$ influx evoked by APs extends into dendritic processes beyond those visualized in the present study.

Dendritic Ca$^{2+}$ channels can contribute to the integration of spatially segregated ANF and PF inputs by modulating synaptic responses in an activity-dependent fashion. Coincident synaptic inputs and activation of dendritic conductances by APs produce changes in synaptic efficacy (Bell et al. 1997; Magee and Johnston 1997; Markram et al. 1997). Activation of N-methyl-D-aspartate receptors, which are present at PF synapses in PCs (Bilak et al. 1996; Manis and Molitor 1996), may contribute to this modulation of postsynaptic responses by augmenting
an AP-evoked dendritic Ca\(^{2+}\) influx (Schiller et al. 1998). Therefore, suprathreshold ANF-mediated inputs in the basal dendrites of a PC could propagate to PF synapses in the apical dendrites, altering the subsequent postsynaptic responses to PF-mediated inputs that were coincidentally active. This modulation could occur independently of coincident inputs: PCs exhibit high rates of intrinsic spontaneous activity (Waller and Godfrey 1994), which would generate a dendritic Ca\(^{2+}\) influx and potentially lead to an ongoing modulation of postsynaptic responses to excitatory inputs.

Although APs, presumably mediated by somatic and axonal Na\(^{+}\) channels, initiate a Ca\(^{2+}\) influx into PC dendrites, the results of the present study suggest that dendritic Na\(^{+}\) channels are not necessary for the retrograde activation of dendritic Ca\(^{2+}\) channels. Individual Na\(^{+}\) channel-mediated APs evoke a Ca\(^{2+}\) influx into the dendrites of hippocampal and cortical PCs (Markram et al. 1995; Spruston et al. 1995), which possess dendritic Na\(^{+}\) channels (Jaffe et al. 1992; Stuart and Sakmann 1994), but not into the dendrites of cerebellar Purkinje neurons (Lev-Ram et al. 1992; Ross and Werman 1987), which lack a sufficient density of dendritic Na\(^{+}\) channels (Stuart and Häusser 1994). In the DCN, a Ca\(^{2+}\) influx into PC dendrites could still be observed during the direct application of TTX to dendritic processes at levels sufficient to partially block somatic APs. Furthermore, dendritic Ca\(^{2+}\) transients could be evoked when a large somatic depolarization was presented in the complete absence of APs during TTX block. Again, these conclusions are limited to observations made from proximal dendrites, and it is possible that the contribution of dendritic Na\(^{+}\) channels is required for a depolarization to spread into distal processes.
Ca\(^{2+}\) Channels in Cartwheel Cell Dendrites

One striking difference between CWCs in this study and morphologically identified CWCs in previous studies (Ding et al. 1999; Golding and Oertel 1996, 1997; Manis et al. 1994; Zhang and Oertel 1993) is the inability of some CWCs to respond with complex spikes to depolarizing stimuli. Otherwise, this subpopulation of simple-spiking CWCs appears to be normal: fast Na\(^{+}\) channel-mediated APs were followed by an afterdepolarization (e.g., figure 10B), as observed in sharp-electrode recordings from CWCs in guinea pig DCN (Manis et al. 1994). The most obvious differences between the present and previous studies are the use of different rodent species (rat vs. mouse, guinea pig and gerbil), the ages of the animals used, and the use of whole-cell recordings instead of sharp-electrode recordings. Bursting units were observed in extracellular recordings from rat DCN (Waller and Godfrey 1994), so it is unlikely that the difference in CWC responses can be attributed solely to the species used. The use of whole-cell recordings could prevent complex spikes by disrupting normal Ca\(^{2+}\) buffering processes, leading to a Ca\(^{2+}\)-dependent activation of K\(^{+}\) channels and/or a Ca\(^{2+}\)-dependent inactivation of Ca\(^{2+}\) channels in some CWCs. Although not representative of the responses typically observed in CWCs, the presence of a simple-spiking CWC subpopulation provided an opportunity to investigate the differences between the electrophysiologic mechanisms underlying the generation of simple and complex spikes.

In contrast to PCs, the dependence of the dendritic Ca\(^{2+}\) influx on Na\(^{+}\) channel-mediated APs depends on the firing mode of CWCs. Although both simple and complex spikes evoke a Ca\(^{2+}\) influx, only complex spikes elicit a large dendritic Ca\(^{2+}\) influx that is not dependent upon Na\(^{+}\) channel activation. The data in this study confirm the existence of Ca\(^{2+}\) channels in CWC dendrites; however, it is unclear from these data whether dendritic Ca\(^{2+}\) channels contribute to
the slow depolarization observed in somatic recordings, or if these channels only generate a Ca\(^{2+}\) influx in response to the slow depolarization initiated by more proximal Ca\(^{2+}\) channels. A correlation between the slow depolarization underlying complex spikes and a dendritic Ca\(^{2+}\) influx suggests that dendritic Ca\(^{2+}\) channels may contribute to the generation of complex spikes. However, repetitive simple spikes evoked similar dendritic Ca\(^{2+}\) transients with little or no evidence of the slow depolarization associated with complex spiking. In addition, blocking Na\(^+\) channels had little or no effect on the threshold for evoking the slow underlying depolarization, as would be expected if this slow depolarization were generated by Ca\(^{2+}\) channels remote to the site of depolarizing current injection. Although further investigation is required, complex spikes are likely to be generated by a coordinated action of somatic and dendritic Ca\(^{2+}\) channels.

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REFERENCES


FIGURE LEGENDS

Figure 1: Fluorescence increases are only evoked by suprathreshold current injection in the proximal apical and basal dendrites of a pyramidal cell (PC). A: Increasing numbers of action potentials (APs) are evoked by increasing the amplitude of 100 Hz current pulses from subthreshold levels. Dashed lines: resting potential of –60 mV with a holding current of –130 pA. Calibration bar: 10 ms, 20 mV and 2 nA. Recordings were obtained using a List EPC-7 in current clamp mode. B: Fluorescence increases are only evoked by suprathreshold somatic current injection that evoked one AP (middle image) or four APs (right image) in the proximal apical (above soma) and basal (below soma) dendrites of a PC. Color bar: indicates ΔF/F values from 0% (blue) to 40% (red). Scale bar: 20 µm. C: Fluorescence profiles elicited by zero (blue lines), one (green lines) and four (red lines) APs along the apical and basal dendrites shown by arrows in B.

Figure 2: Localized Cd$^{2+}$ application reduces the fluorescence increase elicited by a train of somatic APs in PC dendrites. A: Eight APs evoked by a 100 Hz train of somatic current pulses before (left) and during (right) the application of 1 mM CdCl$_2$ via pressure pipette. Dashed lines: resting potential of –65 mV with a holding current of –290 pA. Calibration bar: 10 ms, 25 mV and 2.5 nA. Recordings were obtained using a List EPC-7 in current clamp mode. B: Fluorescence increase evoked by eight APs (left image) is reduced in an apical dendrite near the region of Cd$^{2+}$ application via pressure pipette (right image). The absence of fluorescence changes in the middle of the soma is where the resting fluorescence saturated the camera. Scale bar: 20 µm. Color bar: indicates ΔF/F values from 0% (blue) to 30% (red). C: Cd$^{2+}$
application reduces the background fluorescence (shaded region) obtained from a profile 3 µm to the right of the apical dendrite indicated by the upper arrows in B. D: Cd²⁺ application produces a more extensive reduction of dendritic fluorescence increases elicited by a train of somatic APs. Arrows: spatial extent of the background and dendritic fluorescence reduction due to the first CdCl₂ application. Vertical lines: location of the pressure pipette relative to the fluorescence profiles. E: No fluorescence reduction was observed in a basal dendrite opposite to the pressure pipette location (lower arrows in B) during either Cd²⁺ application.

Figure 3: Profile and time course of fluorescence changes with increasing number of APs from the apical dendrite of a PC. A: Grayscale photomontage of a PC. Apical dendrites are to the upper and lower left of the soma, the basal dendrite is to the right of the soma, and the ependymal surface of the DCN is visible in the upper left corner. Scale bar: 20 µm. B - D: Fluorescence profiles elicited by 4 APs (B), 2 APs (C) and 1 AP (D) averaged across 3 stimulus repetitions from the apical dendrite indicated by the arrows in A. Dashed lines: fluorescence profiles scaled to the profile elicited by 8 APs (shaded region duplicated in B - D) for direct comparison. Scaling factors are 1.49 for 4 APs, 2.74 for 2 APs and 5.80 for 1 AP. E – H: Comparison of the time course of fluorescence changes (bottom traces) to the duration of the AP train (top traces). Dotted lines: resting potential of –63 mV with a holding current of –100 pA. Calibration bar in F (10 ms, 25 mV and 2.5 nA) applies to current and voltage traces in E - H. Recordings were obtained using an Axoprobe 1A. Fluorescence changes were averaged across three trials from the proximal (solid lines) and from the distal (shaded regions) dendritic regions indicated by the circles in A and vertical lines in B - D. Horizontal bars: indicate the duration of
the current pulses on the time scale of the fluorescence time course. Calibration bar in F indicates the time scale (30 ms) of the fluorescence time courses in E – H.

Figure 4: Localized TTX application has little or no effect on the fluorescence increase unless APs are blocked. A: Grayscale photomontage of a PC. The apical dendritic arbor is above the soma, the basal dendrite is below the soma, and the axon is to the right of the soma. A pressure pipette containing 10 µM TTX was placed near the apical dendrite. Scale bar: 20 µm. B: Seven APs were evoked by a 100 Hz train of somatic current pulses before (top) and during (middle) the first application of TTX via pressure pipette; a second TTX application 10 s later resulted in a block of all but one of the APs (bottom). Dotted lines: resting potential of –67 mV with –390 pA holding current. Calibration bar: 10 ms, 20 mV and 2 nA. Recordings were obtained using a List EPC-7 in current clamp mode. C: The first TTX application had little or no effect on the fluorescence profile (black line) when compared to the control fluorescence profile (shaded region) averaged across three trials prior to TTX application. D: The second TTX application blocks APs and reduces the dendritic fluorescence profile. The reduced fluorescence profile is scaled by a factor of 2.28 (dashed line) for direct comparison to the fluorescence profile elicited under control conditions.

Figure 5: Blocking somatic APs with TTX blocks fluorescence increases. A: Grayscale photomontage of a PC. The apical dendritic arbor is above the soma; two basal dendrites can be seen below and to the right of the soma. Scale bar: 20 µm. B: Six APs evoked by a 67 Hz train of somatic current pulses (upper traces) were blocked by the addition of 1 µM TTX to the superfusate (lower traces). Two small APs were evoked as the TTX was being added to the
superfusate (middle traces). Dotted lines: resting potential of –62 mV with –340 pA of holding current. Calibration bar: 10 ms, 20 mV and 2 nA. Recordings were obtained using a List EPC-7 in current clamp mode. C: Addition of 1 µM TTX to the superfusate blocks APs and reduces the fluorescence increase (solid line) elicited before TTX application (shaded region). Some of the fluorescence increase is still evoked by two small APs elicited during the wash-in of TTX (dashed line). Fluorescence profiles were averaged across five trials before and after TTX wash-in; data from an individual profile is shown for the two APs evoked during the wash-in of TTX.

Figure 6: Fluorescence increases can be recovered with a large somatic depolarization when somatic APs are blocked by TTX. A: Grayscale photomontage of a PC. The apical dendritic arbor is above the soma; the basal dendritic arbor is to the lower right. Scale bar: 20 µm. B: Eight APs evoked by a 100 Hz train of somatic current pulses (upper left) were blocked by the addition of 1 µM TTX to the superfusate (lower left). Increasing the duration of somatic current pulses to 80 ms during TTX exposure (lower right) produces small active events (arrow) mediated by Ca^{2+} conductances. Dotted lines: resting potential of –65 mV with –160 pA of holding current. Calibration bar: 10 ms, 20 mV and 5 nA (upper left); 10 ms, 5 mV and 5 nA (lower left and right). Recordings were obtained using an Axoprobe 1A. C: TTX blocks APs and eliminates the fluorescence increase. Increasing the amplitude of a single 80 ms somatic current pulse to 1200 pA (lower dashed line) and to 2500 pA (upper dashed line) produces a substantial recovery of the original fluorescence profile. Fluorescence profiles were averaged across three trials before and during TTX application and across two trials during fluorescence recovery with increased current injection.
Figure 7: Propagation of a somatic depolarization can continue past a local block of Ca$^{2+}$ channels while Na$^{+}$ channels are blocked. A: Grayscale photomontage of a PC. The apical dendritic arbor is above the soma, the basal dendritic arbor is below the soma, and the axon is to the right of the soma. A pressure pipette containing 1 mM CdCl$_2$ was placed near the apical dendrite. Scale bar: 20 µm. B: Eight APs evoked by a 100 Hz train of somatic current pulses (top) were blocked by 1 µM TTX (middle); increasing the amplitude and duration of somatic current pulses produces small active events mediated by Ca$^{2+}$ conductances (bottom). Dotted lines: resting potential of -66 mV with –170 pA of holding current. Calibration bar: 10 ms, 20 mV and 8 nA (top); 10 ms, 10 mV and 8 nA (middle and bottom). Recordings were obtained using an Axoprobe 1A. C: Increasing the amplitude and duration of somatic current pulses in TTX produces a partial recovery of the original fluorescence profile. D: The recovered fluorescence evoked by increasing the amplitude and duration of somatic current injection in the presence of TTX (dashed line duplicated from C) is observed distal to the site of Cd$^{2+}$ application (solid line). Data from an individual fluorescence profile during localized Cd$^{2+}$ application are shown in D; otherwise, profiles were averaged across three trials for each condition shown.

Figure 8: A complex spike elicits a TTX-insensitive fluorescence increase in cartwheel cell (CWC) dendrites. A: Grayscale photomontage of a CWC. Scale bar: 20 µm. B: A complex spike was evoked by a small somatic current pulse (top left). Addition of 1 µM TTX to the superfusate blocked the fast APs and augmented the duration of the underlying slow depolarization (top right). Dotted lines: resting potential of -77 mV with –110 pA of holding current. Calibration bar: 20 ms, 20 mV and 2 nA. Recordings were obtained using a List EPC-7 in current clamp mode. The fluorescence time courses under control conditions (bottom left) and
in the presence of TTX (bottom right) from the dendritic regions indicated by the circles in A are superimposed for direct comparison. Horizontal bars: indicate the estimated duration of the underlying slow depolarization on the time scale of the fluorescence time course. Calibration bar for voltage and current traces indicates the time scale (60 ms) of the fluorescence time courses. C: TTX had little effect on the fluorescence increase (solid lines) compared to the control fluorescence profiles (shaded regions). Fluorescence time courses in B and profiles in C were averaged across four trials before and during TTX application.

Figure 9: The slow depolarization underlying the complex spike is responsible for the fluorescence increase in cartwheel cell dendrites. A. Grayscale photomontage of a CWC. The recording pipette was not withdrawn immediately after electrophysiologic recordings had been terminated. Scale bar: 20 µm. B. A complex spike evoked by a small somatic current pulse. Dotted line: indicates a resting potential of -74 mV with no holding current. C. Addition of 1 µM TTX to the superfusate blocked the fast APs; the underlying depolarization was elicited in some trials (left) but not in others (right). Dotted lines: resting potential of -71 mV with no holding current. Calibration bar: 10 ms, 20 mV and 1 nA in B - C. Recordings in B – C were obtained using a List EPC-7 in current clamp mode. D: No fluorescence increase was observed in TTX (solid lines) unless the slow depolarization underlying the complex spike was elicited (dashed lines). Fluorescence profiles were averaged across three control trials, five TTX trials with a slow depolarization, and eight TTX trials without a slow depolarization. A change in the focal plane occurred during the application of superfusate containing TTX, which may account for some differences in the profiles in D.
Figure 10: TTX block of fast APs eliminates the fluorescence increase in the dendrites of a simple-spiking CWC. A: Grayscale photomontage of a CWC. The ependymal surface of the DCN extends along the upper portion of the image. Scale bar: 20 µm. B: Four APs evoked by a 50 Hz train of somatic current pulses (upper left) were eliminated by the addition of 1 µM TTX to the superfusate (upper right). Small APs were observed during recovery following the washout of TTX (lower left and lower right). Arrow: indicates afterdepolarization that follows the first AP under control conditions. Dotted lines: resting potential of –77 mV with −150 pA holding current. Calibration bar: 10 ms, 20 mV and 2 nA. Recordings were obtained using a List EPC-7 in current clamp mode. C: Addition of 1 µM TTX to the superfusate blocks APs and eliminates the majority of the fluorescence increase (solid line) evoked under control conditions (shaded region). A recovery of fluorescence was observed when one AP (lower dashed line) and three APs (upper dashed line) were evoked during the washout of TTX. Fluorescence profiles were averaged across six control and TTX trials; data from individual profiles are shown for the small APs evoked during the washout of TTX.

Figure 11: Localized Cd\textsuperscript{2+} application reduces the fluorescence increase elicited by APs in CWC dendrites. A: Grayscale photomontage of a CWC. A pressure pipette containing 1 mM CdCl\textsubscript{2} was placed distal to the visible edge of a dendritic process. The ependymal surface of the DCN can be seen in the upper left corner of the image. Scale bar: 20 µm. B: Four APs evoked by a pair of somatic current pulses were unaffected by the first (not shown) or second of consecutive Cd\textsuperscript{2+} applications 10 s apart. Dotted lines: resting potential of -77 mV with –220 pA of holding current. Calibration bar: 10 ms, 20 mV and 2 nA. Recordings were obtained using a List EPC-7 in current clamp mode. C: Cd\textsuperscript{2+} application produces a localized reduction in the dendritic
fluorescence profile compared to the control dendritic fluorescence change averaged across three trials prior to Cd$^{2+}$ application. A substantial recovery of dendritic fluorescence was observed during an individual trial after localized Cd$^{2+}$ application (long dashed line).
Figure 2 - Molitor & Manis

A

control Cd\(^{2+}\) puff

B

C

-6

0

6

\(\Delta F/F\) (%)
apical background

D

-20

0

20

\(\Delta F/F\) (%)
apical dendrite

E

-20

0

20

\(\Delta F/F\) (%)
basal dendrite

distance from soma (µm)

Figure 2 - Molitor & Manis

A

control Cd\(^{2+}\) puff

B

C

-6

0

6

\(\Delta F/F\) (%)
apical background

D

-20

0

20

\(\Delta F/F\) (%)
apical dendrite

E

-20

0

20

\(\Delta F/F\) (%)
basal dendrite

distance from soma (µm)
Figure 4 - Molitor & Manis

A

B

ccontrol

1st TTX puff

2nd TTX puff

C

\[ \Delta F/F (\%) \]

control

1st TTX puff

D

\[ \Delta F/F (\%) \]

ccontrol

2nd TTX puff

TTX \rightarrow control

distance from soma (\(\mu m\))
Figure 5 - Molitor & Manis

A

B

C

control
TTX wash-in
TTX

apical
basal

ΔF/F (%) vs. distance from soma (µm)

control
TTX wash-in
TTX
Figure 6 - Molitor & Manis
Figure 7 - Molitor & Manis

A

B

control

TTX

TTX + stim

C

control

TTX

TTX + stim

D

TTX

Cd²⁺ puff

TTX + stim

TTX + stim
Figure 8 - Molitor & Manis
Figure 9 - Molitor & Manis
Figure 10 - Molitor & Manis

A

B

control            TTX

wash - 1 AP        wash - 3 APs

C

ΔF/F (%) vs. distance from soma (µm)

- control
- TTX
- wash (1 & 3 APs)
- wash (1 & 3 APs)
Figure 11 - Molitor & Manis

A

B

control

2nd Cd\(^{2+}\) puff

C

1st Cd\(^{2+}\) puff

2nd Cd\(^{2+}\) puff

wash

\(\Delta F/F\) (%)

distance from soma (\(\mu m\))