Coupling of Calcium Homeostasis to Axonal Sodium in Axons of Mouse Optic Nerve

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INTRODUCTION

Traditionally, translation of electricity to biochemistry is thought to take place at the nerve terminal of a neuron where electricity (the action potential) is translated into biochemistry via calcium-mediated vesicular release of neurotransmitters. Prior to the nerve terminal, the axon has been regarded as a high-speed conduit whose major role is transmission of electricity rather than translation of electricity to biochemistry. This picture of axons is no longer tenable, as recent studies have firmly established activity-dependent calcium influx into mammalian axons, including unmyelinated axons from rat neocortical pyramidal neurons (Schiller et al. 1995), cerebellar Purkinje cells (Callewaert et al. 1996), cultured dorsal root ganglion cells (Lüscher et al. 1996), neonatal rat optic nerve (Sun and Chiu 1999), and rat vagus nerves (Wächter et al. 1998). Activity-dependent calcium signals have also been detected from myelinated axons in the rat optic nerves (Lev-Ram and Grinvald 1987). The physiological role of axonal calcium influx remains unknown. In developing axons of the rat cerebellar interneurons, Forti et al. (2000) observed local calcium hot spots, which they speculated, might represent functional clusters of voltage-dependent Ca2+ channels marking the future position of presynaptic terminals. A local rise in calcium may trigger actin/cytoskeletal assembly (Bentley and O’Connor 1994; Lankford et al. 1996). Edwards and Cline (1999) hypothesize that local axonal calcium influx may regulate the addition of new branch points in growing retinal axons. However, in mature axons such as the mammalian optic nerves where varicosities or branches are absent, the functional role of calcium influx along the entire length of axon remains unclear. Pathologically, excessive elevation of axonal calcium is injurious to axons (for review, see Stys et al. 1995a,b). One of the best-studied mammalian CNS white-matter tracts where calcium has been implicated in axonal pathology is the mammalian optic nerve (Stys et al. 1995). This nerve can be isolated without neuronal contaminations and is highly amenable to experimental studies, including calcium imaging (Krieglstein and Chiu 1993; Sun and Chiu 1999), electrophysiology and ischemia (Stys et al. 1995). These studies have led to the identical of physiological and pathological calcium-influx pathways. In particular, the reversal of the Na/Ca exchanger in ischemic axons has been proposed to produce damaging calcium influx into axons (Stys et al. 1995).

In this report, we examined the mechanisms for calcium clearance in axons of optic nerves. Because of the technical difficulty of staining axons in these nerves with calcium dyes for direct visualization of axonal calcium signals, very little is known about the calcium clearance mechanisms in axons of mammalian optic nerves under physiological conditions, including such basic issues as how these axons clear a calcium load following repetitive nerve activity and how the clearance is regulated. In this paper, we removed a major technical difficulty in studying calcium signaling in the optic nerve axons by devising a method to selectively stain pure population of axons in these nerves with calcium indicators, thereby allowing the first direct visualization of axonal calcium signals in these nerves in physiological time scales. We then applied this technique to examine some very basic properties of calcium clearance in these axons. We found a strong coupling between axonal [Na+] and axonal [Ca2+], and suggest that this coupling is best explained if the Na/Ca exchanger is a key

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player in calcium extrusion following physiological nerve activity.

METH O DS
Selective axonal staining and calcium imaging

Optic nerves, excised between the eye and the chiasm, were freshly obtained from mice of two age groups, neonatal (P5–P10) and adult (3–8 wk), and laid down on the bottom of an experimental 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 (Fig. 1A). 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 a high-K (140 mM), low-calcium (0 calcium plus 1 mM EGTA) solution containing 4–5 μl of the cell impermeant form of various calcium dyes (2–8 mM). The axons were stained by diffusion of the dye from the cut end. A standard loading time of 3 h was allowed before experiments began. At the end of the loading period, there was typically a spatial gradient of dye along the axis of the nerve, with the resting dye fluorescence highest at the loading end, and smallest at the other end (the stimulating end). We typically performed calcium imaging in the middle of the nerve trunk, ~500–1,000 μm away from the dye-loading pipette. The dyes were allowed to remain in the loading pipette (also serve as the recording pipette) during the entire experiment. During a typical 1- to 2-h experiment, the calcium fluorescence baseline measured at the middle of the nerve was quite stable in most studies (see Fig. 1). We believed that this stable baseline was due partly to our use of the high-molecular-weight, dextran-conjugated Oregon Green bis-(o-aminophenoxy)-N,N,N',N'-tetraacetic acid (BAPTA)-1, which has restricted dye mobility and excellent retention in the axons without leaking. While a slowly changing baseline fluorescence was sometimes observed in some experiments, these slow baseline changes can be easily distinguished from fast calcium fluorescence changes induced by the various pharmacological (veratridine, ouabain, or monensin) used in this study. In most of the figures, we reported the calcium responses as either ΔF/ΔF₀ or ΔF/F₀, which normalizes differences in background fluorescence due to differences in dye loading.

Confocal fluorescence imaging of axonal calcium

Calcium images were viewed with a ×40 (Olympus) objective lens on a Noran Odyssey confocal system (Madison, WI). Throughout the course of our study, we tried four membrane-impermeant calcium indicators: Oregon Green 488 BAPTA-1 (MW = 1114) with or without conjugation to the high-molecular-weight dextran [MW = 10,000; Kᵣ = 170 nM, Kₑ values from Handbook of Fluorescence Probes (6th ed.), Molecular Probes], Oregon Green 488 BAPTA-2 (Kᵣ = 580 nM), Magnesium Green (Kᵣ = 6,000 nM), and Oregon Green 488 BAPTA-5N (Kᵣ = 20,000 nM). We finally chose the high-affinity, dextran-conjugated Oregon Green BAPTA-1 due to its excellent dye retention and signal-to-noise ratio. The disadvantage of this high-affinity dye is potential distortion of calcium signal kinetics due to dye saturation. The low-affinity dyes were used in this study mostly as a control to assess dye-saturation as a potential source of...
error in the interpretation of our data. All dyes were used at a concentration of 5–10 nM in the loading pipette. The actual axonal dye concentration at the site of optical recording was estimated to be ~0.013 of that at the loading pipette (see RESULTS). Most of the experiments were performed with the Noran Odyssey System where the dyes were excited with an argon laser at 488 nm and confocal fluorescence images monitored with a 500-nm long-pass emission filter. The average fluorescence signal from the whole field (15,000 μm² area) was collected on-line at near video rate (30 Hz) and stored for off-line analysis; image acquisition and on-line calculations were controlled through the Metamorph software (Universal Imaging). The disadvantage of the Noran system is poor time resolution (the calcium signal was sampled every 30–40 ms). To achieve higher time resolution where needed, some experiments were performed with a Prairie Technology System (NED) that collects nonconfocal calcium signals every 500 μs. In most figures, intracellular calcium concentration was reported as F/F₀ or ΔF/F₀ without calibration for absolute values. Experiments were either done at room temperature (22°C) or at 36°C. Temperature was controlled by a DC-thermistor-based system.

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. Compound action potential (CAP) data were analyzed using pClamp 6.0 software (Axon Instruments, Foster City, CA).

Solution and drugs

The optic nerves were normally bathed in a Ringer solution that contained (in mM) 129 NaCl, 3 KCl, 1.2 NaH₂PO₄, 2.4 CaCl₂, 1.3 MgSO₄, 3 HEPES, 20 NaHCO₃, and 10 glucose. Calcium-free solutions were prepared by replacing Ca²⁺ with Mg²⁺ and by adding EGTA (1 mM); pH was adjusted to 7.4 with NaOH or HCl as necessary. All compounds were from Sigma.

RESULTS

This paper consists of two sections. In the first section, we will describe a technique for selective staining of axons in the mouse optic nerve and provide arguments as well as experimental evidence that we have indeed succeeded in selectively staining axons. This section is an important technical prerequisite to the second section, which uses this staining technique to examine several basic properties of calcium clearance mechanism in the mouse optic nerve axons.

Technique for selective staining of axonal population

In the rat optic nerve, the mean diameter of the axons remains ~0.2 μm for the first postnatal week and increases rapidly to ~1 μm in adult (>P28 days after birth) when the axons are fully myelinated (Foster et al. 1982). No morphometric data exist for the mouse optic nerves, the nerves used in this paper, and the developmental profile for the axon diameters is assumed to be similar to rats. An important prerequisite for a rigorous analysis of axonal calcium homeostasis is selective staining of axons with calcium indicators. In our previous studies, the cell-permeant form of the dye was injected into the extracellular space in the nerve trunk, which was taken up by both glial cells and axons (Kriegler and Chiu 1993; Sun and Chiu 1999). This staining method is useful for analysis of axonal signals only for short electrical stimulations that evoke only axonal calcium transients. For prolonged electrical stimulations, glial calcium responses are evoked (Kriegler and Chiu 1993) that contaminate the axonal signal, rendering conclusive analysis of axonal calcium homeostasis impossible. In this study, we selectively stained axons by allowing the cell-impermeant form of the calcium dye to diffuse into axons via a nerve cut end for 3 h before experiments began (Fig. 1A). Figure 1B compared optic nerve staining obtained with Kriegler and Chiu (1993)’s method, which stained both axons and glia (left), and that obtained with our current method (right). Our current staining method resulted in no glial cell staining (Fig. 1B, right), both in neonatal and in adult optic nerves. When we first started this study, we used Oregon Green BAPTA-1 (MW = 1,114). One concern we had was that this low–molecular-weight dye may cross gap junctions, allowing dyes that enter damage glial cells at the cut end to spread through glial network to reach the site of optical recording, which is typically 500–1,000 μm away from the site of dye loading. However, we did not observe staining of glial cells at the optical recording site, suggesting that this source of contamination of the axonal signal did not occur. To further exclude dye transfer via glial gap junctions, we switched (in later experiments) to calcium dyes conjugated to the high-molecular-weight dextran (MW = 10,000; Oregon Green BAPTA-1), which cannot penetrate gap junctions (Eckert et al. 1999). Because the site of optical recording is 2.5–5 times longer than the length of the average longitudinal processes of glial cells in mammalian optic nerves (~200 μm) (see Butt and Ransom 1993), transfer of the dextran-conjugated dye through glial processes to the recording site is unlikely.

To further confirm that we have in fact selectively stained axons, we performed the experiment in Fig. 2. We first loaded the calcium impermeant form of the calcium dye (dextran-conjugated Oregon Green) through the cut ends of the nerve with a tight suction pipette, the standard loading method in this paper. After 3 h (A), we evoked a whole-field calcium response with a single compound action potential (†). The nerve was allowed to rest for 10 min, after which adenosine was applied to the bath (Fig. 2A). The important observation is that no adenosine response was seen. The rationale behind the use of adenosine is that glial cells are known to express adenosine receptors, and previous calcium imaging studies from our laboratory (Kriegler and Chiu 1993) have demonstrated adenosine-evoked calcium responses in glial cells of mammalian optic nerves in situ. The fact that no adenosine-evoked calcium response was seen following our standard dye loading suggests that glial cells were not stained in our current study, and that the action potential evoked response (†) is purely axonal in origin. To show that glial cells are indeed viable and can respond to adenosine, we stained glial cells in the same nerve with a second dye loading (Fig. 2B) using the method of Kriegler and Chiu (1993). This was done by injecting into the middle of the nerve trunk, the calcium-permeant form of Oregon Green, which was taken up by both glial cells and axons. Two hours after this second dye loading, glial cells were stained and clearly visible. Further, the background fluorescence was greatly increased, due presumably to additional loading of the axonal population following the second dye loading. To compare the calcium responses following the second loading to that of the first one, we normalized the calcium fluorescence signal to the prestimulation baseline signal. Confirming our previous studies (Kriegler and Chiu 1993), this...
second dye loading, which stained glial cells, produced a robust adenosine response (Fig. 2B). To further exclude the possibility that our standard axonal loading paradigm (i.e., the 1st dye loading), if allowed to stain for an additional 2 h after the first 3 h (the addition time needed for the 2nd loading), would by itself lead to gradual glial staining, we did the control experiment in Fig. 2C and D. Here we employed only the standard axonal loading method (putting the dye in the loading pipette), and applied adenosine twice, one at 3 h (C), and another at 5 h (D) after loading. In neither case was an adenosine response seen (C and D), suggesting our axonal loading method did not lead to glial loading even after extended periods.

Our loading method relies on diffusion of dyes from the cut ends along the axoplasm of axons. Is it reasonable to expect that a dextran-conjugated molecule with a large MW of 10,000 can produce significant diffusion in only 3 h to allow calcium imaging in our studies. For example, McClellan et al. (1994) found that injection of dextran-conjugated Calcium Green in spinal cords resulted in retrograde labeling of neurons 5–14 mm away but only after 4 days. How might it be possible that we observed detectable dye loading in axons 3 h after dye loading? One explanation is that the mode of dye entry may be different. In McClellan et al. (1994), the dextran dye, which is impermeant, will have to be taken up by the cells, presumably via pinocytosis. In our case, we cut the axons, which allowed direct dye entry through the cut ends. Further, our loading pipette is filled with a calcium-free, EGTA-buffered solution intended to keep the cut ends of the axons from re-sealing. Hence, our in vitro loading method may allow faster dye diffusion into axons than in vivo loading. We further estimated the coefficient of diffusion for the dextran dye in our experiments. At a distance of ~540 µm from the loading site (the typical site of optical recording in experiments involving dextran dyes), the resting fluorescence is ~0.013 (n = 3) of the fluorescence in the loading pipette (which contains 2–8 mM of the dye) after a 3-h loading period. From this we estimated a diffusion coefficient (Crank 1956) of ~6.8 µm²/s at room temperature. This is considerably smaller than the coefficient of diffusion of 102 µm²/s measured for fura-2 (Gabso et al. 1997), which is a much smaller molecule. Our estimate suggests a very sluggish diffusion of dextran in our experiments. However, the use of a high dye concentration in the loading pipette evidently allowed sufficient dye diffusion to a recording site ~540 µm away to allow optical recording to be performed after a 3-h loading period.

In most of the experiments, we measured the whole-field calcium response (area ~15,000 µm²) elicited from a large number of axons. One concern is whether the whole-field response misses local heterogeneity and whether it faithfully represents response at the single axon level. We therefore compared the whole-field response simultaneously with several spot responses selected randomly from the same nerve, with each spot having an area that is ~300 times less than the whole field. Figure 1C shows the shape of the whole field (smooth trace) and spot (noisier traces) responses are virtually identical, suggesting the calcium response is spatially homogeneous and that the whole-field response faithfully captures the essence of the response at the single fiber level. Given that whole-field response has excellent signal-to-noise ratio, this measurement was routinely adopted as the primary tool for analysis of axonal calcium homeostasis in this paper.

Prior to our study, Ren et al. (2000) published a method of selective loading of axons in the mammalian optic nerves with calcium indicators by incubating the nerve in a solution containing the cell-impermeant form of the dye. Both that work and our work are in agreement that axons are selectively stained. Collectively, we believe that we have achieved staining of a pure population of axons in the optic nerve for rigorous analysis of axonal calcium homeostasis in this study. In the following section, we will apply this technique to examine several basic properties of axonal calcium clearance in the mouse optic nerves.

Basic properties of axonal calcium clearance mechanisms

4-AP increases evoked calcium transients. To facilitate analysis of calcium responses with acceptable signal-to-noise, in some experiments we applied 4-AP, a blocker of fast K channels, to increase the amplitude of the evoked calcium transients. Figure 3 shows the effects of 4-AP on the calcium transient and compound action potential evoked by a single stimulation for a P6 (top) and an adult (bottom) optic nerve. In both nerves, 4-AP increased the amplitude and duration of the compound action potential (B and E) and concomitantly increased the area of the evoked calcium transient (C and F).
Interestingly, 4-AP has a much more dramatic enhancing effect on the evoked calcium load (area of the calcium transient) in the adult than in the neonatal nerve. For example, 1 mM 4-AP enhanced the calcium load per action potential 80 times in adult (D) versus ~13 times (D) in the neonatal nerve. In experiments where 4-AP was used to increase the calcium transient, a concentration of 1 mM was used.

In this study, we used both neonatal (premyelinated) and adult (fully myelinated) nerves for analysis. Figure 4A shows the averaged calcium transient evoked by a single action potential in normal saline solutions in neonatal and adult nerves. The amplitude of the evoked calcium response is significantly attenuated by myelination (A). However, the shape of the calcium decline is unaffected by myelination (B), suggesting the same calcium clearance mechanism operates throughout development.

**GRADED CALCIUM DECAY FROM PROGRESSIVELY HIGHER CALCIUM LEVEL.** We started our analysis of calcium homeostasis by examining the time course of axonal calcium restoration following repetitive nerve activity. Figure 5A shows superimposed axonal calcium transients evoked in a P27 optic nerve (without 4-AP) by a single action potential and a train of 20 action potentials (10 Hz). On termination of the stimulation, the axonal calcium decays toward baseline (A). Interestingly, the calcium decay is graded according to the calcium level at which the decay began, being slower following the 20 action potentials (which elevated axonal calcium to a higher level) than following the single action potential (B). The calcium decay is not a single exponential, either following the single or 20 action potentials.

**CALCIUM DECAY DEPENDS ON THE NUMBER OF ACTION POTENTIALS.** In the preceding studies, both the calcium level at the start of the calcium decay and the number of action potentials during the tetanus were varied. It is possible that the number of action potentials itself (via axonal Na loading) is an important determinant of the posttetanus calcium decline. This issue was examined in Fig. 6. Figure 6A shows superimposed calcium responses to 1, 20, and 100 action potentials (at 5 Hz) at 22°C for a P8 nerve. The 20-action potential train elevates

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**Fig. 3.** Effects of 4-aminopyridine (4-AP) on action potentials and evoked axonal calcium transients on neonatal (top) and adult (bottom) optic nerves. A and D: dose-response curve for relative calcium load per action potential and 4-AP concentrations. Calcium load is calculated as the area under the entire calcium transient evoked by a single action potential. The dose-response data were fitted with a sigmoidal curve with a $K_d$ of ~65 µM in A, B and E: compound action potentials at various 4-AP concentrations. C and F: corresponding evoked calcium transients. Each calcium transient was evoked by a single action potential. P6 (top) and 10-wk-old adult (bottom) optic nerves. Calcium dye is Oregon Green 488 BAPTA-1.

**Fig. 4.** Comparison of evoked axonal calcium transients in neonatal and adult optic nerves. A: averaged axonal calcium transients evoked by a single action potential in P7 and adult (7 wk) optic nerves. B: comparison of the shape of the neonatal and adult responses from A. Temperature 22°C

**Fig. 5.** Dependence of posttetanus calcium decay on the calcium level reached during a tetanus. A: superimposed axonal calcium responses from a single action potential and a train of 20 action potentials at 10 Hz. The calcium fluorescence is in arbitrary units. B: normalized axonal calcium decay from data in A. The calcium decay was normalized with respect to the calcium level at the end of the stimulation at which the decay started. P27 optic nerves without 4-AP treatment. Temperature 22°C.
calcium to a higher level than the 1-action potential before the calcium decay began. However, for the 100-action potential train, the calcium response during the tetanus first reached a peak then slowly declined, so that by the end of the tetanus (at which the calcium decay began), the axonal calcium level is actually lower than that at the end of the 20-action potential train. The decline of the calcium response during the 100-action potential train may have various causes. First, there may be calcium-induced calcium inactivation, so that the increment of calcium flux per action potential is reduced during the tetanus. Second, there may be gradual axonal Na loading so that the amplitude of the compound action potential is gradually reduced during the tetanus, which leads secondarily to a reduction in the calcium influx per action potential. This gradual reduction of the compound action potential amplitude during a prolonged tetanus is frequently observed (also see Fig. 14), which is consistent with Na loading and is unlikely due entirely to refractory period. When we compared the posttetanus calcium decay following the 1-, 20- and 100-action potential train, we found a progressive slowing of the posttetanus decay (Fig. 6B). This is in spite of the fact the calcium decay started at a lower level at the end of the 100-action potential than at the 20-action potential train. Figure 6C shows posttetanus calcium decay from another experiment at 35°C where the calcium level at the start of the calcium decay actually reduced (from $\Delta F/F_0 = 2.8$ to 2.6 to 1.13) as the action potential number during the tetanus (5 Hz) increased from 20 to 100 to 300 respectively. Figure 6D demonstrates a similar phenomenon using two tetani of different frequency. The first tetanus is brief (4 action potentials/5 Hz), which brought the axonal calcium level to $\Delta F/F_0 \sim 3$ before the calcium decay. This is followed by a second tetanus that is considerably longer but at a lower frequency (40 action potentials/1 Hz), which elevated calcium to a slightly lower level ($\Delta F/F_0 < 3$) before the decay began. The second decay is slower than the first one (Fig. 6D).

These experiments demonstrate that the posttetanus calcium decay is sensitive to the number of action potentials during the tetanus. Because there might be increased axonal Na loading as the number of action potential is increased (as reflected by the gradual decline in the calcium response during the 100-action potential train in Fig. 6A), these observations suggest that axonal Na loading may impede posttetanus calcium recovery.
The residual component in the calcium recovery could be response during the tetanus deleted for clarity of presentation. The control tetanus was brief (20 action potentials at 10 Hz) and produced no significant residual posttetanus component. As the tetanus was increased to 94 action potentials (at 1 Hz), a residual posttetanus component developed that lasted ~10 min before slowly returning to baseline. When the nerve was re-stimulated with the control brief tetanus 18 min later, the residual posttetanus component disappeared, showing reversibility in the development of the persistent component. This argues against irreversible axonal damage due to the long durability in the development of the persistent component. To further test this idea, we examined the effects of pharmacologically increasing axonal \([\text{Na}]_i\) on the calcium clearance. To achieve a similar depolarization of the resting membrane potential induced by the ionophore, there was a reduction in the action potential amplitude. This depolarization may be minimal in low dosage of monensin (or ouabain), the extra-cellular K plays a role in posttetanus calcium extrusion. However, an alternative explanation might be that the membrane depolarization produced by monensin or ouabain (Leppanen and Stys 1997) directly inhibits the Na/Ca exchanger, which is electrogenic. This depolarization may be minimal in low dosage of monensin (4 \(\mu M\)) since the action potential amplitude was not appreciably affected (Fig. 7A). However, in higher doses of ionophore (or ouabain), there was a ~30–50\% reduction of action potential that accompanies the retardation of the posttetanus calcium decay. To achieve a similar depolarization of the resting membrane as in the case of ionophore (or ouabain), the extra-cellular K was increased by 15 mM to produce a ~50\% reduction in the action potential. Figure 9 shows that tetanizing the nerves in a solution with K elevated by 15 mM did not result

**PHARMACOLOGICAL INCREASES IN AXONAL \([\text{Na}]_i\) IMPEDES POSTTETANUS CALCIUM CLEARANCE.** The sensitivity of the posttetanus calcium decay on the number of action potentials suggests that axonal Na loading during the tetanus may impede posttetanus calcium clearance. To further examine this, we bath applied monensin (4–50 \(\mu M\), a Na-ionophore) (Senatorov et al. 2000), ouabain (20–30 \(\mu M\), a Na/K-ATPase inhibitor), and veratridine (a modifier of Na channel inactivation).

**Monensin.** Figure 7 shows an experiment with 4 \(\mu M\) monensin in which the compound action potential (A) and the calcium signal (B) were monitored simultaneously. Posttetanus calcium decay was monitored by evoking a tetanus (20 action potentials/10 Hz) at various times before and after monensin application. The baseline calcium signal prior to the evoked calcium response served also to monitor any changes in resting calcium level. We found three effects of monensin. First, the compound action potential gradually declined in a dose-dependent fashion. Thus the amplitude of the compound action potential was not affected appreciably at 4 \(\mu M\) (Fig. 7A) but greatly reduced at 30 \(\mu M\) (data not shown), an effect that took 5 min to occur. Second, the resting calcium level rose (Fig. 7B). Third, there was a significant retardation of the posttetanus calcium decay that was partially reversible after washing away monensin (Fig. 7C). The first observation of a reduction in the compound action potential can be readily explained by a depolarization of the resting membrane potential induced by the ionophore. The second observation of an elevation in the resting axonal calcium is an interesting one that will be further examined in the following text. Pertinent to our present purpose is the third observation on the monensin-induced retardation of the posttetanus calcium decay, which is consistent with the hypothesis that increasing axonal \([\text{Na}]_i\) impedes calcium clearance.

**Ouabain.** Similar effects were seen after blocking the Na/K-ATPase with 20 \(\mu M\) ouabain (Fig. 8B, 22°C). Thus ouabain reduces the compound action potential, elevates the resting calcium level, and impedes the posttetanus calcium clearance. However, the effect of ouabain, unlike that of monensin, is irreversible. Besides ouabain, K-free solution is an alternative means to block Na/K-ATPase. Figure 8A (36°C) shows that the posttetanus calcium decline was slowed down when the nerves were tetanized in a K-free solution, again suggesting that inhibition of Na/K-ATPase can interfere with posttetanus calcium clearance.

**Veratridine.** Another approach to increase axonal \([\text{Na}]_i\), is to inhibit Na channel inactivation with veratridine. In the presence of veratridine, electrophysiological studies have shown that Na currents activate normally but cannot completely inactivate during an action potential. Further, immediately after an action potential, inward Na currents persist for tens of seconds before shutting down (Barnes and Hille 1988; Ulbricht 1969). This abnormal pattern of excessive Na influx both during and after an action potential causes a dramatic slowing of the calcium decay following a single action potential (Fig. 8C; 5 \(\mu M\) veratridine). Collectively, these experiments with the Na-ionophore, Na pump inhibitor and inhibitor of Na channel inactivation suggest that increases in axonal Na retards posttetanus calcium clearance.

**POSTTETANUS CALCIUM CLEARANCE IS NOT AFFECTED BY K-DEPOLARIZATION.** A simple explanation for the impeding effect of monensin (or ouabain) on the posttetanus clearance is that \([\text{Na}]_i\) loading inhibits the Na/Ca exchanger that normally plays a role in posttetanus calcium extrusion. However, an alternative explanation might be that the membrane depolarization produced by monensin or ouabain (Leppanen and Stys 1997) directly inhibits the Na/Ca exchanger, which is electrogenic. This depolarization may be minimal in low dosage of monensin (4 \(\mu M\)) since the action potential amplitude was not appreciably affected (Fig. 7A). However, in higher doses of ionophore (or ouabain), there was a ~30–50\% reduction of action potential that accompanies the retardation of the posttetanus calcium decay. We therefore tested if a K-mediated membrane depolarization can retard the posttetanus calcium clearance. To achieve a similar depolarization of the resting membrane as in the case of ionophore (or ouabain), the extra-cellular K was increased by 15 mM to produce a ~50\% reduction in the action potential. Figure 9 shows that tetanizing the nerves in a solution with K elevated by 15 mM did not result.

**FIG. 7.** The Na-ionophore monensin retards posttetanus calcium decay. A and B: simultaneous recordings of compound action potential (A) and the calcium signal (B) before, during, and after bath application of 4 \(\mu M\) monensin. C: normalized posttetanus calcium decay from the data in B. The tetanus used was 20 action potentials/10 Hz. Note monensin retards the posttetanus calcium decay that is partially reversible on washing. P8 optic nerves treated with 1 mM 4-AP. Temperature 22°C.
extrusion efficacy of this exchanger is coupled to the transmembrane Na concentration, which is normally high outside and low inside. We therefore tetanized nerves in solutions in which Na was replaced by lithium (Li). Li is permeable to Na channels and should support action potentials. We found that the compound action potentials (both in neonatal and in adult nerves) were gradually abolished in the Li-saline solutions. However, at 10–15 min after Li application (before a complete block of the action potentials), we found a approximate two-fold slowing of the posttetanus calcium decay, consistent with the exchanger playing a role in the calcium extrusion (Fig. 10, A and B). Next, we applied bepridil (10 μM), which has been suggested to inhibit Na/Ca exchangers in ischemic optic nerves (Stys et al. 1995a,b). The compound action potentials also gradually declined in bepridil. However, we found an ~1.5-fold slowing of the posttetanus calcium decay after bepridil application (Fig. 10, A and B). Thus both the bepridil and Li experiments suggest that the Na/Ca exchanger is involved in the posttetanus calcium clearance.

Figure 10 (A and B) summarizes effects of the various drug treatments on the posttetanus calcium decay according to two age groups, neonate (P5–P8) and adult (P60). In both age groups, pharmacological manipulations used to elevate axonal [Na]i (metabolic blocker ouabain, Na ionophore monensin, Na channel inactivation inhibitor veratridine) or to inhibit the Na/Ca exchanger (bepridil, Li replacement of bath Na) all result in a retardation of the posttetanus calcium clearance.

ELEVATION OF AXONAL Na CAUSES AN INCREASE IN RESTING CALCIUM INFLUX INTO AXONS. The preceding studies suggest a coupling between axonal [Na], and calcium clearance following nerve activity. In this section, we examined if axonal [Na] is also coupled to the resting axonal calcium level. As noted in

in a slowing of the posttetanus calcium decline (B), suggesting that membrane depolarization cannot explain the retardation of posttetanus decay seen in Na loading experiments.

Na-free solutions and bepridil

Because we postulate that the Na/Ca exchanger is involved in calcium extrusion after a tetanus, we should be able to slow the extrusion by tetanizing nerves in a Na-free solution. The
nus calcium decline, also caused a rise in the resting axonal channel activation, leading to calcium in the membrane. This membrane depolarization could cause calcium presumably by increasing the sodium conductance of the membrane. The temperature was 22°C.

The preceding text with regard to ouabain and monensin, application of these agents, in addition to retarding the posttetanus calcium decline, also caused a rise in the resting axonal [Ca$$^{2+}$$]. What are the mechanisms underlying this rise in the resting calcium level? There are at least three possibilities. First, increasing axonal [Na$$^+$$] may cause a depolarization, presumably by increasing the sodium conductance of the membrane. This membrane depolarization could cause calcium channel activation, leading to calcium influx into axons. Second, increasing axonal [Na$$^+$$] triggers calcium release from internal stores (Mulkey and Zucker 1992). Third, increasing axonal [Na$$^+$$] causes influx of calcium through the Na/Ca exchanger when it is driven by intracellular Na accumulation to operate in the reversed mode. To raise axonal [Na$$^+$$], we used ouabain and monensin (as used in preceding studies), as well as veratridine (which activates a resting Na in the membrane). The bath solution was completely replaced by lithium (Li).

In the neonates bath application of ouabain (20 μM), monensin (4 μM), and veratridine (5 μM) all caused an elevation of the posttetanus calcium level before and after drug application for the neonates (A) and 10 action potentials/10 Hz in the adult (B) in all drugs except veratridine. In the case of veratridine, a single action potential was used in the neonates and 10 action potentials/10 Hz for the adult.

Higher concentrations of all three drugs were used, including veratridine (data not shown). Bath application of all three agents caused a rise in the resting axonal Ca level within 5–10 min of application. An example for monensin-induced elevation of resting calcium is shown in Fig. 11A for a P8 nerve. We next examined if the drug-induced elevation in resting axonal calcium is due to calcium influx (Fig. 11B). We first switched the bath solution to a calcium-free solution. This caused a slight reduction in the resting calcium level. After ~17 min in the calcium-free solution, we applied 5 μM veratridine. This did not result in any elevation in the resting axonal calcium level. Restoring calcium (2.4 mM) to the bath solution (with veratridine still present) caused an immediate increase in axonal [Ca$$^{2+}$$]. These experiments demonstrate that Na loading causes a resting calcium influx from the extracellular space.

What is the calcium influx pathway? Because all these agents (ouabain, monensin, and veratridine) may cause membrane depolarization (for ouabain, see Leppanen and Stys 1997), one pathway for influx is voltage-gated calcium channel. To examine contribution from voltage-gated calcium channels, we compared [Ca$$^{2+}$$], elevation induced by Na loading to that caused by depolarizing the membrane with a high-K solution. Because we cannot directly measure the absolute resting potential in our setup, we used the amplitude of the compound action potential as an indirect means for comparing membrane depolarizations. This follows from the principle that a depolarized resting potential inactivates Na channels thereby causing a reduction in the amplitude of the compound action potential. Figure 12 shows the experiment. Here, we monitored simultaneously and the amplitude of the compound action potential (A) and the resting axonal [Ca$$^{2+}$$] (B) for a P9 nerve.

In the neonates bath application of ouabain (20 μM), monensin (4 μM), and veratridine (5 μM) all caused an elevation of the resting axonal Ca (Fig. 10C). In the adult, the resting axonal calcium level was not appreciably affected at these drug concentrations for ouabain and monensin (Fig. 10D). However, for the adult, the resting axonal calcium was increased when

![Image](http://jn.physiology.org/)

**FIG. 10.** Summary data for pharmacological manipulation of posttetanus calcium decay (A and B) and resting calcium level (C and D) for neonatal and adult optic nerves. Top: plot of normalized changes in the half-time of posttetanus calcium decay in axons before and after various drug application for P5–P8 (A) and P60 (B) optic nerves. A: drug concentration: 20 μM (ouabain), 4 μM (monensin), 10 μM (bepridil), 5 μM (veratridine). For Li, bath Na was completely replaced by lithium (Li). B: same as A except veratridine was used at a lower concentration of 2 μM because adult nerve action potentials were rapidly blocked by 5 μM. In each drug assay, evoked calcium transients were sampled every 5 min for 4 times to obtain a baseline before drugs were applied. For each drug, posttetanus calcium decay was measured at a fixed time (10–30 min) after application. Calcium transients were evoked with 20 action potentials/5 Hz for the neonates (A) and 20 action potentials/10 Hz in the adult (B) in all drugs except veratridine. In the case of veratridine, a single action potential was used in the neonates and 10 action potentials/10 Hz for the adult. Bottom: plot of the relative changes in the resting axonal calcium level before and after drug application for the neonates (C) and the adult (D). Relative changes in the resting axonal level measured 10–30 min after drug application. * the drug data are significantly different from control (P <0.05). Temperature 22°C.

**FIG. 11.** Axonal Na loading induces axonal calcium influx. A: bath application of Na-ionophore monensin (50 μM) causes an elevation of resting axonal [Ca$$^{2+}$$]. B: calcium elevation induced by Na loading is due to calcium influx. The bath solution was first switched to a calcium-free solution for 20 min before 5 μM veratridine was applied to induce axonal Na loading. Subsequent solutions had calcium either present or absent as indicated. P8 optic nerves. Temperature 22°C.
Figure 12. Resting calcium influx induced by Na loading is not due to voltage-gated calcium channels. Simultaneous recordings of compound action potential (A) and resting axonal calcium signal (B) during various test solution applications. The resting calcium signal was obtained from the baseline calcium signal prior to the calcium transient evoked by each single action potential. Note that B only displays the resting calcium level and not the evoked calcium transient. The first test solution was one in which K was elevated by 15 mM. This was followed by a wash before 5 μM veratridine was applied. P9 optic nerve. Temperature 22°C.

On increasing the bath K by 15 mM, the amplitude of the compound action potential declined to ~10% of the normal value, accompanied by an elevation of resting axonal [Ca²⁺], to F/F₀ = 1.1. The nerve was allowed to recover in normal-K solution before veratridine (5 μM) was applied. Veratridine caused a rise in the resting [Ca²⁺], as well as a decline in the amplitude of the compound action potential. At ~16 min after veratridine application (Fig. 12B, ↓), the resting axonal [Ca²⁺] rose to F/F₀ = 4 while the compound action potential declined to ~40% of the normal value (A, ↑). By making the reasonable assumption that the decline in the compound action amplitude is due to depolarization of the resting membrane potential, we deduced that veratridine caused a larger calcium influx (F/F₀ = 4) than in the K solution (F/F₀ = 1.1) but with less membrane depolarization (40% action potential remaining in veratridine vs. 10% action potential remaining in the K solution). This shows that most of the veratridine-induced calcium influx is unrelated to voltage-gated calcium channels. The same conclusions were reached for ouabain- and monensin-induced calcium influx in similar studies (the corresponding values are F/F₀ = 5.8 and F/F₀ = 3.6 for 30 μM ouabain and 50 μM monensin, respectively). We believe that the most likely candidate for the calcium influx pathway is the Na/Ca exchanger, which is driven to run in the reverse mode under elevated axonal [Na], conditions.

Effect of Mitochondrial Blockers. Besides various calcium extrusion mechanisms located on the plasma membrane (such as Na/Ca exchanger, Ca-ATPase), cytosolic calcium buffers such as mitochondria may influence the decay kinetics of the evoked calcium response. For example, Colegrove et al. (2000) have recently shown that mitochondria in sympathetic neurons participate in buffering fairly modest elevation in cytoplasmatic calcium induced by depolarizations. We therefore examined the role of mitochondria in the optic nerves by applying the mitochondrial blocker carbonyl cyanide p-(trifluoromethoxy) phenyldrazine (FCCP, 1 μM) or carbonyl cyanide m-chlorophenylhydrazine (CCCP, 1 μM) (Colegrove et al. 2000; Fierro et al. 1998).

Figure 13 shows that FCCP causes a rise in the resting axonal calcium level (B) as well as a fall in the amplitude of the compound action potential (A) in the optic nerves. The rise in the resting axonal calcium is due to calcium influx because applying FCCP in the absence of bath calcium did not lead to an elevation in the resting calcium (D). FCCP also caused a slowing of the posttetanus calcium decay that is only partially reversible on washing (C). The effect of FCCP on the resting calcium and the posttetanus calcium decay is thus very similar to that observed after loading axons with Na with monensin and veratridine (Figs. 7 and 11). The rise in resting axonal calcium following FCCP treatment (Fig. 13B) makes it difficult to dissect out a role for mitochondria in the posttetanus calcium decay in the optic nerves. For example, Fierro et al. (1998) also found an FCCP-induced rise in resting calcium in the Purkinje cell, and this complication impedes testing the role of mitochondria in evoked calcium clearance in their study. In other cells where FCCP was used successfully in dissecting out the role of mitochondrial in activity-dependent calcium clearance, no effect of FCCP on the resting calcium was observed (Colegrove et al. 2000).

In our experiments, a likely explanation for the effects of FCCP on optic nerve is that this agent slowly poisons the nerve in a nonspecific fashion, which causes axonal Na accumulation that in turn leads to calcium influx. In other words, all these agents tested in this study (the Na ionophore monensin, the Na channel activator veratridine, and the metabolic poisons ouabain and FCCP) all act through a common pathway of Na accumulation that leads, as hypothesized in our study, to a retardation of posttetanus decay.

Figure 13. Effects of the mitochondrial blocker carbonyl cyanide p-(trifluoromethoxy) phenyldrazine (FCCP) on the resting and evoked axonal calcium signal. A and B: effects of bath application of FCCP on the amplitude of the compound action potential (A) and the corresponding resting axonal calcium fluorescence (B). C: effects of FCCP on the posttetanus calcium decay. The amplitude of the peak calcium response was normalized to compare the posttetanus calcium decay. The response was evoked with a tetanus (20 action potentials at 10 Hz) before, 15 min after FCCP, and 40 min after wash. D: application of FCCP in calcium-free bath solutions (0 calcium plus 1.0 mM EGTA) elicited no elevation in the resting axonal calcium fluorescence. P10 nerve. Temperature 22°C.
EFFECTS OF POSTTETANUS CALCIUM ELEVATION ON EXCITABILITY. Our analysis of axonal calcium reveals a prolonged phase of calcium elevation following a long tetanus (Fig. 6). An interesting issue is whether this protracted posttetanus calcium elevation has any physiological consequences on nerve excitability. We therefore measured posttetanus recovery of compound action potentials with or without calcium in the bath (Fig. 14). Mg$^{2+}$ was added to replace the omitted calcium to minimize effects on the nerve excitability due to screening of surface charges. To produce significant posttetanus calcium elevation, we used a 5-min tetanus at 5 Hz. The amplitude of the compound action potentials was reduced at the end of the tetanus, to 49 and 39% of the control values for Ca-free and normal solutions, respectively (Fig. 14). The posttetanus recovery of the compound action potential is calcium dependent. In the case where calcium is present, the recovery occurred in an initial fast phase (~1 min) followed by a slow one that took ~20 min. In calcium-free bath solutions (i.e., no activity-dependent axonal calcium elevation), the two recovery phases are still present. However, fully recovery to the pretetanus level is achieved faster (~10 min). This suggests that the posttetanus axonal calcium elevation demonstrated in our calcium analysis modulates posttetanus excitability.

DOES INTRODUCTION OF CALCIUM DYES DISTORT AXONAL CALCIUM SIGNALING? One potential source of uncertainty in this study is that the calcium dyes introduced in the axons might distort calcium signaling. We addressed this issue by using different concentrations of calcium dye in the loading, and by using low-affinity calcium dyes.

Different dye loading concentrations. One potential uncertainty in this study is the use of a very high concentration of high-affinity calcium dyes in the loading pipettes (2–8 mM of dextran-conjugated Oregon Green). The intra-axonal dye concentration at the site of optical recording may be excessively high, which could distort the kinetics of the calcium transients.

However, as estimated earlier, the intra-axonal dye concentration at a typical recording site of ~540 μm from the loading pipette after a 3-h loading might be only in the order of ~0.013 of that in the loading pipette (2–8 mM), which translates to 26–104 μM. This intra-axonal dye concentration is clearly in line with other studies of the kinetics of calcium transients using high-affinity dyes, such as fura-2, where a known concentration of ~200 μM is introduced into the cells via a whole cell pipette (Fierro et al. 1998; Koester and Sakmann 2000).

To further examine the possible effects of dye concentrations on the kinetics of calcium transients, we compared the calcium transient kinetics in nerves of similar age after loading with either 8 mM or 250 μM dextran-conjugated Oregon Green in the pipettes (Fig. 15C). For the 8 mM loading, we employed a standard 3-h loading period. However, for the 250 μM loading, we found that we had to load for ~20 h to get a detectable signal. Figure 15C compares the averaged calcium transients evoked by identical stimulation in nerves loaded with these two different dye concentrations in the pipette. The responses were normalized so that they have the same peak. Note that the noisier trace corresponds to the 250 μM loading, which produces a very small signal when compared with the 8 mM loading. It can be seen that the two traces have a similar shape. The slightly slower calcium decline in the 250 μM loading might be related to a Na loading that occurs during a 20-h loading period, which according to our hypothesis should retard posttetanus calcium clearance. The intra-axonal dye concentration following a 20-h, 250 μM loading is likely to be considerably lower than that following the 8 mM loading, because the former signal is much smaller than the latter. This experiment suggests that the amount of dyes in the axons in the current study is unlikely to have a major impact on the calcium kinetics, at least to a degree to invalidate the key conclusions drawn regarding the effect of Na loading on the calcium transient kinetics.

Low-affinity calcium dyes. Most of the experiments in this paper were performed using the high-affinity calcium indicator (Oregon Green 488 BAPTA-1, $K_d = 170$ nM) due to its excellent signal-to-noise ratio. However, one concern with this high-affinity indicator is that part of the slowing of the posttetanus fluorescence decay may be due to dye saturation as calcium rises to a high level during high-frequency stimulation. Dye saturation might slow the dissociation of calcium from the calcium-dye complex (the fluorescence species), thereby producing a slowing of the fluorescence decline that does not reflect real biology. We therefore performed several control experiments using low-affinity calcium indicators. Figure 15A shows that the posttetanus slowing of calcium decay induced by increasing the number of action potentials in the tetanus was reproduced using the low-affinity indicator Magnesium Green ($K_d = 6000$ nM). Figure 15B shows that retardation of the posttetanus calcium decay by monensin was reproduced using another low-affinity indicator Oregon Green 488 BAPTA-5N ($K_d = 20,000$ nM). In general, low-affinity dyes produced much smaller ΔF/F0 values than high-affinity dyes, resulting in poor signal-to-noise ratios. Because these two low-affinity indicators have a $K_d$ for calcium that is 35–117 times less than the high-affinity dye, we conclude that the key observations in this study are not artifacts of dye saturation.
In this paper, we reported a method for selective staining of axonal populations in the mouse optic nerves with calcium dyes and argued, based on the use of a dextran-conjugated dye that is known to be impermeable to gap junctions, and on the lack of an adenosine-induced calcium response (the hallmark of glia), that we have indeed successfully achieved selective axonal staining. We then applied this method to examine several basic properties of calcium clearance mechanisms in these axons and obtained two key observations regarding the clearance mechanisms. The first observation is tetanus-dependent calcium decay. A tetanus that elevates axonal calcium to a higher level leads to a slower calcium clearance. Further, even from a similar calcium level, the posttetanus calcium clearance is slower if it is preceded by a larger number of action potentials. The second observation is that inducing axonal Na loading with pharmacological agents retards posttetanus calcium clearance as well as induces a resting calcium influx into axons. We believe that our observations can be explained by evoking the Na/Ca exchanger as a key player in calcium homeostasis in the axons of the mouse optic nerves; the coupling of calcium homeostasis to axonal [Na], simply reflects the dependence of the operation of the exchanger on the Na gradient across the axolemma. Our postulated role for the Na/Ca exchanger makes excellent functional sense given the intense staining of the exchanger in the axons of the mammalian optic nerves (Steffensen et al. 1997).

**Determinants of the shape of the posttetanus calcium decline**

A key observation in this paper is that the posttetanus calcium decline is multi-exponential, and its shape is dependent on the calcium level and the action potential numbers during the tetanus. Posttetanus slowing of the calcium decline is observed with both high and low affinity calcium indicators, suggesting that it is not related to dye saturation artifacts. Bi-phasic calcium decline following a stimulus-induced calcium load has been observed in various neuronal preparations, including terminals of pyramidal cells of rat neocortex (Koester and Sakmann 2000), mouse cerebellar Purkinje cells (Maeda et al. 1999), and presynaptic terminals of cerebellar granule cells (Regehr 1997).

What determines the shape of the calcium decline after a calcium load? In general, uptake by mitochondria, binding to endogenous axonal calcium buffers, extrusion through axolemmal Ca-ATPase and Na/Ca exchanger will jointly determine the shape of the posttetanus calcium decline. As discussed by Koester and Sakmann (2000), several factors may produce multi-exponential calcium decay. One is the existence of multiple species of endogenous calcium buffers. For example, Maeda et al. (1999) suggested that the bi-phasic calcium decline in Purkinje neurons can be explained by the presence of two endogenous buffers, one with high affinity and the other with low affinity. In their model, raising the calcium level to different levels by increasing the intensity of the tetanus will lead to various degree of saturation of the endogenous buffers, producing a calcium decline whose shape is tetanus-dependent. In the optic nerve axons, we found that increasing the tetanus duration makes the bi-phasic calcium decline more prominent, particularly after 4-AP treatment, which dramatically increases...
the calcium influx per action potential. The complex calcium clearance time course in the mouse optic nerve axons could be explained by the saturation of several distinct as-yet-unidentified endogenous calcium buffer species. In neuronal systems, strong candidates for endogenous buffers include the calcium-binding proteins calbindin-D and parvalbumin. The nature of endogenous buffers in the axons of the mammalian optic nerves has not been explored.

Another factor that can influence the shape of the calcium decline at the site of influx is diffusion along the axoplasm to regions of low calcium. Such diffusion can act as spatial buffering to restore calcium level at the site of entry. After an axon has been fully myelinated, the tiny nodal gap (≈ 1 µm) is presumably the site of local calcium influx. The two long internodes flanking the node might act as a large “low-calcium” sink to spatially dissipate a high calcium increase at the node. The diffusion of calcium ions away from the node to the internode, coupled with calcium extrusion at the node, may produce a multi-exponential calcium decline. However, this spatial diffusion is unlikely to explain the multi-exponential calcium decay seen also in premyleinated axons (P5–P7) where the calcium influx is likely to be uniform over the entire length of the axon.

Besides endogenous buffers and axial diffusion of calcium, saturation of calcium extrusion mechanisms might also produce tetanus-dependent calcium decay (Blaustein 1988). Of the two extrusion pathways, Ca-ATPase and Na/Ca exchanger, which one might produce tetanus-dependent calcium decline? Regehr (1997) examined this issue with computer modeling of calcium decline in the granule cell terminals. He found that Ca-ATPase extrusion does not produce tetanus-dependent calcium decline. That is, increasing the calcium level in the tetanus by increasing the action potential numbers will not affect the shape of the posttetanus calcium decline if Ca-ATPase is the sole source for calcium extrusion. This is contrary to our observation. On the other hand, Regehr (1997) shows that extrusion through the Na/Ca exchanger will produce a tetanus-dependent calcium decline. According to Regehr’s model, following a calcium load, the Na/Ca exchanger will operate in the extrusion mode to extrude calcium, which will cause the initial fast decline in calcium. However, this extrusion also simultaneously brings in Na, which causes axonal [Na], to rise above the normal, pretetanus level. The Na/Ca exchanger will rapidly reach a new equilibrium, with a slightly higher axonal [Na], level and a higher axonal [Ca], level than the pretetanus level. As the calcium load is progressively increased (as happens after 4-AP treatment), the posttetanus axonal [Ca], will recover to a higher level. This predicts a persistent posttetanus calcium elevation whose amplitude should be proportional to the calcium level at the start of the decline as indeed observed in Fig. 6. With the participation of the Ca-ATPase in the extrusion process, this posttetanus, persistent calcium elevation should be slowly dissipated. Thus the behavior of the posttetanus calcium decline observed in this study fits qualitatively with Regehr’s model (Regehr 1997), and argues for a role for Na/Ca exchanger in calcium extrusion following nerve activity. This conclusion is clearly in line with recent immunohistochemical data showing heavy staining of the Na/Ca exchangers in axons of the rat optic nerves (Stefensen et al. 1997).

Na accumulation retards posttetanus calcium clearance

Regehr’s model (Regehr 1997) cannot account for our observation that increasing the number of action potentials also can impede posttetanus decline, even under conditions where there is no increase in the calcium level from which the decline begins. We hypothesize that Na accumulation during tetanus retards posttetanus calcium clearance. This would explain why under certain conditions, the posttetanus decline is dependent on the number of action potentials during the train and not on the calcium level reached at the end of the train. This hypothesis is supported by the observation that elevation of axonal [Na], with pharmacological agents does indeed retard posttetanus calcium decay. What kind of calcium clearance mechanism might be sensitive to axonal [Na]? Extrusion via the Na/Ca exchanger would exhibit such a property. Calcium extrusion is coupled to the Na gradient, and elevation of internal Na is known to inhibit the exchanger (for review, see Styx et al. 1995). Alternatively, elevation of axonal Na might weaken the buffering capacity of endogenous buffers or inhibit calcium uptake into organelles, thus retarding the fall of free calcium in the axon. However, such an effect of Na has not been described. Finally, calcium clearance might be directly inhibited by the calcium elevation that accompanies pharmacological elevation of axonal Na. It is unclear what kind of calcium clearance mechanism might be inhibited by elevated internal calcium. As for the Na/Ca exchanger, increased intracellular calcium is thought to stimulate, rather than inhibit, calcium extrusion (see Styx et al. 1995). On the other hand, high axonal calcium might inhibit the Na/K-ATPase (McGeogh 1990), and the consequential Na loading might retard calcium clearance though the mechanism suggested in this study.

Collectively, we believe that evoking the Na/Ca exchanger provides the simplest explanation to account for the impeding effect of Na loading on the posttetanus calcium decline. Because axonal Na accumulation is frequently associated with metabolic perturbations, they could disrupt calcium homeostasis either by directly inhibiting the Ca-ATPase or indirectly by blocking the Na/K-ATPase. In mammalian retinal rods, blockage of the Na/K-ATPase slows calcium extrusion and affects light adaptation, and inhibition of the Na/Ca exchanger by [Na], accumulation has been evoked to explain the results (Demoit-Care et al. 1995). The idea of a coupling between axonal Na and calcium flux through the Na/Ca exchanger was first proposed for pathological optic nerve axons (Styx et al. 1995a,b). In their model, energy interruption in ischemic optic nerves leads to Na accumulation inside axons; this drives a reversal of the Na/Ca exchanger to deliver injurious calcium influx (Styx et al. 1995a,b). Our present work suggests that calcium homeostasis is coupled to axonal Na even during physiological activity.

Physiological significance

What is the physiological significance of coupling activity-dependent Na influx to postactivity calcium clearance? This coupling might be used to amplify activity-dependent calcium signaling. While it is clear from previous study of optic nerves that there is an activity-dependent calcium influx into axons (Sun and Chiu 1999), this study suggests that the calcium
elevation in the axons will persist longer if its clearance is retarded by activity-dependent Na influx. The net effect is a prolongation of the action of calcium on intracellular processes that are calcium dependent. Such a system would nicely allow a two-pronged pathway to translate electrical activity into intracellular calcium signaling: an activity-dependent calcium influx due to activation of calcium channels and a Na-coupled retardation of postactivity calcium clearance to prolong the action of calcium. We suggest that this amplification of calcium signaling might be important in both development and possibility in adult optic nerves.

During development, the actin/cytoskeleton elements along a growing axon must undoubtedly be undergoing an active phase of assembly. Because actin/cytoskeletal assembly is sensitive to local rise in calcium (Bentley and O’Connor 1994; Lankford et al. 1996), axonal calcium influx might be an important signal for regulating axonal growth in a developing optic nerve. It has been well documented that axonal patterning is dependent on electrical nerve activity (Campbell et al. 1997; Shatz 1990). Interestingly, retinal ganglion cells of the developing retina fire spontaneous action potentials at 10–30 Hz in bursts of 4–15 s (Masland 1977; Meister et al. 1991). We suggest that the associated axonal calcium influx might modulate axonal elongation. Sodium that enters the axons during the action potentials will retard calcium clearance, further augmenting the modulatory effect of calcium on the actin/cytoskeletal elements. Prior to myelination, activity-dependent Na influx occurs presumably uniformly along the axon, thereby augmenting calcium action over the entire growing axon. As the axons become myelinated, the augmenting action of Na influx on calcium signaling might be expected to be restricted to the nodes of Ranvier. Consistent with a developmental role for calcium, we observed that activity-dependent elevation of axonal calcium is reduced by ~10-fold during development (Fig. 4A).

The persistence of activity-dependent calcium influx in the adult nerves, although much attenuated compared with the neonates, raises interesting question as to the role of calcium signaling in mature myelinated nerves. One intriguing role might be to mediate a highly localized calcium signaling near the nodes of Ranvier. It has been shown that glial contact influences neurofilament phosphorylation in the axon in a highly local manner (Brophy 2001; De Waegh et al. 1992). What factors might facilitate calcium signaling at the node of Ranvier? First, the constriction of the axon at the node of Ranvier might transform small calcium influx into a high local calcium elevation. Second, the high density of Na channels at the node of Ranvier will produce a large and highly localized Na influx, which might retard calcium clearance and focally prolong intra-axonal calcium action during action potential propagation. These local calcium changes might modulate cellular processes including phosphorylation of neurofilaments. Our model also has pathophysiological implications. One thesis of our paper is that activity-dependent Na influx prolongs calcium elevation during nerve excitation. In such a system, calcium overload (which is toxic to axons) can be produced either by increasing calcium influx or by modification of the kinetic properties of Na channels. We observed that veratridine, an agent that slows Na channel inactivation, dramatically prolongs axonal calcium elevation associated with even a single action potential (Fig. 8C). This suggests that calcium homeostasis in the optic nerve axons is exquisitely sensitive to Na channel kinetics. Thus any abnormal alteration of Na channel kinetics may be expected to have a profound effect on activity-dependent calcium signaling in these nerves. Whether calcium overload specifically related to action potentials can contribute to axonal degeneration in various pathological conditions remains an intriguing possibility for future investigations.

The retardation of posttetanus calcium clearance by Na influx may also modulate excitability changes following periods of intense activity. The excitability of CNS axons undergoes a complex sequence of changes following repetitive nerve activity (Swadlow and Waxman 1978), and various factors, including electrogenicity of the Na/K pumps (Gordon et al. 1990), have been evoked to explain these changes. Our work (Fig. 14) suggests that the prolonged axonal calcium elevation following repetitive activity may contribute to posttetanus excitability modulation. Our model suggests that posttetanus calcium clearance is retarded by Na influx during nerve activity so that the longer the repetitive activity, the larger the axonal Na accumulation and the longer the posttetanus calcium elevation lasts. Our study (Fig. 14) suggests that the posttetanus axonal calcium elevation acts to suppress excitability, i.e., depression of the compound action potentials. This posttetanus depression of excitability does not appear to be resulting from axonal damage due to a high calcium load because the compound action potentials slowly recovered to the pretetanus level (Fig. 14, ●). How might elevation of axonal calcium depress nerve excitability? One mechanism is that a rise in axonal calcium activates a calcium-activated potassium conductance, which will reduce nerve excitability. However, calcium-activated K channels have not been described for mammalian optic nerves. Alternatively, a rise in axonal calcium may activate a calcium-activated chloride conductance (Barish 1983; Miledi 1982), which will also have the effect of stabilizing the membrane against depolarization, leading to reduced excitability. Interestingly, chloride conductance has been demonstrated to be an important modulator of optic nerve excitability with a large portion of the resting conductance in these axons originating from a chloride conductance (Connors and Ransom 1984). It is unclear if the chloride conductance in the optic nerve axons is calcium sensitive.

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REFERENCES


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