Calcium Microdomains in Aspiny Dendrites

Jesse H. Goldberg,1,* Gabor Tamas,2
Dmitriy Aronov,1 and Rafael Yuste1
1Department of Biological Sciences
Columbia University
New York, New York 10027
2Department of Comparative Physiology
University of Szeged
Szeged
Hungary H-6726

Summary

Dendritic spines receive excitatory synapses and serve as calcium compartments, which appear to be necessary for input-specific synaptic plasticity. Dendrites of GABAergic interneurons have few or no spines and thus do not possess a clear morphological basis for synapse-specific compartmentalization. We demonstrate using two-photon calcium imaging that activation of single synapses on aspiny dendrites of neocortical fast spiking (FS) interneurons creates highly localized calcium microdomains, often restricted to less than $1 \mu$m of dendritic space. We confirm using ultrastructural reconstruction of imaged dendrites the absence of any morphological basis for this compartmentalization and show that it is dependent on the fast kinetics of calcium-permeable (CP) AMPA receptors and fast local extrusion via the Na+/Ca2+ exchanger. Because aspiny dendrites throughout the CNS express CP-AMPA receptors, we propose that CP-AMPA receptors mediate a spine-free mechanism of input-specific calcium compartmentalization.

Introduction

Mammalian dendrites are remarkably diverse in their structure (Ramón y Cajal, 1899). While pyramidal neurons have elaborate dendritic trees covered with spines, most cortical GABAergic interneurons have few or no spines with relatively simpler dendritic geometries (Fairen et al., 1984; Somogyi et al., 1983b). At present, the integrative functions underlying dendritic morphologies are poorly understood (Johnston et al., 1996; Yuste and Tank, 1996).

The shape of spines and their ability to compartmentalize calcium during activation of single excitatory synapses have engendered the view that they subserve synapse-specific postsynaptic biochemical compartmentalization (Koch and Zador, 1993; Wickens, 1988; Yuste and Denk, 1995). The view that spines form the structural basis for synapse-level biochemical signaling predicts that aspiny dendrites are incapable of synapse-specific compartmentalization and plasticity (Cowen et al., 1998; McBain et al., 1999; Yuste and Denk, 1995).

Recently, multiple forms of synaptic plasticity have been demonstrated on aspiny interneurons in hippocampus, amygdala, and cerebellum, and, in all cases, calcium-permeable AMPA receptors (CP-AMPARs) were involved (Alle et al., 2001; Lei and McBain, 2002; Liu and Cull-Candy, 2000; Mahany and Sah, 1998). Interestingly, while calcium influx on dendritic spines is mediated primarily by relatively slow NMDA currents (Kovalchuk et al., 2000; Yuste et al., 1999), aspiny dendrites throughout the CNS appear to be innervated by fast CP-AMPAR synapses. Indeed, dendrites of bushy and stellate cells of the cochlear nucleus, cerebellar stellate cells, amygdaloid, neocortical and hippocampal FS interneurons, retinal AII amacrine cells, dorsal horn interneurons, large striatal cholinergic interneurons, and spino-thalamic projection neurons are all smooth or sparsely spiny and express CP-AMPA receptors (Albuquerque et al., 1999; Angulo et al., 1999; Cheunsuang and Morris, 2000; Gardner et al., 1999, 2001; Gotz et al., 1997; Katsamaru et al., 1988; Koh et al., 1995; Mahany and Sah, 1998; Morkve et al., 2002; Mugnaini, 1985; Otis et al., 1995a, 1995b; Tamas et al., 1997; Tolbert and Morest, 1982; Tolbert et al., 1982; Washburn and Moises, 1992). We wondered if the correlation between lack of spines and expression of CP-AMPARs was not coincidental but represented a unique solution to calcium compartmentalization on aspiny dendritic shafts.

Our goal was to examine the calcium influx associated with the activation of individual synapses on aspiny dendrites. In contrast to pyramidal neurons, dendrites of fast spiking (FS) cortical interneurons are aspiny and express low levels of GluR2, which results in AMPA receptors with high calcium permeability and distinctly fast kinetics (Geiger et al., 1995). In a recent study, we found that CP-AMPARs provided FS cell dendrites with uniquely fast calcium influx kinetics (Goldberg et al., 2003b), and we have further pursued these findings to examine the spatial compartmentalization produced by CP-AMPARs at individual synapses.

We find that activation of single synapses on aspiny dendrites of FS interneurons from layers II/III and V of primary visual and somatosensory neocortex causes remarkably localized ($<1 \mu$m) calcium microdomains in the absence of any morphological structures that would restrict calcium diffusion. This synapse-specific compartmentalization depended on the fast kinetics of CP-AMPARs and on fast membranous extrusion via the Na+/Ca2+ exchanger. Our findings indicate that calcium-based plasticity on aspiny dendrites can be synapse specific and imply that the expression of CP-AMPARs on dendritic shafts represents a novel approach to ensure calcium localization.

Results

Calcium Microdomains during Spontaneous Synaptic Activation on Dendritic Shafts

We imaged calcium signals during both spontaneous and electrically evoked activation of single synaptic contacts on the aspiny dendritic shafts of fast spiking (FS) interneurons to test if spines were necessary for syn-
Figure 1. Calcium Signals Associated with Spontaneous EPSPs Were Fast and Highly Localized on Dendrites of Parvalbumin-Positive FS Interneurons

Slices were bathed in 1 mM 4-AP to increase the frequency of spontaneous EPSPs. (Aa) Top, fast spiking (FS) firing pattern (black) and hyperpolarizing voltage deflection (red), in response to 800 ms depolarizing (black) and hyperpolarizing (red) current injections, bottom. Note the absence of spike frequency adaptation within bursts. (Ab) Parvalbumin (PV) immunopositivity of an FS cell with firing pattern shown in (Aa), left, and green biocytin-filled cell, right. (Ba) Top, image of a targeted horizontal dendrite, 40 μm from the soma, through which line scan, indicated by arrows, was placed. Bottom, line scan image showing spontaneous calcium event. Horizontal scale bars, 1 μm; vertical scale, 400 ms. (Bb) Top, expanded ΔF/F image of the calcium event in (Aa). Vertical scale, 100 ms. Bottom, peak calcium signal (ΔF/F) was plotted as a function of dendritic space, at different times after the peak signal. These plots were well fit by Gaussians. Note that x axes of the ΔF/F versus space traces and the ΔF/F image are identical. (C) Another example of a spontaneous calcium event from a different cell, plotted as in (B). Dendrite was 70 μm from soma. (D) Top, calcium transient (ΔF/F) from line scan in (Ba), plotted above time locked electrical activity. The influx of the calcium transient and the time-locked physiology are labeled in red. (E) Data plotted as in (D), on a smaller time and voltage scale. Note how the influx of the calcium event was synchronous with two EPSPs. Also note the fast kinetics of the calcium signal, τ = 21 ms. Both cells were imaged with 100 μM Fluo-4.

apse-specific calcium localization. FS cells were characterized by their firing pattern during somatic current injection and by their expression of the calcium binding protein parvalbumin (Figure 1A) (Kawaguchi and Kubota, 1993).

To detect calcium events associated with the activation of single synaptic contacts, we recorded from FS interneurons in whole-cell mode and filled them with calcium indicators (see Experimental Procedures). We placed line scans longitudinally through dendritic shafts to examine with high resolution the spatial dynamics of calcium events and to maximize the dendritic surface imaged and, therefore, increase the likelihood of detecting a spontaneous event. In 40 FS cells, we observed
seven spontaneous calcium events at seven branches of four cells (Figure 1). Spontaneous calcium events were highly localized in space (~1 μm) (Figures 1Ba–1Ca). We termed these highly localized calcium transients “calcium microdomains” (Augustine et al., 2003; Llinàs et al., 1992).

When we correlated the calcium signal with the ongoing electrical activity of the cell, we found that the influx of the calcium signal was always time locked to a spontaneous EPSP (Figures 1D and 1E). Spontaneous EPSPs time locked to the influx of calcium events were similar in amplitude to unitary EPSPs (1.12 ± 0.42 mV, n = 9) (Buhl et al., 1997; Geiger et al., 1997), suggesting that they were due to the activation of single synapses.

We converted the line scan images into ΔF/F images (see Experimental Procedures), to quantitatively examine the spatial spread of the microdomains (Figures 1Bb and 1Cb, top). We then plotted the peak calcium accumulation (% ΔF/F) as a function of space along the dendritic shaft, at distinct times after the peak signal. These traces were well fit by Gaussian curves, as predicted by diffusion from an instantaneous point source in a cylinder (Figures 1Bb and 1Cb, bottom) (Delaney et al., 1989; Koch, 1998; Gabso et al., 1997; Helmcen, 1999). The standard deviation of a Gaussian curve from its peak, σ, represents the lateral displacement (in μm), and thus the spatial extent of a calcium event at a given time. We fitted ΔF/F versus space curves until the peak amplitude was less than two standard deviations above the noise and termed the standard deviation of the largest fit “σ max.” At their peak amplitude, which always occurred at the first time point, spontaneous calcium transients were highly localized (Δr peak = 0.76 ± 0.19 μm, n = 7), and σ max, which represents the maximal resolvable spatial extent of the calcium signal, was 1.05 ± 0.38 μm, n = 7.

**Electrical Activation of Single Synapses also Produces Submicron Calcium Compartmentalization**

In order to more systematically measure microdomains and examine their underlying mechanisms, we electrically evoked single synaptic calcium events by delivering single electrical shocks via a fluorescently labeled stimulation electrode placed close to the dendrite (Figures 2A and 2C). During successive single shocks, we observed all-or-none calcium events, reflecting stochastic successes and failures of synaptic transmission at the imaged synapse (Figures 2B and 2D) (Yuste and Denk, 1995). These calcium accumulations were identical to those observed during spontaneous EPSPs and were always highly localized to approximately 1 μm of the dendritic shaft (Δr peak = 0.76 ± 0.25 μm, n = 24). In some cells, we generated trains of action potentials 1 s after synaptic stimulation, well after the decay of the microdomain was complete, to confirm that the entire dendritic segment was in focus and responsive to calcium influx (Figure 2C).

Based on the localization of the calcium events, the unitary-sized EPSPs which occurred during spontaneous influx, and the stochastic nature of evoked signals, we conclude that calcium microdomains were caused by activation of single synapses on aspiny dendrites.

**Calcium Indicators Overestimate the Size of Calcium Microdomains**

One possible mechanism for the localization of microdomains was that the exogenous buffer unphysiologically restricted the signal. We examined the effect of exogenous buffer by quantitatively comparing microdomains in 400 μM versus 100 μM Fluo-4 conditions. We plotted σ as a function of time and found that signals imaged with 400 μM Fluo-4 were significantly less local than those imaged with 100 μM Fluo-4 (Fluo-4 100 μM σ max = 0.84 ± 0.04 μm, n = 21; 400 μM σ max = 1.9 ± 0.22 μm, n = 7, p < 0.05) (Figure 3), indicating that the diffusion of calcium indicator indeed contributed to microdomain size. Indeed, for a Koff of Fluo-4 of ~350 s⁻¹ and a diffusion coefficient of ~200 μm² s⁻¹, the characteristic distance traveled by a calcium bound Fluo-4 molecule during the unbinding process is approximately 0.75 μm. Diffusion of indicator is visible in the 400 μM Fluo-4 ΔF/F image plot (Figure 3A) and is plotted quantitatively in Figure 3C. Thus, the localization of microdomains in our experiments was not due to exogenous buffer distortion; instead, added calcium indicator actually overestimated their size.

**Effect of Calcium Indicators on the Decay of Calcium Microdomains**

In addition to being highly restricted in space, microdomains were unusually fast. We imaged some cells with high temporal resolution (397 Hz) and found that the time to peak was reached in three time points (~7.5 ms) (n = 3). Decays of the calcium transients were also extremely fast—and were not different between 100 and 400 μM Fluo-4 imaging conditions (decay τ = 49.5 ± 28 ms 100 μM Fluo-4; τ = 55.4 ± 28 ms 100 μM Fluo-4). Thus, extra indicator appeared to have a mixed effect on calcium transient decay kinetics. First, added buffer slowed calcium clearance by competing with extrusion pumps for calcium (Sabatini et al., 2002). However, for localized microdomains, added mobile buffer could contribute to subsynaptic clearance by shutting calcium to adjacent dendritic regions.

Although microdomains appeared not to expand in space as a function of time in the 100 μM Fluo-4 conditions (Figure 3), we wondered if undetectable diffusion contributed to the rate of microdomain decay. To test for diffusion, we compared the decay time constants of microdomains to those of calcium transients caused by backpropagating action potentials (bAPs) at identical dendritic sites (as in Figure 2C). We reasoned that since bAPs cause global calcium signals, there is no calcium gradient along the dendritic shaft, and longitudinal diffusion therefore cannot drive calcium clearance. On the other hand, during single synaptic calcium events there is a steep [Ca²⁺] gradient along submicrons of the dendritic branch (Figures 1 and 3). We found that microdomain decays were significantly faster than bAP decays (microdomain τ = 61 ± 36 ms, bAP τ = 235 ± 124, p < 0.05, n = 8), indicating that longitudinal diffusion contributed to subsynaptic calcium clearance. Further, these results demonstrate that FS cell dendrites can in principle read out calcium signals triggered by single synaptic activation with higher temporal resolution than those produced by bAPs.
It is important to note that, even when imaged with diffusible indicators, microdomains were so restricted in space that they were at the limit of our two-photon detection. Previous experiments with our microscope using 0.1 μm calibration beads found that the point-spread function was approximately 0.6 μm (Majewska et al., 2000b). This indicates that microdomains were oversampled in space and that the standard deviations of Gaussian fits represented the upper limit of “true” microdomain size.

Thus, both distortion by exogenous calcium indicators and the spatial filtering of our system overestimated the spatial extent of microdomains. In physiological conditions, calcium could be restricted to the cytoplasm just under the excitatory synapses, which are approximately 0.3–0.5 μm in diameter (Gulyás et al., 1999).

Electron Microscopy of Imaged Dendrites Confirms the Absence of Physical Compartments

For the rest of the study, we pursued the mechanisms of the microdomain localization. Interneuron dendrites have few spines and beads, as well as intracellular structures such as ER and mitochondria (Somogyi et al., 1983a). We wondered whether any of these structures, and particularly subcellular structures invisible to fluorescence imaging, were involved in physically restricting longitudinal calcium diffusion. To address this issue, we performed serial EM reconstructions of dendritic segments on which we imaged microdomains. After imaging, we visualized the imaged dendrites, using biocytin and EM reconstructions (Figure 4). In two experiments where the quality of the imaging and subsequent ultra-
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Figure 4. EM Reconstructions of Imaged Dendrites Confirm the Absence of Physical Diffusional Boundaries

(Aa) Biocytin reconstruction of Layer V FS cell. Dendrites, orange; axon, black. Red dendritic segment represents region of interest in (Ab). (Ab) Two-photon z projection of imaged region of interest of an FS cell filled with 100 μM Fluo-4, left, and corresponding region from the cell reconstruction, right. Boxes indicate the dendritic segment selected for line scan imaging and for EM reconstruction. Scale bar, 20 μM. (Ac) Top, horizontal dendrite of interest with the cartoon of the serial EM reconstruction overlay at the precisely realigned section. Bottom, line scan through dendrite reveals the evoked single synaptic calcium signal. Note how its position appeared aligned to the synapse, as indicated by the red arrow in the cartoon. (Ad) Top, cartoon detail of the serial reconstruction. Dendrite, d, is labeled in green, and terminals, t, in white. The terminal labeled by the “t” corresponds to the terminal of interest in Ac. Bottom, the electron micrograph focusing on the site aligned to the microdomain; arrows indicate synapses.

(B) Data are laid out as in (A). (Bb) Scale bar, 20 μm. (Bc) Top, scale bar, 2 μm; bottom, scale bar, 400 ms. (Bc–Bd) Red arrow points to a candidate synapse aligned with the microdomain.

structural reconstruction were optimal, we failed to observe dendritic beads, interbead necks, or subcellular structures which could physically interfere with longitudinal diffusion, ruling out a morphology-based mechanism for the microdomains. Further, when we compared EM-reconstructed segments with the imaging data, we detected candidate excitatory synapses that were remarkably aligned to the peak position of the microdomain on the line scan (red arrows, Figures 4Ac and 4Bc). Moreover, the lateral size of single synaptic calcium signals was on the same order of magnitude of individual synapses, especially in the less buffered imaging condi-
In the absence of physical diffusional boundaries, we found that MK-801, nor CPA significantly altered the spatial range of the microdomain, represented by Calcium-Permeable AMPA Receptors (CP-AMPARs). Role of Intracellular Stores in Microdomains

Microdomains Are Mediated by Calcium-Permeable AMPA Receptors

In the absence of physical diffusional boundaries, we reasoned that microdomains could be localized by a combination of fast calcium influx and efflux. To pharmacologically isolate the mechanism of single synaptic calcium influx, we electrically evoked microdomains (as in Figure 2) and tested the effect of several drugs on microdomain amplitude and spatial restriction. Neither d-APV (n = 8) nor MK-801 (n = 3), significantly reduced the peak amplitude of microdomains, indicating that NMDA receptor activation was not significantly involved (Figure 5). Addition of DNOX (n = 2) or Philanthotoxin (PhTx) (n = 5 total, n = 3 washout), a specific blocker of CP-AMPARs, reversibly blocked calcium transients, indicating that the calcium influx was mediated by CP-AMPARs. These results suggest that voltage-gated calcium channels (VGCCs) recruited by AMPAR-mediated depolarization did not contribute to single synaptic calcium events. Further, relative to microdomain amplitude at −75 mV, those at −55 mV were decreased by 27% ± 28%, and at −90 mV were increased by 7% ± 11%, n = 5 cells (see Supplemental Data at http://www.neuron.org/cgi/content/full/40/4/807/DC1). These results suggest that VGCCs were not significantly recruited at imaged sites and that the driving force through CP-AMPARs was increased at hyperpolarized potentials. This result is also consistent with the spatial restriction of microdomains, since activated CP-AMPARs are presumably clustered at the synapse, while VGCCs are dispersed throughout the dendritic tree of FS cells (Goldberg et al., 2003b).

Figure 5. Microdomains Are Mediated by Calcium-Permeable AMPA Receptors

(A) Calcium transients, top, and EPSPs, bottom, during control, d-APV (50–100 μM), CPA (50 μM), and PhTx (5 μM) conditions (black, red, green, and blue traces, respectively). Note that the size of the EPSP reflects activation of several synapses on unimaged dendritic sites.

(B) Data plotted as in (A) from a different experiment in control, PhTx, and washout conditions (black, blue, and red, respectively).

(C) Pooled pharmacological results. Line at 100% indicates the peak amplitude of the control signal. Left, effect of d-APV (n = 8) or MK-801 (n = 3), CPA (n = 6), and 20 μM DNOX (n = 2) or PhTx (n = 5) on the peak amplitude of the calcium transient (%ΔF/F). Right, neither d-APV, MK-801, nor CPA significantly altered the spatial range of the microdomain, represented by α_{max}. *p < 0.05; **p < 0.001.

Role of Intracellular Stores in Microdomains

To test if uptake of calcium into internal stores contributed to calcium clearance, and thus to the spatial restriction of microdomains, we examined the effect of CPA (n = 6) on α_{max}. CPA did not significantly change α_{max} (control α_{max} = 1.62 ± 0.44 μm; CPA α_{max} = 1.56 ± 0.26 μm, n = 6) or slow the temporal decay of calcium transients (control τ = 150 ± 130 ms; CPA τ = 190 ± 125 ms, n = 6), although it did reduce their peak amplitude (control ΔF/F [%] = 203 ± 33, CPA ΔF/F [%] = 115 ± 21, p < 0.05, n = 6) (Figure 5C). However, since CPA increases resting calcium levels by blocking uptake into ER, we attribute this reduction to a decrease in the dynamic range of the calcium indicator (Goldberg et al., 2003a, 2003b; Kovalchuk et al., 2000).

Localization of Microdomains Depends on Fast Kinetics of Calcium-Permeable AMPA Receptors

Calcium-permeable AMPA receptors (CP-AMPARs) lacking the GluR2 subunit are unique even among AMPA receptors for having extremely fast kinetics (Geiger et al., 1995; Verdoorn et al., 1991). To test if the kinetics of CP-AMPARs were responsible for the localization of microdomains, we performed experiments in voltage clamp, in the presence of d-APV to isolate AMPARs, and pharmacologically slowed the kinetics of AMPAR-mediated EPSCs with CX-546, a drug which inhibits AMPAR deactivation (Arai et al., 2002).

Addition of CX-546 increased the duration and area of AMPAR-mediated EPSCs and dramatically increased the spatial extent of microdomains (Figure 6). Figure 6A shows the experimental approach to the CX-546 experiments (n = 7, n = 2 washout [see Supplemental Data at http://www.neuron.org/cgi/content/full/40/4/807/DC1]). Horizontally oriented dendrites were targeted, and single synaptic calcium transients were evoked as in Figure 2. Successes were averaged before (Figure 6Ab) and after (Figure 6Ac) addition of CX-546. In the presence of CX-546, microdomains were significantly less localized, as the calcium signal spread from the synaptic source. We quantified the spatial increase of microdomains by plotting ΔF/F as a function of space and time, as in Figures 1 and 3, and compared α_{max} under control and CX-546 conditions (Figures 6B and 6D).
Figure 6. Localization of Microdomains Depends on the Fast Kinetics of CP-AMPARs

(Aa) Horizontal dendrite of interest from layer V FS cell filled with 100 µM Fluo-4. (Ab) Top, control (50–100 µM d-APV) line scan through dendrite in (Aa), average of three successes. Vertical scale, 400 ms. Bottom, ΔF/F image from line scan above. Vertical scale bar, 100 ms. (Ac) Images are presented as in (Ab), after addition of 1 mM CX-546, an inhibitor of AMPAR deactivation. Note how the microdomain spread to adjacent dendritic segment. Average of four successes.

(Ba) ΔF/F versus dendritic space plot for calcium transient in (Ab). (Bb) Data plotted as in (Ba) for the transient in the presence of CX-546. Note that panels in (A)–(B) have the same x-axis.

(C) Top, calcium transients from bracketed regions of line scans in (A), before, black trace, and after, red, addition of CX-546. Bottom, EPSC time locked to the calcium transients above. Note how CX-546 dramatically prolonged the AMPAR-mediated currents.

(D) Data pooled from seven CX-546 experiments. Left, maximal range traces from ΔF/F versus dendritic space plots, normalized to the peaks of their Gaussian fits. Note how CX-546 always increased the longitudinal spread of the calcium signal. Right, CX-546 significantly increased the decay and area of calcium transients and increased τ_max, the standard deviation of the largest Gaussian fits. CX-546 also increased EPSC area and decay but not EPSC amplitude. In all CX-546 experiments, “control” microdomains were in the presence of d-APV, to isolate AMPARs. *p < 0.05, **p < 0.01.
spatial extent of microdomains increased approximately 2-fold (control $r_{\text{max}} = 1.1 \pm 0.4 \, \mu m$, CX546 $r_{\text{max}} = 2.0 \pm 0.6 \, \mu m$, $p < 0.05$, $n = 7$) (Figures 6D and 6E). At the same time, CX-546 increased EPSC decay time constants (control $\tau = 4.6 \pm 1.4 \, ms$; CX-546 $\tau = 46 \pm 26 \, ms$, $p < 0.01$, $n = 7$) and EPSC area (470% ± 230%, $p < 0.05$), indicating that the total calcium injected at the imaged synapse increased greater than 4-fold (Figures 6C and 6E). CX-546 also increased the duration which entered the cell became bound to indicator and that subsequent diffusion of indicator contributed toand/or (2) increased levels of subsynaptic calcium bound indicator diffused to adjacent dendritic regions.

These data further indicate that, under control conditions, calcium extrusion is strong enough to locally handle the CP-AMPAR-mediated microdomains and control the spatial range of the indicator-calcium interaction.

Membranous Calcium Extrusion via the Na+/Ca2+ Exchanger Restricts Microdomains in Space

Calcium influx, efflux, and buffers together control the spatiotemporal dynamics of calcium signals (Koch, 1998; Majewska et al., 2000a). The Na+/Ca2+ exchanger is a low-affinity but high-capacity plasma membrane calcium pump which drives calcium extrusion after synaptic activation (Blaustein et al., 2002; Ranciario-McComb et al., 2000). To test if local calcium extrusion was necessary for microdomain localization, we measured the spatiotemporal dynamics of microdomains after blocking Na+/Ca2+ exchange with 30 μM benzamil. Benzamil increased the spatial spread of microdomains (204% ± 60%, $p < 0.05$, $n = 5$, $n = 1$ washout) and slowed the decay of calcium transients (259% ± 122%, $p < 0.05$, $n = 5$) (Figure 7). Surprisingly, however, the peak amplitude was not significantly changed, suggesting that diffusion of free calcium and not local accumulation of calcium bound indicator and its subsequent diffusion was responsible for the loss of signal localization. While it was possible that the effect of benzamil on microdomain size was via blockade of Na+/Ca2+ exchange at the presynaptic terminal, we did not observe a change in EPSC amplitude (Figure 7), suggesting that the effect of benzamil on microdomains was predominantly postsynaptic.

Numerical Simulations: Extrapolation to Zero Exogenous Buffer

The imaged microdomains, as shown in Figures 1–7, represented the position of calcium bound indicator and not of free calcium. Thus, manipulations which affected the calcium-indicator interaction could not be distinguished from those which affected the distribution of free calcium.

To address this issue, we constructed a computer model using realistic parameters to simulate the spatiotemporal evolution of synaptic calcium influx on a thin aspiny dendrite (Table 1, Figure 8A, and see the Appendix in the Supplemental Data available at http://www.neuron.org/cgi/content/full/40/4/807/DC1). Our first goal was to recreate experimental conditions (100 μM Fluo-4) and observe the behavior of free calcium, as opposed to calcium bound indicator. In Figure 8B, the fractional occupancy of Fluo-4, which approximates $\Delta F/F$, simulated under experimental conditions is plotted as a line scan image next to the actual free calcium signal. The free calcium signal was both an order of magnitude faster and more localized than the simulated fluorescence signal (Figure 8B). This result demonstrated that during experiments most of the calcium ions which entered the cell became bound to indicator and that subsequent diffusion of indicator contributed to subsynaptic calcium clearance and microdomain size.

We next simulated native conditions, by replacing exogenous indicator with the calcium binding protein parvalbumin, which is widely expressed in FS interneurons (Figure 1). Under these conditions, the peak amplitude of free calcium was almost 2-fold larger, suggesting that CP-AMPAR synapses generate micromolar domains of free calcium under normal conditions. This range was on par with our experimentally derived estimates of peak [Ca2+]. (770 ± 400 nM, $n = 3$, range 400–1200 nM), (see Experimental Procedures and Supplemental Data at http://www.neuron.org/cgi/content/full/40/4/807/DC1) (Maravall et al., 2000).

Thus, imaged microdomains misrepresented true calcium signals in two fundamental ways. First, the indicator fluorescence signal overrepresented in space and time the free calcium signal under simulated experimental conditions (Figure 8B). Second, under natural conditions in the absence of indicator, simulated free calcium signals were significantly larger in amplitude yet remained localized (Figure 8C).

Simulated Microdomains Are Highly Sensitive to the Association Rate of Mobile Buffers, the Fixed Buffer Capacity, Dendrite Radius, and the Kinetics of Calcium Influx and Efflux

We next used our model to determine which parameters most profoundly affect the free calcium signal generated at a shaft synapse under physiological conditions, without added indicator. We examined how (1) mobile buffer (“slow” parvalbumin or “fast” Fluo-4), (2) fixed buffer capacity, (3) the kinetics of the calcium influx, (4) extrusion, and (5) dendrite radius affect the spatiotemporal evolution of free calcium.

Parvalbumin has a high affinity for calcium but binds calcium significantly slower than Fluo-4 (Table 1) (Lee et al., 2000b). We found that increasing parvalbumin concentration resulted in faster decays and increased localization of free calcium. However, in separate simulations, we found that the fractional occupancy of parvalbumin changed less than 1% during the rapid CP-AMPAR-mediated calcium influx (data not shown). This demonstrates that slow buffers can be “blind” to fast calcium influxes (Markram et al., 1998) and suggests that the role of parvalbumin in sharpening microdomains in space and time may be more profound during trains of synaptic input.

To explore the role of fast mobile buffers, we simulated free calcium in 0, 10, and 100 μM Fluo-4, in the absence of parvalbumin. Adding small amounts of Fluo-4 significantly decreased peak [Ca2+] and increased the decay rate at the synaptic site, consistent with fast Fluo-4 binding to calcium and facilitated diffusion from the cal-
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Figure 7. Microdomain Localization Depends on Membranous Na⁺/Ca²⁺ Exchange

(Aa) Dendrite of interest from a layer V FS cell filled with 100 μM Fluo-4; horizontal scale bar, 2 μm. (Ab) Control line scan, average of four successes; vertical scale, 400 ms. (Ac) Line scan after addition of benzamil, average of three successes. (Ad) Line scan after benzamil washout. Vertical scale bar in (Ab) applies to all line scans.

(B) Spatiotemporal profile from control, top, and benzamil, bottom, ΔF/F signals. Note that x axes throughout (A)–(B) are identical.

(C) Top, calcium transient from regions of interest indicated by brackets in (A) and time-locked EPSC, bottom, in control (black), benzamil (red), and washout (blue) conditions.

(D and E) Data pooled from five benzamil experiments. (D) Maximal range traces from ΔF/F versus dendritic space plots, normalized to the peaks of their Gaussian fits. Note how benzamil increased the longitudinal range of the calcium signal. (E) Benzamil significantly increased ΔF/F max and the decay of the calcium transient, ΔF/F, but had no significant effect on the peak amplitude (ΔF/F).

Since FS processes are unusually thin, we next examined the effect of dendrite radius on microdomains. We found that decreasing the radius caused an increase in the peak amplitude and an acceleration of the decays of simulated microdomains. This effect was so dramatic that decreasing the radius from 0.4 to 0.2 μm increased calcium clearance.

Interneurons reportedly have significantly larger fixed endogenous buffer capacities, κᵣ, than pyramidal cells (Kaiser et al., 2001; Lee et al., 2000a). In order to accurately simulate κᵣ, we first measured the endogenous buffer capacity (see the Supplemental Data at http://www.neuron.org/cgi/content/full/40/4/807/DC1). We found κᵣ to be 140 ± 70 (n = 8 FS cells), at least twice as large as reported in pyramids (Helmchen et al., 1996; Lee et al., 2000a; Maravall et al., 2000). To kinetically simulate κᵣ, all simulations were conducted in the presence of an immobile species with calmodulin-like binding kinetics (Table 1) (Robertson et al., 1981). Increasing fixed buffer capacity significantly reduced the peak amplitude, slowed the decay of microdomains, and contributed to their localization (Figure 8D). Thus, the high fixed buffer content of interneuron dendrites likely contributed to the subsynaptic localization of calcium in our experiments.

Since FS processes are unusually thin, we next examined the effect of dendrite radius on microdomains. We found that decreasing the radius caused an increase in the peak amplitude and an acceleration of the decays of simulated microdomains. This effect was so dramatic that decreasing the radius from 0.4 to 0.2 μm increased the peak [Ca²⁺] by an order of magnitude (Figure 8E). However, since extrusion is also scaled by S/V ratio, this excess calcium did not significantly diffuse to adjacent sites, and localization of calcium was not particularly sensitive to dendrite radius.

Experimentally, we disrupted microdomain localization by increasing calcium influx or by inhibiting extrusion (Figures 6 and 7). However, it is unclear to what extent these results reflected the range of free calcium ions versus calcium bound indicator. In simulations, increasing the decay time constant of the EPSC caused a large increase of the subsynaptic [Ca²⁺] (Figure 8E). However, although localization was compromised, free calcium remained surprisingly local even when the EPSC decay time constant was 40 ms (Figure 8E). But if we simultaneously reduced either the fixed buffer capacity or Vmax, calcium diffused >1 μm to adjacent dendritic sites (data not shown). Thus, in the presence of local buffering or
strong extrusion, fast calcium influx was not necessary to localize free calcium. By controlling the peak and duration of local \([Ca^{2+}]_o\), however, EPSC kinetics determine the spatial extent of diffusible calcium-bound molecules or proteins and thus the range of a calcium-dependent process.

Calcium extrusion, unlike EPSC kinetics, directly operated to restrict the range of free calcium (Figure 8E). Simulations showed that higher \(V_{\text{max}}\) resulted in faster, smaller, and, more significantly, more localized free calcium signals (Figure 8E).

Thus, the peak amplitude and duration of free calcium signals were especially sensitive to dendrite radius, fixed buffer capacity, the association rate of mobile buffers, and the kinetics of the calcium influx. The spatial extent of free calcium, however, was most sensitive to calcium extrusion and fixed buffer capacity.

**Discussion**

**FS Dendrites Are “Spines-in-Series”**

We examined the spatiotemporal dynamics of calcium signals caused by the activation of single synapses on the aspiny dendrites of fast spiking interneurons. We found that these single-synaptic calcium events (microdomains) are localized in space to synapse-sized compartments (<1 \(\mu\)m), representing the potential for synapse-specific biochemical compartmentalization in the absence of dendritic spines. The localization of microdomains depends on fast calcium influx through calcium-permeable AMPA receptors and on fast membranous efflux via the Na⁺/Ca²⁺ exchanger. In addition, our modeling studies show that endogenous immobile buffers contribute to calcium localization and that thin dendrites facilitate fast handling of large free calcium concentrations. These data help integrate five well-known characteristics of FS interneuron dendrites: (1) they are aspiny, (2) they express CP-AMPARs, (3) they have elevated endogenous buffer capacities, (4) they are particularly thin, and (5) they express parvalbumin (Koh et al., 1995; Lee et al., 2000a; Somogyi et al., 1983a). We show that these factors cooperate to create a fast and highly localized calcium signal. In view of these observations, FS cell dendrites functionally behave as “spines-in-series,” due to their high surface-to-volume ratio and their ability to compartmentalize calcium and calcium-dependent processes at the level of individual synapses. These results indicate that the expression of CP-AMPARs on dendritic shafts provides a “spine-free” mechanism of signal localization and represents a novel approach to single-synaptic calcium signaling.

**Compartmentalized Calcium Accumulations during Activation of Single Synapses on Aspiny Dendrites**

We adopted two experimental strategies to image the calcium influx associated with the activation of single synapses on aspiny FS dendrites. First, we found that spontaneous microdomains were associated with unitary-sized EPSPs, suggesting that they were single-synaptic calcium events (Figure 1). Second, we electrically evoked microdomains and found that successive shocks resulted in all-or-none calcium signals with spatial dynamics identical to those observed during spontaneous activation (Figures 1 and 2). We concluded that the microdomains resulted from the activation of single synapses.

At their peak amplitude, imaged microdomains, which represent calcium bound indicator and not free calcium, were restricted to submicrons of dendritic space (\(\sigma =\)
We systematically examined the effect of fixed and mobile buffers, the kinetics of calcium influx and efflux, and the dendrite radius on the spatiotemporal evolution of microdomains. (A) Schematic of the model. (B) Simulations were performed in the presence of 100 μM Fluo-4 to recreate the experimental environment. Simulated line scan images of the fractional occupancy of Fluo-4, which approximates the free calcium signal, left, and the actual free [Ca^{2+}]_i, right. Note how Fluo-4 occupancy (as reported in line scan images in Figures 1–7) overrepresented the spatial extent of the true free calcium signal. (C) Free [Ca^{2+}]_i under “native” conditions, with 100 μM parvalbumin replacing the exogenous Fluo-4. (D) Simulations were run testing the effect of parvalbumin, left, Fluo-4, middle, fixed buffer capacity, middle, right, on microdomains. Top panels, [Ca^{2+}]_i versus time. Bottom panels, to illustrate the effect of these manipulations on the spatial component of microdomains, we plotted the standard deviation of fitted Gaussians, σ, versus time, as in Figure 3C. Traces terminate at the time when peak [Ca^{2+}]_i reached 300 nM. Thus, the length of plots reflects the duration of the calcium signal. Note differences in axes in different simulation conditions. (E) Results are plotted as in (C), examining (1) dendrite radius, left, (2) EPSC, middle, and (3) Vmax, right. Asterisks indicate values used in control conditions, which were held when other variables were tested (Table 1).

0.70 ± 0.22 μm, n = 21). Thus, even this upper limit estimation of calcium compartmentalization is an order of magnitude smaller than previously observed on dendritic shafts. Local dendritic calcium spikes and activation of many synapses converging onto an individual dendritic branch are localized to approximately 10 μm (Goldberg et al., 2002, 2003b; Schiller et al., 2000; Wei et al., 2001), and NMDAR-mediated single synaptic calcium transients in cultured pyramidal neurons diffused to approximately 5 μm of adjacent dendritic regions (Murthy et al., 2000). Given that excitatory synapses on FS shafts appear at a density of approximately 1–3 per micrometer, and are approximately 0.3–0.4 μm in diameter, we propose that microdomains may underlie synapse-specific calcium signaling (Figure 4).

Microdomains Depend on Fast Calcium-Permeable AMPA Receptors
We conducted serial EM reconstructions of imaged dendrites and confirmed the smooth nature of FS dendrites and showed that there were no diffusional boundaries which physically restricted microdomains in space (Figure 4). Instead, localization of calcium transients depended in part on the kinetically fast CP-AMPAR-mediated calcium influx (Figures 5 and 6). This fast localized influx could be locally handled by extrusion and buffering, effectively bypassing the requirement for a spine morphology-based mechanism of calcium compartmentalization. In our model, τ_{EPSC} was a major determinant of the peak and duration of the calcium signal, two key components in regulating the range of a calcium-
dependent process. The submillisecond kinetics of CP-AMPARs thus seem designed to produce a localized calcium-dependent signal. When we reduced AMPAR deactivation with CX-546, we found that microdomains became less localized in space (Figure 6).

**CP-AMPARs Innervate Aspiny Dendrites throughout the CNS**

This result suggests that while aspiny dendrites can localize CP-AMPAR-mediated calcium-dependent processes, dendritic spines may be necessary to localize those triggered by NMDARs. Consistent with this, while synaptic calcium influx on spines is preferentially mediated by NMDARs (Kovalchuk et al., 2000; Yuste et al., 1999), aspiny dendrites throughout the CNS are generally innervated by CP-AMPAR synapses. We propose that the correlation between lack of spines and CP-AMPARs is not coincidental but represents an alternative approach to synapse-specific calcium signaling.

Specifically, CP-AMPARs innervate aspiny dendrites in the hippocampus, neocortex, striatum, amygdala, retina, spinal chord, and brainstem (Albuquerque et al., 1999; Angulo et al., 1999; Cheunsuang and Morris, 2000; Gardner et al., 1999, 2001; Gotz et al., 1997; Katsumaru et al., 1988; Koh et al., 1995; Mahanty and Sah, 1998; Morkve et al., 2002; Mugnaini, 1985; Otis et al., 1995a, 1995b; Tamas et al., 1997; Tolbert and Morest, 1982; Tolbert et al., 1982; Washburn and Moises, 1992). This correlation is even born out in pyramidal neurons. First, developing pyramidal neurons are aspiny and express CP-AMPARs (Kumar et al., 2002). However, in the first postnatal weeks, the expression of CP-AMPARs wanes, just as spines are being generated, suggesting a progression through two different synapse-specific calcium signaling strategies, a CP-AMPAR and a NMDAR phase. Second, this progression seems to be reversed in postkindled or post-ischemic pyramids. While AMPARs on mature pyramids are largely calcium impermeable, kindling or ischemia induces an AMPAR switch resulting in a higher expression of CP-AMPARs (Pellegrini-Gamto et al., 1992; Prince et al., 2000). Interestingly, kindling or ischemia also decrease spine density (Hasbani et al., 2001; Park et al., 1996; Teskey et al., 1999), and a reduction in spine density has been described in epileptic syndromes (Maultani et al., 1994). Thus, downregulation of GluR2 and spine withdrawal may be jointly regulated processes. Consistent with this, overexpression of GluR2 increases spine density in pyramidal neurons and induces spine assembly in aspiny interneurons (Passafaro et al., 2003). Thus, GluR2 may be a molecular link between the structure of dendrites and their synapse-specific signaling strategies.

**Mobile Buffers Regulate Single Synaptic Calcium Events**

Our experiments demonstrated that mobile calcium indicator overestimated the spatial spread of microdomains by diffusing from the synaptic calcium source. At the same time, our model showed that low concentrations of mobile buffers, either Fluo-4 or the physiologically relevant calcium buffer parvalbumin, accelerated the clearance of subsynaptic calcium. Taking into consideration its high affinity for and slow binding to calcium, PV may be perfectly suited to preserve microdomain localization during repetitive activation of single synapses.

It is interesting to note that PV is expressed in many aspiny neurons innervated by CP-AMPARs, such as in the cochlear nucleus, retinal All amacrine cells, cerebellar stellate cells, and striatal, amygdaloid, hippocampal, and neocortical cortical FS interneurons (Gabriel and Straznicky, 1992; Hamano et al., 1990; Kawaguchi, 1993; Kawaguchi and Kubota, 1995; Lohmann and Friauf, 1996; Ohshima et al., 1991; Sik et al., 1995; Smith et al., 1998). This observation puts smooth dendrites, the expression of CP-AMPARs, and parvalbumin into a single functional framework, subserving spine-free synapse-specific calcium signaling.

**Calcium Signals and Membranous Na⁺/Ca²⁺ Exchange**

As for the efflux, calcium clearance in dendrites proceeds via three main pathways: uptake into intracellular stores via SERCA pumps and extrusion through the plasma membrane via the Ca-ATPase (PMCA) and the Na⁺/Ca²⁺ exchanger. We showed that reduction of membranous calcium extrusion via the Na⁺/Ca²⁺ exchanger and not SERCA blockade had a dramatic impact on the spatial spread but not the peak amplitude of microdomains (Figures 7 and 8). The Na⁺/Ca²⁺ exchanger is a low-affinity but high-capacity pump capable of fast extrusion at high calcium concentrations (Blaustein et al., 2002). Recently, mice lacking NCX2, the major isofrom of the Na⁺/Ca²⁺ exchanger in the brain, have been shown to have enhanced learning and memory and increased susceptibility to LTP (Jeon et al., 2003). Our results illustrate the importance of membranous calcium extrusion, and Na⁺/Ca²⁺ exchange in particular, controlling fast and localized postsynaptic calcium signals on narrow dendritic shafts.

**On Spines and Shafts**

In summary, we have shown that CP-AMPAR innervation of dendritic shafts represents a novel form of synapse-specific calcium signaling in FS interneurons, one that does not require physical boundaries for compartmentalization. What then is the function of dendritic spines? Given the importance of S/V ratio in determining the peak amplitude and duration of a calcium signals (Figure 8E), submicron variabilities in dendritic compartment sizes could make it difficult for a given calcium binding protein to be reliably responsive to a characteristic calcium influx. Although spines could have a raison d'être independent from their calcium dynamics (Yuste and Majewska, 2001), they may reduce S/V variability and preserve the fidelity of calcium-dependent signaling cascades. Moreover, even a localized calcium signal may have a spatially diffuse range of action if the calcium influx is slow and the calcium binding molecule is diffusible, and spines could be specifically designed to compartmentalize cascades triggered by slower NMDAR-mediated calcium influxes. In contrast, the fast CP-AMPAR synapse on thin aspiny FS dendrites appears specially designed not only to generate large amplitude, local calcium signals (Figure 8C) but also localized calcium-dependent processes.
Experimental Procedures

Slice Preparation and Electrophysiology

Experiments were carried out according to the NIH Guide for the Care and Use of Laboratory Animals (NIH publication no. 86-23, revised 1987). Whole-cell recordings in coronal slices of primary visual and somatosensory cortices from P13 to 17 C57BL/6 mice were performed as described (Goldberg et al., 2003a). Mechanisms of synaptic calcium influx were explored with cyclopiazonic acid (CPA) (50 μM), benzamil (30 μM), DNOX (20 μM), d-APV (50-100 μM), CX-546 (1-5 mM), 4-AP (1 mM), philanthotoxin-433, (5 μM), and MK-801 (20 μM). Trolox (100 μM) was added to the bath to reduce phototoxicity. Synaptic inputs to neurons were stimulated electrically using a fluorescently labeled extracellular pipette as described (Goldberg et al., 2003b).

Two-Photon Imaging

Cells were filled with 100 or 400 μM Fluo-4 (Molecular Probes, Eugene, OR), and images were acquired as described (Goldberg et al., 2003a). Imaging was done using a custom-made two-photon laser scanning microscope (Majewska et al., 2000b; Nikolenko et al., 2003), consisting of a modified Flouviuscope (Olympus, Melville, NY) confocal microscope with a Ti:sapphire laser providing 130 fs pulses at 75 MHz (Mira, Coherent, Santa Clara, CA), pumped by a solid-state source (Verdi, Coherent), with a 60 × 0.9 numerical aperture immersion objective (IR1, Olympus).

Analysis

Fluorescence levels of calcium measurements were analyzed using ImageJ (NIH, Bethesda, MD). Calcium transients were corrected for background fluorescence by measuring a nonfluorescent area close to the dendrite. For both calcium transients and ΔF/F images, the relative change of fluorescence of baseline - (ΔF/F) (400 ms prior to synaptic stimulation or prior to spontaneous calcium microdomains) was used as indicator for the change in calcium. After the first time point of the microdomain, ΔF/F versus space traces were averaged from two time points (25.28 ms), and all traces were filtered with a sliding Hanning kernel. Decay time constants of calcium transients and ΔF/F versus space traces were fitted using single exponential and Gaussian algorithms in Igor (Wavemetrics, Lake Oswego, OR), of hippocampal unitary inhibitory postynaptic potentials and the decay time constants, which were photographed and imaged dendritic segments, which were photographed and aligned with the LM micrographs and two-photon images. Utrathin sections were observed at tilting angles of 0–75° to document synapses cut at oblique planes. Three-dimensional reconstruction of dendritic segments was also performed with Neurolucida/Neuroexplorer (MicroBrightfield, Colchester, VT) with oil immersion objective at 1250× magnification. We then made a complete series of ultrathin sections from the imaged dendritic segments, which were photographed and aligned with the LM micrographs and two-photon images. Utrathin sections were observed at tilting angles of 0°–75° to document synapses cut at oblique planes. Three-dimensional reconstructions of dendritic segments were also performed with Neurolucida/Neuroexplorer (Buhl et al., 1994; Tamas et al., 1997). Monoclonal antibodies to parvalbumin (Swant, 1:1000) were applied to characterize fast spiking interneurons. Dual fluorescence labeling of cortical slices was carried out as described (Tamas et al., 2000), using Alexa488-conjugated streptavidin (Molecular Probes) revealing biocytin and CY3-conjugated anti-mouse IgG (Jackson Labs, West Grove, PA, USA) for parvalbumin.

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References


Arai, A.C., Xia, Y.F., Rogers, G., Lynch, G., and Kessler, M. (2002). Fluorescence levels of calcium measurements were ... (NIH, Bethesda, MD). Calcium transients were corrected for with distinct modes of action. J. Pharmacol. Exp. Ther. 303, 1075–1085.


