Action potential initiation and propagation in rat neocortical pyramidal neurons

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1. Initiation and propagation of action potentials evoked by extracellular synaptic stimulation was studied using simultaneous dual and triple patch pipette recordings from different locations on neocortical layer 5 pyramidal neurons in brain slices from 4-week-old rats (P26–30) at physiological temperatures.

2. Simultaneous cell-attached and whole-cell voltage recordings from the apical trunk (up to 700 μm distal to the soma) and the soma indicated that proximal synaptic stimulation (layer 4) initiated action potentials first at the soma, whereas distal stimulation (upper layer 2/3) could initiate dendritic regenerative potentials prior to somatic action potentials following stimulation at higher intensity.

3. Somatic action potentials, once initiated, propagated back into the apical dendrites in a decremented manner which was frequency dependent. The half-width of back-propagating action potentials increased and their maximum rate of rise decreased with distance from the soma, with the peak of these action potentials propagating with a conduction velocity of approximately 0·5 m s^{-1}.

4. Back-propagation of action potentials into the dendritic tree was associated with dendritic calcium electrogenesis, which was particularly prominent during bursts of somatic action potentials.

5. When dendritic regenerative potentials were evoked prior to somatic action potentials, the more distal the dendritic recording was made from the soma the longer the time between the onset of the dendritic regenerative potential relative to somatic action potential. This suggested that dendritic regenerative potentials were initiated in the distal apical dendrites, possibly in the apical tuft.

6. At any one stimulus intensity, the initiation of dendritic regenerative potentials prior to somatic action potentials could fluctuate, and was modulated by depolarizing somatic or hyperpolarizing dendritic current injection.

7. Dendritic regenerative potentials could be initiated prior to somatic action potentials by dendritic current injections used to simulate the membrane voltage change that occurs during an EPSP. Initiation of these dendritic potentials was not affected by cadmium (200 μM), but was blocked by TTX (1 μM).

8. Dendritic regenerative potentials in some experiments were initiated in isolation from somatic action potentials. The voltage change at the soma in response to these dendritic regenerative events was small and subthreshold, showing that dendritic regenerative events are strongly attenuated as they spread to the soma.

9. Simultaneous whole-cell recordings from the axon initial segment and the soma indicated that synaptic stimulation always initiated action potentials first in the axon. The further the axonal recording was made from the soma the greater the time delay between axonal and somatic action potentials, indicating a site of action potential initiation in the axon at least 30 μm distal to the soma.

10. Simultaneous whole-cell recordings from the apical dendrite, soma and axon initial segment showed that action potentials were always initiated in the axon prior to the soma, and with the same latency difference, independent of whether dendritic regenerative potentials were initiated or not.

11. It is concluded that both the apical dendrites and the axon of neocortical layer 5 pyramidal neurons in P26–30 animals are capable of initiating regenerative potentials. Regenerative potentials initiated in dendrites, however, are significantly attenuated as they spread to the soma and axon. As a consequence, action potentials are always initiated in the axon before the soma, even when synaptic activation is intense enough to initiate dendritic regenerative potentials. Once initiated, the axonal action potentials are conducted orthogradely into the axonal arbor and retrogradely into the dendritic tree.

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The response of a neuron to a given synaptic input will depend on where within the neuron this synaptic input is integrated to initiate an action potential. Initial work suggested that action potentials in spinal motoneurons are initiated in the axon initial segment (Eccles, 1964). Later work in hippocampal and cerebellar Purkinje neurons, however, suggested that action potentials can also be initiated in the dendrites (see Johnston, Magee, Colbert & Christie, 1996). Similarly, some studies have concluded that action potentials can be initiated in the dendrites of neocortical pyramidal neurons (Deschenes, 1981; Pockberger, 1991; Kim & Connors, 1993; Regehr, Kehoe, Ascher & Armstrong, 1993; Hirsch, Alonso & Reid, 1995). Other studies, however, have concluded that there is an axosomatic location of action potential initiation in neocortical pyramidal neurons based on either single site dendritic recordings (Amitai, Friedman, Connors & Gutnick, 1993) or dual simultaneous somatic and dendritic or somatic and axonal recordings (Stuart & Sakmann, 1994). The conclusions of Stuart & Sakmann (1994), however, have been questioned, and the possibility raised that capacitive loading or 'washout' of intracellular constituents via the whole-cell recording pipettes may have influenced the site of action potential initiation (Regehr & Armstrong, 1994). Furthermore, it has been suggested that a developmental increase in the dendritic sodium channel density may lead to the initiation of action potentials in the dendrites of layer 5 pyramidal neurons in mature animals (Mainen, Joerges, Huguenard & Sejnowski, 1995). These questions, together with the suggestion that in hippocampal CA1 pyramidal neurons the site of action initiation can shift into the dendrites during high intensity distal synaptic stimulation (Turner, Meyers, Richardson & Barker, 1991; Spruston, Schiller, Stuart & Sakmann, 1995), prompted a reinvestigation of the site of action potential initiation in mature neocortical pyramidal neurons.

Here we address the issue of action potential initiation in electrophysiologically mature (P26–30) layer 5 neocortical pyramidal neurons at physiological temperatures during initiation of action potentials by extracellular synaptic stimulation at different locations along the somato-dendritic axis and with different stimulus intensities. Simultaneous dual and triple patch pipette recordings from the soma, dendrites and axon of the same neuron were made to determine the site of action potential initiation by measuring at which recording site action potentials were recorded first. By sampling the membrane voltage of the same neuron at different locations it was also possible to study how, once initiated, regenerative events spread within pyramidal neurons. The experiments showed that action potentials were always initiated in the axon before the soma even when synaptic stimulation was strong enough to initiate regenerative potentials in dendrites. Once initiated, action potentials propagated orthogradely into the axonal arbor and back into the dendritic tree. Some of these results have been previously published in abstract form (Stuart & Sakmann, 1996).

METHODS

Slice preparation and electrical recording

Experiments were performed on 300 μm thick, sagittal brain slices from somatosensory neocortex of P26–30 Wistar rats prepared as described in the previous paper (Schiller, Schiller, Stuart & Sakmann, 1997). During recording slices were continuously perfused with oxygenated solution of the following composition (mM): 125 NaCl, 25 $NaHCO_3$, 25 glucose, 2·5 KCl, 1·25 NaH_2PO_4, 2 $CaCl_2$ and 1 $MgCl_2$ (pH 7·4 with 95% O_2–5% CO_2), and all experiments were performed at 35 ± 1 °C. Pooled data are expressed as means ± S.D. No correction was made for the junction potential between bath and pipette solutions, which was experimentally determined to be −12 mV.

All chemicals were purchased from Sigma, except APV, which was purchased from Tocris Cookson (Bristol, UK).

Layer 5 pyramidal neurons were identified using infrared illumination combined with differential interference contrast optics and video microscopy, and patch-pipette recordings (cell-attached voltage clamp or whole-cell current clamp; seal resistances > 1 GΩ) were made from the soma, apical dendrite (up to 700 μm from the soma) and/or axon (up to 30 μm from the soma) of the same neuron using identical microelectrode amplifiers (Axoclamp-2A, Axon Instruments). Current and voltage were filtered at 10 kHz and sampled at 50 kHz using a VME bus computer (Motorola Delta series 1147, Tempe, AZ, USA). For both cell-attached and whole-cell recordings, patch pipettes (4–7 MΩ for somatic recordings, 8–10 MΩ for dendritic recordings, 10–12 MΩ for axonal recordings) were filled with a potassium gluconate-based intracellular solution (mM: 120 potassium gluconate, 20 KCl, 10 Hepes, 10 EGTA, 2 Na_2-ATP and 2 $MgCl_2$; pH 7·3 with KOH). During cell-attached recordings the patch pipette was held at −60 mV (i.e. transmembrane potential close to 0 mV) to inactivate voltage-dependent currents. Measurements of resting membrane potential were made immediately following break-in (usually with seconds), before significant dialysis with the intracellular pipette solution, and whole-cell recordings were terminated if the access resistance exceeded 100 MΩ. In some experiments biocytin (5 mg ml^{-1}) was added to the pipette solution and cells subsequently stained with the avidin-biotinylated horseradish peroxidase complex reaction to reveal the cell morphology (see Schiller *et al.* 1997). Recordings were only made from cells where it was possible to follow the apical dendrite or axon from the cell soma to the site of dendritic or axonal recording. Axons were identified by their emergence from the basal part of the soma, myelination (usually starting 25 to 50 μm from the soma), projection to the white matter and antidromic activation ($n = 3$). That dendritic or axonal recordings were made from the apical dendrite or axon was confirmed by the use of fluorescent dyes. The distance of dendritic recordings from the soma was measured from the centre of the soma, whereas axonal recordings were measured from the edge of the soma at the beginning of the axon (i.e. from the axon hillock).

Excitatory synaptic potentials (EPSPs) were evoked by 200 μs pulses (up to 100 V in amplitude) applied to an extracellular stimulating pipette placed either distally (top of layer 2/3) or proximally (layer 4) and approximately 100 μm lateral to the apical dendrite. This stimulation pipette was made from a fire-polished patch pipette with a tip diameter of approximately 10 μm filled with oxygenated extracellular solution. 'Threshold' stimulation was defined as extracellular synaptic stimulation or current injection at an intensity sufficient to initiate somatic action potentials on most trials, whereas 'high intensity' stimulation was defined as stimulation at an intensity up to 5–10 times greater than that

required for somatic action potential initiation. Dendritic current injections used to simulate EPSPs were generated by injection of an exponentially rising and falling voltage waveform (τ_{on}, 0·3 ms; τ_{off}, 3 ms) into the current-clamp input of the Axoclamp amplifier used to make the dendritic recording. Data from these experiments were only used if the dendritic access resistance was less than 30 MΩ, was stable and could be adequately compensated.

Analysis

All data analysis was performed with IGOR (Wavemetrics, OR, USA) on a Macintosh computer. Action potential amplitude was measured from a baseline set at threshold and action potential half-width gives the duration at half amplitude. The 'onset latency' and 'peak latency' between events recorded at different locations is defined as the time difference between the onset or peak of a particular event relative to that of the action potential recorded by the somatic pipette. The time of onset was defined as the time at which the maximum rate of rise of voltage was greater that approximately 20–40 V s^{-1} for dendritic events, or 50 V s^{-1} for somatic and axonal events.

RESULTS

All experiments were made on visually identified layer 5 pyramidal neurons in brain slices from 4-week-old rats at physiological temperatures (35 °C). Recordings were only used if the somatic resting membrane potential was more negative that −60 mV and the amplitude of somatic action potentials, measured from threshold, was greater than 80 mV. A total of forty-five cells met these criteria from thirty-one simultaneous somatic and dendritic, six simultaneous somatic and axonal and eight simultaneous somatic, axonal and dendritic recordings. The average somatic resting membrane potential and action potential amplitude for these cells was −64·3 ± 2·2 mV and 96·8 ± 5·7 mV ($n = 45$), respectively. Somatic input resistance and apparent membrane time constant were 32 ± 3·9 MΩ and 11 ± 0·8 ms, respectively ($n = 5$). In addition, bursts of action potentials in response to somatic current injection or synaptic stimulation were observed in 36% of the neurons. In some

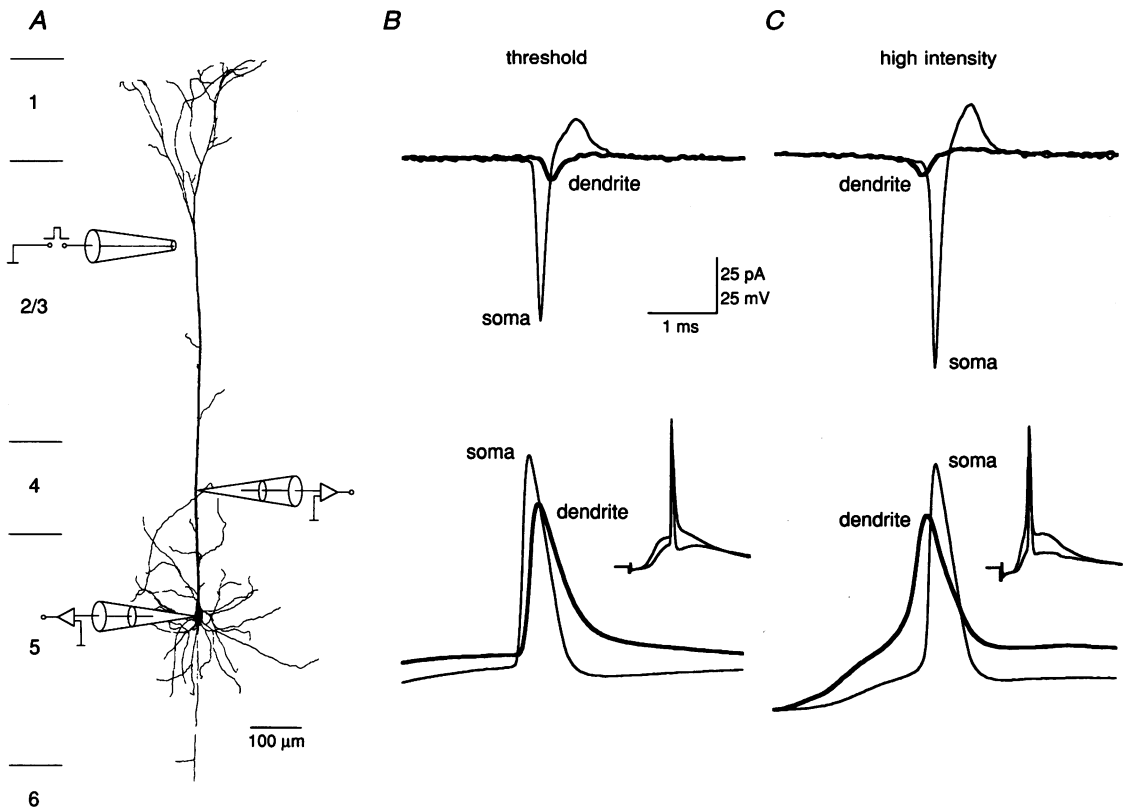

Figure 1. Action potential initiation during synaptic stimulation at different intensities

A, camera lucida drawing showing the approximate location of stimulation and somatic and dendritic recording pipettes during simultaneous somatic and dendritic recording from a neocortical layer 5 pyramidal neuron. Numbers on the left refer to cortical layers. Synaptic stimulation was in upper layer 2/3. Only the initial part of the axonal arbor is shown. *B*, somatic and dendritic (thicker traces) cell-attached (top) and whole-cell voltage (bottom) recordings during threshold intensity synaptic stimulation in layer 2/3. *C*, somatic and dendritic (thicker traces) cell-attached (top) and whole-cell voltage (bottom) recordings during high intensity synaptic stimulation in layer 2/3. All recordings from the same cell. Dendritic recording 175 µm from the soma. The inserts in the lower parts of *B* and *C* show the whole-cell recordings on a reduced time base. Width, 20 ms; height, 125 mV.

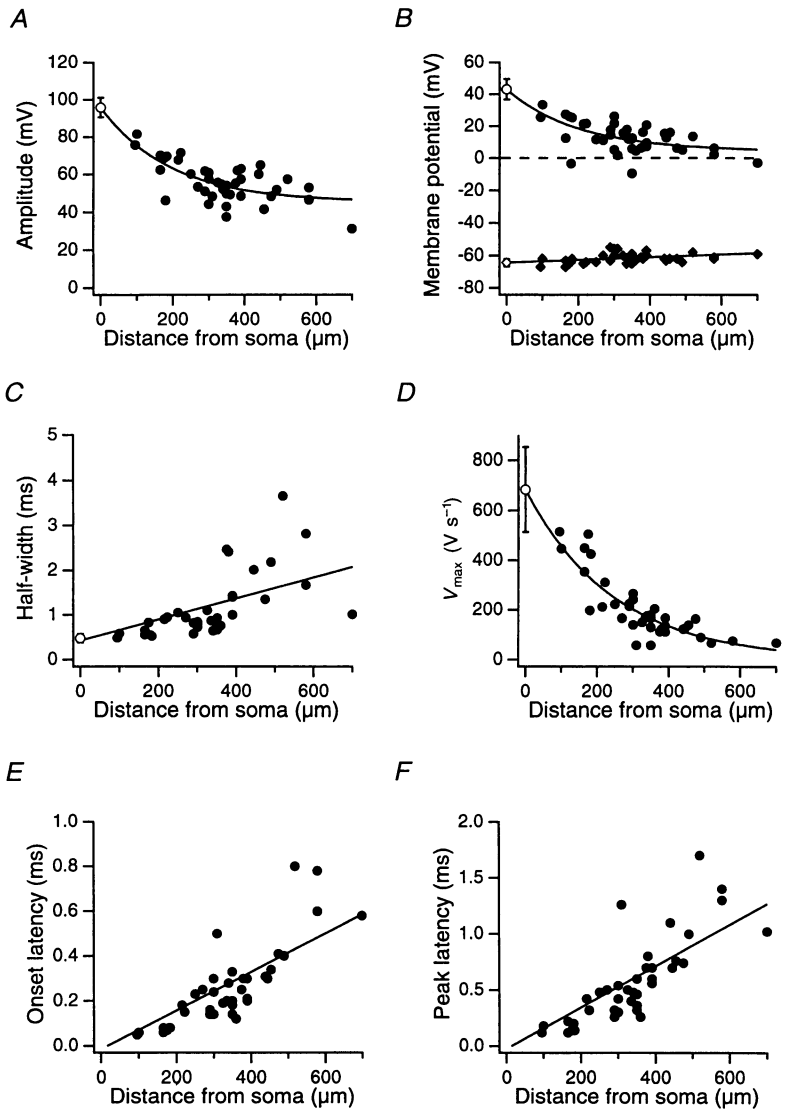

Figure 2. Properties of back-propagating action potentials at different distances from the soma

All data were obtained during simultaneous somatic and dendritic, or somatic, dendritic and axonal recordings at the resting membrane potential during threshold synaptic stimulation in layer 2/3 under conditions where somatic action potentials were initiated prior to any dendritic regenerative response. *A*, amplitude of somatic (○; mean ± S.D.) and back-propagating action potentials at different distances from the soma (●) measured from a baseline set at threshold. The data were fitted to a single exponential with a distance constant of 155 μm (asymptotic amplitude: 50 mV). *B*, membrane potential reached at the peak of somatic (○; mean ± S.D.) and back-propagating action potentials at different distances from the soma (●). The data were fitted to a single exponential with a distance constant of 158 μm (asymptotic amplitude: 9 mV). The resting membrane potential at the soma (◇; mean ± S.D.) and at the different dendritic recording sites (◆) is also indicated. These data were fitted with a linear regression, slope 9 μV μm^{-1}. *C*, width at half-amplitude (half-width) of somatic (○; mean ± S.D.) and back-propagating action potentials at different distances from the soma (●). The data were fitted with a linear regression, slope 2 μs μm^{-1}. *D*, maximum rate of rise (V_{max}) of somatic (○; mean ± S.D.) and back-propagating action potentials at different distances from the soma (●). The data were fitted with a single exponential with a distance constant of 246 μm. *E*, time difference between the onset of somatic and back-propagating dendritic action potentials (onset latency) at different distances from the soma. The data were fitted with a linear regression (forced to go through zero onset latency at 0 μm) whose slope gave a conduction velocity of 1·2 m s^{-1}. *F*, time difference between the peak of somatic and back-propagating dendritic action potentials (peak latency) at different distances from the soma. The data were fitted with a linear regression (forced to go through zero peak latency at 0 μm) whose slope gave a conduction velocity of 0·5 m s^{-1}.

experiments, neurons were filled with biocytin and subsequent staining showed that these cells had apical dendrites which extended to the pia forming an apical tuft ($n = 3$; see Fig. 1*A*). These basic electrophysiological and morphological properties are similar to those previously reported for mature tufted layer 5 pyramidal neurons (see McCormick & Prince, 1987; Kasper, Larkman, Lübke & Blakemore, 1994).

Action potential initiation during threshold and high intensity synaptic stimulation

Simultaneous cell-attached and whole-cell recordings from the soma and apical dendrite of the same layer 5 pyramidal neuron were used to assess the site of action potential initiation during either proximal (layer 4) or distal (top of layer 2/3) extracellular synaptic stimulation (Fig. 1*A*). Extracellular (cell-attached) recording was used to avoid possible effects on action potential initiation of 'wash-out' of intracellular constituents or capacitive loading by the recording pipettes.

Proximal synaptic stimulation always evoked action potentials which were observed to occur first at the somatic recording site, independent of whether action potentials were evoked by threshold or high intensity synaptic stimulation ($n = 5$). Distal synaptic stimulation, however, could shift the apparent site of action potential initiation from close to the soma into the apical dendrites, particularly during high intensity synaptic stimulation (Fig. 1; 10 out of 15 cells). In those cases where it was possible to obtain both cell-attached and whole-cell recordings from the same neurons, the observed site of action potential initiation obtained with the different recording configurations were identical ($n = 7$; see Fig. 1).

These data show that distal, but not proximal, synaptic stimulation can initiate dendritic regenerative responses prior to somatic action potentials, and that the occurrence of this is increased as the intensity of synaptic stimulation increased. One interpretation of this result is that the site of action potential initiation is dependent on the intensity and location of the synaptic input, such that action potential initiation can shift from close to the soma into the apical dendrites during high intensity distal synaptic stimulation, as has been suggested to be the case in hippocampal CA1 pyramidal neurons (Turner, Meyers, Richardson & Barker, 1991; Spruston *et al.* 1995).

Properties of back-propagating action potentials

Following initiation near the soma, action potentials actively propagated back into the dendrites (see Stuart & Sakmann, 1994). The properties of back-propagating action potentials evoked by synaptic stimulation are described in detail below only for those cells where synaptic stimulation evoked somatic action potentials prior to any dendritic response. These cells were chosen for this analysis so as to isolate the properties of back-propagating action potentials from those of dendritically initiated events.

The dependence of back-propagating action potential amplitude on the distance the dendritic recording was made from the soma was similar to that described by Stuart &

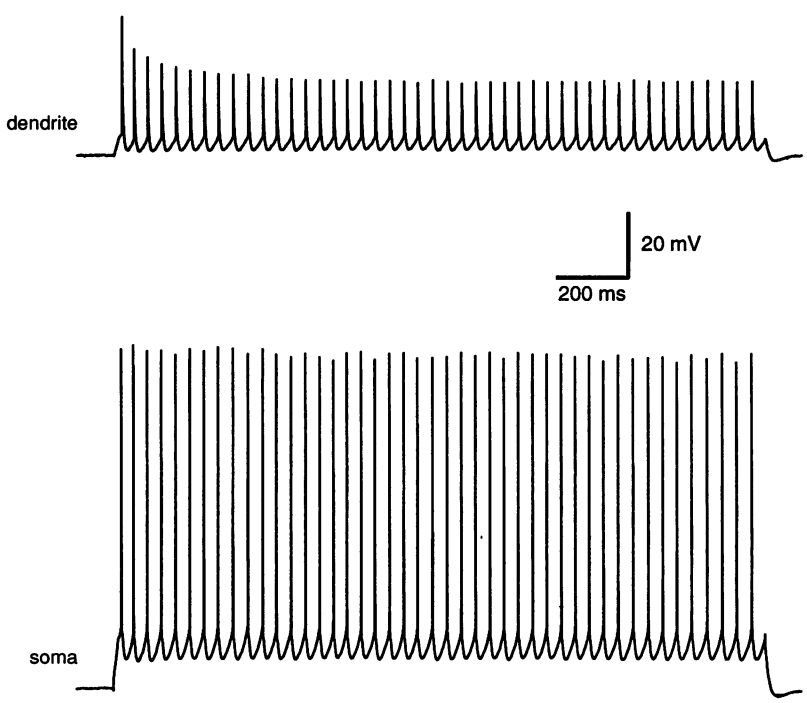

Figure 3. Frequency-dependent attenuation of back-propagating action potentials

Train of action potentials evoked by a long somatic current pulse (1·8 s, 600 pA) during simultaneous dendritic (top) and somatic (bottom) recording 580 μm from the soma.

Sakmann (1994) in 2-week-old animals at room temperature (Fig. 2*A*). Action potentials propagated back into the apical dendrites in a decremental manner, and were still of substantial amplitude 500–600 μm from the soma. Perhaps more important than the amplitude of the voltage change measured from threshold is the absolute dendritic membrane potential reached as this will be the most important determinant of the extent to which back-propagating action potentials activate dendritic voltage-dependent channels or shunt synaptic conductances. The membrane potential reached by back-propagating action potentials at different distances from the soma is shown together with the dendritic resting membrane potential at each dendritic recording site in Fig. 2*B*. At distances up to 700 μm from the soma, the voltage change associated with the back-propagating action potential combined with the dendritic synaptic potential usually depolarized the dendritic membrane potential to or past 0 mV. Note also that the resting membrane potential in the dendrites was slightly more depolarized than at the soma (Fig. 2*B*; on average approximately 4·5 mV more depolarized 500 μm from the soma. This relationship was statistically significant ($P < 0{\cdot}05$). The half-width of back-propagating action potentials increased slightly and their maximum rate of rise (V_{max}) decreased substantially with distance from the soma (Fig. 2*C* and *D*). The latency difference between the onset and peak of somatic and dendritic back-propagating action potentials increased approximately linearly with distance from the soma over the first 700 μm of the apical dendrite (Fig. 2*E* and *F*). The slope of the linear regression fitted to this data gave an approximate conduction velocity of propagation of the onset and peak of action potentials back into the dendritic tree of 1·2 and 0·5 m s^{-1}, respectively (see Fig. 2*E* and *F*).

Recent experiments in hippocampal CA1 pyramidal cells have shown that the amplitude of back-propagating action potentials decreases during a high frequency train, and that failure of back-propagation of action potentials can occur at dendritic branch points (Spruston *et al.* 1995). While no failure of back-propagation of action potentials was

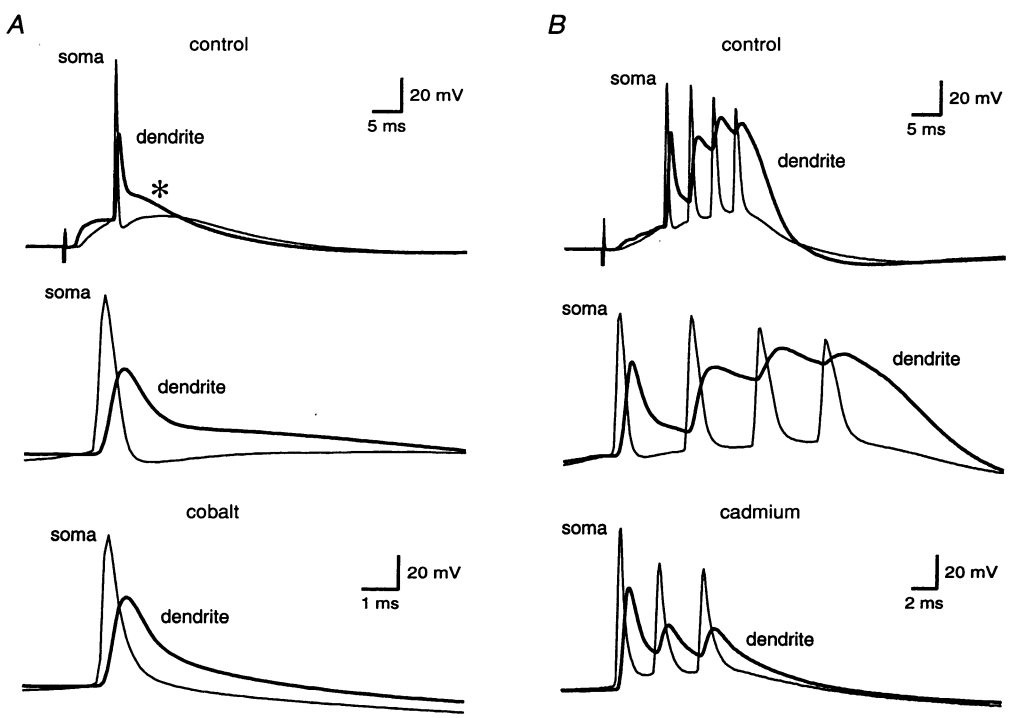

Figure 4. Calcium electrogenesis associated with back-propagating action potentials

A, top: somatic and dendritic (thicker trace) action potentials evoked by threshold synaptic stimulation in layer 2/3. Note the shoulder on the falling phase of the dendritic action potential (*). Middle: same traces as shown in the top on an expanded time scale (see scale bar at bottom). Bottom: effect of $CoCl_2$ (2 mM) on somatic and dendritic (thicker trace) action potentials evoked by a threshold somatic current pulse (200 ms, 500 pA). All recordings from the same cell. Dendritic recording 325 μm from the soma. *B*, top: somatic and dendritic (thicker trace) action potentials evoked by threshold synaptic stimulation in layer 2/3 which evoked a somatic action potential burst. Note the increased amplitude of dendritic action potentials during the burst, despite the decrease in somatic action potential amplitude. Middle: same traces as shown in the top on an expanded time scale (see scale bar at bottom). Bottom: effect of cadmium (200 μM) on somatic and dendritic (thicker trace) action potentials during action potential burst firing evoked by a threshold somatic current pulse (200 ms, 500 pA). All recordings from the same cell. Dendritic recording 390 μm from the soma. Different cell from *A*.

observed at the dendritic recording sites in the present study, there was a clear decrease in the amplitude of back-propagating action potentials during a high frequency (25 Hz) action potential train at distal dendritic recording locations (Fig. 3).

Previous studies have shown that action potentials in the apical dendrites of layer 5 pyramidal neurons are associated with significant dendritic calcium electrogenesis (see Amitai *et al.* 1993; Kim & Connors, 1993). Similarly, back-propagating action potentials recorded in the present study were followed by a shoulder on their falling phase (* in Fig. 4*A*), which was larger the more distal dendritic recordings were made from the soma, and was particularly prominent during bursts of somatic action potentials (Fig. 4*B*). Both the shoulder during the falling phase of dendritic back-propagating action potentials and the larger dendritic response evoked by dendritic back-propagating action potentials during burst firing could be blocked by the application of cadmium (200 μM; $n = 5$), cobalt (2 mM; $n = 4$) or nickel (200 μM; $n = 4$). An example of the effect of cobalt and cadmium on dendritic calcium electrogenesis during single and bursts of somatic action potentials is shown in Fig. 4. Note the reduced amplitude and width of not the first, but subsequent back-propagating action potentials during a high frequency (300 Hz) action potential burst in the presence of cadmium (Fig. 4*B*). These results show that activation of dendritic voltage-activated calcium channels by back-propagating action potentials causes a substantial broadening of the dendritic spike. Furthermore, during burst firing this calcium electrogenesis also increases the amplitude of dendritic back-propagating action potentials.

Properties of dendritic regenerative potentials

In those cases where dendritic regenerative potentials were initiated prior to somatic action potentials following distal synaptic stimulation the further the dendritic recording was made from the soma the longer the time between the onset of the dendritic regenerative potential and somatic action potentials (Fig. 5). This relationship, together with the finding that dendritic regenerative events only occurred during distal, but not proximal, synaptic stimulation indicates that these events are initiated at a distal location, presumably in the apical tuft (see Schiller *et al.* 1997).

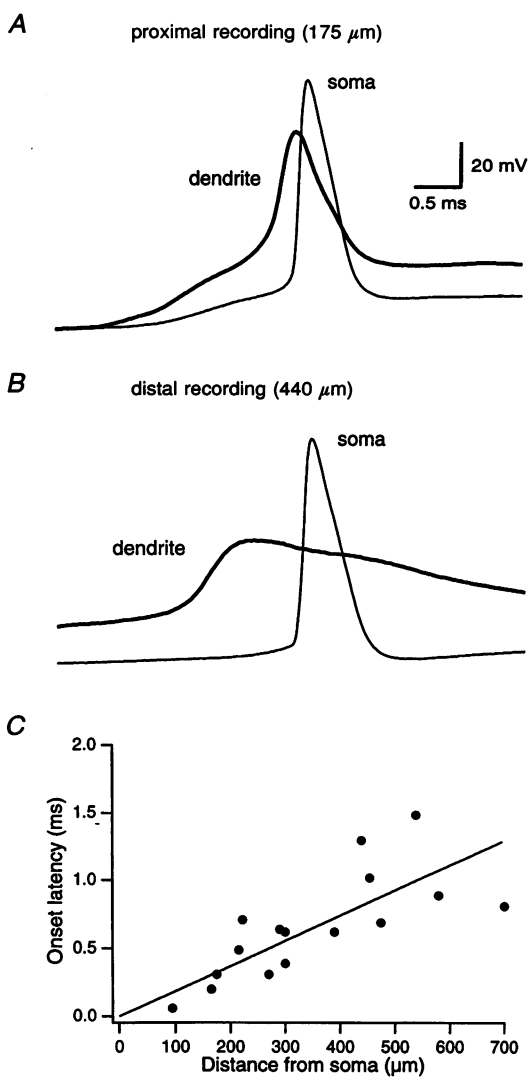

Figure 5. Distal location of dendritic electrogenesis
A, initiation of a dendritic regenerative response (thicker trace) prior to somatic action potentials during proximal dendritic recording (175 μm from the soma; synaptic stimulation in layer 2/3). *B*, initiation of a dendritic regenerative response (thicker trace) prior to somatic action potentials during distal dendritic recording (440 μm from the soma; synaptic stimulation in layer 2/3). Different cell from *A*. *C*, relationship between the time of onset of dendritic regenerative events relative to the onset of somatic action potentials (onset latency) and the distance the dendritic recording was made from the soma for recordings that initiated dendritic regenerative events prior to somatic action potentials. Onset latencies from 3 cells where dendritic regenerative events occurred prior to, but in isolation of, somatic action potentials (see Fig. 8*C*) are not included.

In some cases the apparent site of action potential initiation could fluctuate between the somatic and dendritic recording sites from trial to trial at the same stimulus intensity (Fig. 6*A*). Furthermore, whether dendritic regenerative potentials were initiated prior to somatic action potentials or not could be modulated by depolarization of the somatic (Fig. 6*B*; $n = 5$) or hyperpolarization of the dendritic (not shown; $n = 2$) membrane potential. Thus, an additional factor in determining whether dendritic regenerative potentials will be initiated prior to somatic action potentials will be the level of background excitation and inhibition.

Note back-propagation of somatic action potentials was still observed in those cells where the dendrites initiated regenerative events prior to somatic action potentials, despite the fact that the depolarization associated with dendritic regenerative events might have been expected to inactivate dendritic sodium channels, reducing the ability of action potentials to propagate back into the dendrites. That this was the case can be seen in dendritic recordings were the dendritic response was clearly biphasic, presumably representing first the dendritically initiated regenerative event, and second the back-propagated action potential (see Fig. 6*B*; top). In other recordings a clear separation of dendritically initiated events and back-propagating action potentials was not observed, with the two events merging to form a smooth waveform (see Fig. 6*A*, bottom). Some evidence that dendritic regenerative events may have inactivated dendritic sodium channels comes, however, from more proximal dendritic recordings where following initiation of a dendritic regenerative potential the dendritic component attributable to the back-propagating action potential appeared to be absent (see Fig. 1*C*, bottom).

Conductances mediating dendritic regenerative potentials

To address the issue of which dendritic conductances underlie initiation of dendritic regenerative potentials current injections into the apical dendrite (175–390 μm from the soma) via the dendritic recording pipette were used in an attempt to mimic the dendritic voltage change that occurs during an EPSP (see Stuart & Sakmann, 1995). At threshold, dendritic current injections always evoked action potentials first in the soma (Fig. 7; $n = 8$). Further increasing the amplitude of the dendritic current injection in most cases resulted in the initiation of dendritic

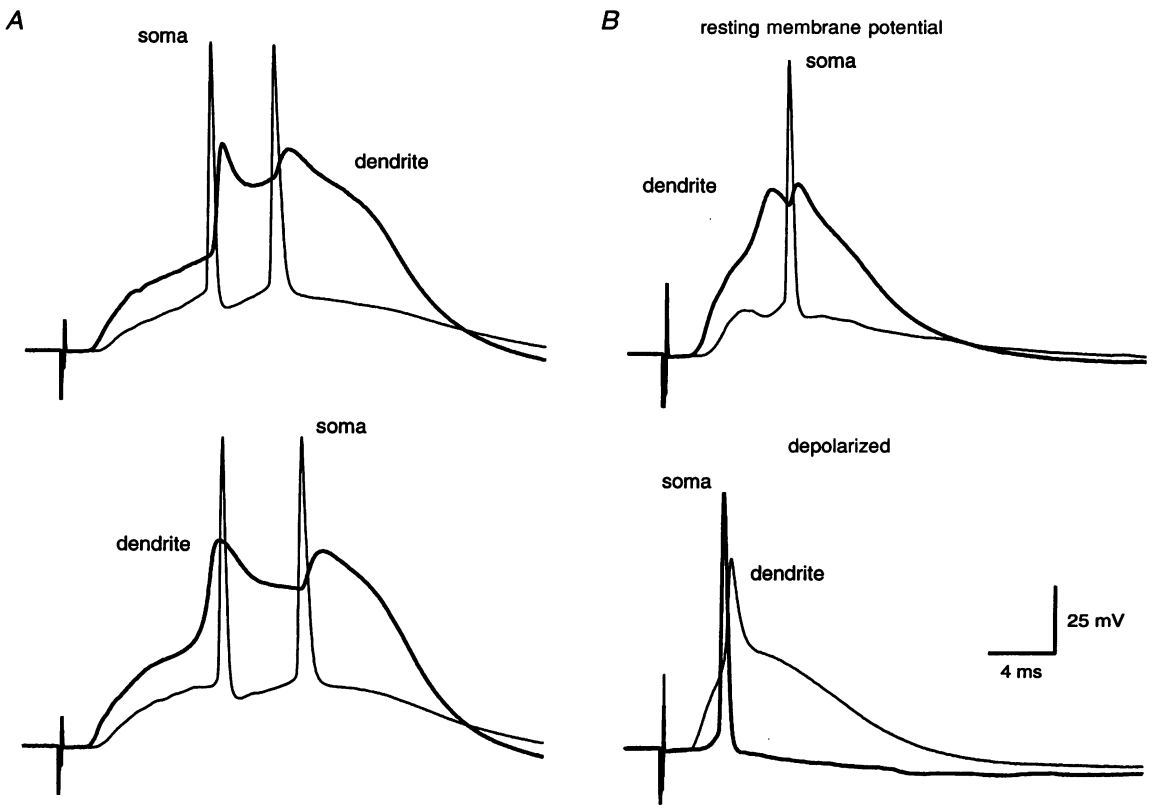

Figure 6. Modulation of the apparent site of action potential initiation

Simultaneous somatic and dendritic (thicker traces) recordings during synaptic stimulation in layer 2/3 (dendritic recording 455 μm from the soma). All recordings from the same cell. *A*, top: somatic action potential initiation prior to dendritic regenerative response. Bottom: initiation of a dendritic regenerative response prior to the first, but not the second, somatic action potential. *B*, top: simultaneous somatic and dendritic (thicker traces) recordings during high intensity synaptic stimulation at the resting membrane potential (different location of synaptic stimulation in layer 2/3). Note the biphasic nature of the dendritically recorded response. Bottom: depolarization of the soma by constant current injection causes the somatic action potential to be initiated prior to a dendritic regenerative response.

regenerative potentials prior to somatic potentials (Fig. 7; 5 out of 8 cells; see also Stuart & Sakmann, 1994). When this occurred the dendritic response was clearly biphasic and similar to that sometimes observed during synaptic stimulation (compare Fig. 7 with Fig. 6*B*, top). Presumably the early dendritic response represents a locally generated regenerative potential initiated close to the dendritic recording pipette in the proximal apical dendrite, and the later response the dendritic voltage change associated with the back-propagating somatic action potential. The application of cadmium (200 μM; $n = 4$) had no effect on the initiation of these dendritic regenerative events (Fig. 7*A*), whereas TTX (1 μM; $n = 3$) completely blocked both dendritic responses and somatic action potentials (Fig. 7*B*). That the application of cadmium in these experiments blocked voltage-dependent calcium channels was confirmed by the complete block of synaptic transmission in these experiments.

Initiation of dendritic regenerative potentials in isolation of somatic action potentials

As shown in Schiller *et al.* (1997), in some recordings dendritic regenerative potentials were observed at stimulation intensities subthreshold for somatic action potential initiation (Fig. 8*B*; $n = 3$), or could appear in apparent isolation of somatic action potentials, preceding them by up to 10 ms (Fig. 8*C*; $n = 3$). In the same neurons at similar stimulation intensities dendritic regenerative events could occur prior to somatic action potentials, apparently synchronized to somatic action potential initiation (Fig. 8*D*; $n = 3$). When initiated in isolation of somatic action potentials, dendritic regenerative potentials attenuated significantly as they spread to the soma, such that the voltage change at the soma in response to these events was difficult to distinguish from that which occurred when distal synaptic stimulation failed to evoke a dendritic regenerative potential (compare Fig. 8*A* and *B*). These results indicate that dendritic regenerative

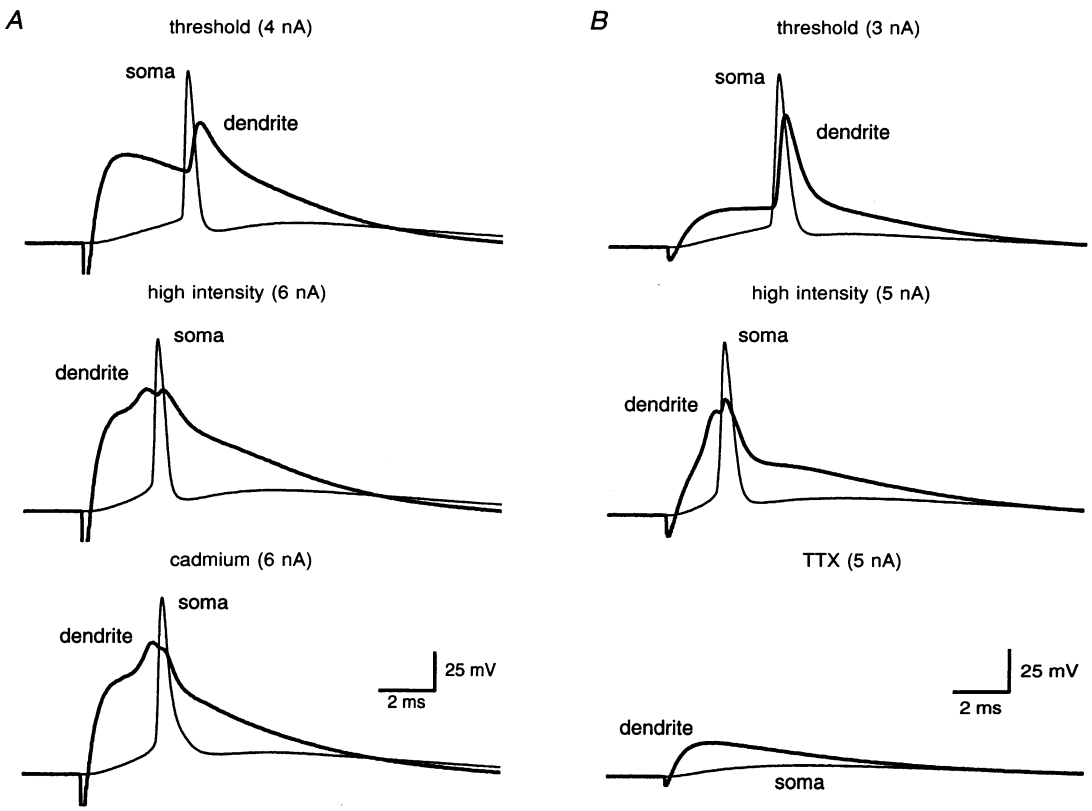

Figure 7. Action potential initiation by simulated EPSPs

Somatic and dendritic (thicker traces) recording of action potentials initiated by a dendritic current injection with the shape of an excitatory postsynaptic current (see Methods). *A*, top: somatic action potential initiation during threshold intensity dendritic current injection (4 nA). Middle: increasing the size of the dendritic current injection (6 nA) initiates a dendritic regenerative response prior to somatic action potentials. Bottom: effect of cadmium (200 μM) on action potential initiation (dendritic current injection 6 nA). Dendritic recording 390 μm from the soma. *B*, top: somatic action potential initiation during threshold intensity dendritic current injection (3 nA). Middle: increasing the size of the dendritic current injection (5 nA) initiates a dendritic regenerative response prior to somatic action potentials. Bottom: effect of TTX (1 μM) on action potential initiation (dendritic current injection 5 nA). Dendritic recording 290 μm from the soma. Different cell from *A*.

potentials undergo significant attenuation as they spread to the soma.

Axonal action potential initiation

To determine the site of action potential initiation, simultaneous recordings were made from the soma and axon (Fig. 9*A*) and action potentials were evoked by distal synaptic stimulation. As previously reported for younger animals (P12–14; Stuart & Sakmann, 1994), synaptic stimulation evoked action potentials which were always observed to occur first at the axonal recording site (Fig. 9*B*; $n = 14$).

Some of the properties of axonal action potentials recorded during simultaneous axonal and somatic recordings are shown in Fig. 10. In some cases action potentials were larger in the axon than the soma (see Fig. 9*B*); however, on average the amplitude of axonal action potentials (recorded up to 30 μm from the axon hillock) was similar to that of somatic action potentials (Fig. 10*A* and *B*). The half-width of axonal action potentials was also similar to that of somatic action potentials (Fig. 10*C*: 0·46 ± 0·06 ms in the axon ($n = 14$) compared with 0·46 ± 0·07 ms at the soma). The rate of rise of axonal action potentials (V_{max}) increased with recording distance from the soma (Fig. 10*D*; this relationship was statistically significant, $P < 0·05$). On average, the V_{max} of axonal action potentials was 826 ± 145 V s^{-1} ($n = 14$) compared with 697 ± 158 V s^{-1} at the soma. Axonal action potentials always occurred before somatic action potentials, with the latency difference between the onset of axonal and somatic action potentials increasing as recordings were made more distal from the soma (Fig. 10*E*). This suggests that the actual site of action potential initiation was in the axon at a distance greater than 30 μm from the axon hillock. The slope of the linear regression fit to this data gave an approximate conduction velocity of action potential onset of 0·4 m s^{-1} (Fig. 10*E*). The latency difference between the peak of somatic and axonal action potentials also increased as axonal recordings were made more distally from the soma (Fig. 10*F*), indicating an approximate conduction velocity of action potential peak of 0·3 m s^{-1}. In some cases ($n = 2$), however, the peak of somatic and axonal action potentials occurred simultaneously, despite a clear difference in action potential onset (Fig. 10*F*). This may have been due to slight damage

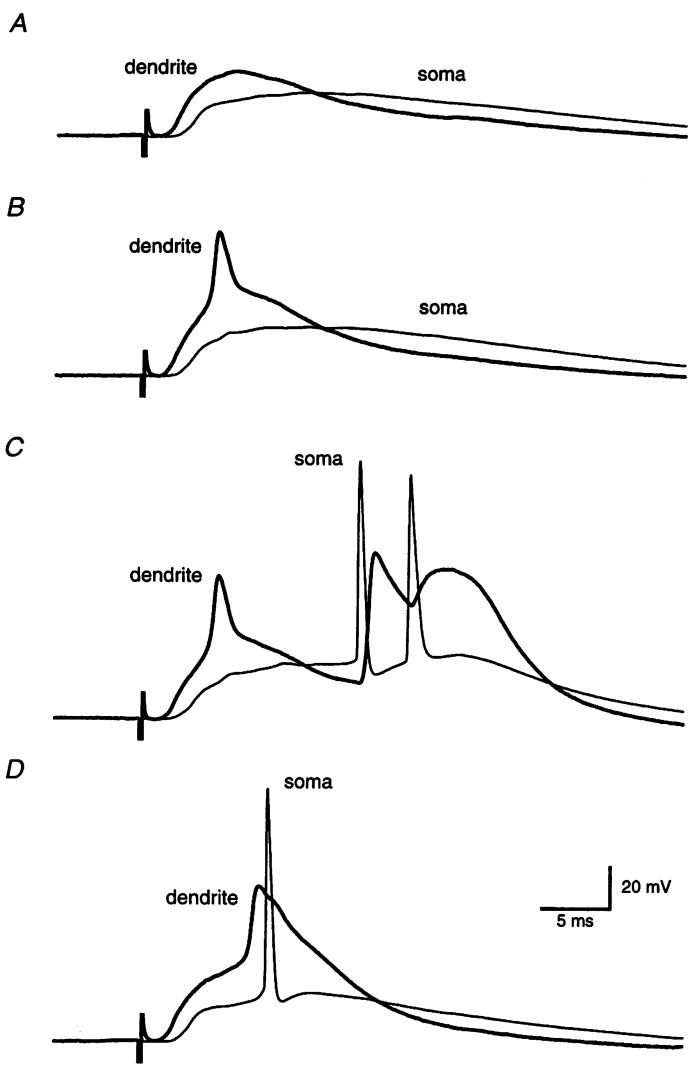

Figure 8. Generation of dendritic electrogenesis in complete and relative isolation of somatic action potentials

Somatic and dendritic (thicker traces) recording during synaptic stimulation in layer 2/3. All recordings from the same cell. Dendritic recording 440 μm from the soma. *A*, subthreshold somatic and dendritic EPSPs. *B*, initiation of a dendritic regenerative potential in the absence of somatic action potentials. *C*, initiation of a dendritic regenerative potential in relative isolation from somatic action potentials. *D*, initiation of a dendritic regenerative potential prior to a somatic action potential.

of the axon during recording, which could also explain the reduced size of axonal compared with somatic action potentials in some cells.

To investigate the site of action potential initiation under conditions where distal synaptic stimulation initiated dendritic regenerative potentials prior to somatic action potentials, simultaneous recordings were made from the soma, dendrite and axon of the same neocortical layer 5 pyramidal neuron (Fig. 11*A*). These experiments showed that at threshold action potentials were always observed first at the axonal recording site, and recorded subsequently by the somatic and then dendritic recording pipettes (Fig. 11*B*; $n = 8$). Distal synaptic stimulation at high intensity was then used to evoke dendritic regenerative potentials prior to somatic action potentials. Under these conditions, the temporal relationship between axonal and somatic action potentials was unchanged (Fig. 11*C*; 6 out of 6 neurons). These experiments therefore show that action potentials are always initiated in the axon before the soma, independent of whether dendritic regenerative potentials are initiated prior to somatic action potentials or not.

DISCUSSION

The experiments described here were designed to locate the site of action potential initiation and propagation during synaptic stimulation of mature layer 5 pyramidal neurons at physiological temperatures. The results show that the site of action potential initiation in these neurons is always in the axon, despite the fact that distal synaptic stimulation can initiate dendritic regenerative potentials prior to somatic action potentials. Once initiated in the axon, action

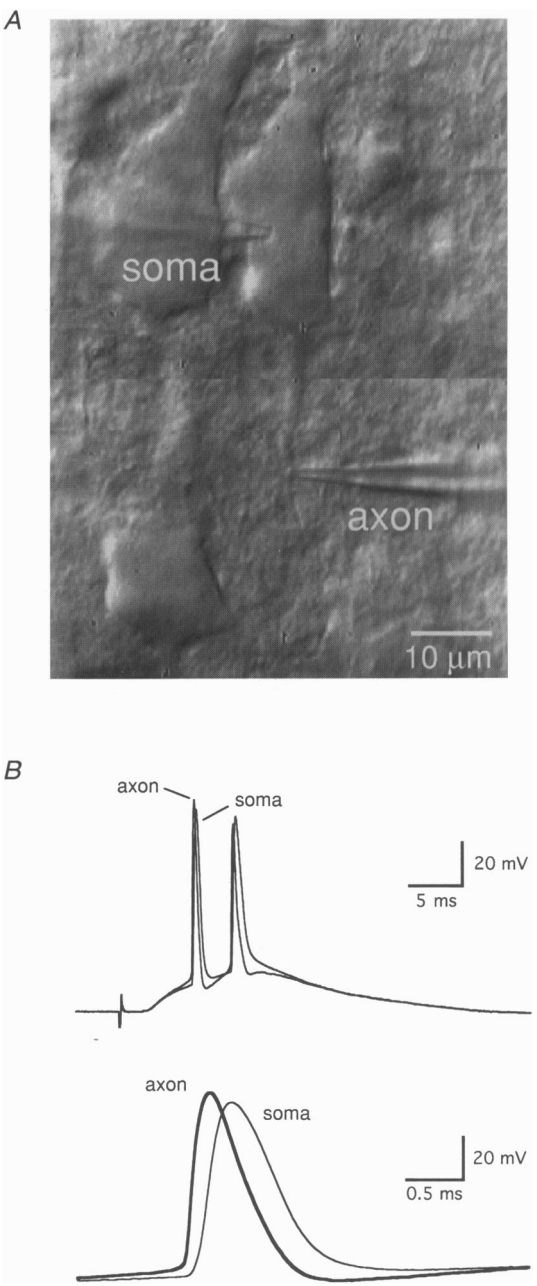

Figure 9. Site of action potential initiation during simultaneous somatic and axonal recording

A, IR-DIC image during a simultaneous somatic and axonal recording from the same layer 5 pyramidal neuron.
B, top: somatic and axonal (23 μm from the edge of the soma) recording during action potential initiation by threshold synaptic stimulation in layer 2/3. Bottom: same recording on an expanded time scale (thicker traces represent axonal recording).

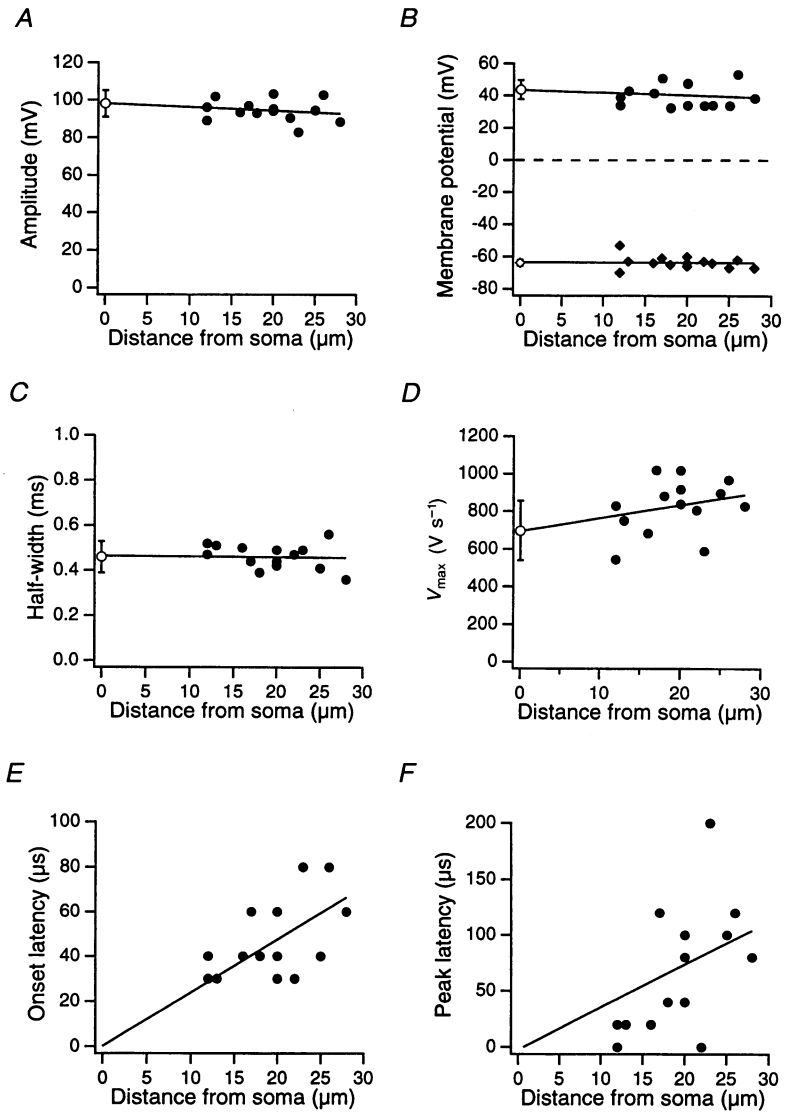

Figure 10. Properties of axonal action potentials at different distances from the soma

All data were obtained during simultaneous somatic and axonal, or somatic, axonal and dendritic recordings at the resting membrane potential during synaptic stimulation in layer 2/3. *A*, amplitude of somatic (○; mean ± S.D.) and axonal action potentials at different distances from the soma (●) measured from a baseline set at threshold. The data were fitted with a linear regression, slope −0·2 mV μm^{-1}. *B*, membrane potential reached at the peak of somatic (○; mean ± S.D.) and axonal action potentials at different distances from the soma (●). The data were fitted with a linear regression, slope −0·2 mV μm^{-1}. The resting membrane potential at the soma (◇; mean ± S.D.) and at the different axonal recording sites (◆) is also indicated. This data were fitted with a linear regression, slope −5 μV μm^{-1}. *C*, width at half-amplitude (half-width) of somatic (○; mean ± S.D.) and axonal action potentials at different distances from the soma (●). The data were fitted with a linear regression, slope −0·3 μs μm^{-1}. *D*, maximum rate of rise (V_{max}) of somatic (○; mean ± S.D.) and axonal action potentials at different distances from the soma (●). The data were fitted with a linear regression, slope 6·9 V s^{-1} μm^{-1}. *E*, time difference between the onset of somatic and axonal action potentials (onset latency) at different distances from the soma. The data were fitted with a linear regression (forced to go through zero onset latency at 0 μm) whose slope gave a conduction velocity of 0·4 m s^{-1}. *F*, time difference between the peak of somatic and axonal action potentials (peak latency) at different distances from the soma. The data were fitted with a linear regression (forced to pass through zero peak latency at 0 μm) whose slope gave a conduction velocity of 0·3 m s^{-1}.

potentials propagated both orthogradely into the axonal arbor and retrogradely into the dendritic tree.

To address the possibility that the site of action potential initiation may change during development (see Mainen *et al.* 1995) all experiments in the present study were conducted on 4-week-old animals. Developmental studies show that the electrophysiological properties of neocortical layer 5 pyramidal neurons are mature by this age (McCormick & Prince, 1987; Kasper *et al.* 1994). Furthermore, at 4 weeks of age the density of both voltage-dependent sodium and calcium channels in neocortical pyramidal neurons has reached that expressed by adult neocortical pyramidal neurons (Cummins, Xia & Haddad, 1994; Lorenzon & Foehring, 1995). The possibility that 'wash-out' of intracellular constituents or capacitive loading by the whole-cell recording pipettes may have affected the site of action potential initiation (see Regehr & Armstrong, 1994) was addressed by comparing action potential initiation during cell-attached recording with that observed during whole-cell recording from the same neuron. That the site of action potential initiation was the same during cell-attached as with whole-cell recording (see Fig. 1) suggests this was not the case.

The main difference between the results of the present study and those of the earlier study on 2-week-old animals (Stuart & Sakmann, 1994) was the enhanced dendritic excitability in 4-week-old animals at more physiological temperatures, which could lead to the initiation of dendritic regenerative potentials during distal synaptic stimulation (see also Schiller *et al.* 1997). This increased dendritic excitability may reflect a developmental increase in the density of dendritic voltage-dependent conductances, although developmental changes in morphology and the passive membrane properties of layer 5 pyramidal neurons (McCormick & Prince, 1987; Kasper *et al.* 1994), or the density and strength of synaptic innervation of the distal apical dendrites may also contribute. In addition, the higher temperatures used in the present experiments (35 °C compared with 22 °C) may also have contributed to the observed increase in dendritic excitability.

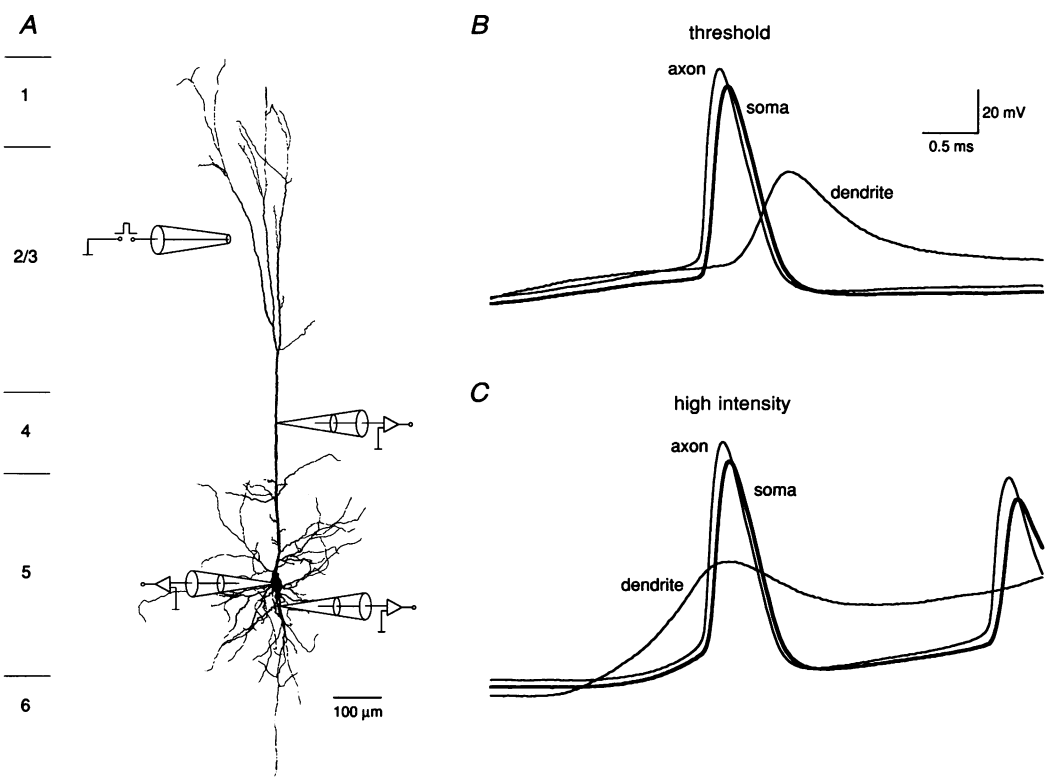

Figure 11. Site of action potential initiation during high intensity synaptic stimulation

A, camera lucida drawing showing the approximate location of stimulation and recording pipettes during simultaneous whole-cell voltage recording from the axon, soma and dendrite of the same layer 5 pyramidal neuron and synaptic stimulation in upper layer 2/3. Numbers on the left refer to approximate borders of cortical layers. *B*, simultaneous recording from the axon, soma (thicker trace) and dendrite after threshold intensity synaptic stimulation in layer 2/3. Same neuron as shown in *A*. *C*, simultaneous recording after high intensity synaptic stimulation which initiated a dendritic regenerative potential prior to the somatic action potential. All traces are from the same experiment. Axonal and dendritic recordings were made 20 and 300 μm from the soma, respectively.

Similar to hippocampal CA1 pyramidal neurons (Spruston *et al.* 1995), back-propagation of action potentials into neocortical dendrites was dependent on somatic action potential frequency, such that there was a decrease in the amplitude of dendritic action potentials during a train of somatic action potentials (Fig. 3). This effect was particularly clear during a high frequency burst of somatic action potentials in the presence of calcium channel blockers (Fig. 4*B*). While the mechanism(s) underlying this observation are unknown, cumulative inactivation of dendritic sodium channels may be involved (Colbert & Johnston, 1996*a*). Failure of action potential back-propagation (see Spruston *et al.* 1995) was not observed at the dendritic recording sites investigated in the present study; however, Ca^{2+} imaging experiments suggest that this may occur in the distal dendrites of the apical tuft (Schiller, Helmchen & Sakmann, 1995).

Axonal action potential initiation

The experiments described here together with results from simultaneous somatic and axonal recordings in cerebellar Purkinje and hippocampal subicular pyramidal neurons (Stuart & Häusser, 1994; Colbert & Johnston, 1996*b*), directly confirm conclusions based on somatic microelectrode recordings that action potential initiation occurs in the axon (see Eccles, 1964). While the exact site of action potential initiation in the axon of neocortical pyramidal neurons is unknown, the increase in the maximum rate of rise and time of onset of axonal action potentials relative to somatic action potentials as recordings were made more distal from the soma (see Fig. 10*D* and *E*) suggests that action potential initiation occurs at a site at least 30 μm distal to the axon hillock. Recent work in hippocampal subicular pyramidal neurons also suggests that action potential initiation occurs in the axon at a site distal from the soma, possibly at the first node(s) of Ranvier (Colbert & Johnston, 1996*b*; see also earlier work in motoneurons by Gogan, Gueritaud & Tyc-Dumont (1983) and Coombs, Curtis & Eccles (1957)). Further evidence is needed to establish if this is the case in neocortical pyramidal neurons.

Dendritic regenerative potentials

The experiments suggest that stimulation of synapses on the distal apical dendrite, particularly at high intensity, can evoke dendritic regenerative potentials initiated in the distal apical dendrites (see also Schiller *et al.* 1997). Once initiated these events propagate to the soma, undergoing significant attenuation. In this respect these dendritic regenerative responses are similar to how Spencer & Kandel (1961) originally described the so called fast prepotentials (FPPs) they observed in hippocampal pyramidal neurons. The dendritically initiated regenerative potentials described here and those in Schiller *et al.* (1997), however, differ from FPPs in that FPPs occur spontaneously and have a fast rise time and decay. Such potentials were not observed. Furthermore, while it was originally thought that FFPs represent initiation of dendritic action potentials, there is now evidence that they are due to action potentials in neighbouring, electrically coupled neurons (MacVicar & Dudek, 1981; Valiante, Velazquez, Jahromi & Carlen, 1995).

Brief dendritic current injections were made into the proximal apical dendrite in an attempt to simulate synaptically evoked dendritic electrogenesis (Fig. 7). At the dendritic recording sites where these current injections were made (175–390 μm distal to the soma), dendritic regenerative potentials were only observed with current injections which were suprathreshold for initiation of somatic action potentials (see Fig. 7). Furthermore, the regenerative potentials initiated by these current pulses were completely blocked by TTX, showing that they were mediated by voltage-dependent Na^+ channels. These findings differ from those described in the preceding paper by Schiller *et al.* (1997), who show that dendritic regenerative potentials evoked by more distally applied (550–940 μm distal to the soma) and longer dendritic current pulses can be evoked under conditions where the soma always remains subthreshold, and which are mediated mostly by dendritic voltage-activated Ca^{2+} channels. This difference is presumably due to the difference in how the regenerative events were initiated by dendritic current injection in the two studies. It seems likely that the short-duration, more proximal dendritic current injections used in the present study would have initated Na^+-dependent dendritic regenerative events more proximally than the long-duration, more distal current injections used in Schiller *et al.* (1997). Given that this was the case, the results from both investigations suggest that dendritic regenerative potentials are mixed Ca^{2+}- and Na^+-dependent potentials which are predominantly mediated by Ca^{2+} channel activation in the distal portions and by Na^+ channel activation in the proximal portion of the apical dendrite.

Relationship between dendritic regenerative potentials and axonal action potentials

While there was, in most cases, a clear temporal relationship between the initiation of dendritic regenerative potentials and the occurrence of somatic action potentials, this was not always the case (Fig. 8; see also Schiller *et al.* 1997). This, together with the finding that distally initiated regenerative potentials attenuate significantly as they spread to the soma, suggests that action potential initiation only occurs in the axon after summation with other synaptic inputs from different parts of the dendritic arbor. That this is the case is clearly demonstrated by the experiments where triple whole-cell voltage recordings were made simultaneously from the dendrites, soma and axon of the same neuron (Fig. 11). These experiments showed that action potentials were initiated in the axon before the soma, and with the same temporal relationship, independent of whether dendritic regenerative potentials occurred prior to somatic action potentials or not. The marked attenuation of dendritic regenerative potentials as they spread to the soma and the axon contrasts with the relative effective propagation of somatic action potentials back into the

dendritic arbor. Such a unidirectional propagation of active potentials within the dendritic arbor is predicted by simulation studies (Rall & Segev, 1987; Mainen *et al.* 1995), and presumably occurs due to impedance mismatches at dendritic branch points and increase in dendritic diameter encountered as a regenerative potential propagates from the dendrites toward the soma (Goldstein & Rall, 1974; Jack, Noble & Tsien, 1983; Rall & Segev, 1987). Non-uniform distributions of dendritic voltage-dependent Na^+, Ca^{2+} and K^+ channels may could also contribute to this attenuation. These findings, and those of Schiller *et al.* (1997), therefore question the interpretation of previous reports of a shift in the site of action potential initiation during high-intensity synaptic stimulation (Turner *et al.* 1991; Spruston *et al.* 1995). They suggest instead that intense distal synaptic stimulation can evoke dendritic regenerative potentials which attenuate substantially as they spread to the soma.

Conclusions and physiological significance

The results from this and the preceding paper (Schiller *et al.* 1997) show that both the distal apical dendrites and the axon of layer 5 pyramidal neurons are capable of initiating regenerative potentials. Dendritic regenerative potentials, however, spread only weakly to the soma and axon. As a consequence, action potential initiation is in the axon even when synaptic input is intense enough to initiate dendritic electrogenesis. Thus the axon is the final site where synaptic integration takes place, providing neurons with a single site where synaptic inhibition will be most effective. Consistent with this idea, anatomical studies show that some classes of inhibitory interneurons form synapses specifically on the axon initial segment (Buhl, Halasy & Somogyi, 1994; Miles, Toth, Gulyas, Hajos & Freund, 1996).

Once initiated, axonal action potentials propagate actively both into the axonal arbor and back into the dendritic tree. Back-propagating action potentials constitute a rapid retrograde signal to the dendritic tree indicating that the neuron has generated an action potential. This retrograde signal is enhanced by dendritic calcium electrogenesis, in particular during bursts of action potentials (Fig. 4). In this way back-propagating action potentials may control, via changes in membrane voltage and $[Ca^{2+}]_i$ transients, both short- and long-term changes in the efficacy of synaptic connections and in addition represent a 'binding signal' for the distributed synaptic contacts of a connection (for review see Stuart, Spruston, Sakmann & Häusser, 1997). The regenerative dendritic potentials observed to occur prior to somatic action potentials during synaptic stimulation of the distal dendrites may have a different function. It has been suggested that ensembles of distant cortical neurons can generate action potentials which are phase locked (Engel, Konig, Kreiter, Schillen & Singer, 1992). While the cellular mechanisms underlying this phase locking are not well established, it seems possible that regenerative potentials in distal dendrites of the nature described here and by Schiller *et al.* (1997) could enhance synchronization by increasing the precision with which cortical neurons respond to common excitatory synaptic input (Softky, 1994). Whether this is the case or not will depend on whether the distal dendritic electrogenesis described above *in vitro* also occurs during the normal *in vivo* operation of the cortex.

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