Action Potentials Induce Uniform Calcium Influx in Mammalian Myelinated Optic Nerves

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Zhang, Chuan-Li, J. Adam Wilson, Justin Williams, and Shing Yan Chiu. Action potentials induce uniform calcium influx in mammalian myelinated optic nerves. J Neurophysiol 96: 695–709, 2006; doi:10.1152/jn.00083.2006. The myelin sheath enables saltatory conduction by demarcating the axon into a narrow nodal region for excitation and an extended, insulated internodal region for efficient spread of passive current. This anatomical demarcation produces a dramatic heterogeneity in ionic fluxes during excitation, a classical example being the restriction of Na influx at the node. Recent studies have revealed that action potentials also induce calcium influx into myelinated axons of mammalian optic nerves. Does calcium influx in myelinated axons show spatial heterogeneity during nerve excitation? To address this, we analyzed spatial profiles of axonal calcium transients during action potentials by selectively staining axons with calcium indicators and subjected the data to theoretical analysis with parameters for axial calcium diffusion empirically determined using photolysis of caged compounds. The results show surprisingly that during action potentials, calcium influx occurs uniformly along an axon of a fully myelinated mouse optic nerve.

INTRODUCTION

Each myelinated axon has two distinct domains (Black et al. 1990; Huxley and Stamplli 1949; Waxman 2002). The first domain occupies a mere 1 μm of the axon length, is referred to as the node of Ranvier, and is the site of excitation. The second domain dominates >99% of the axonal length, is insulated by the myelin sheath, and is referred to as the internode. These two regions are molecularly distinct (Peles and Salzer 2000; Rosenbluth 1976; Scherer and Arroyo 2002). The node is enriched in Na channels, whereas the internode is enriched in K channels with high concentrations of Kv1 channels at the juxtaparanode regions flanking the node (Chiu and Ritchie 1980; Kazarinova-Noyes and Shragger 2002; Ritchie and Rogart 1977; Wang et al. 1993; Waxman and Ritchie 1993). These nodal and internodal regions are also anatomically distinct, exemplified by the wrapping of the internodal axons by the myelin sheath and the differentiation of the glial and axonal membranes in the internode (Arroyo et al. 1999, 2001; Rosenbluth 1995). A major function of demarcating an axon into an insulated (internode) and a noninsulated region (node of Ranvier) is to enable high-speed saltatory conduction through spatial compartmentalization of ionic events, notably the restriction of Na influx at the node of Ranvier (Ritchie and Rogart 1977).

The influx of Na is not the only ionic event that accompanies an action potential in myelinated axons. Recent calcium imaging studies showed that action potentials also induce calcium influx in mammalian CNS axons (Callewaert et al. 1996; Forti et al. 2000; Jackson et al. 2001; Kriegler and Chiu 1993; Lev-Ram and Grinvald 1987; Mackenzie et al. 1996; Mayer et al. 1999; Ren et al. 2000; Sun and Chiu 1999; Verbny et al. 2002; Wachtler et al. 1998). Calcium ions are important intracellular messengers that impact long-term biology (Verbny et al. 2002) and pathology of axons (Stys et al. 1990, 1995; Waxman et al. 2002) and signaling to Na but not to calcium ions.

METHODS

Selective axonal staining

We used a technique developed in our laboratory (Verbny et al. 2002) to selectively stain axons of mouse optic nerves with calcium indicators. The basic principle is to apply, through a tight suction pipette, a high concentration of a cell impermeant calcium dye to the cut end of an excised optic nerve, allowing the dyes to slowly penetrate the axonal cylinders by diffusion and/or axonal transport. We used the cell-impermeant, high-affinity Oregon Green 488 BAPTA-1 (OGB-1, \( K_d = 170 \text{ mM}, MW = 1,114 \)) conjugated to the high molecular weight Dextran (MW = 10,000; Molecular Probes).

Most of the experiments were performed in myelinated axons from adult mice (P30–P60). The optic nerves were excised between the eye and the chiasm and laid down on the bottom of a perfusion chamber. The distal end of the nerve trunk was loosely drawn into a stimulating pipette, and the proximal end drawn tightly into a recording pipette. The nerves were allowed to stabilize for 60 min before dye loading began. For dye loading, the normal saline solution in the recording pipette (the one with the cut end of the nerve tightly drawn in) was replaced either with a high-K (140 mM) or a high-Na (140 mM), low calcium (0 calcium with or without 1 mM EGTA) solution containing 4–5 μl of the cell impermeant form of calcium indicators. The axons were stained by diffusion and/or axonal transport of the dyes from the cut end into the axonal cylinders. We typically performed calcium imaging at a site ~1,000 μm away from the dye-loading pipette. The dyes were allowed to remain in the loading pipette (also serve as the...
DYE LOADING TIME. We varied the dye loading time to achieve enough signal-to-noise ratio to image single axons. In comparing results obtained with 1- and 3-h loading, we found that the 3-h loading gave excellent signal-to-noise ratios and employed this as the standard in this paper. To examine if this 3-h loading causes significant rundown of the nerve, we compared results obtained with 1- and 3-h loading in a few experiments. First, we found that both loading times produced similar results. Thus the key result in this paper, namely, the spatially uniform calcium transients evoked by action potentials (see results, Figs. 5C and Fig. 6B) was observed irrespective of the loading time. Second, we observed little or no change in the conduction velocity (time to peak of the compound action potential measured from the stimulation artifact) during the 3-h loading period, again attesting to the health of the nerve. We sometimes monitored the amplitude of the compound action potential during dye loading and found that it was reduced by ~20% over the 3-h loading period. During subsequent calcium imaging studies lasting typically 1 h, there was little or no reduction in the action potential during 60 min (see Fig. 11C). Third, spatially uniform calcium transients evoked by action potentials were also observed when we reduced the dye concentration in the loading pipette 10 times (from 5 to 0.5 mM, 2 experiments). Collectively, these results suggest that spatially uniform calcium transients evoked by excitation, the key observation in this paper, did not result from pathological development incurred during the 3-h loading period, a time deemed necessary to give sufficient signal-to-noise ratio for calcium imaging of single axons.

NERVE TRANSECTION. The success of the present study relies on selective staining of axons with calcium dyes to the exclusion of glial cells. First, as described in our previous work (Verbny et al. 2002), the use of dextran-conjugated dyes prevents dyes to pass from glial cells at the loading site to glial cells at the site of optical recording via gap junctions. Second, we performed control experiments to address the possibility that dyes from the loading pipette could leak out of the pipette tip, move down through the extracellular space, and be taken up by glial cells through pinocytosis. To address this possibility, we cut out the eye ball in an anesthetized mouse and allowed the axons to degenerate in vivo for 5 days before taking out the nerve and mounting it in the loading pipette for a typical calcium imaging studies. Figure 1A shows the axonal staining from a control optic nerve at a site ~1,000 µm from the tip of the loading pipette. In contrast, no fluorescent signal was observed in the transected nerve at a similar site (Fig. 1B). This suggests that diffusion through the extracellular space is unlikely a pathway to stain glial cells and that the fluorescent signal in Fig. 1A is purely axonal.

Confocal fluorescence imaging of axonal calcium

Calcium images were viewed with a ×40 (Olympus) objective lens coupled to Yokogawa spinning disk system. OGB-1 was used at a concentration of 5 mM in the loading pipette; the actual axonal dye concentration at optical recording site was estimated in our previous study (Verbny et al. 2002) to be ~1.3% of the dye concentration at the loading pipette, which would be ~65 µM. The dyes were excited by an argon laser at 488 nm and confocal fluorescence signals collected through a 500-nm long-pass emission filter. Images of single axons were selected, and calcium images were streamed at a rate of 40 ms between frames. Image acquisition and on-line calculations were controlled through the Metamorph software (Universal Imaging).

**Photolysis of caged calcium compounds**

Caged calcium compound O-nitrophospholyn EGTA (NP-EGTA, tetrapotassium salt) was co-loaded with the calcium indicator OGB-1 (both agents present in the loading pipette) into the optic nerve axons. We used a concentration of 10 mM NP-EGTA in the loading pipette. The pipette solution has calcium omitted, and no EGTA was added. NP-EGTA diffused into the axon cylinders and, because of a high affinity for calcium ($K_d = 80$ nM), binds to calcium in the prephotolysis state. The fraction of NP-EGTA in the axon cylinders that is calcium bound in the prephotolysis state is difficult to determine since the resting calcium level of the axon cannot be easily determined in this study. Uncaging ($K_v$ increases from 80 nM to 1 mM) was achieved by a nitrogen pulse UV laser (model VSL-337ND-S, Laser Science) that produces UV pulses at wavelength of 337.1 nm with a pulse width <4 ns and a peak power of 75 kW. Typically, a train of UV pulses at 25 Hz was applied for 2 s for uncaging. The UV light was delivered onto the nerve sample through fiber optics using an integrated system (Single Path Photolysis Head, Prairie Technologies Madison, WI). This system allows a programmable point or points of UV energy to be directed to a region within the field of view of the microscope objective lens. A patented aiming indicator shows the operator exactly where on the optic nerve the UV energy will be directed. Photolysis was targeted locally to a selected site along an axon, with the area of photolysis being ~4 µm in diameter. The actual size of the photolysis spot was calibrated by directing the UV pulses onto a coverslip coated with black ink. The evaporation of the ink by the UV pulses was used to determine the size of the photolysis spot. UV uncaging of NP-EGTA caused a local rise in free calcium (Ellis-Davies et al. 1996), which is detected by the calcium dye present in the axon cylinders.

**Electrophysiology**

Compound action potentials were evoked by a 125% supra-maximal stimulus applied via the suction electrode to the cut end, and recorded from a second suction electrode at the other cut end. The amplitude of the compound action potential (CAP) data were analyzed using Pclamp 6.0 software (Axon Instruments, CA).

**Computer model for calcium diffusion**

The calcium diffusion data were analyzed using a computer model for calcium diffusion in an elongated cylinder with three diffusible
calcium-binding buffers. The model was simulated using the GENESIS neural simulator (Wilson et al. 1989).

The model consists of an elongated cylinder of diameter 2 μm and a total length of 552 μm. For numerical integration, the axon is divided into 1 μm disks. This model simulates calcium diffusion in the axial direction of the axon following a calcium elevation at specified sites on the axon. Calcium interacts with three buffers according to

\[ \text{Ca}^{2+} + \text{Buffer} \rightleftharpoons \text{Ca}^{2+}\text{Buffer} \]

The diffusion of free Ca, buffer and Ca*buffer between adjacent discs all obey Fick’s Law, which is given in discrete form as

\[ J_{j-\delta} = D_{Cn} \frac{a_i}{\delta} \left[ [\text{Ca}^{2+}]_j - [\text{Ca}^{2+}]_{j+1} \right] \]

where \( J_{j-\delta} \) has units of mol/s, \( a \) is the area of the slice, \( D_{Cn} \) is the diffusion constant for Ca\(^{2+}\), and \( \delta \) is the distance between adjacent slices. The Crank-Nicolson method was used to solve the diffusion equations for Ca\(^{2+}\) and each buffer (Fletcher 1991; Press et al. 1992). In addition, each slice contains a simple pump with a variable time constant for Ca\(^{2+}\) removal, according to the equation

\[ \frac{dC}{dt} = -k \cdot [\text{Ca}^{2+}] - C_{eq} \]

where \( k \) is the time constant, \([\text{Ca}^{2+}]\) is the current calcium concentration, and \( C_{eq} \) is the equilibrium Ca\(^{2+}\) concentration. The time constant \( k \) is set to 1.4×10^{-5}, (de Schutter and Smolen 1998), and \( C_{eq} \) is set to the initial calcium concentration of each slice, 100 nM.

**Simulation of photolysis evoked calcium transient.** To simulate the photolysis data, we introduce a ramp increase in free calcium at a single site in the middle of the axon. Because the photolysis spot is 4 μm, we simulate this by introducing a linear ramp increase in free calcium over 4 μm. In an actual experiment, this increase comes from irreversible destruction of a certain fraction of the caged-calcium compound NP-EGTA via UV photolysis. However, it proves difficult to simulate the release of calcium from NP-EGTA. As an approximation, we assume that calcium undergoes an initial ramp increase at a single site and interacts with the high affinity form of NP-EGTA with \( K_{on} = 8 \times 10^7 \text{ M}^{-1} \text{ s}^{-1} \) and \( K_{off} = 0.4 \text{ s}^{-1} \) (Ellis-Davies et al. 1996) that is uniformly distributed along the axon. The total concentration of NP-EGTA (bound and unbound) is assumed to be 0.13 mM (i.e., 1.3% of 10 mM, the concentration in the loading pipette). Because NP-EGTA is a small molecule, we assume it is highly mobile. We assume that the diffusion coefficient for NP-EGTA (bound and unbound) is similar that of EGTA, which is set at 100 μm²/s (DiGregorio et al. 1999).

**Interaction with endogenous buffer.** The third buffer that calcium interacts with is the endogenous buffer. This compound normally binds calcium with a high affinity, and releases calcium on UV photolysis. As mentioned in the preceding text, it proved difficult to simulate calcium release from NP-EGTA in our model. As an approximation, we assumed that calcium interacts with an endogenous buffer (B) that is assumed to be very immobile with a diffusion coefficient = 30 μm²/s (Gabso et al. 1997). The total concentration (bound and unbound) is 2 mM (DiGregorio et al. 1999). The rates are \( K_{on} = 1.0 \times 10^8 \text{ M}^{-1} \text{ s}^{-1} \), and \( K_{off} = 2.500 \text{ s}^{-1} \) (DiGregorio et al. 1999).

**Resting and peak calcium.** Both concentrations are difficult to determine in our experiments. To simulate the experimental results, we adjusted the parameters for the endogenous buffer and the resting and peak calcium at the end of the ramp. The final resting and peak calcium at the end of the ramp in our model is 100 and 700 nM.

**Solution and drugs.**

The optic nerves were normally bathed in a Ringer solution containing (in mM) 129 NaCl, 3 KCl, 1.2 NaH₂PO₄, 2.4 CaCl₂, 1.3 MgSO₄, 3 HEPES, 20 NaHCO₃, and 10 glucose. All solutions were rigorously oxygenated with 95% O₂-5% CO₂. Calcium-free solutions were prepared by replacing Ca²⁺ with Mg²⁺ to keep the external divalent cation concentration constant, and by adding EGTA (1 mM); pH was adjusted to 7.4 with NaOH or HCl as necessary. 2-Aminoethoxydiphenylborane (2-APB) was purchased from Tocris. OGB-1 (tetrapotassium salt) were purchased from Molecular Probes. All other chemicals were from Sigma. All dye loading and calcium imaging experiments were done at room temperature (20-22°C).

**Results.**

Two models for axonal calcium influx during action potentials

As discussed in the introduction, recent studies from our and other laboratories have established that action potentials induced calcium influx into axons of adult myelinated optic
nerves from mice. Our goal is to examine whether this influx is spatially heterogeneous or homogeneous during nerve excitation. All existing studies on axonal calcium have neither the spatial and time resolution nor the information on in situ calcium diffusion to resolve this issue. Our novel approach is to perform high-speed spatial imaging of axonal calcium in a single axon, use an uncaging methodology to determine the in situ lateral diffusion of calcium ions, and use computer simulations to analyze the calcium signals according to two different models for axonal calcium influx shown in Fig. 2. Most of the experiments were performed on fully myelinated optic nerves from P30–P60 mice. We will first describe the two theoretical models before the empirical findings.

In model 1, calcium influx induced by action potentials occurs only at point sources at the node, followed by calcium spreading axially into the internodes. Using morphological data from mouse optic nerves (Butt et al. 1994), we use an average internodal length of 138 μm, appropriate for myelinated axons from mouse optic nerves at P30–P60 used in our study. This model has three nodes, and given the high speed of action potential propagation, we assume that calcium influx occurs simultaneously at the three nodes, as the time delay for action potential propagation across these three nodes is negligible in the time scale of our current study. In this model, lateral spread of calcium signals from the node into the internode could occur by diffusion, and there are likely cytoplasmic calcium binding proteins that impede this diffusion. Alternatively, nodal calcium could spread to the internode by active propagation using a calcium-induced calcium release (CICR) mechanism. Active propagation of cytoplasmic calcium by these mechanisms can occur at relatively high speed of up to ~260 μm/s (Gordienko et al. 1998), allowing nodal calcium signals to rapidly reach the internodes.

In model 2, the calcium influx induced by action potentials occurs both at the node and throughout the entire internode. This model takes into account recent evidence that calcium channels appear to be present throughout the internodal axon (Brown et al. 2001; Ouardouz et al. 2003). At present, certain types of internodal calcium channels have been shown to be activated during pathological conditions such as ischemia (Ouardouz et al. 2003). Whether internodal calcium channels are activated during normal action potentials is a major unresolved issue and is the concern of the present study.

**Evoked axonal calcium transients show no spatial heterogeneity**

How do we distinguish between these two models? Using a method developed previously in our laboratory (Verbny et al. 2002), we selectively loaded a cell impermeant form of the calcium indicator into axon cylinders of adult myelinated optic nerves, then performed high-speed (40 ms/frame) analysis of the spatial calcium profiles in a single axon evoked by a train of action potentials. The evoked calcium transients have been shown in the previous studies to be due to calcium influx because they are eliminated in calcium-free bath solutions and blocked by the wide spectrum calcium channel blockers (Sun and Chiu 1999; Verbny et al. 2002; Zhang et al. 2004). Figure 3 (bottom) shows an axon segment ~140 μm long with the evoked calcium transients measured at every ~10-μm increment along the axon. In most experiments, the calcium transients are evoked with a standard 2-s action potential train at 25 Hz. The calcium images are captured at one frame every 40 ms sufficient to resolve the rising and falling phase of the calcium transients. The calcium transients were blocked by TTX, showing that they are related to action potentials (data not shown). One possibility is that the calcium transients are due to axonal shrinkage during action potentials. We ruled this possibility out by performing studies using a fluorescence dye that is identical in optical characteristics and molecular weight to our calcium dye, but which is not calcium sensitive (Dextran-conjugated Oregon Green 488, 10,000 MW). When axons were loaded with this calcium insensitive dye, no fluorescence transients were evoked by action potentials (see Fig. 4D, n = 19 axons). Thus the action potential evoked calcium transients are related to calcium changes not to axonal shrinkage.

A striking characteristic of the calcium transients, observed without exception in every axon we examined, is that there is virtually no spatial variation in its amplitude and shape. Although most studies were performed using a 2-s repetitive stimulation, spatially uniform calcium transients were also observed with a single action potential (data not shown). In these studies, we cannot visualize the nodal position. However, since the average internodal length is 138 μm, the 140-μm segment used in this experiment should include at least one node of Ranvier. The observed spatial uniformity in the calcium transients, by itself, cannot distinguish between models 1 and 2. For example, the data would be consistent with model 1 if the lateral diffusion of calcium after nodal entry is very fast so that calcium quickly equilibrates between the node and internode. On the other hand, spatial uniformity in axonal calcium elevation could simply be a direct consequence of model 2, where calcium influx occurs everywhere unrelated to the myelin or nodal position.

**Space constant of calcium attenuation away from a site of local increase is less than the average internodal length**

The decisive factor in distinguishing these two possibilities is to independently measure the rate of axial spread of calcium signal from a node in this same axon to see if the lateral
calcium spread is fast enough to explain the spatial uniformity in the calcium transients. Because there is no easy way to visualize nodes in our preparation, we decide to artificially create a local calcium increase to mimic a nodal calcium elevation and measure the lateral spread of calcium away from it. We solved this problem using localized UV photolysis. Together with the calcium dye, we had co-loaded in this same axon the caged-calcium compound NP-EGTA that releases calcium on UV flashes (Ellis-Davies et al. 1996). The calcium dye and the caged compound, both in their cell impermeant form, are loaded into the axon cylinders using the same loading pipette. We use a photolysis system (Prairie Technology) with the aiming spot for the UV flashes indicated by the white dot (in the middle of the axon); it is \( \sim 4 \, \mu m \) in size. The top row of records (Fig. 3) shows the spatial profile of calcium transients evoked by the point photolysis. The photolysis stimulation lasts 2 s and evokes a calcium transient at the site of photolysis that roughly mimics the calcium transient evoked by action potentials (see Fig. 4). The amplitude of the photolysis-evoked response is largest at the site of photolysis (white dot in the middle of the axon) and then becomes smaller at increasing distance from the site of local increase. Consistent with lateral diffusion, the rise time also declines with distance in most experiments, though in most cases, this is difficult to discern as the signal amplitude quickly attenuates with distance from the photolysis site.

The critical observation in the photolysis is that, as judged from the amplitude of the calcium signal (Fig. 3, top), the space constant of calcium spread away from this hot spot, or “artificial node,” is only \( \sim 30–40 \, \mu m \). This space constant is independent of the location of photolysis along the axon. With an internodal length of 138 \( \mu m \), this lateral spread is clearly insufficient to explain the un-attenuated and spatially uniform calcium response evoked by action potentials along the 140-\( \mu m \) segment.

**Similar diffusion environment for calcium from photolysis and action potentials**

An assumption in the preceding argument is that the calcium released from uncaging diffuses in a similar environment as when calcium was increased using action potentials. There are two reasons to believe that this is the case. First, calcium, whether it comes from NP-EGTA or action potentials, faces the same buffer environment as both photolysis and action potentials were evoked in the same axon loaded with both NP-EGTA and OGB-1. Although both buffers interact with calcium and modify calcium diffusion, this buffered diffusion (to be addressed in details in the following text in computer models) occurs to the same degree whether during photolysis or action potentials. Second, the depletion of NP-EGTA at the site of photolysis appears to be minimal. We used a 2-s train consisting of 50 brief UV pulses to uncage calcium to mimic the 2-s train of 50 action potentials used to evoke calcium elevation. If there were significant depletion of NP-EGTA in the first UV pulse, then calcium will diffuse much earlier in the 2-s UV train because the rest of the train would not contribute to calcium elevation. This would be a very different diffusion profile than the 2-s action potential train, where each action potential presumably contributes to calcium elevation and diffusion occurs throughout the entire 2-stimulation. Thus the key is whether depletion of NP-EGTA occurs and whether each UV pulse causes similar incremental calcium increase.

Figure 4 compares the time course of calcium transient evoked by a 2-s repetitive stimulation at 25 Hz for both UV photolysis (A) and action potentials (B). The averaged peak \( \Delta F/F \) is 0.049 \( \pm \) 0.008 (\( n = 8 \) axons) and 0.082 \( \pm \) 0.011 (\( n = 16 \) axons) for photolysis and action potentials, respectively. The important point is that the rising phase of the photolysis evoked calcium signal is approximately linear (A), suggesting that little or no depletion of NP-EGTA occurred during the 2-s stimulation and that each single UV pulse produced an equal increase in calcium.

**Computer modeling supports uniform, but not punctate, calcium influx**

The argument against model 1 is examined quantitatively using computer modeling. We model longitudinal calcium diffusion in an elongated axon, 2 \( \mu m \) in diameter, in which calcium ions interact with three diffusible buffers: OGB-1, NP-EGTA, and an endogenous buffer. Radial diffusion is ignored. Our approach is to constrain the parameters of the model to fit the space constant data obtained in photolysis, then used the same parameters to calculate predictions of model 1 (with 3 nodes) and compare the predictions with the observations. To minimize the number of free parameters, we fixed the parameters for OGB-1 and NP-EGTA (concentrations, calcium-binding rate constants, and diffusion coefficients) deemed appropriate from other literatures and our current experimental conditions. This leaves the endogenous buffer as the major free parameter adjusted to fit the model to the data with starting values for various parameters associated with the endogenous buffer taken from published works. We started at a resting
calcium level of 100 nM, and adjusted the final calcium level at the end of a 2-s stimulation to 700 nM so that the calculated rising phase of the calcium fluorescence best matches the observed fluorescence transient. In all cases, the predicted concentrations of Ca*OGB-1 were compared with the observed fluorescence data. The final fit of the model to the photolysis data are shown in Fig. 5A, which nicely reproduces the decline of the peak calcium fluorescence signal at increasing distance from the site of photolysis.

Using the same parameters constrained by the fit to the photolysis data, we computed the axonal calcium response evoked by action potentials according to model 1 (nodal influx followed by lateral diffusion). Figure 5B shows the theoretical spatial profile of the amplitude of the calcium response, with three peaks at the three nodes. Deep valleys were seen between nodes, reflecting strong signal attenuation away from the node. According to this model, if one randomly selects axonal segments >138 µm along the axon, one would expect to see amplitude fluctuations of ~70% along the axon (B). This

**FIG. 4.** Comparison of time course of calcium transients evoked by photolysis and action potentials. *Top:* schematic profile of stimulation (2-s, 50-UV pulses or action potentials). *A:* evoked axonal calcium fluorescence transients by photolysis. Measured at site of photolysis. Average of n = 8 axons. *B:* evoked axonal calcium fluorescence transients by action potentials. Average of n = 16 axons. *C:* superimposition of normalized responses from *A* and *B.* *D:* axons loaded with calcium-insensitive fluorescence dyes did not show responses to action potentials (average of n = 19 axons).

**FIG. 5.** Theoretical and observed spatial variation of amplitude of axonal calcium response. *A:* fit of the diffusion model (smooth solid curve) to the photolysis data. The photolysis data are generated from experiments such as Fig. 3 (top) by plotting the normalized peak calcium response at increasing distances from the photolysis site. Data from 9 experiments. *B:* predicted spatial variation in amplitude of the calcium fluorescence evoked by action potentials from model 1 using parameters constrained to fit the photolysis data in *A.* The action potential stimulation is simulated by a ramp increase in calcium from 100 to 700 nM in 2 s simultaneously, and selectively, to the 3 nodes. Calcium elevation in the internodal regions then occurs by diffusion from the nodes. Note response is expressed as [Ca*OBG-1], the calcium bound form of the calcium dye. *C:* observed spatial variation of calcium response evoked by action potentials for 50 randomly selected segments each 140–190 µm long. Calcium response measured every 10 µm increment along each axon.

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prediction clearly contrasts with the observations shown in Fig. 5C. Here the spatial profile of the amplitude of the evoked calcium response, measured at 10-µm increments for a distance of 140–190 µm, is individually plotted for n = 50 randomly selected segments. None of the maximal amplitude fluctuations exceeds 20%, which contradicts the prediction of ~70% from model 1.

Further arguments against model 1 can be made by comparing the time course of the predicted to the observed calcium response. Figure 6A shows the theoretical time course of evoked calcium response at 10-µm increments away from the node. Nodal calcium was assumed to rise in a ramp fashion for 2 s to mimic the experimental conditions. Figure 6B shows the observed calcium transients measured every 10 µm along a 180-µm segment. The predicted amplitude attenuation (A), and the lack of it in the observed traces (B), are recapitulated here. What is particularly revealing is the time course of calcium changes seen when the traces are re-plotted after normalizing to the peak (D and E). At the node, the theory predicts that right after the cessation of the action potential train, the calcium drops immediately. However, as one moves away from the node, the theoretical calcium rises right after the stimulation. This effect is more pronounced as the middle of the internode is approached (for example, see the trace at 70 µm in D).

This predicted behavior for calcium right after the end of the stimulation is clearly contradicted by the observations (E). When the data are normalized to the peak, the responses, obtained every 10 µm over 180 µm, all superimposed (E). In all traces, the observed calcium begins to fall as soon as the action potential train ends. This predicted behavior of calcium response right after the stimulation is a very sensitive test for model 1, even for very short internodal lengths. For example, Butt et al. (1994) gave a range of internodal lengths from 50 to 350 µm; the mean of 138 µm being used in our standard model. Can model 1 explain our data if we recorded from axons with the shortest internodal length of 50 µm?

Figure 6 (C and F) shows the prediction from model 1 using an internodal length of L = 50 µm. Because the nodes are so close together, the prediction shows very little spatial variation in the rising phase (C), making this criterion alone difficult to distinguish it from the observations (B). However, once the calcium behavior following the stimulation is compared, the theoretical prediction (F) and the observation (E) diverge markedly. In fact, at an internodal length of 50 µm with a sampling increment of 10 µm in randomly selected segments, ~50% of the traces should show a calcium rise right after the action potential train. However, in all cases without exceptions, the observed calcium fluorescence declined immediately after stimulation irrespective of spatial location (E). Thus the computer simulations show that model 1 is inconsistent with the observations down to the smallest internodal length (L = 50 µm) based on the anatomical data.

On the other hand, an extremely long internode would also be inconsistent with our data. It could be argued that because we do not know the position of the nodes, some of the calcium imaging studies could have been performed on a very long internode, say several hundred micrometers long, and that calcium transients would be spatially uniform near the middle of a long internode. Computer simulation of model 1 shows that at a distance of 70 µm from a node, the 2-s stimulation produces a calcium fluorescence response that has a significant delay in its rising phase, and does not reach a peak for >10 s (see Fig. 6, A and D). However, none of our calcium responses exhibit a delay in its rising phase (Fig. 6B), and the time to peak is usually ~2 s.

Collectively, the computer model suggests that model 2 best explains our data. The observed spatial uniformity in the evoked calcium transients simply reflects uniform axonal calcium influx, the bulk of which occurs underneath the myelin sheath.

**Sensitivity of model to endogenous buffer**

The major free parameter in our model is the endogenous buffer. Because this buffer can greatly modulate calcium diffusion, we examined the sensitivity of the prediction of the
model to variations in the endogenous buffer. In general, a higher concentration of the endogenous buffer, a higher diffusion coefficient of this buffer, and a higher binding rate constant of calcium to this buffer, will all tend to facilitate calcium diffusion away from a site of local increase. The solid smooth curves in the top row of Fig. 7 shows the final (default) fit of the model to the photolysis data (left, top), the predicted temporal (middle), and spatial (right) calcium profile due to

FIG. 7. Sensitivity of the model to variations in the endogenous buffer. Top: default fit (solid smooth curve) of the model to the fluorescence data. Left: photolysis data. Middle: Temporal calcium fluorescence response to action potentials. Right: spatial calcium fluorescence response to action potentials with 3 nodes. Bottom 4 rows: variation from the default fit by individually varying the concentration of the endogenous buffer (2nd row), diffusion coefficient of the endogenous buffer (3rd row), $K_{on}$ (4th row), and $K_{off}$ (5th row) of the endogenous buffer. For each row, the parameter is either increased ($\times 10$) and decreased ($\times 0.1$) 10 times from the default value.
action potentials according to model 1. The bottom four rows show sensitivity of the model to variation in the four parameters of the endogenous buffer, namely the concentration of the endogenous buffer (2nd row), the diffusion coefficient of the endogenous buffer (3rd row), the $K_{on}$ (4th row), and the $K_{off}$ (5th row).

In each case, the value of parameter is either increased 10 times ($\times 10$) or decreased 10 times ($\times 0.1$) relative to the default value (top). As expected, increases in endogenous buffer concentration, diffusion coefficient, $K_{on}$ and reduction in $K_{off}$ all make the endogenous buffer more efficient in spreading calcium away from the point of local increase as reflected by the increase in space constant of the calcium fluorescence decline from the point of photolysis (left). The corresponding calcium spatial profile for action potentials for model 1 is shown on the right-most column. Notice that based on the right most column alone, we could in principle use model 1 to reproduce the spatial uniformity in calcium transients evoked by action potentials merely by increasing the diffusion coefficient of the endogenous buffer (for example, see 3rd panel in the right-most column showing the effect of increasing the diffusion coefficient of the endogenous buffer by $\times 10$). However, this produces poor fit to the photolysis data (left-most) and the temporal data (middle). The important point here is that if the model is constrained to fit the photolysis data (top left) and the temporal data (top, middle), then the predicted spatial profile during action potentials (top right) shows marked deviation from the uniformity seen in actual experiments. The computation in Fig. 7 thus gives confidence to the choice of parameters used in the final fit.

Sensitivity of model to NP-EGTA concentration and calcium level

We also examined the sensitivity of the model to NP-EGTA concentration and absolute calcium level. For NP-EGTA, we assumed that its concentration in the axon at the site of imaging is 1.3% of the concentration in the loading pipette, with the percentage taken from our previous studies on dextran-conjugated Oregon Green BAPTA that has a much larger molecular weight. We therefore examined the sensitivity of the model to a 10-fold variation in NP-EGTA concentration (either $\times 10$ or $\times 0.1$) from the default value (Fig. 8A). The result shows that the model is not very sensitive to variation of NP-EGTA concentrations. Absolute calcium level is difficult to determine in our preparation, and we used a default value of 100 and 700 nM for the starting and ending calcium level at the end of a 2-second stimulation. Figure 8B shows that doubling (200–1,400 nM) or halving (50 to 350 nM) the starting and ending calcium levels did not significantly alter the prediction of the model.

Effect of calcium-free bath solutions and cadmium

If internodal calcium channels account for the axonal calcium influx, then these channels should not be easily accessible to blockers and ionic exchange manipulations in the bath as the channels are covered by the myelin sheath.

Figure 9 (A and B) shows the effects on the activity-evoked calcium transients in adult optic nerves (C; P30–P60) before and during bath application of 100 $\mu$M Cd$^{2+}$, a wide-spectrum calcium channel blocker. This caused a slow block of the calcium transients (A) without any significant effect on the amplitude of the action potential (B). In contrast, the block of the calcium transient is much faster in neonatal, premyelinated P7 optic nerves (● in A). We also tested the effect of removing bath calcium. This caused a faster abolishment of the evoked calcium transients in the premyelinated axons than in the myelinated axons (C) without a significant effect on the action potential amplitude (D). One possibility to account for the slow block of the calcium transients by Cd$^{2+}$ is that Cd$^{2+}$ slowly enters the axons and quenches the fluorescence of the calcium dye. There are two arguments against this possibility. First, if this were the case, we should observe a reduction in both the evoked calcium transients and the resting calcium fluorescence. However, Cd$^{2+}$ only blocks the evoked calcium transients without affecting the resting fluorescence level. Second, we directly tested if Cd$^{2+}$ quenches fluorescence of calcium dyes in a cell-free system. We started with a 500 $\mu$L of 100 nM Ca buffer solution ($pH = 7.2$ in 100 mM KCl, calcium calibration buffer kit 1 from Molecular Probes) with 65 $\mu$M of the salt form of the dextran-conjugated calcium dye OGB-1, the estimated dye concentration at the axonal site of imaging. We then measured the calcium fluorescence before and 15 min after adding Cd$^{2+}$ (10 $\mu$M, 1 experiment, and 100 $\mu$M, 2 experiments) to the solution. Cd$^{2+}$ was introduced by adding 20 $\mu$L of a concentrated stock solution to the 500 $\mu$L of calcium dye solution to yield the final desired Cd$^{2+}$ concentration and allowed 15 min for

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**Fig. 8.** Sensitivity of the model to O-nitrophenyl EGTA (NP-EGTA) and absolute calcium level. A: spatial profile for action potential-evoked calcium transients calculated according to model 1 when the NP-EGTA concentrations are changed 10-fold (●: either $\times 10$ or $\times 0.1$) from the default (—). B: similar calculations for changing the absolute starting and ending calcium level during action potential stimulation. Default (—): calcium starts at 100 nM and goes to 700 nM during a 2-s stimulation. Ca double (●:): calcium starts at 200 nM and goes to 1,400 nM. Ca halved (•:): calcium starts at 50 nM and goes to 350 nM.
equilibration. We found no quenching of the calcium fluorescence. Hence, quenching of fluorescence of calcium indicators cannot explain the block of evoked calcium transients by Cd\(^{2+}\). Thus the best explanation is that Cd\(^{2+}\) slowly enters the periaxonal space and blocks evoked calcium influx from the extracellular space. The periaxonal space of myelinated axons is known to be accessible to large ions such as La\(^{3+}\) (Hirano and Dembitzer 1969) and even microperoxidase (Feder 1971). Collectively, these results are consistent with the calcium influx sites confined in a diffusion-restricted compartment, presumably underneath the myelin sheath in the internodal region.

**Effect of blocking CICR**

It is possible model 1 could still be correct if a CICR mechanism (through calcium stores operated by ryanodine and IP3 receptors) exists that allow rapid propagation of intracellular calcium from the node to the internode. If this is true, then blocking the node-to-internode calcium propagation with CICR blockers should result in highly localized activity-dependent calcium elevation at the nodes. However, we found that the evoked axonal calcium elevation remains spatially uniform after blockage of intracellular calcium stores with 60 \(\mu\)M ryanodine [a blocker of ryanodine receptor-operated stores in optic nerves (Ouardouz et al. 2003)] and 100 \(\mu\)M 2-APB [a blocker of the IP3 receptor-operated stores (Dobrydneva and Blackmore 2001)]. The results are shown in Fig. 10A. Interestingly, CICR blockers caused a ~50% reduction in the amplitude of the evoked calcium transients (Fig. 10B) without an effect on the action potential amplitude (Fig. 10C), suggesting a CICR component in the amplitude of the evoked calcium transient.

**L-type calcium channels are not responsible for the evoked calcium transients**

Recent studies (Ouardouz et al. 2003) have shown that internodal L-type calcium channels are activated in ischemic axons. Do internodal L-type calcium channels mediate the calcium transients during normal physiological excitation? We therefore examined the effect of the L-type calcium channel blocker nifedipine (10 and 100 \(\mu\)M) on the action potential evoked calcium transients. Figure 11 shows that there is no significant reduction in the evoked calcium transients after 60-min bath application of nifedipine (\(n = 4\) experiments, \(P > 0.05\) for 100 \(\mu\)M; \(n = 4\) experiments, \(P > 0.05\) for 10 \(\mu\)M). Each experiment involved measurement of the total fluorescence from an area of 400 \(\times\) 400 \(\mu\)m, which reflects the average response from many axons. Because nifedipine is lipophilic, it is likely that nifedipine did gain access to the internodal axon during our extended incubation period. Note that at high nifedipine concentration (100 \(\mu\)M), there is a slight (but statistically insignificant) reduction in the action potential (A) due presumably to nonspecific inhibition of Na channels. Collectively, we conclude that L-type calcium channels play little or no role in the calcium transients.

**DISCUSSION**

The myelin sheath functions to speed up action potential propagation while simultaneously economizes on ionic exchange associated with excitation by spatially restricting ionic fluxes, such as Na ions, to the nodes of Ranvier. The novel finding in this study is that myelination does not compartmentalize calcium influx during action potentials. Rather, using single-axon calcium imaging, we observed spatially uniform calcium transients evoked during nerve activity. This finding raises numerous fundamental ques-
tions regarding the functional consequence of the global nature of calcium influx in myelinated axons during normal nerve activity and the mechanisms by which internodal influx sites, though covered by the myelin sheath, are activated by nodal action potentials.

Distribution of calcium channels in a myelinated axon

Voltage-gated calcium channel is a potential candidate for mediating the calcium transients evoked by action potentials because both calcium-free bath solutions and a wide spectrum calcium channel blocker, Cd\(^{2+}\), abolished the calcium transients. Unlike Na channels, which are localized at the node, voltage-gated calcium channels appear to exhibit a more uniform distribution along a myelinated axon. Immunohistochemistry has revealed that L-type calcium channels are broadly distributed in myelinated rat optic nerves (Brown et al. 2001). However, in that work, there is no high-resolution data to show that the channels are inserted and functional on the axolemma. More recent high-resolution EM immunohistochemistry has revealed multi-focal distribution of L-type calcium channels. Foci of L-type calcium channels and ryanodine receptors are present along myelinated axons of the rat spinal cord (Ouardouz et al. 2003). On the other hand, in myelinated nerve fibers in the molars of rats, immunostaining for the L-type calcium channel Cav1.2 is strong at the nodes of Ranvier and faint in the internodal regions (Westenbroek et al. 2004). It would of interest to perform calcium imaging studies on the molar nerves to see if there is a corresponding localization of calcium transients during action potentials.

Different types of calcium channels are activated in pathology and normal physiology

Internodal L-type calcium have been demonstrated to be activated during ischemia and contribute to axonal degeneration (Ouardouz et al. 2003). How might L-type calcium channels be activated under the myelin sheath during ischemia? One possibility is that nodal depolarization is inappropriately transmitted to the internodal membrane during ischemia. For example, there could be a partial loosening of the paranodal seal during ischemia, which allows membrane depolarization to be excessively transmitted to the internode. Another possibility is that ischemia causes a metabolic perturbation, allowing K ions to accumulate in the periaxonal space under the myelin sheath. Because the periaxonal space restricts ionic exchange, a prolong depolarization of the internodal axolemma could result, which in turn leads to activation of internodal L-type calcium channels.

FIG. 11. Nifedipine does not block action-potential-evoked calcium response. Effects of bath application of nifedipine (100 and 10 μM) on compound action potentials (A and C) and evoked calcium transient (B and D). Data were normalized with respect to the predrug value. Action potentials and calcium transients were evoked by stimulation (2 s, 50 pulses) every 5 min for 1-h drug incubation. The plots show results at 30 and 60 min after drug application. The data at 30 and 60 min, though reduced, are not statistically different from the control. n = 4 experiments in each drug concentration. Each experiment represents total fluorescence collected from an area of 400 × 400 μm.
In contrast, in our study, L-type calcium channels do not appear to be the primary pathway mediating calcium influx in a normal nerve during action potentials. First, L-type calcium channel requires high voltage for activation, and it is unlikely that, wrapped by myelin, the internodal axon can experience enough depolarization to activate these channels. Even though there is electrotonic interaction between the nodal and the internodal axon (Barrett and Barrett 1982; Blight and Someya 1985; Chiu and Ritchie 1984), the degree of direct transmission of membrane depolarization from the node to the internodal axon may not be sufficient to activate the internodal L-type calcium channels. For example, theoretical calculations showed that abolishment of an internodal resting membrane potential will depolarize the node by ~20 mV (Chiu and Ritchie 1984), suggesting a limited electronic interaction between the node and the internodal axons that may not be sufficient for activating high-voltage-activated L-type calcium channels on the internodal axon. Second, nifedipine has no significant effects on the calcium transients ~60 min of incubation period. This lack of nifedipine effect on the calcium transients in the mature myelinated optic nerve is also observed in neonatal, remyelinated optic nerve (Sun and Chiu 1999). Hence, L-type calcium channels are unlikely to be a major contributor to calcium transients during action potentials, both in the neonatal nerves and in mature myelinated nerves.

**Diffusion of calcium in photolysis and action potentials**

An assumption in our study is that calcium released from uncaging diffuses in a similar environment as when calcium was increased using action potentials. However, there may be a difference between these two modes of calcium increase, and that is Na entry in action potentials but not in photolysis. Sodium has been shown to positively modulate Ca release from ryanodine receptors (Allard and Rougier 1992; Hu et al. 2003). It is conceivable that during action potential generation, the additional entry of Na potentiates CICR, allowing a faster axial calcium propagation than that indicated by point photolysis. However, there is no realistic way to test this because it would involve comparing calcium data evoked by photolysis to calcium data evoked by action potentials generated without Na influx. Nevertheless, the conclusion of our work is unaffected by this possible uncertainty for two reasons. First, calcium transients evoked by action potentials remain spatially uniform following CICR blockage (Fig. 10A). If calcium influx occurs primarily through nodes of Ranvier during action potentials (model 1) and calcium rapidly propagates axially via a CICR mechanism augmented by Na, then blocking CICR should reveal localized Ca elevation corresponding to nodal positions averaging 138 μm. However, the observed spatial profile for action potential-evoked calcium transients remains spatially uniform after CICR blockage (Fig. 10A). Second, the predicted spatial profiles for these transients (with CICR blocked), if obtained, would have strong deviation from uniformity. All predictions from model 1 were based on parameters extracted from point photolysis data obtained from axons with intact CICR. If CICR were blocked in point photolysis (which we have not done), the efficacy for axial calcium spread in point photolysis studies should be reduced. This should produce a shorter space constant for calcium decline than that shown in Fig. 5A. Consequently, the prediction for the spatial calcium profiles during action potentials, with CICR blocked, should have deeper valleys than that shown in Fig. 5B, further exacerbating the discrepancy between the prediction of model 1 and the observation (Fig. 10A).

**Mechanisms for internodal calcium influx**

Other mechanisms could be mediating the internodal calcium influx. First, as discussed in the preceding text, the cadmium sensitivity of the calcium transients suggests calcium channels are involved. However, because the membrane depolarization of the internodal axon via electronic interaction with the node is unlikely large, internodal calcium channels might need to be of a subtype that has a low threshold for voltage activation. If global axonal calcium signaling is a special functional requirement in a myelinated axon, then insulating by the myelin sheath may be circumvented by increasing the sensitivity of axonal calcium signaling in the internodes. For example, deployment of yet-to-be identified low-threshold calcium channels may facilitate activation of internodal calcium channels. Further, calcium signaling can be amplified by coupling calcium channels to ryanodine receptors and to a CICR mechanism as in the case for L-type calcium channels (Ouardouz et al. 2003). Modest activation of internodal calcium channels may be translated to large axonal calcium elevations through triggering intracellular calcium release. We observed that blockage of CICR reduces activity-dependent calcium transients, consistent with the existence of an intracellular release mechanism to boost internodal calcium signaling.

Second, Na/Ca exchangers are electrogenic and could mediate internodal calcium influx if conditions are met for a reversal of these exchangers. These conditions include a low concentration of Na in the periaxonal space between the myelin and the internodal axon, and a high periaxonal calcium concentration. If the internodal Na/Ca exchangers are near equilibrium in a resting nerve, any depolarizations transmitted from the nodal to the internodal axon during action potentials could mediate calcium influx via reversal of the exchanger. Because cadmium also blocks the Na/Ca exchanger, the sensitivity of the calcium transient to cadmium cannot be used to reliably distinguish between voltage-gated calcium channels and Na/Ca exchangers. Na/Ca exchangers have uniform distribution in mammalian myelinated optic nerves (Steffensen et al. 1997).

Third, K accumulation in the periaxonal space during repetitive nerve activity could evoke calcium influx. Theoretical calculations show that internodal K channels can produce significant K accumulations in the periaxonal space (Chiu 1991). K accumulation in the periaxonal space might allow a slow buildup of internodal membrane depolarization during repetitive activity, causing internodal calcium channel activation and/or reversal of the Na/Ca exchangers. Channel activation by K depolarization might translate repetitive action potentials to cumulative calcium signaling. The restricted diffusion space underneath the myelin is suitable for summation of internodal calcium channel activation, whereas the presumably rapid K clearance at the node would not be indicative for calcium signal integration to occur (Chiu 1991).

Finally, action potentials could trigger oligodendrocytes to release neuroactive substances that target receptors on the...
internodal axolemma. Calcium-permeable nicotinic acetylcholine receptors are present on axons of the optic nerve (Zhang et al. 2004), and other yet-to-be identified ligand-gated ion channels might mediate internodal calcium transients. The argument against this is that ligands might be released only in response to repetitive action potentials, whereas in our case, we have observed spatially uniform calcium transients in response to a single action potential (data not shown).

Nodal calcium channel density appears to be small compared with the internode

Computer simulations show that during an action potential, there is a sharp spatial gradient of membrane depolarization along a myelinated axon (Zhou and Chiu 2001). The large membrane depolarization at the node is sharply attenuated as the internodal axon is reached. How does this spatially heterogeneous voltage profile arise to a spatially uniform calcium transient? One explanation is that the calcium channels/receptors that mediate the calcium transient are present only at the internode. This inference may be difficult to verify using immunostaining technique because the nodal gap is only 1 μm compared with an internodal axon that is ~200 times longer. If these calcium channels/receptors are sparsely distributed, it may be difficult to verify its absence in a mere 1 μm axonal gap against a background internodal length of 200 μm. Detection of local elevation of channel density at the node relative to the internode will be easy with immunohistochemical technique, but not when the channel density is high in the internode but low in the node. We need also to point out that in the molar myelinated nerves of the adult rat, there is nodal clustering of calcium channels (Westenbroek et al. 2004), a result that appears to be at variance with our finding. However, this discrepancy could be due to a difference in nerves because no nodal clustering of calcium channels is observed in the mammalian optic nerves (Brown et al. 2001).

Function of internodal calcium signaling

Our results show that transduction of electrical activity into axonal calcium signaling is locally direct, global in extent and unaffected by the myelin wrapping, a scenario in contrast to the spatially heterogeneous Na+ signaling imposed by the myelin. Why the difference in spatial signaling between these two ions? In the case of Na+, restriction of ionic fluxes to the node makes functional sense as this produces great economy of ionic usage that lessens the metabolic burden accompanying action potentials. Further, Na+ fluxes in the internodes would be unnecessary as the internodes do not participate in fast electrical signaling. However, in the case of calcium ions, internodal calcium signaling may be important for other cellular functions. Internodes comprise 99% of the axonal length, and any axonal processes that require interaction with calcium ions along the entire axon would greatly benefit from direct calcium influx in the internode. Such processes may include calcium-mediated modulation of axonal transport (Breuer et al. 1992), phosphorylation of neurofilaments (Brophy 2001; De Waegh et al. 1992) and actin/cytoskeletal assembly (Bentley and O'Connor 1994; Lankford et al. 1996). Further, calcium in an important stimulator of mitochondrial oxidative metabolism (Robb-Gaspers et al. 1998), and internodal calcium signaling can translate nerve activity to metabolic needs. Mitochondrial dysfunction underlies axonal degeneration in multiple sclerosis (Andrews et al. 2005; Mutzaers and Carroll 1998), and whether internodal calcium signaling is linked to mitochondrial dysfunction in axonal degeneration remains to be investigated. Finally, internodal calcium influx might activate calcium-sensitive ion channels in myelinated axons (Jonas et al. 1991), leading to modulation of excitability after extended periods of nerve activity. In the premyelinated optic nerve axons, removal of bath calcium results in modulation of excitability after tetanus stimulation (Verbny et al. 2002). It remains to be seen if blocking internodal calcium channels in mature myelinated axons affects excitability after intense period of nerve excitation.

Collectively, activity-dependent calcium signaling along the entire axon may allow use-dependent modulation of these various processes, affecting biology and development of the axons. If this signaling molecule (Ca2+) is supplied only through the node, then it has to diffuse to 99% of the axon to exert its effect. Diffusion of calcium along axons is slow and likely impeded by endogenous binding proteins (al Baldawi and Abercrombie 1995). Further, the signal would suffer severe spatial attenuation with a space constant of ~30 μm (Fig. 3A), which would compromise delivering calcium ions to long internodes, some reaching 1,700 μm in large CNS axons (Hess and Young 1949). In conclusion, our study unravels a new organization of ionic trafficking in myelinated axons during normal nerve activity whereby the classical restriction of Na+ signaling at the node for fast electrical signaling is complemented by a spatially uniform calcium signaling that appears to be adapted for use-dependent global functional modulation. The physiological activation of internodal calcium channels demonstrated in this study must be under careful control, as pathologic overdrive of internodal calcium channels leads to axonal death (Ouardouz et al. 2003).

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