Calcium action potentials restricted to distal apical dendrites of rat neocortical pyramidal neurons

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1. Simultaneous whole-cell voltage and Ca^{2+} fluorescence measurements were made from the distal apical dendrites and the soma of thick tufted pyramidal neurons in layer 5 of 4-week-old (P28–32) rat neocortex slices to investigate whether activation of distal synaptic inputs can initiate regenerative responses in dendrites.

2. Dual whole-cell voltage recordings from the distal apical trunk and primary tuft branches (540–940 μm distal to the soma) showed that distal synaptic stimulation (upper layer 2) evoking a subthreshold depolarization at the soma could initiate regenerative potentials in distal branches of the apical tuft which were either graded or all-or-none. These regenerative potentials did not propagate actively to the soma and axon.

3. Calcium fluorescence measurements along the apical dendrites indicated that the regenerative potentials were associated with a transient increase in the concentration of intracellular free calcium ($[Ca^{2+}]_i$) restricted to distal dendrites.

4. Cadmium added to the bath solution blocked both the all-or-none dendritic regenerative potentials and local dendritic $[Ca^{2+}]_i$ transients evoked by distal dendritic current injection. Thus, the regenerative potentials in distal dendrites represent local Ca^{2+} action potentials.

5. Initiation of distal Ca^{2+} action potentials by a synaptic stimulus required coactivation of AMPA- and NMDA-type glutamate receptor channels.

6. It is concluded that in neocortical layer 5 pyramidal neurons of P28–32 animals glutamatergic synaptic inputs to the distal apical dendrites can be amplified via local Ca^{2+} action potentials which do not reach threshold for axonal AP initiation. As amplification of distal excitatory synaptic input is associated with a localized increase in $[Ca^{2+}]_i$ these Ca^{2+} action potentials could control the synaptic efficacy of the distal cortico-cortical inputs to layer 5 pyramidal neurons.

A dendritic arbor with active membrane conductances could, in principle, amplify excitatory postsynaptic potentials (EPSPs) and process synaptic inputs locally in the dendrites (Shepherd & Brayton, 1987; Cauller & Connors, 1992; Mel, 1993; Softky, 1994). The dendritic membrane of pyramidal neurons contains active conductances such as voltage-activated Na^+, Ca^{2+} and K^+ channels and previous studies have shown that apical dendrites of pyramidal neurons can sustain action potentials (Spencer & Kandel, 1961; Wong, Prince & Basbaum, 1979; Turner, Meyers, Richardson & Barker, 1991; Reuveni, Friedman, Amitai & Gutnick, 1993; Amitai, Friedman, Connors & Gutnick, 1993; Kim & Connors 1993; Regehr, Kehoe, Ascher & Armstrong, 1993; Stuart & Sakmann, 1994; Andreasen & Lambert, 1995; Spruston, Schiller, Stuart & Sakmann, 1995). Experimental evidence for the contribution of active dendritic conductances to integration of synaptic input is mostly indirect (for review see Johnston, Magee, Colbert & Christie, 1996). Current recordings from cell-attached patches of dendrites and calcium fluorescence imaging have demonstrated the voltage-dependent activation of dendritic Ca^{2+} and Na^+ channels during sub-threshold synaptic stimulation mostly in the proximal parts of apical dendrites (Markram & Sakmann, 1994; Magee & Johnston, 1995; Magee, Christofi, Miyakawa, Christie, Lasser-Ross & Johnston, 1995). The contribution of active dendritic conductances to the amplitude and time course of EPSPs in the distal dendrites of the apical tuft is of particular interest for pyramidal neurons in layer 5. The tuft dendrites are distant (> 600 μm) from the axonal site for action potential initiation and distally evoked postsynaptic potentials (PSPs) will be strongly attenuated at the soma (Cauller & Connors 1994).

We have addressed the issue of the contribution of voltage-dependent Ca^{2+} channels to subthreshold dendritic and somatic membrane potential changes and initiation of action potentials by measuring simultaneously the membrane potential in distal apical dendrites and the soma of neocortical neurons and the associated Ca^{2+} fluorescence

transients in mature (P28–32) rats. The results indicate that synaptic stimulation in layer 1–2 or focal depolarization of the distal apical dendrites can initiate Ca^{2+} action potentials which remained locally restricted to the distal dendrites and evoke a local dendritic $[Ca^{2+}]_i$ transient. Some of these results have been previously published in abstract form (Schiller, Schiller & Sakmann, 1996).

METHODS

Slice preparation and electrical recording

Somato-motor neocortical brain slices (300–350 μm in thickness) were prepared from 26- to 34-day-old rats (P26–34). Prior to decapitation rats were anaesthetized by intraperitoneal pentobarbitone (20–25 mg) and perfused intracardially with extracellular solution (4 °C, 1–3 min). Thick tufted pyramidal neurons in layer 5 were visualized using infrared illumination (λ_{max}, 780 nm) and differential interference contrast optics (Zeiss water immersion lens; numerical aperture, 0·9) combined with video microscopy. Simultaneous whole-cell voltage recordings using borosilicate pipettes from soma and dendrites of the same neuron were made as previously described (Stuart, Dodt & Sakmann, 1993). In neurons, in which the apical dendrites could not be followed for more than 550 μm from the soma, first somatic whole-cell recording was established (with a pipette containing 100 μM Calcium Green-1, and the fluorescence image of dendrites was used to localize the distal apical dendrites and to establish dendritic whole-cell voltage recording.

Simultaneous somatic and dendritic recordings from the same neuron were confirmed by injection of hyperpolarizing current applied to the soma or the dendrite and by visualizing the fluorescence dye in the neuron and the two recording pipettes. Somatic (3–5 MΩ resistance) and dendritic (7–10 MΩ) recording pipettes were filled with (mM): 115 potassium gluconate, 20 KCl, 2 Mg-ATP, 2 Na_2-ATP, 10 Na_2-phosphocreatine, 0·3 GTP, 10 Hepes and 0·1 Calcium Green-1 (CG-1) at pH 7·2. In some experiments biocytin (5 mg ml^{-1}) was added to the pipette solution. Whole-cell voltage recordings were made with Axoclamp-2B amplifiers (Axon Instruments). During dendritic current injections the series resistance was less than 40 MΩ. The extracellular solution contained (mM): 125 NaCl, 25 $NaHCO_3$, 25 glucose, 3 KCl, 1·25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, pH 7·4. All experiments were performed at 35 ± 0·5 °C. Synaptic stimulation was made with monopolar platinum–iridium electrodes and a stimulus isolator (World Precision Instruments, Sarasota, FL, USA) placed either distally from the soma (layer 1 or upper layer 2) or proximally (layers 5 or 6) and 100–200 μm lateral to the apical dendrite. The data were acquired using a VMEbus Motorola Delta series 1147 (Tampa, FL, USA) with a sampling rate of 10 kHz.

Fluorescence measurements

High speed $[Ca^{2+}]_i$ fluorescence measurements were performed using the calcium-sensitive fluorescent dye Calcium Green-1 (CG-1; K_d, 315 nM; Molecular Probes) and a frame transfer cooled CCD camera (ATC-5, Photometrics, Tucson, AZ, USA). The indicator dye was excited at 488 nm and fluorescence measurements (acquisition rate was 100 Hz) were collected sequentially from small regions of interest (ROI; width, 3–5 μm; length, 10–25 μm). Background subtraction and bleach correction were performed as previously described (Schiller, Helmchen & Sakmann, 1995). All fluorescence values are given as $\Delta F/F$ as a percentage ($\Delta F/F = 100 \times (F - F_0)/F_0$, where F is the fluorescence measured at different times and F_0 is the average baseline fluorescence).

All chemicals were purchased from Sigma except APV and CNQX, which were purchased from Tocris Cookson, Bristol, UK.

Histological procedures and reconstruction of neurons

After recording, slices with biocytin-filled neurons were fixed for 12–24 h in cold 100 mM phosphate-buffered (PB) solution (pH 7·4), containing 1 % paraformaldehyde and 1 % glutaraldehyde. Thereafter, the slices were rinsed several times in PB solution. Sections were then run through an ascending series of dimethylsulphoxide (DMSO) at the following concentrations: 5, 10, 20 and 40 %, diluted in 100 mM PB solution. After five to six rinses in PB solution, sections were incubated overnight at 4 °C in avidin-biotinylated horseradish peroxidase according to the manufacturer's protocol (ABC-Elite, Vector Labs, Peterborough, UK). The dendritic and axonal arborization of neurons was reconstructed with the Neurolucida package (MicroBrightField, Inc., Colchester, VT, USA).

RESULTS

Regenerative depolarizing potentials restricted to distal apical dendrites

Dual whole-cell voltage recordings from the distal apical trunk or a primary tuft branch (550–940 μm distal to the soma) and the soma of the same pyramidal neuron (Fig. 1*A*) revealed that synaptic stimulation via an electrode located in layer 1–2 has different effects on the dendritic and somatic membrane potential depending on the intensity of stimulation. At a low stimulus intensity, less than 50 % of the intensity required for initiation of somatic action potentials, a composite postsynaptic potential (PSP) was recorded by the dendritic pipette which was strongly attenuated as it spread to the soma (Fig. 1*B*). When the stimulus intensity was increased to about 60 % of the intensity for somatic action potential initiation, distal synaptic stimulation evoked an all-or-none dendritic potential that did not, however, propagate actively to the soma, where depolarization remained subthreshold (Fig. 1*B*). This dendritic potential overshot a baseline set at 0 mV, with an absolute dendritic membrane potential at the peak of 4·4 ± 2·5 mV (mean ± S.E.M., $n = 12$). This implies that the synaptically evoked dendritic potentials are regenerative, rather than being mediated solely by large composite excitatory postsynaptic potentials (EPSPs). Regenerative dendritic potentials evoked by distal synaptic stimulation and restricted to apical dendrites were observed in thirteen of fifteen dual dendritic and somatic voltage recordings (10 recordings were from the distal apical trunk close to the bifurcation and 3 were from primary tuft branches). The regenerative potentials were on average 64 ± 1·2 mV in amplitude and were relatively long lasting with an average half-width of 11·2 ± 1·8 ms ($n = 12$), compared with less than 1 ms for action potentials evoked and recorded by the somatic pipette.

To examine whether intrinsic electric membrane properties of apical dendrites could account for the initiation of the distal regenerative potentials and their attenuated spread to the soma, distal dendrites were depolarized briefly by current injection. Figure 1*C* illustrates that depolarization of a primary tuft branch could also initiate all-or-none

distal dendritic regenerative potentials which remained subthreshold at the soma.

Similar regenerative potentials were not observed in dual whole-cell voltage recordings made from the soma and the proximal portion of the apical dendrite (up to 400 μm from the soma, $n = 29$) as long as the composite PSP at the soma remained subthreshold (not shown). At higher stimulus intensities, however, dendritic regenerative potentials could precede somatic action potentials (see Stuart, Schiller & Sakmann, 1997).

$[Ca^{2+}]_i$ transients restricted to distal apical dendrites

To compare the electrical excitability of distal tuft dendrites and of the proximal apical dendrite, fluorescence imaging of dendritic $[Ca^{2+}]_i$ transients along the apical dendrite (Schiller *et al.* 1995) was combined with dual-voltage recordings from the same neuron. Synaptic stimulation was made via electrodes which had their tips located either close to the distal branches (layers 1–2) or to the proximal apical dendritic branches (layers 5–6). Fluorescence imaging indicated that stimulation of distal synapses in layers 1–2, which elicited a subthreshold PSP at the soma, could evoke a large $[Ca^{2+}]_i$ transient that peaked in the distal apical branches and gradually decreased in amplitude to undetectable levels along the main apical trunk (Fig. 2*A*). The distal dendritic $[Ca^{2+}]_i$ transients (measured at a dendritic region more than 600 μm away from the soma) were significantly larger than those evoked by either synaptic stimulation of the proximal apical dendrite (100–300 μm lateral to the soma, Fig. 2*B* and *C*) or by action potentials initiated in the axon (by somatic current injection) which then back-propagate into the distal dendrites (average peak $\Delta F/F$ amplitude of $16 \pm 15\%$ and average $\Delta F/F$ decay time constant of 338 ± 54 ms, $n = 6$). Thus, synaptic stimulation which evoked regenerative all-or-none potentials in distal dendritic branches, but only subthreshold PSPs at the soma, elicited a $[Ca^{2+}]_i$ transient which remained largely localized to the distal apical dendrites. This result further demonstrates the distal initiation of the dendritic regenerative potential and its restricted propagation.

Amplification of distally evoked PSPs and dendritic $[Ca^{2+}]_i$ transients by local regenerative potentials

To characterize further these distal regenerative potentials, we examined the dependence of the dendritic voltage and $[Ca^{2+}]_i$ transients on stimulus intensity. Figure 3*A* illustrates the increase in the amplitude of the dendritic

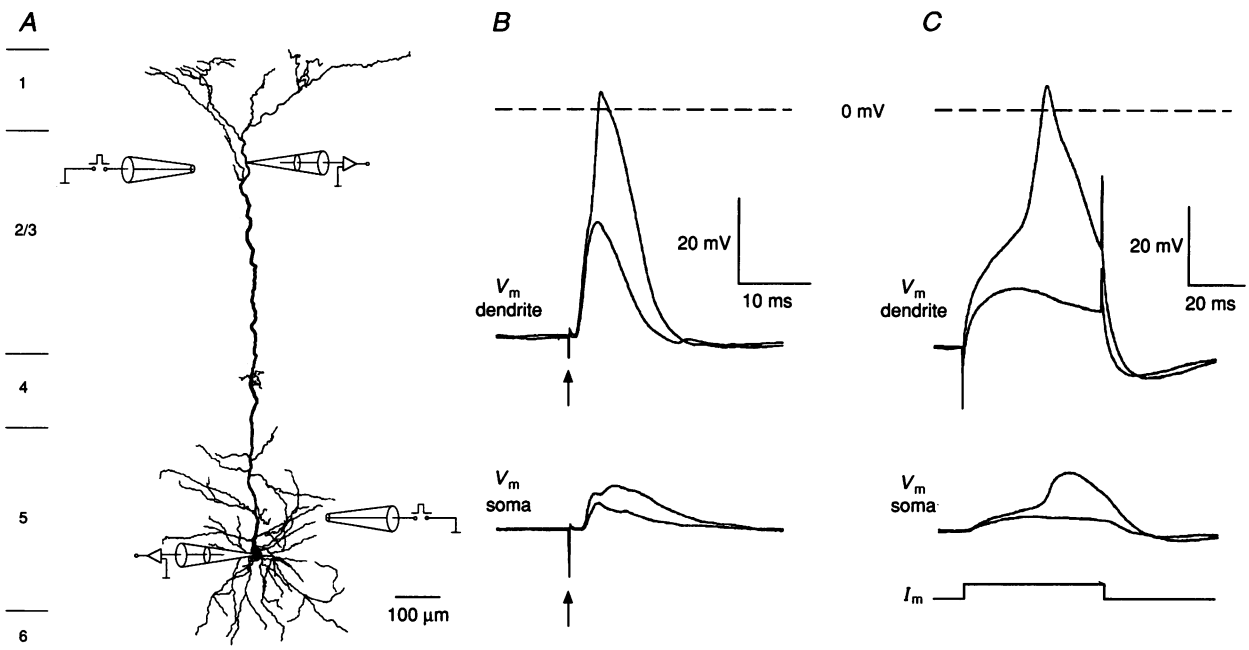

Figure 1. Synaptically evoked dendritic regenerative potentials restricted to distal apical dendrites

A, reconstruction of soma and dendritic arbor of a thick tufted pyramidal neuron in layer 5 from a 28-day-old rat (P28) after filling with biocytin. The location of the cortical layers and schematic drawings of a proximal (layer 5–6, lower right) and distal (layer 1–2, upper left) stimulating electrode and of the somatic (left) and dendritic (right) recording pipette are shown. *B*, regenerative potentials evoked by distal synaptic stimulation and recorded simultaneously from a primary dendritic branch of the apical tuft (920 μm distal to the soma) and the soma of the same neuron. Note that although a regenerative potential was initiated in the distal dendrite the depolarization at the soma remained subthreshold. *C*, voltage transients in dendrite and soma evoked by current injection (I_m) into a distal apical dendritic branch. Site of current injection was 920 μm distal to the soma.

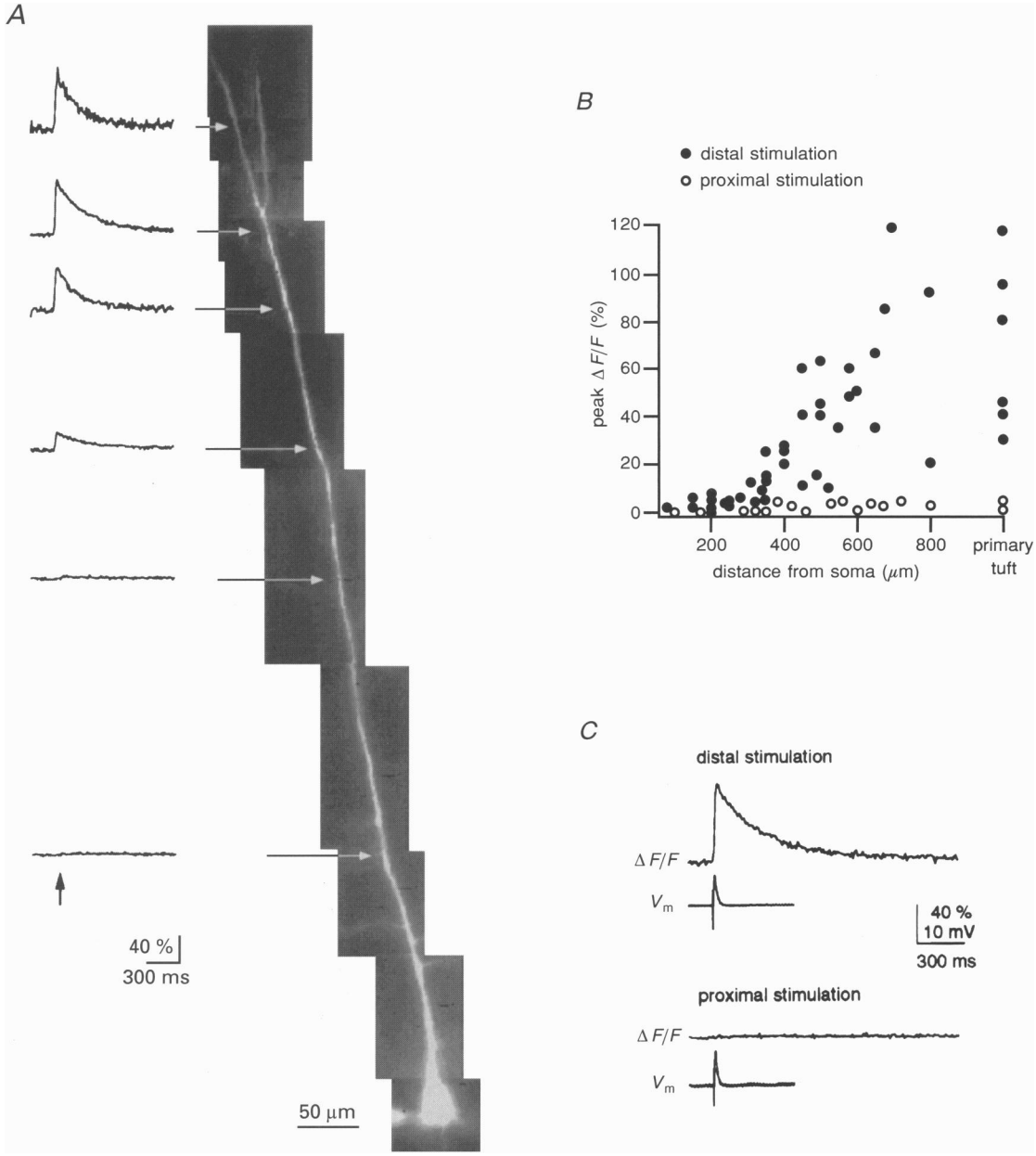

Figure 2. Spatial distribution of $[Ca^{2+}]_i$ transients evoked by local dendritic regenerative potentials in apical dendrites

A, fluorescence image of a neuron loaded with Calcium Green-1 (100 μM) under resting conditions. $[Ca^{2+}]_i$ fluorescence transients evoked by distal synaptic stimulation which was sub-threshold at the soma (time of stimulation indicated by arrow at the lowermost record) are shown on the left for different locations along the apical dendrites. Fluorescence signals from different dendritic regions of interest (ROIs) are presented as $\Delta F/F$ (as a percentage). Note that the peak of the transient decreases in the distal portion with distance from the tuft branches until signals become undetectable in the proximal portion of the apical dendrite. *B*, spatial profile of the peak $[Ca^{2+}]_i$ fluorescence transients evoked in apical dendrite by either distal (●) or proximal (○) synaptic stimulation. In all experiments, synaptic potentials remained sub-threshold at the soma. The peak fluorescence increase measured in different dendritic ROIs sequentially is plotted as a function of distance from the soma. The data for distal and proximal synaptic stimulation were obtained from 15 and 5 neurons, respectively. *C*, calcium fluorescence transients (upper traces) measured in the distal trunk (690–710 μm from soma) evoked by distal and proximal synaptic stimulation. The corresponding synaptic potentials recorded at the soma are shown in the bottom traces. Distal synaptic stimulation resulted in large fluorescence transients in the distal apical dendrite, while proximal synaptic stimulation evoked no significant fluorescence increase. Fluorescence recordings were made within 100–200 μm of the tip of the stimulating electrode.

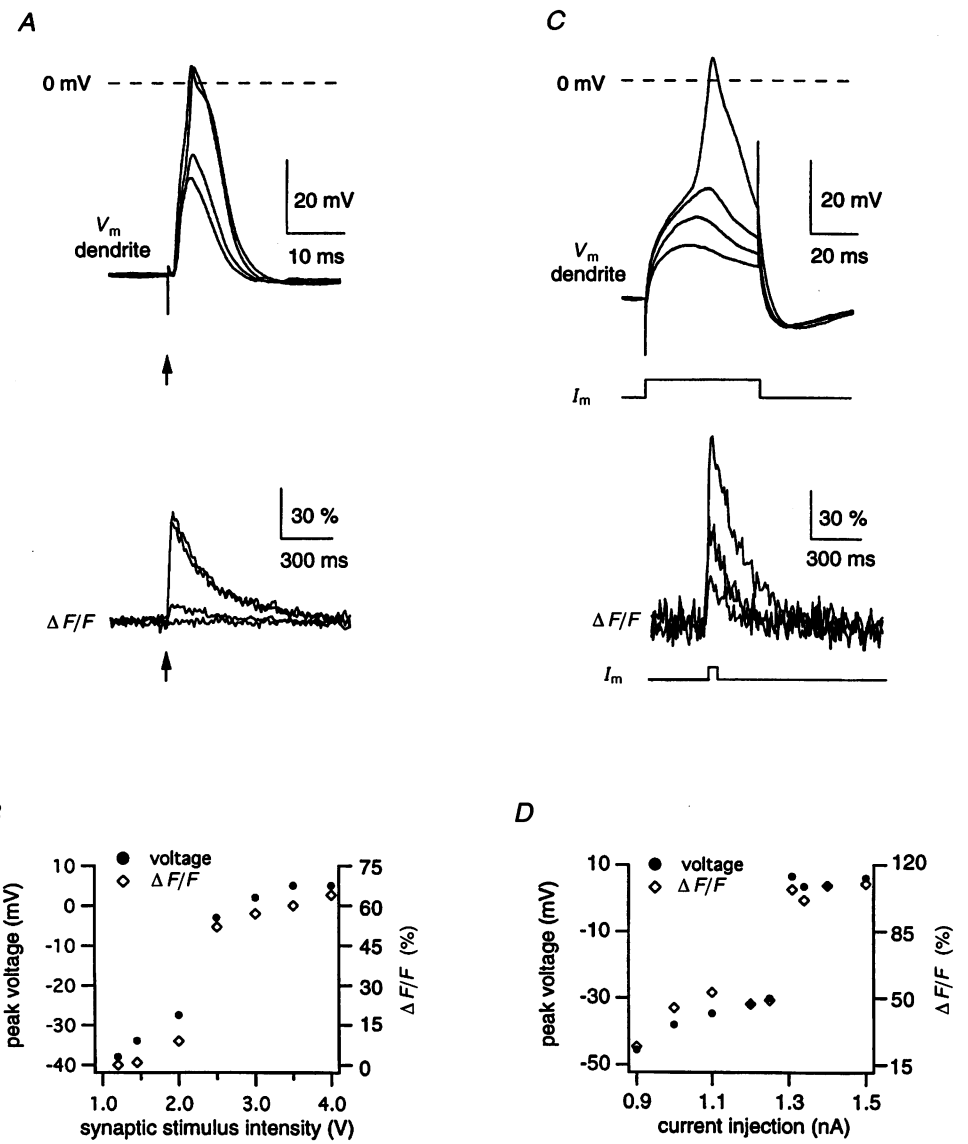

Figure 3. Initiation of distal dendritic regenerative potentials by distal dendritic current injection

A, upper traces: voltage responses and initiation of a local regenerative potential evoked by distal synaptic stimulation with different stimulus intensity. Lower traces: $[Ca^{2+}]_i$ fluorescence transients (presented as $\Delta F/F$, as a percentage) in a primary tuft branch evoked by distal synaptic stimulation with increasing intensity of stimulation. Fluorescence was measured from a ROI close to the dendritic recording pipette, 890–910 μm from soma. Arrow indicates the time of stimulation. All stimulus intensities used were subthreshold at the soma. *B*, peak dendritic voltage responses (●, left scale) and peak $[Ca^{2+}]_i$ fluorescence transients (◇, right scale) are plotted as a function of stimulus intensity. PSPs more positive than −26 mV initiated dendritic regenerative potentials. Concomitant with initiation of regenerative potentials, fluorescence transients increased in a step-like fashion. *C*, upper traces: voltage responses and initiation of local dendritic regenerative potentials by dendritic current injection via the dendritic pipette. Simultaneous whole-cell recording from a primary tuft branch (800 μm distal to the soma) and the soma of the same neuron during injection of current pulses (50 ms) with different amplitude (0·9–1·3 nA) via the dendritic pipette. Note that the distal dendritic regenerative potentials did not initiate somatic action potentials. Lower traces: $[Ca^{2+}]_i$ fluorescence transients (presented as $\Delta F/F$, as a percentage) in a primary tuft branch evoked by distal current injection of different amplitudes via the dendritic pipette. Fluorescence was collected from a region of interest (810–825 μm from the soma) close to the dendritic pipette. The corresponding peak voltage and $[Ca^{2+}]_i$ transient responses in the dendrite are shown in panel *D*. *D*, peak dendritic voltage responses (●, left scale) and peak $[Ca^{2+}]_i$ fluorescence transients (◇, right scale) are plotted as a function of the amplitude of current injection. Responses more positive than −30 mV initiated dendritic all-or-none events associated with a step-like increase of the fluorescence signal.

regenerative potential (upper traces, V_m) and in the amplitude of the concomitant $[Ca^{2+}]_i$ transient (lower traces, $\Delta F/F$) in distal dendrites when the intensity of synaptic stimulation was increased. At all intensities, the stimulation evoked only subthreshold PSPs at the soma (not shown). The peak amplitude of both dendritic voltage and fluorescence transients increased in a step-like fashion when a threshold value for initiation of dendritic regenerative potential was reached (Fig. 3*B*). Similar findings were observed in nine additional neurons.

Simultaneous measurement of dendritic membrane potential (Fig. 3*C*, upper traces) and dendritic $[Ca^{2+}]_i$ transients (Fig. 3*C*, lower traces) evoked by current injection into distal dendrites via the recording pipette showed that a transient increase in $[Ca^{2+}]_i$ fluorescence was detectable already at dendritic membrane potentials below −30 mV (Fig. 3*D*). Both, the membrane voltage and the $[Ca^{2+}]_i$ fluorescence transient increased abruptly when the stimulus intensity was increased and threshold was reached (Fig. 3*C* and *D*). The average threshold potential for the initiation of all-or-none events was $-26{\cdot}4 \pm 1{\cdot}9$ mV in seven recordings made 700–820 μm for the soma (Fig. 3*C*). The largest increase in dendritic $[Ca^{2+}]_i$ amplitude occurred concomitantly with the initiation of all-or-none regenerative potential (Fig. 3*C* and *D*). The peak of the $[Ca^{2+}]_i$ fluorescence transient did not increase further with increasing stimulus intensity even when a somatic action potential was initiated by the stimulus, possibly reflecting significant non-linearity of the fluorescence indicator. Similar imaging results were obtained in one additional neuron.

Conductances mediating distal dendritic regenerative potentials

Addition of Cd^{2+} (200 μM) to the bath solution blocked both dendritic regenerative potentials and dendritic $[Ca^{2+}]_i$ transients initiated by distal dendritic current injection (Fig. 4, $n = 7$). This indicated that these responses were caused by the activation of voltage-dependent Ca^{2+} channels and supports the view that the dendritic regenerative potentials represent local Ca^{2+} action potentials. Voltage-dependent Na^+ channels contributed also to the initiation of the dendritic action potentials, as addition of TTX (1 μM) to the bath solution increased the threshold for action potential initiation by distal current injection. Dendritic regenerative potentials could still be initiated by distal synaptic stimulation when voltage-activated Na^+ channels were blocked by dialysis of the neuron with QX-314 (Fig. 5*A*; 5–10 mM, $n = 3$) or in the presence of TTX (1 μM) following distal dendritic current injection (Fig. 5*B*, $n = 3$).

Subthreshold dendritic voltage responses elicited by proximal dendritic current injection (less than 500 μm away

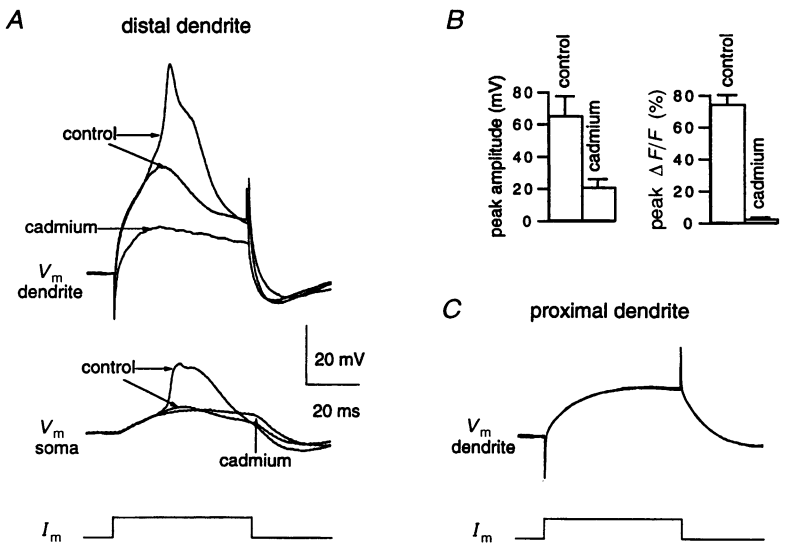

Figure 4. Local distal dendritic regenerative potentials are mediated by voltage-dependent calcium channels

A, effect of Cd^{2+} on dendritic regenerative potentials evoked by current injection into distal dendrite. Depolarizing current injections (50 ms, 1·1 and 1·2 nA in the two control traces and 1·2 nA in the presence of cadmium) delivered via the dendritic pipette (recording from a primary tuft branch, 800 μm from the soma) evoked a subthreshold dendritic regenerative response and local distal all-or-none potential (upper traces). Both responses were blocked by addition of 200 μM Cd^{2+} to the extracellular solution. Simultaneous somatic recordings indicated that the voltage contribution of the distal dendritic all-or-none potential is blocked after the addition of Cd^{2+} (bottom traces). *B*, effect of Cd^{2+} (100–200 μM) on the peak of the dendritic voltage change and calcium fluorescence transients (mean ± S.E.M.; $n = 7$). The control values represent measurements where the stimulus was just suprathreshold for the generation of dendritic all-or-none potential (upper traces marked control in Fig. 4*B*).

from the soma) were not significantly affected by the addition of Cd^{2+} (200 μM) to the bath solution. Thus, we found no evidence for Ca^{2+}-mediated amplification of subthreshold synaptic potentials in the proximal apical trunk, consistent with the results of Stuart & Sakmann (1995).

Calcium action potentials in distal dendrites mediated by coactivation of AMPAR and NMDAR channels

Addition of 2-amino-5-phosphonovaleric acid (APV 50–100 μM), an antagonist of *N*-methyl-D-aspartate receptors (NMDARs) blocked the initiation of dendritic action potentials (Fig. 6*A* and *B*), and attenuated the peak amplitude of the associated $[Ca^{2+}]_i$ transients (Fig. 6*C* and *D*; $n = 9$). Initiation of both Ca^{2+} action potentials by distal synaptic stimulation and the associated $[Ca^{2+}]_i$ transients were completely blocked by addition of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPAR) antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM) to the extracellular solution (Fig. 6*B* and *D*; $n = 3$). CNQX also reduced the amplitude of synaptically evoked composite PSPs in dendrites and the soma (not shown).

Figure 6*E* and *F* illustrates that the initiation of dendritic regenerative potentials by distal synaptic stimulation at 60% of threshold intensity for somatic action potential initiation was blocked by application of APV (50 μM). Thus, under physiological conditions, the depolarization at the soma evoked by distal synaptic stimulation (Fig. 6*E*) did not represent a passively attenuated PSP. More likely, it reflected a PSP which was amplified in the distal dendrite by a local Ca^{2+} action potential. As a consequence, the $[Ca^{2+}]_i$ transient in distal dendrites, evoked by synaptic stimulation which remained subthreshold at the soma and which was sensitive to APV, represented primarily Ca^{2+} inflow via dendritic voltage-dependent Ca^{2+} channels.

The dependence of local distal dendritic action potential initiation and the associated $[Ca^{2+}]_i$ transient on activation of NMDA receptors probably resulted from the relatively slow activation and inactivation kinetics of NMDA receptors. Thus, it is possible that a train of AMPA-mediated EPSPs could also evoke Ca^{2+} action potentials in distal apical dendrites.

A biphasic somatic PSP was typically elicited when a distal dendritic Ca^{2+} action potential was initiated, but the delay between the two phases varied in different experiments (compare Figs 1*B* and 6*E*).

Dendritic and axonal initiation sites for action potentials

Figure 7*A* and *B* illustrates that somatic action potentials were initiated by the summed depolarization of the composite PSP and the attenuated dendritic Ca^{2+} action potential. Under these conditions, the somatic action potential followed the dendritic action potential (Fig. 7*A*). The time of occurrence of the synaptically evoked dendritic action potential with respect to the somatic action potential was dependent on activation of NMDAR channels. Addition of APV (50 μM) eliminated initiation of a distal dendritic Ca^{2+} action potential and only a somatic action potential was initiated which back-propagated into the distal apical dendrite (Fig. 7*B*). Ca^{2+} action potentials initiated by dendritic current injection also spread, strongly attenuated, to the soma and if the somatic depolarization was of sufficient amplitude it could initiate Na^+ action potentials, presumably originating in the axon (see Stuart *et al.* 1997), which back-propagated into the distal dendrite (Fig. 7*C*). Thus, the initiation site controlling the impulse output of the neuron remained in the axo-somatic region (see also Stuart *et al.* 1997).

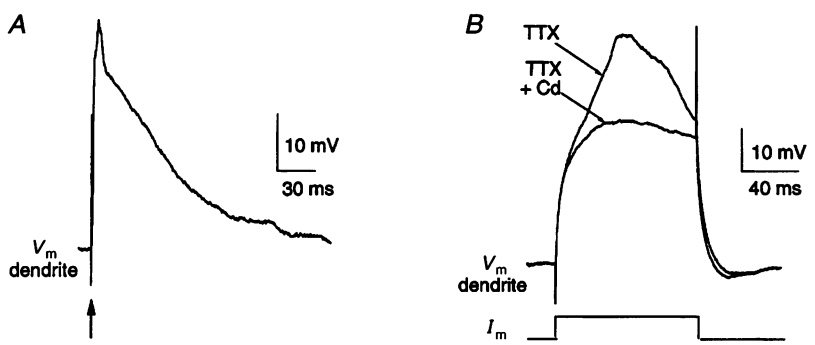

Figure 5. Voltage-dependent sodium channels contribute to the initiation of local distal dendritic regenerative potentials

A, dendritic regenerative potential evoked by distal synaptic stimulation when QX-314 (10 mM) was included in the dendritic recording pipette (primary tuft branch, 700 μm distal to the soma). The resting membrane potential under this condition was −29 mV. *B*, dendritic regenerative potential evoked by current injection into distal dendrite. Depolarizing current injections (1·18 nA, 100 ms) applied via the dendritic pipette (700 μm from the soma) evoked a regenerative response which was blocked by addition of 200 μM Cd^{2+} to the extracellular solution.

DISCUSSION

The observations described here indicate that synaptic stimulation of the afferent fibres which form synapses on the distal apical dendrites of layer 5 pyramidal neurons can initiate local Ca^{2+} action potentials associated with dendritic $[Ca^{2+}]_i$ transients. Both Ca^{2+} action potentials and $[Ca^{2+}]_i$ transients remained largely restricted to the distal parts of the apical dendrites. Thus, these neurons have at least two regions where regenerative potentials can be initiated. One

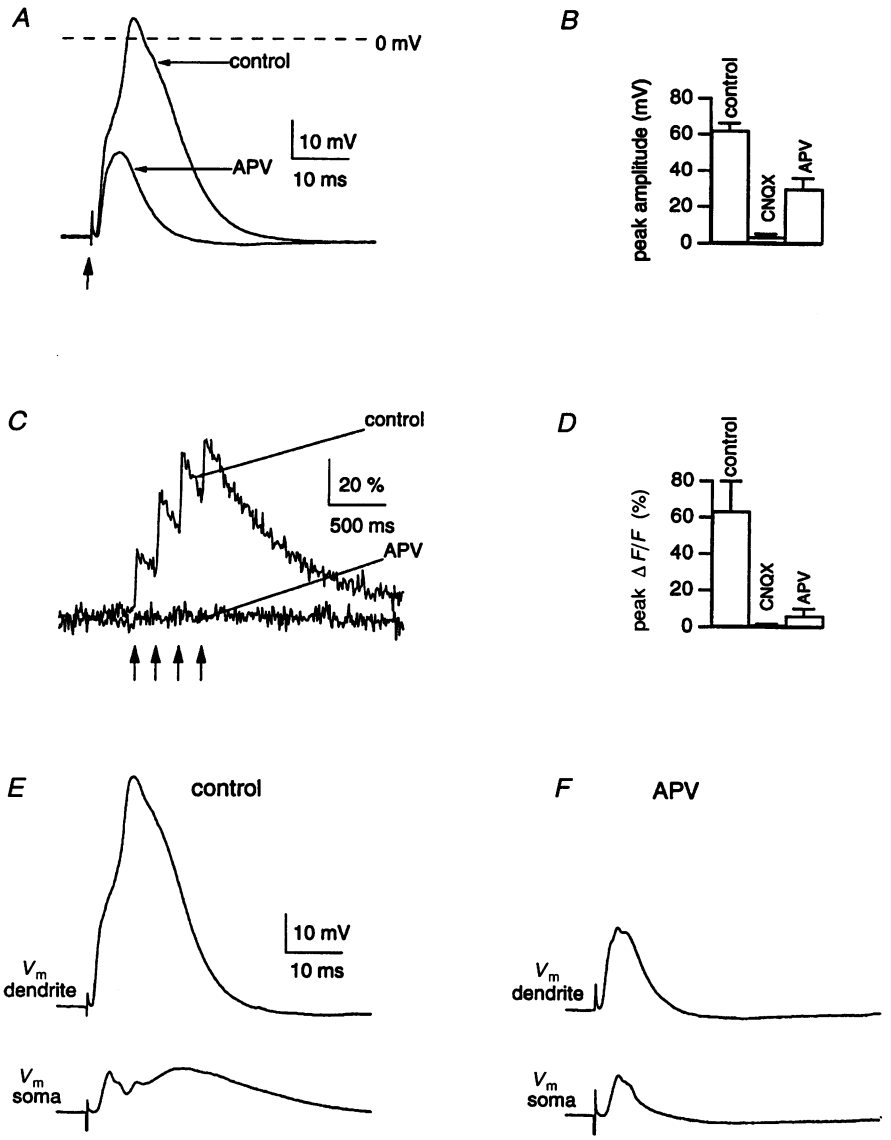

Figure 6. Initiation of synaptically evoked distal dendritic regenerative potentials and associated calcium influx by coactivation of AMPA and NMDA receptors

A, effect of APV on initiation of dendritic regenerative potentials. Simultaneous whole-cell recordings from a distal apical dendrite (600 μm distal to the soma) and the soma (not shown) of the same neuron, during distal synaptic stimulation (timing indicated by arrow) under control conditions and after addition of APV (100 μM) to the bath solution. *B*, averaged (mean ± S.E.M.) peak voltage evoked by distal synaptic stimulation under control conditions and after addition of CNQX ($n = 3$, 20 μM), or APV ($n = 5$, 50–100 μM) to the bath solution. *C*, effect of APV on amplitude of dendritic Ca^{2+} fluorescence transients (presented as $\Delta F/F$, as a percentage) evoked by distal synaptic stimulation (timing indicated by arrows, 5 Hz for 0·8 s). The Ca^{2+} fluorescence transients were recorded from a ROI in the distal apical trunk close to the dendritic recording pipette (710–725 μm distal to the soma). *D*, average effect (mean ± S.E.M.) of CNQX ($n = 3$) and APV ($n = 8$) on the peak dendritic calcium fluorescence transients. *E*, simultaneous dendritic and somatic recording of stimulus-evoked regenerative dendritic potential (upper trace) and subthreshold depolarization at the soma (lower trace). *F*, same experiment following application of 50 μM APV. Note that both the late somatic voltage component and the local dendritic Ca^{2+} action potential were blocked by APV.

site is located in the axon (Eccles, 1964; Stuart & Sakmann, 1994; Stuart *et al.* 1997); the other site (or sites) is located in the distal apical dendrites. The failure of the distally initiated Ca^{2+} action potentials to propagate actively through the soma to the axonal initiation site could be due to an inhomogeneous distribution of voltage-dependent Na^+, Ca^{2+} and K^+ channels in apical dendrites in combination with the long electrotonic distance between the apical tuft and the axon initial segment. Furthermore, the dendritic morphology of layer 5 pyramidal neurons is such that it does not favour propagation of regenerative events from the distal dendrites to the soma (Goldstein & Rall, 1974; Rall & Segev, 1987; Regehr *et al.* 1993). Concomitant activation of inhibitory inputs at the soma and the proximal dendrites by extracellular stimulation may further contribute to the compartmentalization of the apical dendritic tree (Kim, Beierlein & Connors, 1995; Miles, Toth, Gulyas, Hajos & Freund, 1996).

Dendritic regenerative potentials

Several studies have addressed the question of whether action potentials can be initiated in the dendrites of neocortical pyramidal neurons. Recordings from dendrites demonstrated that active conductances in the main apical trunk can support propagation of action potentials (Amitai *et al.* 1993; Kim & Connors, 1993; Stuart & Sakmann, 1994; Stuart *et al.* 1997). Amitai *et al.* (1993), Stuart & Sakmann (1994) and Stuart *et al.* (1997) concluded that dendritic action potentials were initiated first in the axon and then back-propagated into the apical dendrite. Kim & Connors (1993), however, suggested that these action potentials were initiated in dendrites.

The local Ca^{2+} action potentials observed in the distal apical dendrites described here were initiated in the dendrites. At more proximal dendritic recording sites regenerative potentials detected with threshold distal synaptic

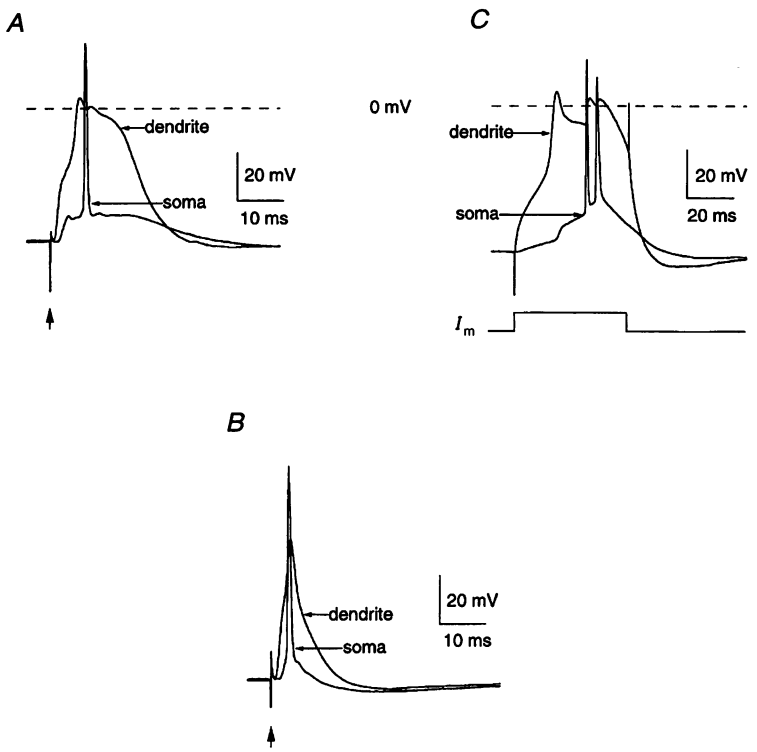

Figure 7. Initiation of distal dendritic and axonal action potentials

A, initiation of action potentials in a distal apical dendrite and axonal action potential initiation evoked by intense distal synaptic stimulation (indicated by arrow). Simultaneous dual recording from the soma and distal apical trunk (740 μm distal to the soma) of the same neuron. Distal synaptic stimulation evoked a dendritic Ca^{2+} action potential, which preceded the somatic action potential. *B*, effect of APV on initiation of distal dendritic and somatic action potentials evoked by distal synaptic stimulation. Addition of APV (50 μM) blocked initiation of dendritic action potentials, shifting the site of the earliest action potential initiation to the soma (for control see *A*). In this experiment, after the addition of APV, the initiation of the somatic action potential occurred earlier compared with control conditions. This time shift was, however, not observed in all experiments. It could be caused by the lack of activation of inhibitory interneurons when APV is present. *C*, distal dendritic action potential initiated by distal dendritic current injection (1·3 nA, primary tuft branch 800 μm distal to the soma) and recorded concomitantly at the soma and the tuft branch. The depolarizing current injection evoked a dendritic action potential that was attenuated strongly at the soma. It initiated, however, a burst of somatic action potentials.

stimulation represented action potentials back-propagating from the axonal initiation site (Stuart & Sakmann, 1994; Stuart *et al.* 1997). Nevertheless, with higher intensity of distal synaptic stimulation the dendritic regenerative potentials were observed as subthreshold depolarizations at the somatic recording sites (Fig. 7*A–C*) which did not actively propagate to the soma and axon. Dendritic regenerative potentials are most likely to be mediated by voltage-dependent Ca^{2+} and Na^+ channels. The fact that distally evoked, predominantly Ca^{2+} dependent regenerative potentials did not propagate actively to the soma could indicate that an impedance mismatch between distal and proximal parts of the apical dendrite prevents the effective activation of Na^+ channels in the proximal dendrite and propagation of dendritic action potentials to the soma (see also Stuart *et al.* (1997) for discussion). As a result, axonal action potential initiation following distal synaptic stimulation was controlled by the summation of the attenuated distal Ca^{2+} action potential and the synaptic depolarization from other inputs.

Dendritic $[Ca^{2+}]_i$ transients in neocortical pyramidal neurons

From calcium imaging experiments Yuste, Gutnick, Saar, Delaney & Tank (1994) reported increased accumulation of Ca^{2+} in the middle portion of the main apical trunk evoked by either long trains of axonally initiated action potentials, or intense synaptic stimulation in different cortical layers (layers 1–4), in young and mature rats (P14–30). The dendritic calcium accumulation observed by Yuste *et al.* (1994) is different from the $[Ca^{2+}]_i$ transients described here as the Ca^{2+} influx evoked by the distal dendritic action potentials peaked in the tuft branches, was evoked only by distal synaptic stimulation (layer 1–2) and not by a wide range of stimulation patterns, and was unique to mature (P28–32) rats. The enhanced Ca^{2+} influx into the main apical trunk described by Yuste *et al.* (1994) probably represented the calcium-dependent component of back-propagating action potentials observed previously (Amitai *et al.* 1993; Kim & Connors, 1993; Stuart *et al.* 1997).

The distal dendritic Ca^{2+} action potentials described here appear to be developmentally regulated, as little or no rise in dendritic Ca^{2+} influx in the distal apical dendrites was observed in Ca^{2+} imaging experiments on P14 rats as long as the depolarization of the soma remained subthreshold ($\Delta F/F$ smaller than 10%; J. Schiller, unpublished observations).

Amplification of EPSPs in pyramidal cells

Several studies addressed the contribution of active dendritic conductances in amplification of EPSPs. Based on somatic recordings Cauller & Connors (1992) demonstrated that synaptic inputs in layer 1 are capable of generating large, fast EPSPs at the soma, and hypothesized that EPSPs in these distal synapses are amplified by active dendritic currents. Calcium imaging experiments (Markram & Sakmann, 1994; Magee *et al.* 1995) and dendritic cell-attached patch clamp current recordings (Magee & Johnston, 1995) showed activation of dendritic Ca^{2+} and Na^+ channels during subthreshold synaptic stimulation.

Amplification of EPSPs in pyramidal neurons of neocortical layer 5 has been directly investigated previously by Stuart & Sakmann (1995). These experiments, which used relatively proximal (up to 500 μm distal to the soma) dendritic current injections to simulate EPSPs and to assess the effect of different pharmacological blockers, suggested that EPSPs were amplified largely by axosomatic Na^+ channels, with insignificant contributions by dendritic voltage-dependent Ca^{2+} channels. Similarly, in the present study, no evidence was found for the involvement of dendritic Ca^{2+} channels during proximal dendritic current injections which remained subthreshold at the soma. Nevertheless, strong suprathreshold stimulation can initiate a large regenerative response in the proximal apical dendrite mediated by both voltage-dependent Na^+ and Ca^{2+} channels (for details see Stuart *et al.* 1997).

In the present experiments, EPSPs generated on the distal apical dendrites which remain subthreshold at the soma were amplified in an all-or-none way by dendritic Ca^{2+} channels. This amplification required large EPSPs, approximately 20 mV in amplitude at the dendritic recording site, before significant amplification of EPSP by voltage-sensitive dendritic Ca^{2+} channels was observed (see Fig. 3). This suggests that EPSP amplification will only occur when synaptic inputs from multiple afferents are synchronized.

It is interesting to note that the local, graded regenerative dendritic response has only a small effect on the somatic membrane potential (Fig. 2*A*). The graded amplification in the dendrites may serve mainly as a signal which locally sets the electrical properties of the membrane via activation of Ca^{2+}-activated channels or it may relieve the Mg^{2+} block of NMDA receptors in neighbouring synapses (Eilers, Augustine & Konnerth, 1995).

These observations, together with earlier ones (Stuart & Sakmann, 1995), show that the ability of different voltage-activated channels to amplify EPSPs in neocortical layer 5 pyramidal neurons depends on the size of the synaptic depolarization, the number of synaptic contacts activated and their degree of synchronization as well as on which part of the dendritic arbor synaptic contacts are located.

Distal dendritic calcium action potentials in cerebellar Purkinje neurons

In cerebellar Purkinje cells Ca^{2+} action potentials localized to dendrites have been observed under suprathreshold conditions at the soma (Llinás & Sugimori, 1980*a*,*b*; Tank, Sugimori, Connor & Llinás, 1988; Lev-Ram, Miyakawa, Lassar-Ross & Ross, 1992; Miyakawa, Lev-Ram, Lasser-Ross & Ross, 1992). Furthermore, $[Ca^{2+}]_i$ transients localized to a small region of the dendrite were evoked by subthreshold synaptic stimulation, but contrary to the results in layer 5 pyramidal neurons, no corresponding voltage change was observed (see Eilers *et al.* 1995). Thus, it is not clear whether

Purkinje cells can produce dendritic Ca^{2+} action potentials under subthreshold condition at the soma.

Functional significance of calcium action potentials in distal dendrites

EPSPs arising in the distal apical dendrites were amplified locally if they were large enough to activate voltage-dependent Ca^{2+} channels. Associational cortico-cortical afferents preferentially innervate the apical tuft branches of layer 5 pyramidal neurons (Pandya & Yeterian, 1985; Zeki & Shipp, 1988; Cauller & Connors, 1994). Hence, the distal dendrites of the tuft could be the primary site of integration of those synaptic inputs which project to cortical pyramidal neurons from other cortical areas. This will only be the case, however, if a sufficiently large number of these afferent inputs are activated synchronously.

The current injected into distal dendrites necessary for the initiation of Ca^{2+} action potentials ranged from 0·8 to 1·1 nA for about 10 ms (see Fig. 1*C*). Depending on the estimate of the size and duration of a quantal conductance increase (Jonas, Major & Sakmann, 1993; Markram, Lübke, Frotscher, Roth & Sakmann, 1997), either only a few or several dozens of contacts must be activated for amplification of EPSPs by dendritic Ca^{2+} action potentials to occur. The characteristics of synaptic connections formed by individual cortico-cortical afferents innervating distal apical dendrites are, as yet, unknown and therefore estimates of the number of synchronously active cortico-cortical afferents necessary for EPSP amplification by Ca^{2+} action potentials remain speculative.

Two previous studies addressed whether localized dendritic action potentials are elicited in response to physiological stimuli *in vivo*. Hirsch, Alonso & Reid (1995) reported the existence of dendritic calcium action potentials evoked by visual stimulation in layer 4 of the cat striate cortex, consistent with a previous study in CA1 hippocampal pyramidal neurons (Spencer & Kandel, 1961). Svoboda, Denk, Kleinfeld & Tank (1997) found, however, no evidence for dendritic Ca^{2+} action potentials in layer 2 of the rat somatomotor cortex during whisker stimulation. The lack of dendritic action potential initiation may be due to the stimulus used, which activated the thalamo-cortical afferents that innervate mostly the basal and proximal apical dendrites (White, 1989). The present results imply that initiation of distal dendritic action potentials depends on activation of feedback cortico-cortical afferents innervating the distal tuft dendrites.

In addition to their function in synaptic integration the synaptically elicited, dendritic Ca^{2+} action potentials evoked a localized $[Ca^{2+}]_i$ transient in the distal dendrites. As a rise in $[Ca^{2+}]_i$ is thought to be required for the induction of certain forms of synaptic plasticity (Bliss & Collingridge, 1993, for review), local Ca^{2+} action potentials in distal dendrites could control specifically the efficacy of cortico-cortical excitatory synaptic inputs depending on the degree of their synchronous activity. It remains to be investigated whether the appearance of this functionally almost independent distal dendritic compartment is developmentally regulated and whether it is dependent on postnatal cortical activity.

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