High Safety Factor for Action Potential Conduction Along Axons But Not Dendrites of Cultured Hippocampal and Cortical Neurons

PAUL J. MACKENZIE AND TIMOTHY H. MURPHY

Kinsmen Laboratory of Neurological Research, Departments of Psychiatry and Physiology, University of British Columbia, Vancouver V6T 1Z3, Canada

Mackenzie, Paul J. and Timothy H. Murphy. High safety factor for action potential conduction along axons but not dendrites of cultured hippocampal and cortical neurons. J. Neurophysiol. 80: 2089–2101, 1998. By using a combination of Ca$^{2+}$ imaging and current-clamp recording, we previously reported that action potential (AP) conduction is reliably observed from the soma to axonal terminals in cultured cortical neurons. To extend these studies, we evaluated Ca$^{2+}$ influx evoked by Na$^+$ APs as a marker of AP conduction under conditions that are expected to lower the conduction safety factor to explore mechanisms of axonal and dendritic excitability. As expected, reducing the extracellular Na$^+$ concentration from 150 to ~60 mM decreased the amplitude of APs recorded in the soma but surprisingly did not influence axonal conduction, as monitored by measuring Ca$^{2+}$transients. Furthermore, reliable axonal conduction was observed in dilute (20 nM) tetrodotoxin (TTX), despite a similar reduction in AP amplitude. In contrast, the Ca$^{2+}$ transient measured along dendrites was markedly reduced in low Na$^+$, although still mediated by TTX-sensitive Na$^+$ channels. Dendritic action-potential evoked Ca$^{2+}$ transients were also markedly reduced in 20 nM TTX. These data provide further evidence that strongly excitable axons are functionally compartmentalized from weakly excitable dendrites. We conclude that modulation of Na$^+$ currents or membrane potential by neurotransmitters or repetitive firing is more likely to influence neuronal firing before AP generation than the propagation of signals to axonal terminals. In contrast, the relatively low safety factor for back-propagating APs in dendrites would suggest a stronger effect of Na$^+$ current modulation.

INTRODUCTION

A number of recent studies contributed to the emerging view that neurons are comprised of compartments of differing electrical excitability, both in the generation and propagation of active conductances. Dual-electrode and imaging studies suggest that action potential (AP) generation does not normally occur in dendrites (Hausser et al. 1995; Stuart and Sakmann 1994); in contrast, the axon at or near the hillock is the site of generation, suggesting greater excitability in the axon. APs that are initiated in the axon can invade dendrites and back-propagate along them in a manner that is sensitive to branching and decrements over distance (Stuart et al. 1997). In contrast, single APs conduct reliably along axons to terminals, indicating that the distal axon is also strongly excitable (Allen and Stevens 1994; Lüscher et al. 1996; Mackenzie et al. 1996; Stevens and Wang 1995). Several lines of evidence suggest AP conduction may fluctuate and be modifiable in an activity-dependent manner. First, conduction block was observed in the spinal cord, particularly after extended periods of high-frequency activity (Lüscher et al. 1994; Wall 1995). Second, Na$^+$ channels, which play a dominant role in AP generation and propagation, can be modulated by second messengers, including protein kinases and G-proteins (Catterall 1993; Li et al. 1993; Ma et al. 1994). Third, several recent reports suggest the possibility that blockade of AP conduction may contribute to fluctuations of synaptic responses. For example, intense activity in principal cells of the cerebellum and hippocampus results in decreased inhibition onto those cells from presynaptic inhibitory cells (Llano et al. 1991; Piter and Alger 1994). In hippocampus, for example, intense postsynaptic activity can lead to failure of evoked but not spontaneous inhibitory postsynaptic currents through postsynaptic Ca$^{2+}$ influx, a retrograde messenger, and possibly involving presynaptic axonal conduction block (Alger et al. 1996). In cerebellar Purkinje cells, fluctuations of inhibitory currents can occur as a result of a presynaptic mechanism downstream of AP generation, possibly including fluctuations in presynaptic depolarization (Vincent and Marty 1996).

Although membrane excitability can be modified, it is unclear how such changes could affect neuronal function. For example, Na$^+$ channel modification could influence AP generation, the propagation of APs to terminals, or the local depolarization of presynaptic terminals. Furthermore, it is unclear whether different neuronal compartments are equally sensitive to changes in Na$^+$ channel function. For example, somatic and dendritic compartments are reported to have similar Na$^{2+}$ channel densities and yet differ in excitability (Hoffman et al. 1997; Magee and Johnston 1995; Schwendt and Crill 1995; Stuart and Sakmann 1994). By using Ca$^{2+}$ imaging, we assessed the excitability of the CNS axon under limiting conditions expected to reduce the safety factor (lowering of the external Na$^+$ concentration). These fine distal axonal branches of mammalian CNS neurons are not directly accessible to patch-clamp or intracellular electrodes. Although brain slice preparations would be preferable for this study, Freguelli and Malinow (1996) found that high concentrations of extracellular Ca$^{2+}$ and intracellular fura-2 were required to reliably view Ca$^{2+}$ dynamics in single boutons in response to a single AP in a slice preparation. Studies performed in cerebellar slices (Calawaert et al. 1996) with more physiological conditions were restricted to initial segments of axons and required averaging of trials and were unable to resolve single boutons.
By using cultures of cortical neurons in which Ca\(^{2+}\) dynamics at single boutons can be reliably measured (Mackenzie et al. 1996), we observe that despite a 60% reduction in somatic spike amplitude reliable conduction of single and pairs of APs was maintained along main and collateral branches of axons. In contrast, along dendrites, although active propagation persisted, the Ca\(^{2+}\) transient, an indicator of the degree of depolarization, was greatly reduced. These results suggest that along the axon there is a large degree of conduction safety leading to reliable terminal depolarization and Ca\(^{2+}\) influx. Consequently, agents that modify Na\(^{+}\) channel function, such as modulating neurotransmitters, are unlikely to exert their effects by altering axonal conduction reliability. In the dendrite, a graded relationship exists between the weakly propagating AP and Ca\(^{2+}\) influx.

**METHODS**

Hippocampal and cortical neurons and glia were dissociated from 17- to 18-day gestation rat fetuses (separate cultures were prepared from the 2 regions) placed in culture and allowed to mature for 17–26 days in vitro as previously described (Murphy and Baraban 1990), with the exception that the plating medium L-cystine concentration was supplemented to 300 μM. The whole cell recording configuration in current-clamp mode (Hamill et al. 1981) was used to fill neurons with fluo-3/fura-2 (Minta et al. 1989; Ragu et al. 1989) and to generate APs by current injection. The patch pipette solution contained (in mM) 0.75 fluo-3 K\(^+\) salt, 1 fura-2 (used to view basal fluorescence), 122 K\(^+\) MeSO\(_4\), 20 NaCl, 5 Mg-ATP, 0.3 GTP, and 10 N-2-hydroxyethylpiperazine-N\(^{\prime}\)-2-ethanesulfonic acid (HEPES), pH 7.2. Imaging was performed with a 100 × 1.3 NA Zeiss objective on a Zeiss Axiosvert microscope with a custom-made stage that permits movement of the preparation during patch-clamp recording. The following extracellular solution was used to isolate the effects of AP stimulation from spontaneous synaptic activity (in mM): 137 NaCl, 5.0 KCl, 2.5 CaCl\(_2\), 1 MgCl\(_2\), 0.34 Na\(_2\)HPO\(_4\) (7 H\(_2\)O), 10 NaHEPES, 22 glucose, 1 NaHCO\(_3\), 20 μM picrotoxin, 100 μM d, l-2-amino-5-phosphonovaleric acid, and 3 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), pH 7.4.

Experiments were performed in current-clamp mode with an Axopatch 200A. To synchronize voltage recordings with video frames, we used a sampling rate of 200 as, which may have underestimated the peak of the AP. Resting membrane potential ranged from −55 to −65 mV; a calculated liquid junction potential of 12 mV was not corrected for (Neher 1995a). Membrane potential was adjusted to −60 mV by injection of a small negative or positive current, and overshooting APs were produced by injection of positive current (5-10 ms duration). In all trials, records of membrane potential were inspected carefully to determine whether the AP changed shape over time or whether stimulation failed to elicit APs. Experiments were terminated if a progressive change in the AP shape or the membrane potential occurred. After establishment of whole cell configuration, data were not collected for 10–15 min to allow diffusion of the Ca\(^{2+}\) indicators into fine processes. Pyramidal-type neurons were used for imaging experiments and were identified by their somatic size, shape, and prominent dendritic process. The axon of the neuron was identified according to the criteria outlined by Mackenzie et al. (1996) and positioned optimally for imaging.

**Perfusion of low-sodium solution**

Computer-triggered solenoid valves (Neptune Research, Northboro, MA) were used to control a gravity-fed double-barreled θ tube (900-μm diam; flow rate 0.75 ml/min) containing normal and low Na\(^+\) solutions. By using a ×2.5 objective and a phenol red containing test solution, the θ tube was placed −4 mm from the center of the chamber so that its contents rapidly exchanged the bath solution of an area (−4 mm\(^2\)) surrounding the cell of interest. As shown in Fig. 1A (not to scale), a compartment of the θ tube contained normal Na\(^+\) (150 Na\(^+\) ) Hanks balanced salt solution (HBSS); the other side contained low Na\(^+\) HBSS (40 mM; ionic substitution with N-methyl-D-glucamine). Figure 1 illustrates that the local perfusion system is able to alter the ionic composition of the extracellular environment surrounding the neuron; this was rapid and reversible. Examination of somatic records of membrane potential revealed that the perfusion system established a low Na\(^+\) environment around the soma of the cell (Fig. 1B, note truncated AP). However, it was also important that the solution was exchanged on distal axonal and dendritic processes. The following points argue that distal axonal and dendritic processes experienced rapid and reversible solution exchange. First, we used a large-diameter θ tube that perfused the solution and hence quickly exchanged it, over several millimeters, clearly encompassing the areas where most distal measurements were made (−500 μm from the soma). Second, after placement of the cell of interest into the field of view but before establishing a whole cell recording, we used a phenol red–containing HBSS in one barrel of the perfusion system to confirm both rapid delivery from one barrel and efficient clearance from the other. Third, the dendrite served as a positive control because dendritic regions that were as distal as axonal regions imaged were also affected by low Na\(^+\) perfusion. Fourth, a zero Ca\(^{2+}\) solution used in the perfusion system blocked Ca\(^{2+}\) transients evoked by APs in distal branches without altering AP generation in six of six neurons (data not shown). Trials of 150 Na\(^+\) and 60 Na\(^+\) were alternated with a 15-s intertrial interval; solution exchange for the following trial occurred at the beginning of this 15-s period. (By using the phenol red–containing medium, we calculated that the [Na\(^+\)]\(_{ext}\) after perfusion was 80 mM within 330 ms and reached a steady state of 60 mM within 2.5 s.) Between trials of data acquisition, cells were bathed with buffer containing 150 Na\(^+\). By using the double-barreled perfusion system, we observed a small (2–5%) increase in basal fluo-3 fluorescence under low Na\(^+\) conditions (presumably caused by blockade of Na\(^+\)/Ca\(^{2+}\) exchanger). This increase in basal fluorescence was ~10% of the increase observed in response to 1 AP and therefore did not appreciably saturate the Ca\(^{2+}\) indicator. This is illustrated for one axonal bouton in Fig. 1 (F), which shows changes in raw fluo-3 fluorescence in response to two somatic APs for an axonal site 174 μm from the soma. In contrast, in preliminary experiments in which continuous (bath) application of low Na\(^+\) medium was used, we observed a robust rise in basal Ca\(^{2+}\) concentration, thus requiring the θ tube application method.

**Imaging and analysis**

A fiberoptically coupled intensified charged-coupled device camera with a Gen III intensifier tube was used for all experiments (Stanford Photonics, Palo Alto, CA) in combination with an Epix 4 M12–64 MB frame grabber board (Northbrook, IL). Images and electrophysiological data were analyzed together on a Pentium computer with custom routines written in IDL (Research Systems, Boulder, CO). Change in fluo-3 fluorescence (excitation 480/40 nm) was used to measure axonal and dendritic Ca\(^{2+}\) dynamics in response to Na\(^+\) APs elicited at the soma (Jaffe et al. 1992; Markram et al. 1995). These intracellular Ca\(^{2+}\) transients reflect AP conduction through an imaged axonal segment because of the following observations: complete block of axonal Ca\(^{2+}\) transients in 1 μM TTX (Fig. 8B), the Ca\(^{2+}\) signal was proportional to the number of APs delivered (Fig. 2C) (Mackenzie et al. 1996), the Ca\(^{2+}\) transients were blocked by removing extracellular Ca\(^{2+}\)
(Mackenzie et al. 1996), and a lack of Ca\(^{2+}\) transients after stimulation that was subthreshold for AP generation (Figs. 2C and 6A) (Mackenzie et al. 1996). Furthermore, it is likely that, given the high indicator concentrations used, the Ca\(^{2+}\) signal is dominated by Ca\(^{2+}\) influx rather than handling (Neher 1995b). To view processes under baseline conditions and to correct for process volume and cell loading, the fluorescent Ca\(^{2+}\) indicator furaptra (excitation 380 nm) was coinjected with fluo-3 into cells. Furaptra was chosen for its high fluorescence signal at basal Ca\(^{2+}\), its low affinity for Ca\(^{2+}\) that produces little buffering, and its well-distinguished spectra that did not contaminate fluo-3 signals (Ragu et al. 1989). Plots of fluo-3 fluorescence versus time were produced off-line from single video frames corresponding to 33-ms intervals. After analysis, data were discarded if basal Ca\(^{2+}\) levels were found to rise significantly during the course of an experiment or if progressive changes in the amplitude of the Ca\(^{2+}\) transient were observed because of factors such as spike broadening. The integral of the Ca\(^{2+}\) transient was termed the Ca\(^{2+}\) response. Ca\(^{2+}\) responses were calculated for individual trials for 1.4 \(\mu\)m\(^2\) regions along dendrites and axons (including individual terminals). Percentage change in fluo-3 fluorescence \((\Delta F/F) \times 100\) was calculated by dividing the change in fluo-3 fluorescence (mean \(F\) over 333 ms after stimulation – baseline \(F\)) by the mean fluo-3 fluorescence measured during baseline. This \(\Delta F/F\) measurement was used for analyses in which fluctuations were measured within individual sites across trials. For analyses of the spatial profile of axonal and dendritic Ca\(^{2+}\) transients, responses were corrected for differences in compartment volume and potential irregularities in illumination or detection by dividing the change in fluo-3 fluorescence by background-subtracted basal furaptra fluorescence and expressed as a ratio of change in fluo-3 divided by furaptra fluorescence \((\Delta F_{\text{axod}}/F_{\text{axod}})\). A single exponential fit was used to determine the decrement of the Ca\(^{2+}\) transient along a region of dendrite.

To detect possible conduction failure in 60 Na\(^{+}\), two analyses were applied. First, for each site, we compared the average Ca\(^{2+}\) response across trials in 150 Na\(^{+}\) to average Ca\(^{2+}\) response in 60 Na\(^{+}\) (paired \(t\)-test). For a given axonal or dendritic site, this test would be most sensitive to consistent decrements in the amplitude of Ca\(^{2+}\) transients across trials. At dendritic sites, a large (in some cases complete) decrement of the Ca\(^{2+}\) response in 60 Na\(^{+}\) was...
observed. In contrast, at axonal sites, we observed a small albeit significant decrease in Ca\(^{2+}\) response in 60 Na\(^+\) (~90\% of 150 Na\(^+\)). However, it is possible that tests evaluating differences in the mean are not sensitive to a single or a few aberrant responses and is therefore insensitive to possible intermittent failure in one or a few trials. In contrast to the mean, the variance of the Ca\(^{2+}\) response across trials is sensitive to single or a few outlying observations. We therefore applied an F-test (ratio of the variance across trials in 150 Na\(^+\) to the variance across trials in 60 Na\(^+\)) to detect occasional conduction failures. To verify that this method was sensitive to single failures, for each experiment, simulated data sets were created for each site that were identical to Ca\(^{2+}\) responses and 150 mM extracellular Na\(^+\) (alternating trials). Delivery in 150 Na\(^+\) but with a known number of failures created in the data set by subtracting the mean response from individual trials. This “failure test” allowed us to confirm that, had conduction failures (and consequent Ca\(^{2+}\) transient failure) occurred, the analysis would have the sensitivity to detect them. This statistical ability to detect a single conduction failure was used as a criterion to select axonal sites with adequate signal to noise properties for analysis; sites that were excluded showed no sign of failure but were simply too noisy to conclusively demonstrate a lack of failure within the number of trials performed. Neighboring sites on small contiguous segments of axon (3–20 \(\mu\)m in length) with low signal to noise were averaged in some experiments to improve the signal to noise ratio.

**RESULTS**

**Reliable axonal conduction in 150 and 60 mm Na\(_{ext}\)**

Conduction reliability along axonal and dendritic processes was monitored by measuring Ca\(^{2+}\) transients in these compartments after AP generation at the soma (Fig. 1A). Figure 1B illustrates 38 overplotted AP waveforms in 60 and 150 mM extracellular Na\(^+\) (alternating trials). Delivery of extracellular solution containing 60 Na\(^+\) resulted in a reversible 60% decrease in the amplitude of the AP. Figure 1C illustrates a typical experimental sequence, with alternating trials of one and two APs (1 and 2 AP) in 60 and 150 Na\(^+\). For two AP the interval between the steps was 50 ms. In 60 Na\(^+\), input resistance to somatic current steps was not significantly different from control solution (Fig. 1, D and E), and basal fluo-3 fluorescence was not appreciably altered in 60 mM Na\(^+\) because of the rapid and reversible perfusion (Fig. 1F, and see METHODS).
To assess the reliability of AP propagation along axons in 60 Na\(^+\), APs were generated and recorded at the soma, and intracellular Ca\(^{2+}\) transients were measured (monitoring changes in fluo-3 fluorescence) at sites along the axon. Figure 2A shows a basal fluorescence image of a segment of main and secondary axon. Figure 2B illustrates superimposed traces of fluo-3 fluorescence versus time at 3 axonal sites in 150 versus 60 Na\(^+\). As we reported previously, in 150 mM Na\(^+\), single APs reliably evoked Ca\(^{2+}\) transients on every trial (Mackenzie et al. 1996). In 60 mM Na\(^+\), Ca\(^{2+}\) transients persisted despite a truncation of the AP, suggesting reliable axonal conduction in 60 Na\(^+\). Figure 2C plots the integral of the Ca\(^{2+}\) transient across trials for one and two APs in 60 and 150 Na\(^+\) for an axonal site. Ca\(^{2+}\) responses in 150 and 60 Na\(^+\) were >2 SDs above baseline variation (Fig. 2C). To investigate whether axonal Ca\(^{2+}\) responses in 150 Na\(^+\) varied with distance, we performed a regression analysis (Ca\(^{2+}\) response vs. distance) on 27 sites from 4 cells that were within 90–175 μm of the soma. The slope of the regression line was 0.08 ± 0.1%/μm (i.e., 8% increase in ΔF\(_{380}/F\(_{380}\) over 100 μm), indicating a small but nonsignificant tendency toward increased Ca\(^{2+}\) responses at more distal sites in 150 mM Na\(^+\) (regression coefficient R = 0.12, P = 0.55).

Reliable AP conduction as assayed by Ca\(^{2+}\) transients was also observed at highly branched tertiary processes that contained most of the synaptic boutons (Fig. 3). These varicose boutons, previously characterized as functional vesicular release sites, exhibited larger Ca\(^{2+}\) transients than neighboring regions of axon (Mackenzie et al. 1996). Figure 3 plots the mean Ca\(^{2+}\) transient at three presumed boutons after one and two AP in both 150 and 60 mM extracellular Na\(^+\). Consistent with the proximal axon, reliable conduction was observed on synaptic bouton-rich tertiary axonal branches, both in 150 and 60 Na\(^+\). A slightly reduced (~10%) Ca\(^{2+}\) transient in 60 Na\(^+\) was observed at both proximal and distal components (>160 μm) of the axon; the degree of reduction did not differ between compartments (P > 0.05, unpaired t-test, distal 59 sites vs. 46 proximal sites from 8 neurons).

Conduction reliability along axons was statistically tested with two analyses (n = 8 neurons). First, at each axonal site, the difference in average Ca\(^{2+}\) transient (60 vs. 150 Na\(^+\)) was evaluated with a t-test. Across 105 sites pooled from 8 neurons, the mean Ca\(^{2+}\) response in 60 Na\(^+\) was significantly lower than the response in 150 Na\(^+\) (90 ± 15% of response in 150 Na\(^+\); P < 0.001, paired t-test). This is illustrated in the plot of the mean Ca\(^{2+}\) response in 60 versus 150 Na\(^+\) shown in Fig. 4A for one AP, where a larger number of observations is below the unity line (P < 0.01, x\(^2\)), indicating a general tendency toward smaller responses in 60 Na\(^+\). The analysis by t-test thus indicated that the mean CA-evoked Ca\(^{2+}\) transients in 60 Na\(^+\) were only minimally affected. However, it was unclear whether this analysis would detect a failure of conduction within a small number of trials because the paired t-test is somewhat insensitive to single outlying observations. In contrast, a comparison of variance in 150 and 60 Na\(^+\) would be sensitive to intermittent failure (see METHODS). Axonal sites with high signal to noise ratio were identified, and a F-test was applied to compare the variance in the amplitude of Ca\(^{2+}\) transients across trials in 150 and 60 Na\(^+\) at these high signal to noise sites. This variance analysis revealed increased variance at only 3 of 105 axonal sites; hence intermittent axonal conduction failure was not observed under the low Na\(^+\) condition expected to promote failure.

**Axonal sites have sufficient signal to noise ratio to detect intermittent failure**

To determine whether the variance analysis would have been sensitive to occasional conduction failure, we created simulated failure data sets from 150 Na\(^+\) data. For each axonal site, the mean Ca\(^{2+}\) response was subtracted from a single trial to create data with one (artificial) failure (Fig. 4C). As predicted, adding one simulated failure to the con-
FIG. 4. Group data showing reliable axonal conduction in 60 mM Na\(^+\). A: mean Ca\(^{2+}\) response (\(\Delta F_{\text{trial}} / F_{\text{ref}} \times 100\%\)) in 60 mM Na\(^+\) (y-axis) vs. mean response in 150 mM Na\(^+\) (x-axis). Pooled data from 105 axonal sites from 8 neurons for 1 AP. Diagonal line represents unity (response in 60 mM Na\(^+\) = response in 150 mM Na\(^+\)). B: coefficient of variation (CV = SD/mean) in 60 mM Na\(^+\) vs. CV in 150 mM Na\(^+\). Diagonal line represents unity (equal CVs in the 2 conditions). C: Ca\(^{2+}\) response integrals evoked by 2 APs at an axonal site plotted across 7 trials in the following conditions: ○, 150 Na\(^+\) (real data set); □, 60 Na\(^+\) (real data set); ▲, simulated failure data (constructed from 150 Na\(^+\) data by subtracting the mean Ca\(^{2+}\) response, in 150 mM Na\(^+\) trials, from trial 6). A significant increase in variance between 150 Na\(^+\) (real) and the “failure” data set was observed (F-test, \(P < 0.05\)) as indicated by the asterisk. Without the simulated failure, no significant difference in variance was observed between 150 and 60 Na\(^+\) at this axonal site, indicating reliable axonal conduction.

Control (150 Na\(^+\)) data set only minimally perturbed the mean response (not reaching statistical significance by \(t\)-test). In contrast to the analysis of the mean, creation of a single failure resulted in a significant increase in variance at 38 of 105 axonal sites from 8 neurons (4 hippocampal and 4 cortical), indicating that it was possible to detect a single failure at these sites had one occurred. This analysis indicated that the 38 sites had signal to noise properties that were sufficient to detect a single failure. When 60 Na\(^+\) medium was compared with 150 Na\(^+\) medium (simulated failure removed), only 3 of the 38 axonal sites showed a significant increase in variance (\(P < 0.05\)). Thus, at most sites where we were confident that we would have reliably measured increased variance, no increase in variance was seen in low Na\(^+\), suggesting no increase in the number of conduction failures. Analysis of conduction reliability of two APs was carried out similarly, except that single failure simulated data sets were created by subtracting one-half of the mean response from one trial (equivalent to 1 AP). For 2 APs, 15 sites from 5 neurons were found to have sufficient signal to noise to permit detection of a failure of 1 AP. No significant difference in variance was observed when APs evoked in 60 and 150 Na\(^+\) medium were compared at these sites. Therefore, at most axonal sites that were determined to be capable of detecting conduction failure, no indicative increase in variance was observed.

At many axonal sites, the signal to noise ratio was too poor to conclusively demonstrate no significant change in variance with a single AP conduction failure (6–12 trials). To compare variance in the two conditions, the coefficient of variation (CV = SD/mean) of Ca\(^{2+}\) responses in 60 Na\(^+\) was plotted versus CV in 150 Na\(^+\), as shown in Fig. 4B. We used the CV to control for the slight decrease in variance expected in the 60 Na\(^+\) group caused by the smaller average Ca\(^{2+}\) response (Fig. 4A). A tendency toward increased variance in 60 Na\(^+\) would appear as a shift of the CV distribution above the unity line. CVs were distributed randomly above and below the unity line (54 above, 51 below; \(P > 0.05\), \(\chi^2\)). Therefore, at all axonal sites, there existed no general tendency toward a greater Ca\(^{2+}\) response variance in 60 Na\(^+\) than in 150 Na\(^+\). We did not observe statistically significant differences between cortical and hippocampal pyramidal neurons in the magnitude of the axonal Ca\(^{2+}\) response in high or low Na\(^+\). Analysis of the variance across trials thus suggested no increase in conduction failure in 60 or 150 mM Na\(^+\) in both hippocampal and cortical axons (data not shown). Therefore in both cell types we observed Ca\(^{2+}\) influx along the axon attributed to reliable and non-nutrient AP conduction in both 150 and 60 mM Na\(^+\).

Dendritic calcium transients evoked by APs are reduced by lowering Na\(_{ext}\). Because axonal conduction (as assayed by Ca\(^{2+}\) imaging) was reliable under conditions of lower external Na\(^+\), we
next investigated whether propagation of single or pairs of APs along dendrites was altered by similar manipulations. Figure 5A, left panel, shows a basal fluorescence image (furaptra excitation wavelength) of a representative pyramidal neuron indicating the main dendrite where Ca\(^{2+}\) measurements were made. Ca\(^{2+}\) transients evoked by single and paired APs were measured along the length of the dendrite (with the 480-nm excitation wavelength of fluo-3 for dynamic Ca\(^{2+}\) measurements). Figure 5B illustrates traces of average dendritic Ca\(^{2+}\) transient (evoked by 2 AP) versus time in 150 and 60 Na\(^+\). In 150 Na\(^+\), dendritic Ca\(^{2+}\) transients decreased with distance from the soma with a length constant of 150 \(\mu\)m (Fig. 5C). However, the decrement with distance of the Ca\(^{2+}\) response along the dendrite was greater in 60 Na\(^+\) than in 150 Na\(^+\) (length constant 53 \(\mu\)m, Fig. 5D) after either one or two AP.

To investigate whether dendritic Ca\(^{2+}\) responses for group data in 150 Na\(^+\) varied with distance, we performed a regression analysis (Ca\(^{2+}\) response vs. distance) on 30 sites from 4 cells that were within 75–160 \(\mu\)m of the soma. The dendritic Ca\(^{2+}\) response in 150 mM Na\(^+\) was negatively correlated with distance \((R = -0.78, P < 10^{-6};\) decrementing 43% over 100 \(\mu\)m). This differs from the spatial profile of the group axonal Ca\(^{2+}\) responses, which did not change significantly with distance. Figure 6 presents group data summarizing the Ca\(^{2+}\) responses in 60 mM Na\(^+\) (as a ratio of response in 150 mM Na\(^+\)) in both the dendrite and axon. Along the axon (Fig. 6A), Ca\(^{2+}\) influx in response to both one and two APs was unchanged in 60 mM Na\(^+\). In contrast, along the dendrite (Fig. 6B), a reduction in the 60 Na\(^+\)/150 Na\(^+\) response ratio was observed over a distance of 40–160 \(\mu\)m for both one and two AP.

**Ca\(^{2+}\) transients in distal dendrites are mediated by TTX-sensitive Na\(^+\) channels**

Because low Na\(^+\) medium selectively decreased the Ca\(^{2+}\) responses along the dendrite, we sought to further characterize the residual dendritic Ca\(^{2+}\) response. To explore whether the remaining Ca\(^{2+}\) response in 60 Na\(^+\) depended on regenerative potentials mediated by TTX-sensitive Na\(^+\) channels, dendritic Ca\(^{2+}\) transients to subthreshold and suprathreshold stimulation were measured in 1 \(\mu\)M TTX. Figure 7A illustrates the Ca\(^{2+}\) response averaged over a region of dendrite...
30–80 μm from the soma (more distal regions would produce a weak signal). The threshold for AP generation in this cell, determined before addition of TTX, and again after washout, was ~600 pA (5 ms of current injection). Figure 7A illustrates the average dendritic Ca$\text{^2+}$ response, in TTX, of one or two 5-ms current steps of 300, 700, or 1,000 pA. Ca$\text{^2+}$ responses to 300-pA current injection were not significantly different from baseline (P > 0.30, t-tests). After 700- and 1,000-pA stimulation, Ca$\text{^2+}$ responses were significantly above baseline variation (P < 0.05, t-tests). After washout of TTX, suprathreshold stimulation that now generated APs resulted in Ca$\text{^2+}$ responses that were 15- to 20-fold greater than responses in TTX, as illustrated in Fig. 7A, (note 6-fold change in scale on y-axis). Again, no measurable Ca$\text{^2+}$ responses were observed after one or two 300-pA current steps (apparently below threshold). Figure 7B illustrates the spatial profile of Ca$\text{^2+}$ responses along the same region of dendrite after two current steps of 1,000 pA in TTX, 700 pA after washout (suprathreshold: 2 AP), and 300 pA after washout (subthreshold). Figure 7B illustrates that Ca$\text{^2+}$ responses to somatic current injection in TTX were markedly reduced along the length of the dendrite (Fig. 7B, inset). Thus the spatially decreasing responses observed under control and low Na$\text{^+}$ conditions were dependent on TTX-sensitive Na$\text{^+}$ channels. Similar results were observed in two other cells. A Ca$\text{^2+}$ spike did not contribute an appreciable component to the residual Ca$\text{^2+}$ response nor was there evidence of a Ca$\text{^2+}$ spike in the somatic voltage records (data not shown).

Conduction safety factor in axonal and dendritic compartments in dilute TTX

To address potential concerns that the low Na$\text{^+}$ manipulation could also affect other Na$\text{^+}$-dependent processes such as pumps and exchangers (Bouron and Reuter 1996), we used a low dose of TTX (20 nM) to attenuate the Na$\text{^+}$ spike to a similar degree as 60 mM external Na$\text{^+}$ (range 45–57% amplitude reduction in 20 nM TTX). Figure 8A illustrates examples of Ca$\text{^2+}$ transients along the dendrite and axon in response to one AP. In the dendrite, the Ca$\text{^2+}$ response at distances >100 μm from the soma was significantly reduced in dilute TTX (compared with control) and decreased with further distance. In contrast, the Ca$\text{^2+}$ response in the axon at distances >100 μm from the soma persisted in 20 nM TTX. In the axon, the Ca$\text{^2+}$ response was reversibly blocked by 1 μM TTX. After TTX washout, Ca$\text{^2+}$ transients were fully restored in both the axon and dendritic compartments. Figure 8B presents group data of the normalized Ca$\text{^2+}$ response from 23 sites from 5 cells (axon) and 39 sites from 5 cells (dendrite) that were >100 μm from the soma.

Reliable axonal AP propagation in neurons from hyperpolarized potentials

A-type K$\text{^+}$ channels have been shown to regulate excitability in dendrites of CA1 pyramidal neurons by limiting AP generation and limiting AP back-propagation in dendrites (Hoffman et al. 1997). A recent report also suggested that A-type K$\text{^+}$ channel could regulate AP conduction in the axon (Debanne et al. 1997). We therefore examined axonal AP conduction from hyperpolarized potentials expected to remove inactivation of the A-type K$\text{^+}$ channels and thereby increase their effect (Segal and Barker 1984; Wu and Barish 1992), possibly leading to conduction failure. Single or pairs of APs were elicited by somatic current steps from −60 or −100 mV. Figure 9A illustrates a basal fluorescence image of three representative secondary and tertiary axonal sites. Figure 9B plots traces of fluo-3 fluorescence versus time in response to pairs of APs from resting and hyperpolarized potentials. No difference in the Ca$\text{^2+}$ transient was observed at hyperpolarized potentials. Figure 9C illustrates traces of membrane potential recorded at the soma after APs evoked from −60 and −100 mV. Figure 9D plots the average Ca$\text{^2+}$ transient elicited by 1 AP from −100 mV versus average Ca$\text{^2+}$ transient from −60 mV for 10 axonal sites from the neuron illustrated in Fig. 9A. The average Ca$\text{^2+}$ response from −100 mV was not significantly different from the −60 mV response (P > 0.05, t-test). Similar results were obtained in five of five neurons.
We explored the propagation of APs along axons and dendrites of hippocampal and cortical neurons by measuring Ca\(^{2+}\) transients along distal regions of axon and dendrite following somatic current steps. We previously reported that single somatic APs reliably result in Ca\(^{2+}\) influx along all branches of axons. This Ca\(^{2+}\) influx occurred predominantly through high-voltage activated Ca\(^{2+}\) channels and was enhanced at distal functional release sites along secondary and tertiary axonal branches (Mackenzie et al. 1996). Here we compared the reliability of axonal and dendritic propagation under normal and limiting conditions expected to reduce conduction safety factor. Axons and dendrites act as separate compartments of differing excitability in normal external Na\(^+\); furthermore AP conduction is differentially sensitive to a reduction in the flux of Na\(^+\) ions along axonal and dendritic compartments.

**Reliable axonal conduction of truncated APs in low Na\(^+\) or dilute TTX**

All axonal sites continued to show reliable Ca\(^{2+}\) transients after somatic APs in reduced extracellular Na\(^+\); this was observed on distal portions of axon as far as we could resolve (>400 μm from the soma) on both main and collateral branches (Figs. 2 and 3). Despite a substantial decrease in AP amplitude in 60 Na\(^+\), Ca\(^{2+}\) transients were still reliably observed at axonal terminals (Fig. 1, B and C). Two analyses were used to test for the occasional failure of the axonal Ca\(^{2+}\) response to a single AP. First, we analyzed 38 sites with sufficient signal to noise to detect intermittent failure (Fig. 4C); at these sites variance analysis indicated that intermittent AP failure does not occur, which is consistent with Dityatev and Clamann (1996). Second, for all 105 sites from 8 cells examined, we measured the CV in 150 Na\(^+\) sites versus the CV in 60 Na\(^+\) and found no consistent tendency toward increased variance in low Na\(^+\) (Fig. 4B), suggesting reliable AP conduction in 60 mM Na\(^+\).

At most axonal sites a small decrease in Ca\(^{2+}\) transient amplitude was observed in 60 mM Na\(^+\) (Figs. 3 and 4). However, this decreased Ca\(^{2+}\) response was more likely due to decreased Ca\(^{2+}\) influx in the terminal than to conduction failure for four reasons. First, Ca\(^{2+}\) transients in 60 Na\(^+\) were only reduced by 10%, were significantly greater than noise (no stimulation), and did not show failure (Figs. 2 and 4). Second, a response decrement with distance was not observed (Fig. 2). Third, at all sites, Ca\(^{2+}\) transients evoked by two AP in 60 Na\(^+\) were greater than those evoked by one AP in 150 Na\(^+\) (Figs. 2 and 3). Fourth, Ca\(^{2+}\) responses to one AP in 60 Na\(^+\) were significantly above noise levels (Figs. 2 and 3). Thus, in 60 mM Na\(^+\), APs successfully conducted to axonal sites. Rather than reflecting conduction failure, the decreased axonal Ca\(^{2+}\) transient in 60 Na\(^+\) may have reflected a direct effect of the Na\(^+\) substitute on the Ca\(^{2+}\) channels or could have been a result of a decreased depolarization in 60 Na\(^+\). The reduction in AP amplitude may have limited the activation of specific classes of Ca\(^{2+}\) channels.

Axonal Ca\(^{2+}\) responses to two APs were less than twofold...
larger than Ca\(^{2+}\) responses to one AP (~90% of an expected doubling of the single AP response). However, it is unlikely that this could be due to intermittent conduction failures because the analysis of response variance (Fig. 4) showed no increased variance in response to two APs versus one AP, indicating reliable conduction. In addition analysis of single sweeps (Figs. 2 and 4) indicates that with two APs the Ca\(^{2+}\) response does not drop to the level mediated by a single AP. Therefore, although there is some attenuation of the Ca\(^{2+}\) signal mediated by a second AP, which could be attributed to a variety of factors including dye saturation or Ca\(^{2+}\) channel inactivation (Forsythe et al. 1998), it cannot be accounted for by intermittent failure.

To address potential concerns that the low Na\(^{+}\) manipulation could also affect other Na\(^{+}\)-dependent processes such as pumps and exchangers, we also used dilute TTX (20 nM) to attenuate the Na\(^{+}\) spike to the same degree as 60 mM external Na\(^{+}\). Ca\(^{2+}\) responses after somatic APs also persisted under these conditions (Fig. 8), confirming reliable axonal conduction.

Our results therefore suggest that modulation of Na\(^{+}\) channels may influence whether the neuron reaches threshold as well as the degree of presynaptic terminal depolarization rather than the conduction of APs along axons. However, other ions such as Ca\(^{2+}\) and K\(^{+}\) may play a role in influencing axonal conduction safety, particularly during high-frequency trains, as demonstrated recently in dorsal root ganglion neurons (Lüscher et al. 1996). To investigate the role of A-type K\(^{+}\) channels, we compared AP-evoked Ca\(^{2+}\) transients from resting and hyperpolarized potentials. Hyperpolarized potentials would be expected to remove steady-state inactivation of A-type K\(^{+}\) channels and increase their effect (Segal and Barker 1984; Wu and Barish 1992). We observed no change in the magnitude of Ca\(^{2+}\) transients (Fig. 9), suggesting that an (inactivated) A-type K\(^{+}\) conductance played little role in the modulation of axonal AP conduction. This contrasts with a recent report that an A-type K\(^{+}\) conductance regulates axonal AP conduction in hippocampal CA3-CA1 cell pairs (Debanne et al. 1997). However, because our method is limited to imaging only a portion of the axon at a time, conduction failure could have been occurring on possibly more distal branches that we were not imaging but that would have been detected in the study of Debanne et al. Furthermore different patterns of axonal branching could have contributed to the differences observed. As for dendritic AP conduction, recent findings of Hoffman et al.
FIG. 9. Reliable axonal AP conduction from hyperpolarized potentials. A: basal fluorescence image illustrating secondary and tertiary axonal branches and associated boutons ~250 μm from the soma (hippocampal). B: traces of change in Δ fluo-3/fura-2 (ΔF_{480}/F_{380}) fluorescence vs. time after 2 APs elicited from −60 mV (thin trace) and −100 mV (thick trace). Traces are average of 8 (−60 mV) and 11 trials (−100 mV) for 3 axonal sites. C: traces of somatic membrane potential illustrating 2 APs 50 ms apart elicited from −60 mV (left) or from −100 mV (right). D: average Ca^{2+} transient (−100 mV) vs. average Ca^{2+} response (−60 mV). Diagonal line represents unity where the −60 mV response equals the −100 mV response.

(1997) demonstrated the regulation of dendritic excitability by an A-type K⁺ channel. We acknowledge that a potential role for Ca^{2+} ions could have been lessened by the high concentration of fluo-3 that was required to achieve a sufficient signal to noise ratio in recordings. Therefore it is possible that exogenous Ca^{2+} buffering may have reduced the activation of Ca^{2+}-dependent K⁺ channels, thereby increasing the reliability of AP conduction. To determine whether conduction reliability was enhanced by Ca^{2+} buffering, we increased the contribution of K⁺ channels during AP repolarization by using a low (1 mM) extracellular K⁺ medium (to increase the K⁺ driving force). We did not observe AP failure in the axon in three of three neurons in preliminary experiments. Additional experiments in high extracellular Ca^{2+} medium (5 mM) were performed to directly increase Ca^{2+} influx (and potential activation of K⁺ channels) with no apparent effect on conduction failure (n = 4 neurons; data not shown).

**Decreased dendritic AP propagation in low external sodium**

We measured Ca^{2+} transients caused by back-propagating APs along the main dendrite of cortical and hippocampal neurons in 150 and 60 Na⁺. In proximal dendritic regions (~30–50 μm from the soma), the Ca^{2+} transients were similar in magnitude to Ca^{2+} responses in the axon (Figs. 2 and 5), consistent with reports by Calleweart et al. (1996) and Llano et al. (1997). In the dendrite, the Ca^{2+} response decreased with distance from the soma with a length constant of 150–220 μm (Figs. 5–7). The degree of attenuation of the Ca^{2+} transient evoked by a single AP along the apical dendrite appears to vary according to the cell type, age, and recording conditions (Schiller et al. 1995, 1997; Spruston et al. 1995; Svoboda et al. 1996; Yuste et al. 1994), presumably reflecting the differential distribution and activation of ion channels and shunting conductances (Jaffe et al. 1992; Migliore 1996; Tsubokawa and Ross 1996; Westenbroek et al. 1992). Rather than focus on factors controlling dendritic back-propagation, we chose to compare dendritic and axonal sensitivity under conditions of low safety factor (low extracellular Na⁺). In proximal dendritic regions (~30 μm from the soma), AP-evoked Ca^{2+} responses in 60 Na⁺ were reduced by ~10% when compared with responses in 150 Na⁺. This slight attenuation observed at the proximal dendrite is likely due to the effects of Na⁺ substitution and not conduction, as discussed previously. However, when more distal regions were examined, the amplitude of the Ca^{2+} response
decreased with distance in 150 Na\(^+\) and to a greater extent in 60 mM Na\(^-\) (Figs. 5 and 6).

To test whether the residual Ca\(^{2+}\) transient in 60 Na\(^+\) depended on Na\(^-\) channels, we measured Ca\(^{2+}\) responses along the dendrite to suprathreshold somatic current steps in the presence of 1 \(\mu\)M TTX in 150 Na\(^+\). In TTX, Ca\(^{2+}\) responses were considerably attenuated along the length of the dendrite; responses were <5% of the 150 Na\(^+\) response obtained after washout of TTX (Fig. 7). The spatial profile in TTX contrasted with the profile in 60 Na\(^+\), where Ca\(^{2+}\) transients at more proximal dendritic regions were considerable, up to 90% of their amplitude in 150 Na\(^+\) (Fig. 5). Therefore most of the Ca\(^{2+}\) response that remained in 60 Na\(^+\) depended on TTX-sensitive Na\(^+\) channels. Dendritic Ca\(^{2+}\) responses to APs differed from axonal responses in their spatial profile in normal external Na\(^+\) and in their sensitivity to a reduction of Na\(^+\) influx.

### Electrical compartmentalization of the neuron

Previous studies contributed to the emerging view that the neuron is composed of compartments of differing excitability (for review see Stuart et al. 1997). Cortical and hippocampal dendrites are capable of local electrogenesis, however this usually stops short of an all-or-none AP. In contrast AP generation results in faithful propagation along the axon (Allen and Stevens 1994; Lüscher et al. 1996; Mackenzie et al. 1996; Stevens and Wang 1995) and back-propagation along dendrites where the amplitude decreases with distance (Spruston et al. 1995; Stuart and Sakmann 1994; Stuart et al. 1997). We report here a marked difference in conduction reliability between axons and dendrites of hippocampal and cortical neurons. The propagation of APs at low frequency occurs in the axon even under conditions of lower external Na\(^+\)). This resistance to external 60 Na\(^+\) was unique to the axon and supports the suggestion that the axon functions as a more excitable electrical compartment (Mainen et al. 1995; Rapp et al. 1996). This considerable degree of safety in the mechanism of AP propagation suggests that in the axon the AP, once generated, is indeed all or none and that Na\(^+\) channel modulation is not likely to play a major role in AP conduction. However, as larger current steps were required to generate an AP in 60 Na\(^+\), we acknowledge a possible role of Na\(^+\) channel modulation at the sites of AP initiation such as the axon hillock. Taken together, this suggests that modulation of Na\(^+\) channels or leakage channels that affect membrane resistance may have a greater impact on the AP generation or on the local depolarization of terminals than on conduction safety. In dendrites, in low Na\(^+\), APs resulted in Ca\(^{2+}\) transients over a more limited distance along the dendrite. These results in the dendrite are consistent with previous results suggesting a graded, modifiable dendritic AP (Jaffe et al. 1992; Spruston et al. 1995; Tsubokawa and Ross 1996). Our results support the hypothesis that regulation of dendritic Na\(^+\) channels can control the spatial extent of dendritic electrogenesis, presumably for both forward synaptic integration and for retrograde AP propagation (Colbert et al. 1997; Jung et al. 1997; Mainen et al. 1995; Rapp et al. 1996).

We thank Drs. J. Seamans, S. Wang, and J. Winslow for helpful comments on the manuscript.

This work was supported by grants from the Medical Research Council of Canada and the EILB foundation to T. H. Murphy, an EILB and Medical Research Council scholar. P. J. Mackenzie is supported by a Medical Research Council studentship. Address reprint requests to T. H. Murphy.

Received 3 March 1998; accepted in final form 9 July 1998.

### REFERENCES


Jung, H.-Y., Mickus, T., and Spruston, N. Prolonged sodium channel


