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Dendritic calcium transients evoked by single back-propagating action potentials in rat neocortical pyramidal neurons

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1. Dendrites of rat neocortical layer V pyramidal neurons were loaded with the Ca\(^{2+}\) indicator dye Calcium Green-1 (CG-1) or fluo-3, and the mechanisms which govern action potential (AP)-evoked transient changes in dendritic cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_d\)) were examined. APs were initiated either by synaptic stimulation or by depolarizing the soma or dendrite by current injection, and changes in fluorescence of the indicator dye were measured in the proximal 170 \(\mu m\) of the apical dendrite.

2. Simultaneous two-pipette recordings of APs from the soma and apical dendrite, and dendritic fluorescence imaging indicated that a single AP propagating from the soma into the apical dendrite evokes a rapid transient increase in fluorescence indicating a transient increase in [Ca\(^{2+}\)]\(_d\). At 35–37 °C the decay time constant of the fluorescence transient following an AP was around 80 ms.

3. Voltage-activated Ca\(^{2+}\) channels (VACCs) of several subtypes mediated the AP-evoked fluorescence transient in the proximal (100–170 \(\mu m\)) apical dendrite. The AP-evoked fluorescence transient resulted from Ca\(^{2+}\) entry through L-type (nifedipine sensitive; 25%), N-type (\(\omega\)-conotoxin GVIA sensitive; 28%) and P-type (\(\omega\)-agatoxin IVA sensitive; 10%) Ca\(^{2+}\) channels and through Ca\(^{2+}\) channels (R-type) not sensitive to L-, N- and P-type Ca\(^{2+}\) channel blockers (cadmium ion sensitive; 37%).

4. The decay time course of the dendritic fluorescence transient was prolonged by the blockers of endoplasmic reticulum (ER) Ca\(^{2+}\)-ATPase, cyclopiazonic acid and thapsigargin, suggesting that uptake of Ca\(^{2+}\) into the ER in dendrites governs clearance of dendritic Ca\(^{2+}\).

5. The decay time course of the fluorescence transient was slightly prolonged by benzamil, a blocker of plasma membrane Na\(^+\)--Ca\(^{2+}\) exchange and by calmidazolium, a blocker of the calmodulin-dependent plasma membrane Ca\(^{2+}\)-ATPase, suggesting that these pathways are less important for dendritic Ca\(^{2+}\) clearance following a single AP. Neither the mitochondrial uncoupler carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP) nor the blocker of Ca\(^{2+}\) uptake into mitochondria, Ruthenium Red, had any measurable effect on the decay time course of the fluorescence transient.

6. Dendritic fluorescence transients measured during trains of dendritic APs began to summate at impulse frequencies of 5 APs s\(^{-1}\). At higher frequencies APs caused a concerted and maintained elevation of dendritic fluorescence during the train.

7. It is suggested that dendritic [Ca\(^{2+}\)]\(_d\) transients evoked by back-propagating APs represent a retrograde signal sent to dendrites which may alter both the receptive and the integrative properties of the dendritic compartment depending on the impulse activity of the neuron.

Understanding the mechanism of synaptic integration is a prerequisite for understanding the function of different classes of neurons in information processing and encoding. Postsynaptic potentials from synapses in distal dendrites of neocortical pyramidal neurons are integrated as they spread towards the soma and may evoke APs in the initial axon segment if a threshold depolarization is reached. The manner in which synaptic input is integrated by the
dendritic tree is dependent on the passive cable properties of the neuron (see Spruston, Jaffe & Johnston, 1994). Opening and/or closing of ion channels in the dendritic membrane introduces non-linear effects on the integrative process (see Spruston et al. 1994). In particular, the opening of VACCS may exert a profound effect on synaptic integration. In the short term dendritic Ca$^{2+}$ inflow may amplify depolarizing potentials (Deisz, Fortin & Zieglgänsberger, 1991; Markram & Sakmann, 1994). Intracellular Ca$^{2+}$ may also interact with ion channels, neurotransmitter receptors and intracellular biochemical pathways to exert long-lasting effects on many cellular responses which are coupled to neuronal electrical activity (see Henzi & MacDermott, 1992).

An increase in dendritic [Ca$^{2+}$] via VACCS has been shown in Purkinje neurons of the cerebellum (Ross & Werman, 1987), pyramidal neurons of the hippocampal CA1 region (Regehr, Connor & Tank, 1989), neurons of deep cerebellar nuclei (Muri & Knöpfel, 1994) and neocortical neurons (Markram & Sakmann, 1994). In hippocampal neurons, the elevation of proximal dendritic [Ca$^{2+}$] has been shown to occur during APs evoked and recorded from the soma and depends on the presence of Na$^+$ channels on dendrites (Jaffe, Johnston, Lasser-Ross, Lisman, Miyakawa & Ross, 1992). This suggested that APs may propagate back into dendrites to trigger Ca$^{2+}$ entry. Recently, using two-pipette recording from the same neuron, back-propagation of APs from the axon initial segment into dendrites of layer V pyramidal neurons of the neocortex was demonstrated (Stuart & Sakmann, 1994). Furthermore, back-propagation, as opposed to propagation from dendrites towards the soma, is the most probable direction of AP propagation under physiological conditions (Stuart & Sakmann, 1994). This voltage signal in dendrites represents the result of summed synaptic potentials arising in dendrites. To elucidate the function of AP-evoked dendritic Ca$^{2+}$ entry in generating a 'retrograde signal', it is essential to obtain time-resolved measurements of [Ca$^{2+}$] transients evoked by back-propagating APs and to determine the factors contributing to dendritic Ca$^{2+}$ entry and removal.

We have used Ca$^{2+}$-sensitive indicator dyes to measure Ca$^{2+}$ entry in dendrites of identified layer V pyramidal neurons of the neocortex by recording an increase in fluorescence relative to basal fluorescence to determine (a) whether a single AP propagating back into the apical dendrite is sufficient to trigger detectable Ca$^{2+}$ entry through VACCS, (b) the time course of the rise in [Ca$^{2+}$] and removal of Ca$^{2+}$, (c) the subtypes of VACCS which mediate Ca$^{2+}$ entry into dendrites, (d) the mechanism of Ca$^{2+}$ removal from the dendritic cytosol, and finally (e) the impulse rate of APs in pyramidal neurons that would cause merging of the discrete dendritic Ca$^{2+}$ signals that are generated by a single back-propagating AP.

**METHODS**

**Electrophysiology**

Methods similar to those described previously were used (Stuart, Dott & Sakmann, 1993). Briefly, Wistar rats (12–16 days) were rapidly decapitated (in some cases following ether or halothane anaesthesia) and neocortical slices (sagittal; 200 μm) were cut and incubated (20–22 °C) in standard bicarionate Tyrode solution (composition (mm): 125 NaCl, 25 NaHCO3, 2.5 KCl, 1.25 NaH2PO4, 25 glucose) containing 4 mm CaCl2 and 4 mm MgCl2 to reduce background synaptic activity. Layer V pyramidal neurons from the somatosensory cortical areas were identified using infrared differential interference contrast (IR-DIC) video-microscopy on an upright microscope (Zeiss-Axioplan, fitted with x40-W/0.75NA objective lens). The microscope stage was oriented so that the apical dendrite was parallel to the scanning direction of the fast mirror of a confocal laser scanning microscope (CLSM, Phoibos 1000; Molecular Dynamics, Sunnyvale, CA, USA). Somatotopic (5–10 MΩ pipette resistance) and dendritic whole-cell (8–12 MΩ pipette resistance) recordings were made with an EPC-7 and an EPC-9 amplifier (List Electronic, Darmstadt, Germany), respectively. Electrophysiological data were captured with an Apple Macintosh computer and commercial software (Pulse; Heka Electronic, Ratingen, Germany). Since long voltage records (2.56 s) were required to correlate with the imaging (see below), samples were collected every 400 μs. The full amplitude of APs, especially at higher temperatures, may therefore not be represented in the records shown. In the rise time experiments, however, samples were collected every 200 μs. Data of a group of experiments are presented as means ± s.e.m., unless otherwise noted. Dendritic recordings were from apical dendrites 70–170 μm from the soma. For current-clamp recordings, partial bridge balancing was performed using the series resistance compensation of the EPC-9. Neurons were recorded with pipettes containing (mm): 100 potassium gluconate, 20 KCl, 4 Mg-ATP, 20 phosphocreatine, 50 U ml$^{-1}$ creatine phosphokinase, 0.3 GTP, 10 Hepes (pH 7.3, 310 mosmol$^{-1}$). Neurons typically had resting membrane potential ($V_m$) levels from −60 to −55 mV and AP threshold potentials around −40 mV. The input resistances of these pyramidal neurons were 50–120 MΩ. To evoke APs by afferent stimulation, a monopolar tungsten electrode, of 2–3 MΩ resistance, was placed in layer II and stimulus pulses of 10–50 V were applied for 10–50 μs. An enhancement or reduction of accommodation was defined as a more than 50% change in the number of APs evoked by a 1 s depolarizing pulse which resulted in five to seven APs under control conditions.

The temperature of the recording chamber was changed by adjusting the gravity-enabled flow-rate of Tyrode solution that was preheated to 50 °C and bubbled with 95% O₂–5% CO₂. Teflon tubing was used to reduce gaseous escape and the pH of the solution when entering the bath was 7.2–7.4, suggesting that the partial pressure of CO₂ and hence probably O₂, was adequate for physiological viability. The temperature was monitored by either a thermocouple device, a small thermometer or both, the probes of which were placed near the slice. In these experiments [Ca$^{2+}$]o was 2 mm and [Mg$^{2+}$]o was 1 mm.

**Calcium fluorescence imaging**

Calcium Green-1 (20, 50 or 100 μm; Molecular Probes; 488 nm excitation; 515–560 nm emission; 530 nm peak; $K_{ex}$ 243 nm; forward rate constant $k_f$, 0.57 × 10$^9$ s$^{-1}$; backward rate
constant $k_n$, $139 \text{s}^{-1}$; at 20–22°C; \cite{erber-hach-1991} or fluo-3 (100 \mu M; Molecular Probes; 488 nm excitation; 515–560 nm emission; 530 nm peak; $K_d$, 461 nm; $k_0$, 992 $\times 10^5 \text{ms}^{-1}$; $k_n$, 424 $\text{s}^{-1}$; \cite{lattanzio-barteschi-1991}) was loaded into neurons and fluorescence ($F$) was recorded by CLSM controlled by a Personal Iris computer (model 4D35, Silicon Graphics, Mountain View, CA, USA). Confocality increased sampling errors that arise from the fact that dendrites were not equally in focus along their entire lengths, and hence experiments were performed in the non-confocal mode. All lines laser power (456, 488 and 514 nm) was set to 20 mW and the photomultiplier tube (PMT) voltage was adjusted to accommodate the dynamic range of change in fluorescence from basal fluorescence. The power of the 488 nm laser line that illuminated the neuron after passing through the wavelength selection filter, dichroic beam splitter, ocular, lens and slice is estimated to be less than 1 mW. In most experiments this meant that the fluorescence sampled from the soma saturated the PMT. The dendrite was scanned in the ‘line-scan mode’ where the laser beam was directed to scan along a single line along the length of the dendrite (20 ms per line scan; first 10 ms of the scan is used for data collection and the remaining 10 ms is taken to reset the scanner; 1.5 \mu m pixel spacing; pixel sample rate of 75 \mu s). Typically 128 consecutive line scans were executed to produce a scan series with dendrite distance on the y-axis, time on the x-axis and fluorescence in PMT units (0–255) on the z-axis. PMT values are referred to as ‘F’ or ‘fluorescence’ in the text. The apical dendrite was scanned up to a distance of 170 \mu m away from the soma. Stimuli to neurons were applied after 500 ms of basal fluorescence ($F_{basal}$) recording ($F_{basal}$ for each pixel point along the dendrite). Typical autofluorescence values were 3–9 PMT units while $F_{basal}$ values averaged from the proximal apical dendrite ranged from 60 to 120 PMT units.

The maximal changes in fluorescence recorded following phototoxic damage caused by long (60 s) exposures of the dendrite to the laser beam or following application of 20 mM K+ and 10 \mu M N-methyl-d-aspartate to the bath was greater than 100% \DeltaF. The changes in [Ca2+] measured are therefore below the [Ca2+] that may have saturated the indicator dye. Bleaching of the indicator dye during the 128 line scans was evident in about 20% of experiments (1–2% \DeltaF per second). In these experiments a control scan series without stimulation was used to correct for bleaching. The amplitude of the \DeltaF transient recorded in 4 mM MgCl2 and 4 mM CaCl2 was about 80% of the amplitude of the \DeltaF transient recorded in 1 mM MgCl2 and 2.5 mM CaCl2 (data not shown).

Off-line analysis of fluorescence transients

Binary images were transferred to either a VME-bus computer (Motorola, Delta series 1147, Tempe, AR, USA) where the entire image (each pixel point along the dendrite) was normalized to $F_{basal}$ (\% \DeltaF = $([F - F_{basal}] / F_{basal}) \times 100$) for display purposes (and to determine the rise time of Ca2+ entry) or to an Apple Macintosh computer for off-line analysis of fluorescence records. Images were imported into the NIH (National Institutes of Health, Bethesda, MD, USA) image program, the fluorescence values of dendritic regions of interest (see below) were measured and wave data were reimported into and analysed with Igor software (Igor Wave metrics, Lake Oswego, OR, USA). Single exponentials were fitted from the peak of a \DeltaF transient to the last point in the scan. In some cases either two to five separate scans were averaged or the dendritic region analysed was subdivided into sections and the sections were averaged individually. In most experiments fluorescence records were normalized to $F_{basal}$, by \% \DeltaF = $([F - F_{basal}] / F_{basal}) \times 100$. In experiments where $F_{basal}$ changed after treatment, fluorescence records were normalized to control (pre-treatment) fluorescence records. Both former and latter representations of the fluorescence records are referred to in the text as ‘\DeltaF transient’.

Dendritic fluorescence transient analysis

The entire length of the dendrite (166.4 \mu m) was seldom perfectly sampled whereby the dendrite was within the scan path of the laser beam and equally focused for the entire length. A restricted scan could not be performed and the scan series therefore contained variable lengths of inaccurately sampled dendrite. Only dendritic regions that were focused and where the laser scan path was within 1 pixel spacing (1.3 \mu m) from the edge of the dendrite were included in the analysis. Dendritic regions analysed were around 50–100 \mu m in length in about 80% of the experiments and below 50 \mu m or above 100 \mu m in 20% of the experiments. Selected dendritic regions were still characterized by small regions (1–10 \mu m) where the baseline fluorescence was variable. While a gradual decrease in baseline fluorescence is typical from the soma to the distal dendrite as a result of volume changes, local variations were most likely to have been due to changes in sampling from the centre to the edge of the dendrite. Peaks of the \DeltaF transients were normalized for these baseline fluctuations (see above). The effect of this sample fluctuation on the decay time constant of the \DeltaF transient of the selected dendritic region was analysed by subdividing the area into four to eight subsections (11–7–5.2 \mu m) and determining the decay time constants of \DeltaF transients of these subsections. The decay time constant of the mean \DeltaF transient of a dendritic region selected showed a standard deviation of 8.6 ± 3.9% (mean ± s.d.; n = 9) and the decay time constant of the mean \DeltaF transient was 8.9 ± 5.1% below the decay time constant of the \DeltaF transient with the slowest decay (assuming the slowest decay reflects optimal sampling). The \DeltaF transient decay time constant may therefore be underestimated by about 10% using this method. The same dendritic region was used in experiments investigating changes induced by pharmacological agents or by temperature.

Blockade of voltage-activated Ca2+ channels

Nifedipine (Calbiochem; 20 \mu M), \omega-conotoxin GIVA (Peptide Institute (PI), Osaka, Japan; 5 \mu M), \omega-agatoxin IVA (PI; 1 \mu M) and CdCl2 (Sigma; 500 \mu M) were used to block Ca2+ channels. Somatic whole-cell recordings were obtained in this set of experiments. The time required for optimal loading of the proximal ~150 \mu m of the dendrite with CG-1 was less than 10 min (plateau in $F_{basal}$ and peak of the \DeltaF transient). Neurons were therefore loaded with indicator dye for 15 min and then antagonists were applied sequentially in an additive manner within a further 15–20 min. The concentration of the blocker was elevated to the desired level within 10 s by rapid superfusion of 0.5 mM antagonist, at twice the desired concentration, directly over the slice (bath volume was ~0.5 ml), followed by continuous superfusion (~0.3 ml min$^{-1}$) with the desired concentration. In a separate population of neurons (20 \mu M nifedipine; n = 5), the maximal blocking effect using this application system was found to occur within 1 min. The transient (two to five trials averaged) was therefore measured after 1–2 min of application. The \DeltaF transient of the proximal 100–170 \mu m of the apical dendrite was analysed (80–100 \mu m dendritic regions are represented in each experiment). For quantification of the peak of a \DeltaF transient three points around
the peak were averaged. The component of the $\Delta F$ transient that was blocked by a compound was calculated by subtracting the measured $\Delta F$ transient after a compound from the measured $\Delta F$ transient before. To determine the significance of blocker effects, the amplitude of the control $\Delta F$ transient was paired with the amplitude of the $\Delta F$ transient after application of a blocker. The $\Delta F$ transient after application of a blocker was calculated by subtracting the relevant component of the $\Delta F$ transient from the control $\Delta F$ transient.

Errors in the estimates of Ca$^{2+}$ channel subtypes contributing to the $\Delta F$ transient may have arisen from four major sources. Firstly, the higher [Ca$^{2+}$]$_i$ and [Mg$^{2+}$]$_i$ were effective in reducing background synaptic activity, but may have affected the action of blockers. Calcium channel blockers were therefore used at above saturating concentrations. Secondly, high concentrations of blockers may have caused partly non-specific inhibition and hence may have caused an over- or underestimation of some components, depending on the order in which the inhibitors were added. Blockers were applied in rotating order in an attempt to minimize this effect. Thirdly, although a small effect on the duration of the AP is not likely to cause large changes in the $\Delta F$ transient (see the effect of TEA for comparison) such an effect could not be excluded. Fourthly, since the peak of the [Ca$^{2+}$]$_i$ transient is not likely to be represented by the peak of the $\Delta F$ transient, the quantitative effects of Ca$^{2+}$ channel blockers represent only approximations. Finally, the results rest on the assumption that drug action is uniform along the length of the dendrite, which may not necessarily be the case.

Other compounds used
Thapsigargin (Calbiochem; 5 $\mu$M) was used in the superfusate and was placed in the pipette for local application. Cyclopiazonic acid (CPA; Sigma; 30 $\mu$M), carboxyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP; Sigma; 10 $\mu$M), benzamil-HCl (RBI Biochemicals, Köln, Germany; 200 $\mu$M), 6-cyano-7-nitroquinolinoxaline-2,3-dione (CNQX; Toecis Neuramin, Bristol, UK; 20 and 50 $\mu$M) and d(-)-2-amino-5-phosphonopentanoic acid (d-AP5; Toecis; 50 and 100 $\mu$M), tetrodotoxin (TTX; Sigma; 1 $\mu$M) were bath applied. Heparin (Sigma; 150 units mg$^{-1}$; 7 mg ml$^{-1}$), Ruthenium Red (Sigma; 100 $\mu$M) and calmidazolium (Calbiochem; 72 $\mu$M) were placed into the pipette solution. TEA-Cl (Aldrich, Steinheim, Germany; 10 mM) was placed in Tyrode solution and the NaCl concentration was reduced accordingly by 10 mM. Thapsigargin, CPA and FCCP were dissolved in dimethyl sulphoxide (DMSO) and diluted in external solution, just before the experiment, such that the DMSO content was 0.05% of the superfusion solution. DMSO at 0.1% had no effect on the [Ca$^{2+}$]$_i$ transient ($n = 3$).

RESULTS

Single back-propagating action potentials evoke a transient rise in dendritic [Ca$^{2+}$]$_i$

Synaptically generated dendritic [Ca$^{2+}$]$_i$ transients
Action potentials evoked by synaptic stimulation in neocortical layer V pyramidal neurons are initiated in the axon initial segment and then back-propagate into dendrites (Stuart & Sakmann, 1994). As the AP invades the apical dendrite, at least as far as 500 $\mu$m from the soma, its amplitude decreases (~10% per 100 $\mu$m) and its duration increases (~20% per 100 $\mu$m) (Stuart & Sakmann, 1994). To study the determinants of the peak amplitude and time course of the dendritic [Ca$^{2+}$]$_i$ evoked by a single back-propagating AP we performed whole-cell voltage recordings from the soma and apical dendrite and loaded the neuron with the Ca$^{2+}$ indicator dye CG-1. Figure 1.4a shows a dendritic and a somatic pipette sealed to a neuron loaded with CG-1. The fluorescence ($F$) of the dendritic region indicated by the two arrows is shown in Fig. 1.4b and the normalized fluorescence ($\Delta F$) in Fig. 1.4c. An AP was evoked by electrically stimulating afferents in layer II–III and was recorded first in the soma and then, with a delay of 600–700 $\mu$s per 100 $\mu$m, in the dendrite ($n = 9$) similar to the results obtained when dendrites are filled with Ca$^{2+}$ buffering solution (Stuart & Sakmann, 1994). Following an AP, the dendritic fluorescence increased transiently to a peak and then decayed to baseline in a manner temporally correlated with the AP (Fig. 1B). For the rest of the study fluorescence transients along proximal dendritic regions were averaged (see Methods).

[Ca$^{2+}$]$_i$ transient generated by APs following somatic and dendritic current injection

To examine the $\Delta F$ transient evoked by the change in the voltage caused by the AP and to exclude a possible contribution from synaptically evoked changes in fluorescence, we initiated single APs in the soma. Figure 2A shows the $\Delta F$ transient; the APs that produced this $\Delta F$ transient were initiated in the soma–axon and recorded in soma and dendrite as shown in Fig. 2B and on an expanded time scale in Fig. 2C. An AP-evoked $\Delta F$ transient was recorded in all sixty-eight neurons examined. The neurons were located within 50 $\mu$m of the surface of the slice and the amplitude of the $\Delta F$ transient decreased markedly when neurons deeper into the slice were examined ($n = 5$). A similar $\Delta F$ transient was also measured when a back-propagating AP was evoked by current injection through the dendritic pipette ($n = 135$). The peak of the single AP-evoked $\Delta F$ transient ranged from 8 to 20% $\Delta F$ (mean, 13% $\Delta F$; $n = 50$) in CG-1-loaded neurons and from 36 to 69% $\Delta F$ (mean, 52% $\Delta F$; $n = 9$) in fluo-3-loaded neurons.

In two-pipette recordings from the same neuron, current injection into the dendrite evoked the AP first near the somatic pipette and then at the dendritic pipette ($n = 9$), suggesting that back-propagation also occurred following current injection into the dendrite (see also Stuart & Sakmann, 1994). The dendritic $\Delta F$ transient evoked by somatic current injection was abolished by the Na$^+$ channel blocker TTX (1 $\mu$m; $n = 7$), supporting the view that Na$^+$ channel-dependent APs triggered Ca$^{2+}$ entry. The $\Delta F$ transient did not result from synaptic feedback, since the AP-evoked $\Delta F$ transient persisted when the slice was perfused with the AMPA and NMDA receptor blockers CNQX and d-AP5, respectively ($n = 6$).
Dendritic calcium transients

Figure 1. Time course of ΔF transient correlated with time course of back-propagating AP evoked by synaptic stimulation

A a, a layer V pyramidal neuron loaded with 50 μM CG-1 from both dendritic and somatic pipettes. The image represents a partial, maximal intensity, confocal reconstruction of the neuron. A b, dendrite in the region between the arrows was scanned 128 times at a sampling rate of 20 ms per sweep to produce a scan series that represents the fluorescence (F) along the dendrite (y-axis) with time (x-axis). Afferents in cortical layer II–III were electrically stimulated 500 ms after the start of the scan to produce an AP, which was recorded simultaneously in the soma and dendrite (lower traces; d, dendrite; s, soma; APs are truncated for display). An increase in fluorescence is from dark towards light on the grey-scale. A c, the scan series shown in A b was normalized with respect to Fbasal (average F 500 ms before the stimulus for each pixel position along the dendrite) and is referred to as a ΔF transient. The amplitude of the ΔF transient is quantified in terms of % ΔF which is represented by a progressive change towards white on the grey-scale. A b and A c are shown to illustrate the scan series in the raw fluorescence form and after normalization (for graphical quantification of amplitude see B). Scale bar represents distance along the dendrite (20 μm) and applies to A a–c. B, the normalized scan series in A c presented three-dimensionally. The arrow points to dendritic locations away from the soma up along the dendrite. C, the somatic and dendritic membrane potentials recorded simultaneously with the ΔF transient are shown in the Vm traces. The lines drawn from part B indicate the time period represented by the voltage trace in C. Bath temperature was 24 °C.
Time course of AP-evoked dendritic $[\text{Ca}^{2+}]_i$ transients

Rise time course
To examine the $\Delta F$ within a few milliseconds following the AP, the fluorescence at different time points with respect to the onset of the AP was measured. A region of the dendrite (4-8 $\mu$m) was selected and aligned such that the CLSM samples this region at a defined time in the scan cycle (Fig. 3A and B). This time point was correlated off-line with the time from the AP onset. At 24°C, the fluorescence reached a 0–90% peak $\Delta F$ within 6–8 ms ($n = 5$) in CG-1-loaded neurons. To examine the extent to which the time constant for equilibrium of the indicator influenced the time for the fluorescence to peak, comparable experiments were performed with fluo-3, which has a 2.5-fold smaller time constant. The 0–90% rise time in fluo-3-loaded neurons ranged from 5.5 to 6.5 ms ($n = 5$) and the results were not significantly different from those measured with CG-1 (Fig. 3C). At 35°C the 0–90% time for the $\Delta F$ to peak was 1.5–2.5 ms in five fluo-3-loaded neurons, suggesting that the AP-evoked Ca$^{2+}$ influx lasted less than 1 or 2 ms. The temperature coefficient ($Q_{10}$) for the rise time of the fluorescence is approximately 3.

Decay time course
At 20–22°C the $\Delta F$ transient relaxed to basal levels with a time constant of $348 \pm 14$ ms ($n = 50$; dendrites loaded with 50 or 100 $\mu$M CG-1). Since VACCs would have

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**Figure 2.** Time course of $\Delta F$ transient evoked by back-propagating AP evoked by somatic depolarization

A, scan-series representing the normalized $\Delta F$ transient before, during and after an AP was evoked by somatic current injection. Vertical orientation ($y$-axis or dendritic distance) of the scan series indicated by the dotted line in the schematic diagram on the right. Positions of dendritic and somatic pipettes are also illustrated. Scale bar represents 20 $\mu$m of apical dendrite. B, APs recorded simultaneously in soma (s) and dendrite (d). C, same records as in B on an expanded time scale. The lines from B indicate the time period of the voltage trace in C. Neuron loaded with 50 $\mu$M CG-1, 24°C.
Figure 3. Rise time course of AP-evoked ΔF transients

A, dendritic region analysed. The image represents a partial surface-shaded confocal reconstruction of the dendrite and may overestimate the diameter of the dendrite; scale bar, 20 μm. A dendritic region of 4-8 μm in length that was located 120 μm distal to the soma was analysed (468 μs required to record F). Each data point was collected about once every minute (15–25 data points per neuron). B, the depolarizing pulse was triggered in increments of 1 ms (+ or −) with respect to the scan onset to initiate an AP earlier or later. The onset of each scan occurs on the falling flank of the scan signal. The time difference from one scan signal (e.g. the 12th scan signal) to the onset of an AP (three downward arrows) is measured off-line to determine the time before/after the AP that the dendritic region (described in A) was analysed. Where the scan onset occurred after an AP, 250 μs was added to the measurements and subtracted where the scan onset occurred before to partly correct for the 468 μs required to sample the dendrite. C, rise time course of ΔF at 24 °C. The fluorescence values were normalized to the maximum fluorescence obtained in the second line scan to facilitate comparison of the rise time course between fluo-3 and CG-1 experiments. The data from representative fluo-3- (○) and CG-1-loaded (●) neurons were binned in 0.5 ms intervals and the mean F is plotted as a function of time with respect to the AP onset. A single exponential is fitted from the data point at t = 0 to the last data point (continuous line). The exponential is extrapolated to zero at t = 0. The 0–90% rise time is 6 ms for fluo-3 and 7 ms for CG-1. D, rise time of ΔF at 35 °C. The data from a representative cell at 35 °C is shown. The fluorescence values were normalized with respect to F_{basal}. The AP that initiated the fluorescence response is normalized to the maximum ΔF. The 0–90% rise time is 2 ms.
opened and closed within a few milliseconds of the AP (Llinás, Sugimori & Simon, 1982), the relatively slow decay of the $\Delta F$ transient probably reflects dendritic Ca\(^{2+}\) clearance mechanisms. The decay of the AP-evoked $\Delta F$ transient may be prolonged if an increase in [Ca\(^{2+}\)]\(_i\) induces Ca\(^{2+}\) release from intracellular stores. To determine the possible contribution of Ca\(^{2+}\)-induced Ca\(^{2+}\) release to the $\Delta F$ transient, two potential receptors on which Ca\(^{2+}\) may act to evoke Ca\(^{2+}\) release, inositol trisphosphate (IP\(_3\)) and ryanodine receptors, were blocked. Neurons were loaded with heparin to block the IP\(_3\) receptor (7 mg ml\(^{-1}\); Worley, Baraban, Supattapone, Wilson & Snyder, 1987) or with Ruthenium Red (100 $\mu$M; McPherson et al. 1991) to block the ryanodine receptor. The decay time constant of the $\Delta F$ transient was unaffected in heparin-loaded (385 $\pm$ 56 ms; $n = 3$; not shown) or in Ruthenium Red-loaded neurons (331 $\pm$ 31 ms; $n = 5$). The decay of the $\Delta F$ transient therefore represents the decay of dendritic [Ca\(^{2+}\)]\(_i\) levels to basal levels. The release of Ca\(^{2+}\) from intracellular stores is negligible.

The decay time constant for the relaxation of the $\Delta F$ transient decreased to 98 $\pm$ 15 ms at 34 $^\circ$C in six experiments (Fig. 4A), the $Q_{10}$ of the decay time constant being 2-6-3-1 (mean 2-8; Fig. 4B). The $Q_{10}$ of the $k_b$ (1-52) of the indicator dye is less than that of the $\Delta F$ transient ($k_b$, $Q_{10} = 1-65$; $K_d$, $Q_{10} = 0-92$) (Eberhard & Erne, 1991). In neurons where the temperature was decreased from 34 to 24 $^\circ$C ($n = 6$) the measured peak of the $\Delta F$ transient increased by about 10% (Fig. 4A). This may be due to a slowing of the onset of clearance mechanisms which may enable [Ca\(^{2+}\)]\(_i\) to rise higher or the low temperature may enable a more accurate measurement of the peak.

A [Ca\(^{2+}\)]\(_i\) indicator dye necessarily interacts with Ca\(^{2+}\) entering the dendrite. The extent to which the decay of the ‘naive’ [Ca\(^{2+}\)]\(_i\) transient (in the absence of exogenous buffer) was affected by the indicator dye was therefore examined. One effect of the indicator dye, at high concentrations, is to prolong the decay of the [Ca\(^{2+}\)]\(_i\) transient (Sala & Hernandez-Cruz, 1990). When CG-1 concentration was reduced to 20 $\mu$M the decay time constant was not significantly different (355 $\pm$ 31 ms; 20–22 $^\circ$C; $n = 5$) from the decay time constant in control cells recorded with 50 or 100 $\mu$M CG-1. When the concentration of CG-1 was increased to 500 $\mu$M the decay time constant increased to 520–760 ms ($n = 11$). The decay was not significantly limited by the rate of Ca\(^{2+}\) dissociation from CG-1 (139 s\(^{-1}\) at 20–22 $^\circ$C; or a calculated time constant of about 7 ms (1/139 s\(^{-1}\)) if Ca\(^{2+}\) were removed instantaneously) since another indicator dye, fluo-3, which has a larger off-rate than CG-1, did not

![Figure 4. Decay time course of AP-evoked $\Delta F$ transients](image-url)

**A** Upper traces show averaged $\Delta F$ transients (6 cells) recorded first at 34 $^\circ$C and then at 24 $^\circ$C. The $V_m$ trace represents simultaneously recorded dendritic membrane potential from a representative neuron. The time course of current injection ($I_m$) is shown below the $V_m$ trace. Smooth curves superimposed on the decay of $\Delta F$ transients represent single exponentials. **B** Means $\pm$ s.d. of fitted time constants of $\Delta F$ transients from neurons recorded at 20 ($n = 15$), 22 ($n = 16$), 24 ($n = 6$), 29 ($n = 2$), 34 ($n = 6$) and 37 $^\circ$C ($n = 5$). Neurons were loaded with either 50 or 100 $\mu$M CG-1. The region of interest was 50–100 $\mu$m in length and more than 50 $\mu$m distal to the soma.
report a significantly faster $\Delta F$ transient (328 ± 38; $n = 9$; 20–22 °C). Indicator dyes with smaller $K_d$ values may also compete with immobile endogenous Ca$^{2+}$ buffers and thereby act as a shuttle for Ca$^{2+}$ to bypass the endogenous buffers to the clearance mechanisms (Sala & Hernandez-Cruz, 1990), and hence the decay of the $\Delta F$ transient may become more rapid. Such an effect cannot be ruled out but it appears to contribute little to the observed $\Delta F$ transient since in neurons loaded with fluo-3, which has a higher $K_d$ than CG-1, the decay of the $\Delta F$ transient was not slower than in neurons loaded with CG-1.

**Modulation of dendritic Ca$^{2+}$ inflow by channel blockers**

The experiments reported so far show that, in neurons loaded with the Ca$^{2+}$ indicator dye CG-1, an AP propagating into the apical dendrite evokes a transient change in dendritic Ca$^{2+}$ fluorescence. This suggests that the AP activated VACCs in the dendritic membrane. We therefore examined the effect of increasing the AP duration on the $\Delta F$ transient and the nature of the Ca$^{2+}$ channel subtypes that mediate the Ca$^{2+}$ inflow.

**Effect of K$^+$ channel block by TEA**

Modulation of the AP waveform may be an effective mechanism to alter the amount of Ca$^{2+}$ inflow evoked by an AP (see McCobb & Beam, 1991). To examine the effect on the $\Delta F$ transient of increasing the AP duration, the K$^+$ channel blocker TEA was applied by bath perfusion or by local perfusion of the apical dendrite. Bath-applied TEA increased the duration of dendritic APs, delayed full repolarization by 40–100 ms and increased the peak of the $\Delta F$ transient to 158 ± 21 % of control ($n = 3$; 100 μM CG-1). The effect of TEA on the peak of the AP-evoked $\Delta F$ transient was smaller than expected from the potentially large increase in the Ca$^{2+}$ current caused by an increase in the AP duration (see McCobb & Beam, 1991). To confirm that the peak was not limited by saturation of CG-1 by Ca$^{2+}$, the experiments were repeated in neurons loaded with the indicator dye fluo-3, which has a higher $K_d$ than CG-1. In these experiments TEA increased the peak of the $\Delta F$ transient to 166 ± 11 % of control (Fig. 5; $n = 6$; 100 μM fluo-3; $P < 0.05$; Student's paired $t$ test). In neurons loaded with either CG-1 or fluo-3 the peak of the $\Delta F$ transient was also prolonged by TEA, which

![Figure 5. Effect of K$^+$ channel block by TEA on the AP-evoked ΔF transient](image)

Average $\Delta F$ transients measured in control neurons, during application of TEA (10 mM) and after wash (upper traces). Averages of 6 neurons loaded with 100 μM fluo-3. Records of representative APs ($V_m$) before and during TEA application on the same time scale as the $\Delta F$ transients are superimposed (middle traces). Same records on an expanded time scale are shown in the lower traces. 20–22 °C. The region of interest was 50–100 μm in length and more than 50 μm distal to the soma.
resulted in longer half-decay times for the $\Delta F$ transient (up to 2 times longer than control).

While bath application had a marked effect on the AP recorded in the dendrite ($n = 5$) and in the soma ($n = 3$), local application of TEA to the dendrite (20 mM) did not broaden the dendritic AP, nor did it increase the local dendritic $\Delta F$ transient ($n = 4$). The broadened AP recorded in the dendrite following bath application of TEA does not necessarily suggest that TEA blocked K$^+$ channels on the dendritic membrane since the AP also represents a passive component spreading electronically from the soma. On the other hand, the lack of an effect on the dendritic AP following local application of TEA does not imply that TEA did not block K$^+$ channels on the dendrite since local AP broadening may have been shunted by the normal AP in the rest of the neuron.

**Effect of Ca$^{2+}$ channel block**

In most neurons, Ca$^{2+}$ entry across the surface membrane is mediated by several subtypes of VACCs. To find out which channels mediate the AP-evoked increase in dendritic $[\text{Ca}^{2+}]_i$, we measured the amplitude of the $\Delta F$ transient before and after application of selective Ca$^{2+}$ channel blockers. $F_{\text{base}}$ and the $\Delta F$ transient were stable for more than 40 min ($91 \pm 2\%$ of control; $n = 8$) as illustrated in

![Figure 6. Effect of Ca$^{2+}$ channel blockers on AP-evoked $\Delta F$ transient](image)

A, single AP-evoked $\Delta F$ transients at different time intervals after establishing whole-cell recording with the somatic pipette demonstrates stability of $\Delta F$ transients. B, effect of successive and additive applications of Ca$^{2+}$ channel blockers on $\Delta F$ transient. Records following application of $\omega$-agatoxin IVA (ATX; 1 $\mu$M), $\omega$-conotoxin GVIA (CgTX; 5 $\mu$M), nifedipine (Nif; 20 $\mu$M) and Cd$^{2+}$ (500 $\mu$M) and after wash are shown. C, records of APs recorded simultaneously with $\Delta F$ transients in B. Neuron was loaded with 100 $\mu$M CG-1 from somatic pipette for 15 min before compounds were applied (20–22 °C). The dendritic region of interest was 30–70 $\mu$m in length and 40–100 $\mu$m distal to the soma.
Fig. 6A. In seven experiments, we applied, in an additive manner and in rotating order (see Methods), nifedipine to block L-type, \(\omega\)-conotoxin GVIA to block N-type and \(\omega\)-agatoxin IVA to block P-type Ca\(^{2+}\) channels (Zhang et al. 1993). The recently reported Q- and R-type channels may also have been affected at the concentration of \(\omega\)-agatoxin IVA and \(\omega\)-conotoxin GVIA used, respectively (Zhang et al. 1993). Cadmium ions (500 \(\mu\)M) applied in four of these experiments blocked the remaining \(\Delta F\) transient. Figure 6B illustrates the decrease in amplitude of the \(\Delta F\) transient during successive applications of these blockers in one experiment.

The L-, N- and P-type Ca\(^{2+}\) channel blockers did not have a significant effect on \(R_{\text{total}}\), while Cd\(^{2+}\) reversibly reduced it by 10–30\% (\(P < 0.05\); paired \(t\) test). Neither the resting \(V_m\) nor the time course of the dendritic AP were significantly affected by the blockers (Fig. 6C). The L-, N- and P-type Ca\(^{2+}\) channel blockers had no consistent effect on AP frequency accommodation individually but when applied together, accommodation was reduced. Cadmium ions caused a clear reduction in accommodation in all experiments.

We determined the contribution of different Ca\(^{2+}\) channel subtypes by subtracting the \(\Delta F\) transient recorded after a given application of a blocker from the \(\Delta F\) transient recorded before and averaging the differences between these \(\Delta F\) transients (Fig. 7). The N-component (that portion of the \(\Delta F\) transient removed by \(\omega\)-conotoxin GVIA) had an amplitude of 28 ± 3\% of control (Fig. 7A; \(P < 0.05\); paired \(t\) test). The L-component was 25 ± 3\% of the peak of the \(\Delta F\) transient (Fig. 7B; \(P < 0.05\)) and the P-component was 10 ± 3\% (Fig. 7C; \(P < 0.05\)). The residual R-component (the component insensitive to L-, N- \& P-type Ca\(^{2+}\) channel blockers and mediated by other Ca\(^{2+}\) channels) was 37 ± 4\% of the control \(\Delta F\) transient (Fig. 7D; \(P < 0.05\)). The decay time constants of the averaged N- and P-type channel components were almost 2-fold larger than those mediated by L- and R-type channels (Fig. 7A and D).

Duration of the [Ca\(^{2+}\)]\(_{\text{cyt}}\) transient is determined by Ca\(^{2+}\) clearance

The duration of the \(\Delta F\) transient appears to be determined by dendritic Ca\(^{2+}\) clearance mechanisms, because (as described above) the decay time constants were unaffected by substances that block release of Ca\(^{2+}\) from intracellular stores. These clearance mechanisms were examined with blockers of Ca\(^{2+}\) extrusion to the extracellular space or of Ca\(^{2+}\) uptake into storage organelles.

**Plasma membrane Na\(^{+}\)-Ca\(^{2+}\) exchange**

Benzamil (200 \(\mu\)M), a compound that blocks the plasma membrane Na\(^{+}\)-Ca\(^{2+}\) exchange (Kaczorowski, Barros, Dethmers & Trumble, 1985), increased the decay time constant of the \(\Delta F\) transient from 322 ± 49 to

![Figure 7. Several Ca\(^{2+}\) channel subtypes mediate AP-evoked dendritic \(\Delta F\) transient](image-url)
515 ± 83 ms (Fig. 8A; n = 5; 2 min after application; P < 0.05; paired t test). The decay rate in the presence of benzamil was similar 15 min after application. The peak of the averaged ΔF transient was reduced by 7% in the presence of benzamil (not shown). Benzamil had no significant effect on Fbasal (as determined from the scans in which the ΔF transient was recorded; Fbasal was not monitored continuously), the amplitude or the duration of the dendritic AP. The compound did, however, cause a transient depolarization of 2–3 mV in four of five experiments. Benzamil also increased the input resistance, measured by the voltage response to a current pulse via the dendritic pipette, by 5–30% in three experiments. A marked effect of benzamil was an enhancement of AP frequency accommodation. The effects of benzamil on the decay of the ΔF transient and on AP frequency accommodation were partially reversed following a 10 min wash. While benzamil is a relatively selective blocker for the plasma membrane Na⁺–Ca²⁺ exchange, it cannot be ruled out that the effect on the decay of the ΔF transient was secondary to another effect of benzamil. Nevertheless, it does appear that dendrites are still capable of rapidly clearing Ca²⁺ that entered during a single AP when the Na⁺–Ca²⁺ exchange is blocked.

**Plasma membrane Ca²⁺-ATPase**

To determine the contribution of the Ca²⁺-ATPase on the plasma membrane, five neurons were loaded via the recording pipette with calmidazolium (72 μM), a potent inhibitor of calmodulin-activated enzymes such as this Ca²⁺-ATPase (Van Belle, 1981). Resting Vm, input resistance, and APs were recorded; Fbasal was not monitored continuously, the amplitude or the duration of the dendritic AP. The compound did, however, cause a transient depolarization of 2–3 mV in four of five experiments. Benzamil also increased the input resistance, measured by the voltage response to a current pulse via the dendritic pipette, by 5–30% in three experiments. A marked effect of benzamil was an enhancement of AP frequency accommodation. The effects of benzamil on the decay of the ΔF transient and on AP frequency accommodation were partially reversed following a 10 min wash. While benzamil is a relatively selective blocker for the plasma membrane Na⁺–Ca²⁺ exchange, it cannot be ruled out that the effect on the decay of the ΔF transient was secondary to another effect of benzamil. Nevertheless, it does appear that dendrites are still capable of rapidly clearing Ca²⁺ that entered during a single AP when the Na⁺–Ca²⁺ exchange is blocked.

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**Figure 8. Effect of blocking plasma membrane extrusion and mitochondrial uptake of Ca²⁺ on time course of ΔF transient**

A, average ΔF transients (n = 5) before and after block of the Na⁺–Ca²⁺ exchange by benzamil (200 μM) are superimposed. Star designates ΔF transient recorded in the presence of blocker. B, average ΔF transient (n = 5) of neurones loaded with a blocker of the plasma membrane Ca²⁺-ATPase, calmidazolium (72 μM). Control ΔF transient (average of records from 50 control neurons) is superimposed. C, average ΔF transients (n = 5) before and after application of mitochondrial uncoupler, FCCP (10 μM). Cb, average ΔF transient (n = 5) in neurons loaded with Ruthenium Red (100 μM), a blocker of mitochondrial Ca²⁺ uptake. Control ΔF transient is superimposed. Neurons were loaded with either 50 or 100 μM CG-1 (20–22 °C). The smooth curves superimposed on the decay of the ΔF transients represent fitted single exponentials. All traces represent single AP-evoked ΔF transients. Neurons were loaded and APs were evoked via the dendritic pipette in most neurons and in about 25% of neurons by a somatic pipette (the effect of compounds was not dependent on location of loading pipette). Peaks of ΔF transients are normalized to those of control ΔF transients. The dendritic region of interest was 40–100 μm in length and 50–100 μm distal to the soma.
resistance, AP amplitude and duration and AP frequency accommodation were comparable to those of control neurons. The decay time constant of the $\Delta F$ transient was, however, increased (Fig. 8B; 505 ± 63 ms compared with 348 ± 14 ms in control neurons; $P < 0.05$; independent $t$ test; 10–20 min after establishing whole-cell recording conditions). This was the maximal effect of calmidazolium because the decay was not prolonged further after 1 h of whole-cell recording. Since the effect of calmidazolium may have been mediated by the blockade of other calmodulin-dependent enzymes, it is not certain that the plasma membrane Ca$^{2+}$-ATPase is involved in clearing dendritic Ca$^{2+}$. What the result does indicate, however, is that the dendrite is still capable of clearing Ca$^{2+}$ rapidly when the Ca$^{2+}$-ATPase on the plasma membrane is inactivated.

**Mitochondrial Ca$^{2+}$ uptake**

The role of mitochondrial Ca$^{2+}$ uptake in clearing Ca$^{2+}$ from the dendritic cytoplasm was examined by bath applying a mitochondrial uncoupler, carbonyl cyanide $p$-(trifluoromethoxy)phenylhydrazone (FCCP; Jenson & Rehder, 1991) or loading dendrites with Ruthenium Red, via the pipette, to block Ca$^{2+}$ uptake (Moore, 1971). The application of FCCP (10 $\mu$M) had no effect on $V_m$, AP shape, input resistance, AP frequency accommodation or the $F_{basal}$ and peak of the $\Delta F$ transient in five experiments. The decay time constant of the $\Delta F$ transient was also unaffected (Fig. 8C; $337 \pm 30$ ms in control vs. $373 \pm 51$ ms after FCCP). Loading neurons with Ruthenium Red (100 $\mu$M) was also without effect on membrane properties and on the [Ca$^{2+}$]$_i$ transient in five experiments (Fig. 8C). The lack of effect of FCCP and Ruthenium Red on the decay of the $\Delta F$ transient suggests that mitochondria do not participate in clearing Ca$^{2+}$ from the dendritic cytoplasm following inflow after a single AP.

**Endoplasmic reticulum Ca$^{2+}$-ATPase**

The endoplasmic reticulum (ER) may serve as a Ca$^{2+}$ store and intracellular source of Ca$^{2+}$ (Duce & Keen, 1978; Neering & McBurney 1984). Calcium ions are taken up into ER by means of a specific Ca$^{2+}$-ATPase (see Henzi & MacDermott, 1992). We therefore examined the importance of the ER in clearing dendritic Ca$^{2+}$ by applying two relatively specific blockers of the ER Ca$^{2+}$-ATPase, cyclopiazonic acid (CPA; Seidler, Jona, Vegh & Martonosi, 1989) and thapsigargin (Thastrup, Cullen, Drobsak, Hanley & Dawson, 1990).

Bath application of CPA (30 $\mu$M) had no effect on $V_m$ or on AP shape in nine experiments but increased input resistance by 16 ± 3% in six experiments, enhanced AP frequency accommodation in two experiments and blocked accommodation in one experiment. CPA increased the decay time constant of the $\Delta F$ transient to 208% of control (Fig. 9A; 382 ± 40 to 795 ± 77 ms; $n = 9$; $P < 0.05$; paired $t$ test). This effect of CPA on the $\Delta F$ transient was partially reversed upon washing (546 ± 39 ms; $P < 0.05$; paired $t$ test). $F_{basal}$ at the time when the $\Delta F$ transient was measured (about 3 min after application) was higher than in control (89 ± 11 PMT units in control vs. 111 ± 13 PMT units after CPA; $P < 0.05$; paired $t$ test). Since $F_{basal}$ was not monitored continuously, a transient increase in the [Ca$^{2+}$]$_i$ may have occurred undetected.

Bath application of thapsigargin (5 $\mu$M) had no effect on membrane properties but caused a 2- to 3-fold increase in the decay time constant of the $\Delta F$ transient in three experiments (Fig. 9B; 394 ± 25 ms in control vs. 1048 ± 176 ms after thapsigargin; $P < 0.05$; paired $t$ test). The effect of thapsigargin on the $\Delta F$ transient was not reversible. $F_{basal}$ at the time that the $\Delta F$ transient was measured was increased (102 ± 12 PMT units in control vs. 136 ± 21 PMT units; $P < 0.05$; paired $t$ test).

To limit the exposure of the dendrite to UV light, long lasting line scans were not carried out and complete decay to baseline was not recorded in the presence of CPA or thapsigargin. The effect of these compounds may therefore be underestimated. The peak of the $\Delta F$ transient was reduced 20–50% by thapsigargin and was increased 10–20% by CPA (not shown). One possible explanation for the effects on the peak may be that CPA enabled the $\Delta F$ transient to reach higher levels by blocking the fastest clearance mechanism and that this effect could not be seen with thapsigargin because of a non-specific effect on VACs.

To confirm that dendritic Ca$^{2+}$-ATPase was important for Ca$^{2+}$ clearance, thapsigargin was applied locally to the dendrite and the effect on the $\Delta F$ transient examined (Fig. 9Ca–d). The control response on both sides of the recording pipette are shown in Fig. 9Cc and Fig. 9Cd. An application pipette containing thapsigargin (5 $\mu$M) was then lowered to position 2 (Fig. 9Ca) and thapsigargin was applied locally to the dendrite under visual control. An AP was initiated within 1–2 min of application. The resulting $\Delta F$ transient at positions 1 and 2 are shown in Fig. 9Cc and Cc, respectively. The decay of the $\Delta F$ transient was prolonged only in the region where thapsigargin was applied (position 2). $F_{basal}$ was raised by 17% in position 2. When the $\Delta F$ transient was examined further from the application pipette, the decay rate was found to be progressively faster (not shown). After about 5 min of local thapsigargin application, the decay of the $\Delta F$ transient at position 1 was also prolonged. These effects of local thapsigargin application were similar in each of three experiments.

**Dendritic [Ca$^{2+}$]$_i$ transients during physiological electrical activity**

At physiological temperature, the duration of the dendritic $\Delta F$ transient is about two orders of magnitude larger than the duration of the AP. This suggests that during bursts of APs at high impulse rates, the [Ca$^{2+}$]$_i$
Figure 9. Effect of blockers of endoplasmic reticulum Ca\textsuperscript{2+}-ATPase on time course of \(\Delta F\) transient

A, bath application of CPA prolongs the decay of AP-evoked \(\Delta F\) transient. Average \(\Delta F\) transients of 9 neurons. B, bath application of thapsigargin prolongs decay of the \(\Delta F\) transient. Average \(\Delta F\) transients of 3 neurons. Ca, photomicrograph of apical dendrite with whole-cell recording pipette sealed to dendrite (dendritic pipette) for loading of dendrite with CG-1 and with application pipette containing thapsigargin. Application pipette was lowered close to the dendrite after control scan. About 3 mbar of positive pressure was used to apply thapsigargin locally. Cb, schematic diagram shows location of recording and application pipettes with respect to the cell body and the dendritic positions (1 and 2) where the \(\Delta F\) transient was measured. Cc, superimposed \(\Delta F\) transients measured in dendritic position 1 before and during thapsigargin application. The star indicates the record obtained during thapsigargin application. Cd, same as for Cc, but applying to measurement of dendritic position 2. The smooth curves superimposed on the decay of \(\Delta F\) transients in A and B represent fitted single exponentials (20–22°C). Peak of \(\Delta F\) transients are normalized to control \(\Delta F\) transients. The dendritic region of interest was 40–100 \(\mu\)m in length and 50–100 \(\mu\)m distal to the soma.
transients evoked by individual APs would merge. The changes in dendritic fluorescence during trains of APs were therefore examined. The maximal sustained impulse rate of these neurons was measured and then the effect of trains of APs at progressively higher frequencies on the dendritic ΔF transient was examined.

The impulse rate in response to current injection, examined at 37 °C (Fig. 10A and B), showed a linear increase with a slope of about 60 APs s⁻¹ nA⁻¹ in three experiments (Fig. 10C). Higher impulse rates could not be measured, since current injection above 800 pA resulted in a transient burst of five to ten APs followed by rapid accommodation. A reasonable estimate of maximal impulse rates for these neurons therefore seems to be around 30 APs s⁻¹. The effect of driving layer V pyramidal neurons synaptically at different frequencies on the change in dendritic fluorescence is shown in Fig. 11. Single AP-evoked ΔF transients began to overlap at 5 APs s⁻¹ (Fig. 11A). At 10 APs s⁻¹ the overall overlap was considerable but the maximal %ΔF level measured was slightly higher than at 5 APs s⁻¹. At 30 APs s⁻¹, the maximal %ΔF level measured was more than twice that of the peak amplitude of the ΔF transient evoked by a single AP. The change in fluorescence was maintained for the duration of the impulse train. High frequency impulse rates caused similar fluorescence responses in dendrites loaded with either CG-1 (n = 5) or fluo-3 (n = 5; not shown).

**DISCUSSION**

The aim of this study was to examine dendritic [Ca²⁺]ₗ transients evoked by back-propagating APs. We filled neurons with fluorescent Ca²⁺ indicators and measured fluorescence transients in proximal apical dendrites evoked by APs. The results indicate that a single back-propagating AP evokes a discrete [Ca²⁺]ₗ transient in the apical dendrite of neocortical layer V pyramidal neurons where [Ca²⁺] rises to a peak within a few milliseconds of an AP and then decays back to resting level with a time constant of around 80 ms at 35–37 °C. The results further indicate that AP evoked [Ca²⁺]ₗ transients begin to merge at an impulse frequency of 5 APs s⁻¹. Above this rate, trains of APs cause a concerted and maintained elevation of dendritic [Ca²⁺]ₗ. In the following sections the cellular mechanisms which underlie the dendritic [Ca²⁺]ₗ transients are discussed.

**Rise time of [Ca²⁺]ₗ transients after a single AP**

As the AP propagates into the dendrite, it causes rapid entry of Ca²⁺. The ΔF transient measured by CG-1 and fluo-3 reaches a peak about 5–6 ms after the onset of the AP when examined at 24 °C and in about 2 ms at 35 °C in fluo-3-loaded neurons. It is likely, however, that this time to peak is an underestimate of the time taken for a rapid initial [Ca²⁺]ₗ transient to reach a peak which would escape detection by the fluorescent indicator. The

![Figure 10. Current–AP frequency relation of layer V pyramidal neuron](image)

Frequency of APs during 150 (A) and 650 pA (B) current injection through somatic pipette; 37 °C. C, relationship between size of injected current and frequency of APs. AP frequency was determined by counting the number of APs during the last 1 s of the 2 s current injection. Differences in AP amplitude may partly reflect sampling error of the faster AP time course at the higher temperature.
measured time to peak is probably also an overestimate of the duration of the Ca\(^{2+}\) influx. Nevertheless, the measured rise time course of \(\Delta F\) is consistent with an earlier proposal that most of the Ca\(^{2+}\) enters during the repolarization phase of the AP (Llinás, Steinberg & Walton, 1981).

**Decay of [Ca\(^{2+}\)]\(_i\) transient after a single AP**

The amplitude of the peak of the \(\Delta F\) transient is likely to be an underestimate of the relative size of the [Ca\(^{2+}\)]\(_i\) peak. It may, depending on the properties of endogenous Ca\(^{2+}\) buffers, reflect a quasi-equilibrium reached between Ca\(^{2+}\) and indicator when rapid Ca\(^{2+}\) inflow has ceased and the much slower clearance of Ca\(^{2+}\) has just begun. The question then arises whether the decay in \(\Delta F\) reflects the decay of [Ca\(^{2+}\)]\(_i\). The calcium indicator CG-1 undergoes a 14-fold increase in fluorescence intensity when bound to Ca\(^{2+}\). The fluorescence increase was measured as a percentage increase from basal dendritic fluorescence in an attempt to normalize for differences in \(F_{basal}\) between different neurons which may have arisen from differences in the region of the dendrite sampled, PMT voltage gain, dye concentration and basal [Ca\(^{2+}\)]\(_i\). The mean \(\Delta F\) transient peak recorded was less than 13% of the maximal change in fluorescence of the indicator dye, suggesting that the single AP-evoked [Ca\(^{2+}\)]\(_i\) reached during the peak and the decay was not in the non-linear range of the Ca\(^{2+}\)-indicator binding curve where CG-1 is saturated with Ca\(^{2+}\). Since the indicator binds free cytosolic Ca\(^{2+}\) and thus acts as an additional exogenous Ca\(^{2+}\) buffer, the decay time course of the \(\Delta F\) transient is only an approximation of changes in dendritic [Ca\(^{2+}\)]\(_i\). Exogenous buffers may distort the time course of the [Ca\(^{2+}\)]\(_i\) transient depending on the affinity, capacity and mobility of the endogenous buffers (see Sala & Hernandez-Cruz, 1990), which are unknown for dendrites at present.

Several lines of evidence suggest that the indicator dye distorted the 'naive' [Ca\(^{2+}\)]\(_i\) transient only slightly at the indicator concentration used (50 or 100 \(\mu\)M). Firstly, neither the peak nor the decay time constant changed during the time course of an experiment, suggesting that the endogenous buffer capacity of the dendrite was not significantly altered during whole-cell recording. A rapid initial washout of mobile buffers within the first 5 min after establishing the whole-cell recording configuration.

![Figure 11. Dendritic \(\Delta F\) response during high frequency synaptic stimulation](image)

*A*, the \(\Delta F\) transient (upper trace) caused by 5 Hz electrical synaptic stimulation of afferents in layer II–III evoking a train of 5 APs (\(V_m\), lower trace). *B*, dendritic \(\Delta F\) transients during 1, 5, 10, 18 and 30 Hz synaptically evoked APs; 37°C. Neuron loaded with 100 \(\mu\)M CG-1. The synaptic stimuli were delivered within 1 s (10–50 V, 50 \(\mu\)s) through a stimulating pipette placed in layer II–III. The \(\Delta F\) transients were corrected for bleaching by subtracting a control fluorescence record without stimulation. The dendritic region of interest was 40–60 \(\mu\)m in length and more than 80 \(\mu\)m distal to the soma. Vertical scale bar in *A* also applies to *B*. 
could, however, have occurred undetected. Secondly, the interaction between CG-1 and Ca\textsuperscript{2+} is rapid enough to detect an even faster \([Ca\textsuperscript{2+}]\) transient than those measured at 20–22°C. CG-1 detected faster transients when the temperature was raised (less than 80 ms decay time constant). This cannot be explained only by an increased backward rate of Ca\textsuperscript{2+} from CG-1 since the \(Q_{10}\) for the backward rate measured \textit{in vitro} is only 1.52 (Eberhard & Erne, 1991). Thirdly, the \(\Delta F\) transient recorded when dendrites were loaded with lower concentrations (20 \(\mu\text{m}\)) was not different. Fourthly, two different indicator dyes, CG-1 and fluo-3, with different rates of Ca\textsuperscript{2+} binding and unbinding showed comparable decay time constants for the \(\Delta F\) transient. It is likely, therefore, that the decay of the \(\Delta F\) transient at 20–22°C is not significantly different from that of the ‘naive’ \([Ca\textsuperscript{2+}]\) transient.

The physiological variability of the decay rate of the \(\Delta F\) transient may, however, mask small changes (up to 15%) in the decay time constant of the \(\Delta F\) transient when dye concentrations are reduced or when indicator dyes with different backward rates are used. One explanation for the comparable results when CG-1 concentration was decreased or when fluo-3 was used could be that the endogenous buffer capacity in apical dendrites of neocortical pyramidal neurons is significantly higher than, for example, \textit{Aplysia} neurons where it is 30 (Blumenfeld, Zablow & Sabatini, 1992).

**K\textsuperscript{+} channels and dendritic Ca\textsuperscript{2+} entry**

Bath application of a K\textsuperscript{+} channel blocker, TEA, broadened the dendritic AP severalfold and increased the amplitude of the dendritic \([Ca\textsuperscript{2+}]\) transient by 60–70\%. On the other hand, local application of TEA to a small segment of the dendrite did not increase the duration of the dendritic AP nor the locally measured \(\Delta F\) transient. This may be because there are fewer K\textsuperscript{+} channels in the apical dendrite than in the soma and/or because a local broadening of the AP is ‘shunted’ by the rest of the active neuronal membrane. Since K\textsuperscript{+} channel openings are observed in cell-attached recordings from dendrites (not shown) the latter possibility is the more likely. This could imply that a locally restricted activation or inactivation of dendritic K\textsuperscript{+} channels, for example by neurotransmitters, is not likely to constitute a powerful mechanism for altering the AP-evoked dendritic Ca\textsuperscript{2+} entry. Instead inactivation of K\textsuperscript{+} channels distributed throughout the neuron could be a more effective means of altering the dendritic \([Ca\textsuperscript{2+}]\) transient.

**Contribution of Ca\textsuperscript{2+} channel subtypes to peak of \([Ca\textsuperscript{2+}]\) transient**

The amplitude of \([Ca\textsuperscript{2+}]\) at the peak, relative to \([Ca\textsuperscript{2+}]\), during the slower decay, is underestimated by the peak of the \(\Delta F\) transient, depending on the time course of Ca\textsuperscript{2+} inflow and on the properties of endogenous buffers, both of which are not known at present. Changes in the peak of \(\Delta F\) transients may, however, be proportional to changes in the total amount of Ca\textsuperscript{2+} entering the dendrite (see Methods). The peak of the \(\Delta F\) transient evoked by a single AP is dependent on the opening of at least four subtypes of VACs in the dendritic membrane. Three of them are high voltage-activated (HVA) Ca\textsuperscript{2+} channels (L-, N- and P-types). The presence of both high and low VACs has been reported in the rat sensorimotor cortex (Sayer, Schwindt & Crill, 1990). Immunohistological experiments have localized L-type (Ahlijanian, Westenbroek & Catterall, 1990), N-type (Westenbroek, Hell, Warner, Dubel, Snutch & Catterall, 1992) and P-type (Hillman, Chen, Aung, Cherksey, Sugimori & Llinás, 1991) Ca\textsuperscript{2+} channel immunoreactivity in cortical neurons and it has been suggested that L-type channels are located predominantly in the soma and proximal dendrites while N-type channels are located mostly in distal dendrites (Ahlijanian \textit{et al}. 1990). Our results are not contradictory to this view, since we examined the average \([Ca\textsuperscript{2+}]\) transient in the proximal portion (100–170 \(\mu\text{m}\)) of the apical dendrite where \(L\)- and \(N\)-type channel distributions may overlap.

The functional and molecular properties of the Ca\textsuperscript{2+} channel which mediates the R-component of the \([Ca\textsuperscript{2+}]\) transient are not yet known. One possibility is that an additional subtype of HVA Ca\textsuperscript{2+} channels is present in the dendritic membrane. Inflow of Ca\textsuperscript{2+} via Na\textsuperscript{+}–Ca\textsuperscript{2+} exchange may also contribute to the R-component. However, since the Ca\textsuperscript{2+} channel antagonists applied together blocked the \(\Delta F\) transient completely, the contribution is likely to be small. Another possibility is that the R-component is mediated by a novel low voltage-activated (LVA) Ca\textsuperscript{2+} channel since an EPSP-evoked \([Ca\textsuperscript{2+}]\) transient in dendrites may amount to about 30% of the AP-evoked \([Ca\textsuperscript{2+}]\) transient (Markram & Sakmann, 1994). The R-component is probably not mediated by T-like Ca\textsuperscript{2+} channels, which are expected to be inactivated at the resting membrane potentials above \(−70\) mV (Sayer \textit{et al} 1990).

The contribution of each channel subtype to the AP-evoked \([Ca\textsuperscript{2+}]\) transient could be determined by a number of factors including channel density, and activation and deactivation kinetics. The recording conditions, such as membrane potential, may favour the detection of Ca\textsuperscript{2+} inflow through some channel subtypes and not through others. The values obtained for the relative contribution of the different Ca\textsuperscript{2+} channel subtypes may therefore be significantly different when the neuron undergoes prior hyperpolarization or prolonged depolarization, or during bursts of APs. Furthermore, if the Ca\textsuperscript{2+} channels involved have different \(Q_{10}\) values then the relative contributions of each channel type may be different at 37°C.
Function for multiple dendritic Ca\textsuperscript{2+} channels

High voltage-activated Ca\textsuperscript{2+} channels are not likely to be activated significantly at membrane potentials reached during subthreshold summation of EPSPs (below \(-40\) mV). Under physiological conditions, one function of the HVA Ca\textsuperscript{2+} channels may therefore be to transduce the signal conveyed by the back-propagating AP into a dendritic [Ca\textsuperscript{2+}] transient. On the other hand, the function of LVA Ca\textsuperscript{2+} channel(s) in dendrites is likely to extend beyond that of the HVA channels since these channels may also be activated from lower membrane potentials (around \(-50\) mV) during subthreshold EPSPs (Markram & Sakmann, 1994) and following strong hyperpolarization (Sayer et al. 1990; Deisz et al. 1991). The observation that application of extracellular Cd\textsuperscript{2+} reduces \(R_{basal}\) at resting \(V_m\) below \(-50\) mV further suggests that a persistent Ca\textsuperscript{2+} conductance may be present in dendrites. Multiple Ca\textsuperscript{2+} channel subtypes in dendrites may therefore provide a mechanism to transduce the voltage signals of EPSPs and APs into longer lasting intracellular messages depending on sub- or suprathreshold electrical activity (see also McCobb & Beam, 1991).

Clearance of dendritic Ca\textsuperscript{2+}

The decay of the \(\Delta F\) transient was analysed as a single exponential. While this exponential may reflect the largest component of the \(\Delta F\) transient, smaller components with a slower decay time constant are not excluded. The decay time constant of the \(\Delta F\) transient was about 350 ms at 20–22°C. The indicator dye would prolong the [Ca\textsuperscript{2+}] transient progressively more as the temperature is increased, and thus the \(C_0\) for the decay of the \(\Delta F\) transient is probably higher than 2–8, suggesting that the decay time constant at 37°C could be even less than 80 ms. The clearance of Ca\textsuperscript{2+} from the dendritic cytoplasm was significantly slowed when the Ca\textsuperscript{2+}-ATPase on the ER was blocked. The fact that two relatively selective blockers, thapsigargin and CPA, produced similar results supports the view that the effects on the [Ca\textsuperscript{2+}] transient caused by thapsigargin and CPA were due to a block of the ER Ca\textsuperscript{2+}-ATPase. Uptake of Ca\textsuperscript{2+} by the ER also occurs in dorsal root ganglion cells (Neering & McBurney, 1984) and sympathetic (Lipscombe, Madison, Poenie, Reuter, Tsien & Tsien, 1988) and cerebellar neurons (Brorson, Bleakman, Gibbons, Miller, 1991). Furthermore, anatomical and immunohistological studies have localized both smooth ER and Ca\textsuperscript{2+}-ATPase in dendrites of neurons (see Villa et al. 1992) and ER and Ca\textsuperscript{2+}-ATPase are located in all cortical layers (see Miller, Verma, Snyder & Ross, 1991). Dendrites of cerebellar Purkinje cells are particularly rich in ER Ca\textsuperscript{2+}-ATPase (see Villa et al. 1992) and in this context it is interesting that the decay time constant at 32–34°C of Arsenazo III-measured \(\Delta F\) transients in these neurons is also very fast (less than 50 ms; Miyakawa, Lev-Ram, Lasser-Ross & Ross, 1992).

On the time scale and amplitude of the [Ca\textsuperscript{2+}] transient evoked by a single AP the other potential clearance pathways appear to be less important. They may become more significant as ER stores fill, for example, during high-frequency electrical activity. Mitochondria, with a ‘set-point’ for Ca\textsuperscript{2+} sequestration of 0.5–1 \(\mu\)M, may also play a more significant role clearing larger [Ca\textsuperscript{2+}] loads on a much slower time scale (Werth & Thayer, 1994). A further consideration is that if the clearance mechanisms involved have different \(Q_0\) values then the relative contributions of each mechanism at 37°C may be different from that reported here.

Dendritic [Ca\textsuperscript{2+}]\textsubscript{h} changes during ‘physiological’ electrical activity

Neuronal impulse rates in the unstimulated somatosensory and visual cortical areas of the rat vary between 0 and 10 APs s\textsuperscript{-1} and during sensory stimulation increase to peak around 20–50 APs s\textsuperscript{-1} (Burne, Parnavelas & Lin, 1984; Simons, Carvell, HERSHEY & Bryant, 1992). The AP-evoked rise in dendritic [Ca\textsuperscript{2+}]\textsubscript{h} began to accumulate at impulse frequencies above 5 APs s\textsuperscript{-1} suggesting that a burst of APs evoked during sensory stimulation would cause a concerted elevation of dendritic [Ca\textsuperscript{2+}]\textsubscript{h} that would be maintained for the duration of the burst and subsequently would decay rapidly to resting levels. This elevation of dendritic [Ca\textsuperscript{2+}]\textsubscript{h} may alter the receptive and integrative properties of the dendritic compartment by Ca\textsuperscript{2+} interacting with neurotransmitter receptors and dendritic ion channels, respectively. To further determine the functional significance of AP-evoked dendritic Ca\textsuperscript{2+} influx, [Ca\textsuperscript{2+}]\textsubscript{h} transients in more distal dendrites and in spines and the effect they have on synaptic integration have to be investigated.


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