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Na^+-Dependent Sources of Intra-Axonal Ca^{2+} Release in Rat Optic Nerve during *In Vitro* Chemical Ischemia

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The contribution of intracellular stores to axonal Ca^{2+} overload during chemical ischemia *in vitro* was examined by confocal microscopy. Ca^{2+} accumulation was measured by fluo-4 dextran (low-affinity dye, $K_D \approx 4$ μM) or by Oregon Green 488 BAPTA-1 dextran (high-affinity dye, $K_D \approx 450$ nM). Axonal Na^+ was measured using CoroNa Green. Ischemia in CSF containing 2 mM Ca^{2+} caused an ~3.5-fold increase in fluo-4 emission after 30 min, indicating a large axonal Ca^{2+} rise well into the micromolar range. Axonal Na^+ accumulation was enhanced by veratridine and reduced, but not abolished, by TTX. Ischemia in Ca^{2+}-free (plus BAPTA) perfusate resulted in a smaller but consistent Ca^{2+} increase monitored by Oregon Green 488 BAPTA-1, indicating release from intracellular sources. This release was eliminated in large part when Na^+ influx was reduced by replacement with *N*-methyl-D-glucamine ($NMDG^+$; even in depolarizing high K^+ perfusate), Li^+, or by the application of TTX and significantly increased by veratridine. Intracellular release also was reduced significantly by neomycin or 1-(6-[(17β-methoxyestra-1,3,5 [10]-trien-17-yl) amino] hexyl)-^1H-pyrrole-2,5-dione (U73122) (phospholipase C inhibitors), heparin [inositol trisphosphate (IP_3) receptor blocker], or 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(^3H)-one (CGP37157; mitochondrial Na^+/Ca^{2+} exchange inhibitor) as well as ryanodine. Combining CGP37157 with U73122 or heparin decreased the response more than either agent alone and significantly improved electrophysiological recovery. Our conclusion is that intra-axonal Ca^{2+} release during ischemia in rat optic nerve is mainly dependent on Na^+ influx. This Na^+ accumulation stimulates three distinct intra-axonal sources of Ca^{2+}: (1) the mitochondrial Na^+/Ca^{2+} exchanger driven in the Na^+ import/Ca^{2+} export mode, (2) positive modulation of ryanodine receptors, and (3) promotion of IP_3 generation by phospholipase C.

Key words: **axon; Na/Ca exchanger; IP_3; ryanodine; endoplasmic reticulum; mitochondria; phospholipase C**

Introduction

Ischemia in CNS white matter causes axonal Na^+ influx through noninactivating Na^+ channels. Accumulation of axoplasmic Na^+ is critical for promoting cellular injury; coupled with severe K^+ depletion that results in large membrane depolarization, high Na^+_i stimulates reverse Na^+/Ca^{2+} exchange and axonal Ca^{2+} overload (Stys et al., 1992; Stys and Lopachin, 1998). Ca^{2+} overloading in turn triggers processes that are detrimental to affected cells (Schanne et al., 1979; Kostyuk et al., 2000; Sattler and Tymianski, 2000; Rizzuto, 2001). In addition to an extracellular source of Ca^{2+}, many excitable cells contain enough bound or sequestered ion to increase $[Ca^{2+}]_i$ markedly (Stys et al., 1997; Kristian and Siesjo, 1998) if these intracellular pools of Ca^{2+} [e.g., endoplasmic reticulum (ER), mitochondria] are released. Results obtained in our laboratory indicate that intracellular Ca^{2+} overload because of anoxia in rat optic nerve is attributable primarily to Ca^{2+} influx from the extracellular space (Stys et al., 1992; Stys and Lopachin, 1998; Stys, 2004). During *in vitro* ischemia, however, removal of external Ca^{2+} failed to protect spinal cord dorsal columns; instead, under these conditions, Ca^{2+} was released from the ER in amounts sufficient to cause severe damage via a mechanism similar to "excitation–contraction coupling" in skeletal muscle (Ouardouz et al., 2003). Given the potentially differing responses of central white matter tracts to injury in different regions of the CNS [e.g., optic nerve vs dorsal column (Jiang et al., 2002)] or among species [e.g., mouse vs rat (Tekkok et al., 2003)], we investigated the contribution of intracellular Ca^{2+} stores to axoplasmic Ca^{2+} rise during *in vitro* ischemia in rat optic nerve with the use of high-resolution confocal laser-scanning microscopy. Our results indicate that a significant portion of ischemic axoplasmic Ca^{2+} increase originates from the ER and from mitochondria; unexpectedly, this Ca^{2+} release is strongly dependent on axonal Na^+ influx, similar to the dependence of extracellular Ca^{2+} entry on Na^+ (for review, see Stys, 2004).

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Materials and Methods

Adult Long–Evans male rats were anesthetized with 80% CO_2/20% O_2 and decapitated. Optic nerves were dissected out and immersed in Ca^{2+}-free artificial CSF (aCSF) at 4°C. Nerves were placed in an interface perfusion chamber and loaded with fluorescent dyes. For Ca^{2+} and Na^+ imaging, the nerves were placed in a custom-built perfusion chamber, mounted on an upright Nikon (Tokyo, Japan) C1 confocal laser-scanning microscope. Imaging was performed at 36°C with a 60 or 40× immersion objective maintained at the same temperature to avoid cooling of the sample. Fluorescence changes were normalized to average basal

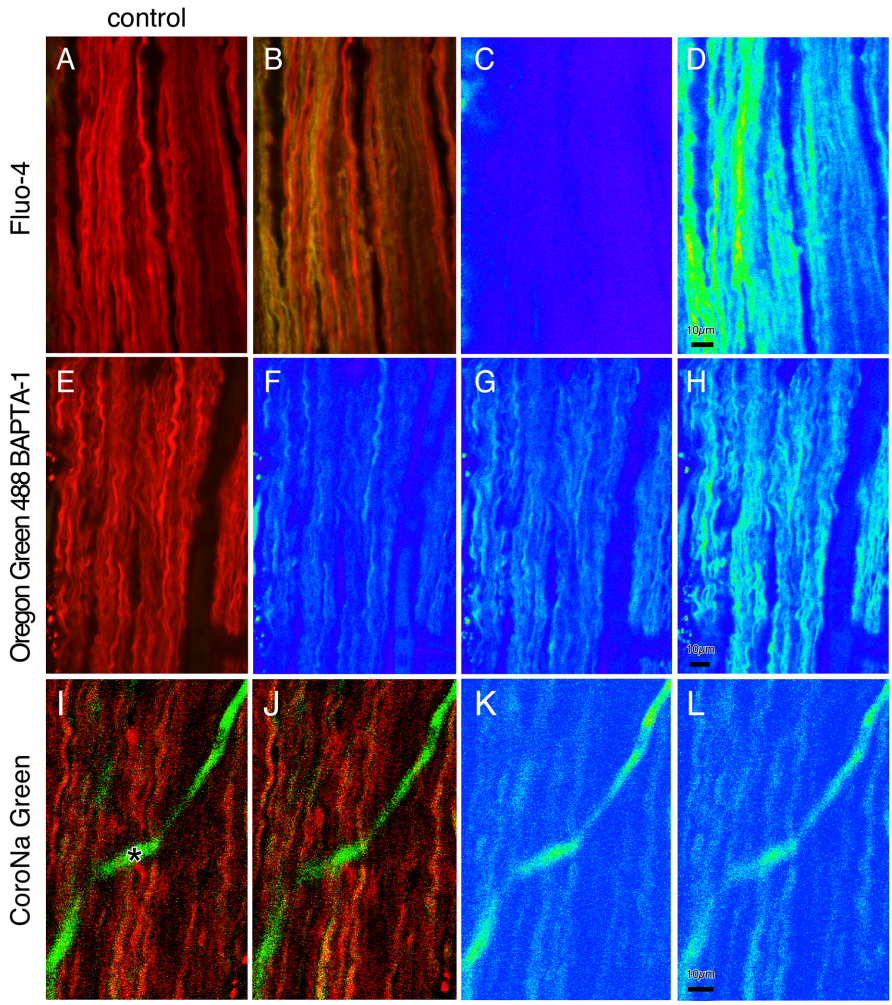

Figure 1. Confocal images of live optic nerve before and during ischemia. Axons were coloaded with red ion-insensitive Alexa Fluor 594 dextran for visualization of axonal profiles and with either low-affinity fluo-4 dextran (K_D, 4 μM; top row) to demonstrate larger increases in axonal Ca^{2+} (***A***, before ischemia; ***B***, 30 min in ischemic buffer; ***C***, ***D***, Ca^{2+}-sensitive fluorescence in pseudocolor) or high-affinity Oregon Green 488 BAPTA-1 dextran (K_D, 500 nM; middle row) to demonstrate contributions of intracellular Ca^{2+} stores in 0 Ca^{2+} (plus BAPTA) conditions (***E***, pre-ischemia image; ***F***, same image in pseudocolor; ***G***, after 7 min in 0 Ca/BAPTA ischemic buffer; ***H***, after 15 min in Ca^{2+}-replete ischemic buffer). The bottom row demonstrates an Na^+-sensitive CoroNa Green fluorescence increase in ischemia (***I***, before ischemia; ***J***, 30 min in ischemic buffer; ***K***, ***L***, Na^+-dependent fluorescence in pseudocolor). The solid green structure (asterisk) is a dye-filled capillary.

levels and reported as a ratio of the signal collected from ion-sensitive and ion-insensitive fluorophores plotted against time. Electrophysiological measurements of compound action potentials (CAPs) were performed by using suction electrodes as described previously (Stys et al., 1991).

The aCSF contained the following (in mM): 126 NaCl, 26 $NaHCO_3$, 3.0 KCl, 1.25 NaH_2PO_4, 2.0 $MgSO_4$, 2.0 $CaCl_2$, and 10 glucose. Solutions were bubbled continuously with 95% O_2/5% CO_2, pH ≈7.4. As Ca^{2+} indicators, two fluorescent probes were used: fluo-4 dextran ($K_D \approx 4$ μM) or Oregon Green 488 BAPTA-1 dextran ($K_D \approx 450$ nM). Na^+ changes were measured by CoroNa Green. For visualization of axonal profiles, the nerves were coloaded with red Alexa Fluor 594 dextran. The dextran-conjugated dyes were restricted to the axoplasmic compartment and loaded optic nerve fibers to >1 mm from the cut end after 1.5 h, likely by axoplasmic transport (Ren et al., 2000; Verbny et al., 2002; Ouardouz et al., 2003). The red ion-independent fluorescence was used to outline regions of interest (ROIs) from which green Ca^{2+}- or Na^+-dependent fluorescence was measured. Because larger axons were more easily distinguishable, ROIs represent mainly larger (>1.5 μm in diameter) fibers. The loading buffer contained a low Na^+ concentration [NaCl was replaced by 126 mM of *N*-methyl-D-glucamine (NMDG)] and a low Ca^{2+} concentration ($CaCl_2$ was omitted) to mimic intra-axonal concentrations. Dye concentrations in the loading pipette included the following: 150 μM for fluo-4 dextran, 75 μM for Oregon Green 488 BAPTA-1 dextran, 1.4 mM for CoroNa Green, and 100–200 μM Alexa Fluor 594 dextran. Because loading efficiency was estimated to be only ~1% (Verbny et al., 2002), the final concentration of Ca^{2+} dyes in the axon was probably only several micromolar. The imaging was performed at a relatively constant distance (~1.2 mm) from the cut end to minimize the effects of spatial dye gradients. Chemical ischemia was induced by using NaN_3 (2 mM), which causes rapid inhibition of mitochondrial complex IV (Petersen, 1977; Malek et al., 2005), and 0 glucose (replaced with 10 mM sucrose) in the perfusate. At the conclusion of every 0 Ca^{2+} experiment, chemical ischemia was induced in the presence of high Ca^{2+} (2 mM) CSF to confirm that axonal Ca^{2+} increased; this control was deemed important to ensure that the absence of a Ca^{2+} rise as a result of manipulations was real and not a technical failure (e.g., inadequate dye loading, etc.). Minimally, three nerves were studied per treatment group, with approximately three ROIs chosen per nerve and typically six axons per ROI. All fluorescent dyes were purchased from Invitrogen (San Diego, CA). BAPTA (tetrapotassium salt, with KCl concentration adjusted accordingly) was purchased from A.G. Scientific (San Diego, CA). Neomycin, heparin (Sigma, St. Louis, MO), and an oxygen-bridged dinuclear ruthenium amine complex (Ru360; Calbiochem, La Jolla, CA) were dissolved directly into aCSF or loading solution. Next, 1-(6-[(17β-methoxyestra-1,3,5 [10]-trien-17-yl) amino] hexyl)-^1H-pyrrole-2,5-dione (U73122), ryanodine, and 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(^3H)-one (CGP37157) (Tocris Bioscience, Ellisville, MO) were dissolved first in DMSO and then added to aCSF. Cyclosporin A (Sigma) was dissolved in ethanol. All drugs were applied 15 min before the onset of 0 Ca^{2+} perfusate (see below) and continued throughout ischemia.

Statistics. All data are expressed as the means ± SD. Statistical differences were calculated by ANOVA with Bonferroni's correction for multiple comparisons or ANOVA with Dunnett's test for multiple comparisons with a common control group. Student's *t* test was used for single comparisons between two groups. Reported *n* values represent the number of individually analyzed axons.

Results

Axonal Ca^{2+} changes during ischemia in normal and Ca^{2+}-free CSF

To assess the extent of Ca^{2+} rise during *in vitro* ischemia in optic axons, we initially used the low-affinity Ca^{2+} indicator fluo-4 dextran. Images were taken during perfusion in normal aCSF (containing 2 mM Ca^{2+}) to determine the basal level of fluorescence and then during ischemia (0 glucose plus 2 mM NaN_3) at 1 min intervals.

Figure 1 shows representative images of optic axons before and after ischemia. Red fluorescence represents Ca^{2+}-insensitive dextran dye loaded to outline axon cylinders from which ROIs were derived for analysis of Ca^{2+}- or Na^+-dependent fluorescence. Panels *A* and *B* show a substantial increase in green Ca^{2+}

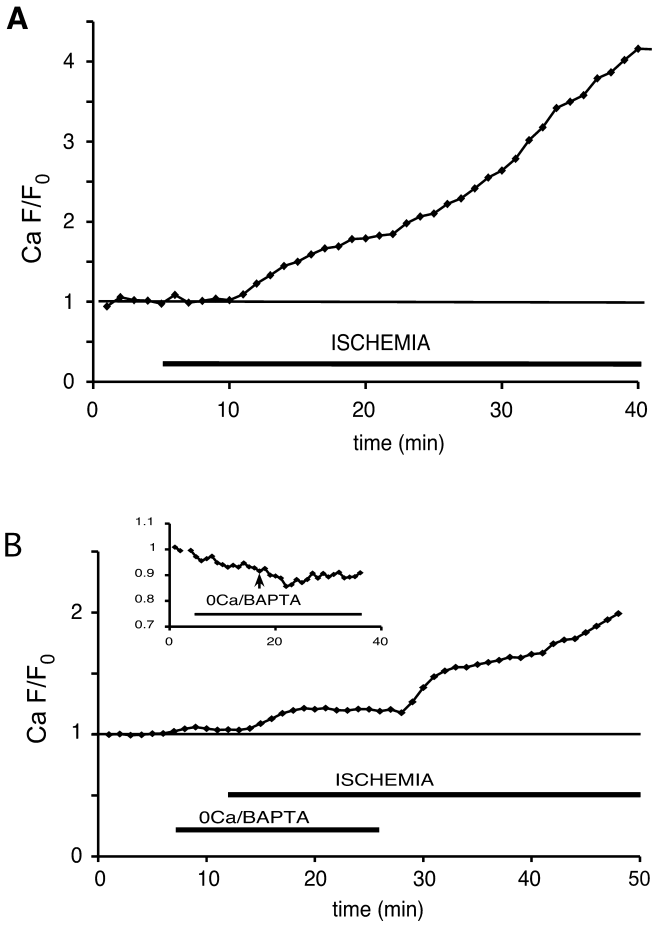

Figure 2. Time course of normalized axonal green/red fluorescence ratio showing a 3.5-fold Ca^{2+}-dependent fluorescence increase over baseline after 30 min of ischemia in 2 mM Ca^{2+} with fluo-4 as the Ca^{2+} indicator (***A***). In 0 Ca^{2+} (plus BAPTA) perfusate, the ischemia evoked a 19% increase with Oregon Green 488 BAPTA-1 dextran as the Ca^{2+} indicator (***B***), indicating a release of this ion from intra-axonal compartments. The inset demonstrates a fluorescence decrease in control experiments without ischemia, indicating that perfusion with the 0 Ca^{2+}/BAPTA solution gradually reduced axoplasmic [Ca^{2+}].

signal after 30 min of chemical ischemia (*C* and *D* show the Ca^{2+} signal in pseudocolor). The middle panels show ischemic axonal Ca^{2+} rise in 0 Ca^{2+} perfusate with the use of the higher-affinity Oregon Green 488 BAPTA-1 indicator. The bottom panels show axonal Na^+ increase (green signal with CoroNa Green indicator) as a function of ischemia (for more details, see the legend to Fig. 1). The fluorescence changes were normalized to the average basal fluorescence before the application of the ischemic buffer, and the change in fluorescence (F/F_0) was plotted against time (Fig. 2). There was a substantial increase in Ca^{2+}-dependent fluorescence, which began after an average lag time of 5–6 min after ischemia was introduced and increased to ~3.5 times control after 30 min (Fig. 2*A*).

This Ca^{2+} accumulation may originate from two sources: (1) influx of extracellular Ca^{2+} and (2) release of Ca^{2+} from intracellular stores. To determine the contribution of the latter and anticipating a smaller [Ca^{2+}] increase when bath Ca^{2+} was removed, we loaded optic nerves with the higher-affinity Ca^{2+} indicator Oregon Green 488 BAPTA-1 dextran instead. The nerves were pretreated with 0 Ca^{2+} (plus 0.75 mM BAPTA) for 5 min to remove any residual external Ca^{2+} and then perfused in an ischemic 0 Ca^{2+} (plus BAPTA) buffer. We needed to ensure that for all experiments in 0 Ca^{2+} perfusate there was minimal extracellular Ca^{2+} remaining that could contribute to any observed intracellular Ca^{2+} increases. To simulate the extracellular environment near the surface of optic nerves from which images were acquired, we dissolved Ca^{2+}-sensitive and Ca^{2+}-insensitive dextran dyes in a bead of 2% agar and imaged them 20 μm below the surface (a depth typically used for optic nerve imaging) as the perfusate was switched from normal (2 mM) Ca^{2+} to 0 Ca^{2+}/EGTA. Ca fluorescence decreased to ~50% after ~4 min of perfusion with the 0 Ca^{2+}/EGTA solution. If we assume that at 2 mM the dye was saturated, the half-maximal fluorescence implies that the free [Ca^{2+}] in the agar bead dropped to $\approx K_D$ level, which is 400–500 nM. Therefore, we are confident that our standard paradigm of preapplying 0 Ca^{2+}/BAPTA solution for 5 min before ischemia effectively eliminated virtually all contribution of Ca^{2+} influx from the extracellular space. We switched to BAPTA as the Ca^{2+} chelator to avoid the possibility of reduced Ca^{2+} binding capacity with EGTA in an acidified extracellular environment during ischemia, known to occur in optic nerve (Ransom et al., 1992). Representative confocal images are shown in Figure 1 (middle row). In Ca^{2+}-free conditions, the green fluorescence began to rise within 1–2 min of ischemia onset (Fig. 1*G*) and reached its maximum (19 ± 6% increase) after ~7 min. When Ca^{2+} was reintroduced, the fluorescence rose further, indicating there was an additional dynamic range of the dye, which could report additional Ca^{2+} increases from influx across the axolemma. It should be noted that in control experiments without ischemia, Ca^{2+}-dependent fluorescence decreased by ~9% below baseline (Fig. 2*B*, inset). For this reason, the reported 19% increase in the above experiment and in all subsequent studies performed in 0 Ca^{2+}-BAPTA perfusate probably underestimated the true Ca^{2+} rise.

Axonal Na^+ accumulation during ischemia

To explore the hypothesis that Na^+ accumulation may modulate Ca^{2+} release, we studied the temporal profiles of axonal Na^+ changes as a function of the same manipulations that modulated Ca^{2+} release from internal compartments (see below). Axonal Na^+ measurements were performed under identical experimental conditions as Ca^{2+} measurements except that the axons were loaded with the Na^+-sensitive dye CoroNa Green along with red dextran-conjugated Alexa Fluor 594. Unfortunately, there is no commercially available Na^+ indicator conjugated to dextran. As a result, in contrast to dextran-conjugated dyes, the intensity of axonal CoroNa Green fluorescence decreased with time, likely attributable to loss of this low-molecular-weight (MW) dye (MW 586 vs 10 kDa dextrans) by export and/or leakage. The rate of CoroNa Green fluorescence decay was determined first in control experiments without ischemia and was found to be approximately linear over the time required for our measurements (~50 min), decreasing by ~1.5%/min (Fig. 3*A*). All experiments therefore included a 20 min baseline from which a linear regression was determined, allowing for extrapolation of expected fluorescence loss for the duration of the study. Raw data (Fig. 3*B*) thus were adjusted, yielding fluorescence changes approximately corrected for dye loss (Fig. 3*C*).

Na^+-dependent fluorescence began to increase within 3–4 min of ischemia onset and continued for the 30 min duration of the experiment, reaching 58 ± 10% of control (Fig. 3*C*). Increasing Na^+ channel permeability with veratridine (50 μM), an alkaloid that inhibits voltage-dependent Na^+ channel inactivation (Catterall, 1980), accelerated and amplified axonal ischemic Na^+ accumulation (95 ± 8%). Conversely, blocking Na^+ channels with TTX (1 μM) significantly reduced (25 ± 5 vs 58 ± 10% after

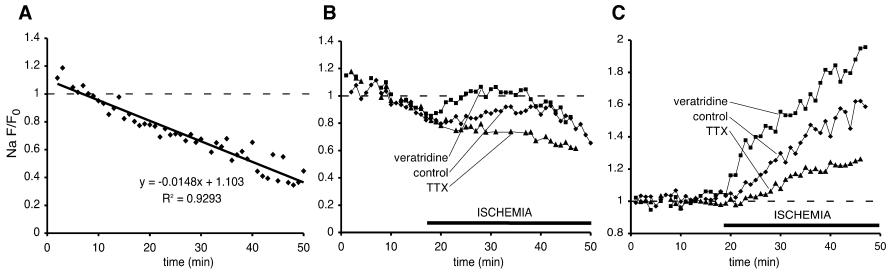

Figure 3. Time course of normalized green/red (Na^+-sensitive CoroNa Green/Na^+-insensitive) fluorescence ratio. ***A***, Approximately linear decrease in axoplasmic fluorescence in nonischemic conditions (each point represents the mean from ~30 axons). ***B***, Reversal of the fluorescence decline during chemical ischemia. ***C***, Fluorescence changes adjusted for loss of the Na^+-sensitive dye, demonstrating the net rise of axoplasmic [Na^+] during ischemia (traces represent the means from 60–90 axons). Activating Na^+ channels with veratridine further exacerbated axonal Na^+ loading during ischemia, whereas blocking these channels with TTX reduced, but did not abolish completely, axonal Na^+ accumulation. Dashed lines indicate baseline fluorescence.

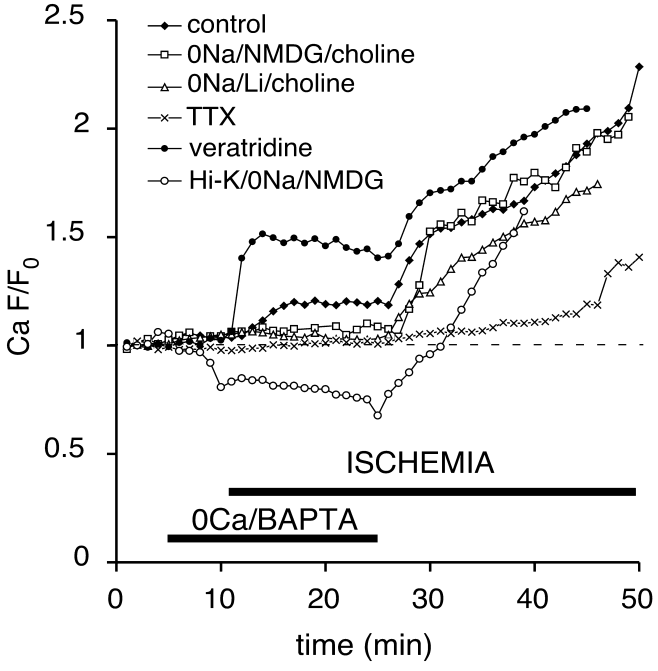

Figure 4. Effects of Na^+ influx on Ca^{2+}-dependent fluorescence changes during ischemia in 0 Ca^{2+} (plus BAPTA) perfusate. Combining 0 Ca^{2+} with 0 Na^+ (126 mM of NaCl replaced with the impermeant $NMDG^+$ ion and 26 mM of $NaHCO_3$ with choline bicarbonate) in the perfusate or the application of 1 μM TTX in large part prevented any Ca^{2+} increase. Preapplication of Li^+-substituted 0 Na^+ perfusate (126 mM of NaCl replaced with LiCl and 26 mM of $NaHCO_3$ with choline bicarbonate) in 0 Ca^{2+} (plus BAPTA), which allows axonal depolarization to occur (in contrast to the impermeable NMDG ion), also prevented ischemic Ca^{2+} rise. Veratridine (which increased Na^+ influx) (Fig. 3) markedly increased the ischemic Ca^{2+} response. Forcing depolarization with 40 mM K^+ in the absence of Na^+ did not promote ischemic Ca^{2+} rise, indicating that it is mainly the Na^+ influx, and not axonal depolarization, that triggers the release of Ca^{2+} from intracellular compartments. The dashed line indicates baseline fluorescence.

30 min of ischemia), but did not eliminate completely, the axonal Na^+ rise, indicating that voltage-gated TTX-sensitive Na^+ channels were the major, but not the only, source of axonal Na^+ influx during ischemia.

Intracellular Ca^{2+} release is Na^+ dependent

Axonal Na^+ accumulation during anoxia/ischemia may drive a number of pathological Ca^{2+}-dependent cascades (e.g., promotion of mitochondrial Ca^{2+} release via reversal of its Na^+/Ca^{2+} exchanger, neurotransmitter release via reversal of Na^+-dependent transporters and potential stimulation of metabotropic receptors leading to Ca^{2+} release, and promotion of depolarization-induced release from Ca^{2+} stores). To examine the role of Na^+ in mediating Ca^{2+} release from intra-axonal compartments, we reduced Na^+ influx by substituting this cation with impermeable $NMDG^+$ or permeable Li^+ or by blocking voltage-gated Na^+ channels with 1 μM TTX. Ischemia then was applied in 0 Ca^{2+}/BAPTA perfusate. The replacement of Na^+ with $NMDG^+$/choline or blocking voltage-gated Na^+ channels with TTX [treatments that both significantly reduce the degree of ischemic depolarization in optic nerve (Malek et al., 2003)] prevented axonal Ca^{2+} rise almost completely (Fig. 4), with an average fluorescence increase of 1 ± 4 and 4 ± 5% over baseline, respectively. This suggests that Na^+ influx and/or membrane depolarizations promote intracellular Ca^{2+} release in optic nerve axons during ischemia. However, the replacement of bath Na^+ with Li^+, which permeates Na^+ channels (Fontana et al., 1995) and allows for axonal depolarization (Leppanen and Stys, 1997), also abolished ischemic Ca^{2+} increase (1 ± 4%) (Fig. 4). Moreover, so that axonal depolarization during Na^+-depleted conditions could be ensured, perfusion of optic nerve in 40 mM K^+ buffer (Na^+ replaced with $NMDG^+$ and Cl^- partially replaced with impermeable gluconate, maintaining a constant $K^+ \times Cl^-$ product to reduce Donnan-mediated volume changes) did not induce any increase in Ca^{2+} fluorescence during ischemia in 0 Ca^{2+}/BAPTA perfusate (Fig. 4). Together, these observations suggested that, in the optic nerve, Ca^{2+} release from intracellular stores was dependent not on ischemic axonal depolarization per se but on the influx of Na^+ ions. The role of Na^+ entry during 0 Ca^{2+} ischemia was investigated additionally by using veratridine to promote axonal Na^+ accumulation. Application of 50 μM veratridine during 0 Ca^{2+} ischemia caused a marked increase in axonal Ca^{2+} fluorescence (50 ± 8%) (Fig. 4), which parallels the exaggerated axonal overload with Na^+ (Fig. 3), further supporting the notion that intracellular Ca^{2+} release is proportional to ischemic Na^+ influx.

Inositol trisphosphate and ryanodine receptors and the mitochondrial Na^+/Ca^{2+} exchanger are major sources of intracellular Ca^{2+} during ischemia

The primary intracellular Ca^{2+} storage/release organelle in most cells is the ER, possessing two major families of Ca^{2+} release channels: the inositol trisphosphate (IP_3) receptor and ryanodine receptor (RyR) (Berridge, 1998; Rizzuto, 2001). The IP_3 receptor is activated by inositol 1,4,5-trisphosphate, which is generated from phosphatidylinositol 4,5-bisphosphate by phospholipase C (PLC). We investigated the role of the IP_3 receptor in Ca^{2+} release by using three agents acting at distinct points in the IP_3 signaling pathway: U73122, an irreversible antagonist of PLC (Jin et al., 1994); neomycin, which complexes phosphoinositide lipids to render them unavailable as PLC substrates (Schacht, 1976, 1978); and heparin, which inhibits IP_3-induced Ca^{2+} release by binding to IP_3-binding sites (Worley et al., 1987; Supattapone et al., 1988). U73122 and neomycin were bath-applied. Because heparin is a large polysaccharide and is therefore incapable of crossing cell membranes, it was loaded intra-axonally along with the dextran-conjugated dyes. Each experiment was performed in

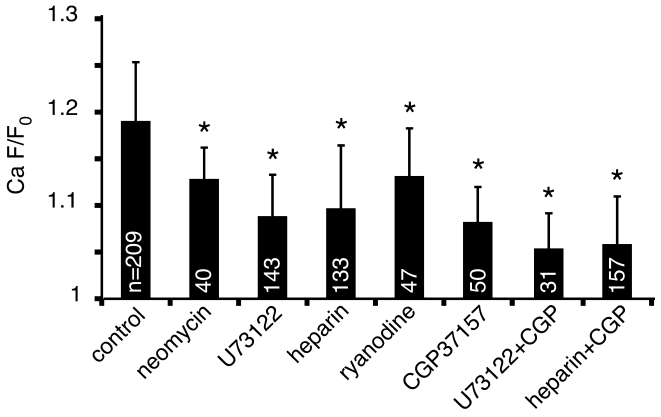

Figure 5. Bar graph summarizing the effects of ER and mitochondrial Ca^{2+} release blockers (measured at the point of maximum Ca^{2+}-dependent fluorescence increase, which occurred within 5–10 min after chemical ischemia was applied). Neomycin (300–500 μM), U73122 (20 μM), heparin (85 mg/ml), ryanodine (60 μM), and CGP37157 (20 μM) all significantly suppressed ischemic Ca^{2+} increases (all in 0 Ca^{2+} plus BAPTA perfusate; *$p < 0.01$ compared with drug-free control). Combining U73122 or heparin with CGP37157 reduced the Ca^{2+} rise even more. White numbers within the bars represent the number of individual axons analyzed for each treatment. Error bars indicate SD.

0 external Ca^{2+} (plus BAPTA), as explained in Materials and Methods. As shown in Figure 5, each of these three antagonists reduced the peak Ca^{2+} response by ~50% compared with drug-free controls ($p < 0.01$), suggesting that ischemia-induced Ca^{2+} release is mediated in part by the IP_3 signaling pathway. There remained a substantial component of Ca^{2+} release, however, that was independent of IP_3 receptors.

Another important ER intracellular Ca^{2+} release channel is the RyR, activated either by Ca^{2+} itself or by depolarization via coupling with voltage-gated Ca^{2+} channels (Berridge, 1998; Rizzuto, 2001). At high concentrations (≥50 μM), ryanodine blocks RyRs (Solovyova et al., 2002). In our experiments, the application of ryanodine (60 μM) resulted in a significant decrease in the ischemic Ca^{2+} response in 0 external Ca^{2+} perfusate (13 ± 5 vs 19 ± 6% without ryanodine; $p < 0.01$).

Mitochondria are also capable of storage and release of intracellular Ca^{2+} (Bernardi, 1999; Rizzuto et al., 1999). Under physiological conditions, Ca^{2+} efflux from mitochondria is mediated primarily by the mitochondrial Na^+/Ca^{2+} exchanger. In addition, under certain conditions, the Ca^{2+} uniporter and the permeability transition pore may serve as conduits of Ca^{2+} release (Gunter et al., 2000; Montero et al., 2001). To determine the specific route through which Ca^{2+} could exit from mitochondria in the optic nerve, we tested three blockers: CGP37157, an inhibitor of mitochondrial Na^+/Ca^{2+} exchange (Chiesi et al., 1988); Ru360, an inhibitor of the mitochondrial Ca^{2+} uniporter (Matlib et al., 1998); and cyclosporin A, a permeability transition pore blocker (Broekemeier et al., 1989; Crompton et al., 1999). Pretreatment with CGP37157 reduced the Ca^{2+} fluorescence increase by ~50% from 19 ± 6 to 8 ± 4% ($p < 0.01$). Cyclosporin A and Ru360 did not reduce the peak Ca^{2+} signal significantly: 15 ± 7 and 16 ± 7%, respectively ($p > 0.05$). Together, these results indicate that the mitochondrial Na^+/Ca^{2+} exchanger accounts for the majority of mitochondrial Ca^{2+} release during ischemia.

If IP_3 receptors and the mitochondrial Na^+/Ca^{2+} exchanger account for almost one-half of the Ca^{2+} rise, blocking both should reduce the Ca^{2+} rise even further if the two pathways are independent. Indeed, a combination of U73122 or heparin with CGP37157 reduced the ischemic axonal Ca^{2+} increase to 5 ± 4% ($p < 0.05$; U73122 vs U73122 plus CGP37157; ANOVA with Bonferroni's correction for multiple comparisons) and 6 ± 5% ($p < 0.01$; heparin vs heparin plus CGP37157), respectively (Fig. 5), confirming that these two sources are the major contributors to intracellular release during ischemia and that they appear to operate independently. To assess whether this restraint of Ca^{2+} rise has functional implications, we performed electrophysiological CAP recordings on optic nerves treated with U73122 plus CGP37157 (both 20 μM) during a 1 h exposure to *in vitro* chemical ischemia (95% N_2/5% CO_2 atmosphere plus glucose replaced with sucrose), followed by reperfusion. Because Ca^{2+}-free bath readily removes Ca^{2+} from axoplasm in optic nerve (see Discussion) and is itself therefore highly neuroprotective (Stys et al., 1990), this experiment was performed in normal Ca^{2+}-replete CSF. Despite the large source of available extracellular Ca^{2+}, the combination of U73122 plus CGP37157 improved electrophysiological recovery from 5 ± 3% ($n = 8$ nerves) of control CAP area in ischemia/DMSO to 17 ± 8% ($n = 12$) in the drug combination ($p < 0.001$).

Discussion

Most studies indicate that ischemic intracellular Ca^{2+} overload in neurons is attributable primarily to Ca^{2+} influx from the extracellular space (Lipton, 1999; Arundine and Tymianski, 2003; Paschen, 2003). Although the role of external Ca^{2+} is well established, less is known about the sources and release mechanisms of intracellular stores of Ca^{2+} during ischemia. Nevertheless, intracellular Ca^{2+} stores are known to contribute to cytosolic Ca^{2+} rise in ischemic neurons (Mitani et al., 1993; Zhang and Lipton, 1999), and inhibition of this release may be protective (Yano et al., 2001; Wang et al., 2002). Very little is known about such mechanisms in myelinated axons.

In the present study, ischemic Ca^{2+} accumulation was measured by a low-affinity dye when extracellular Ca^{2+} was maintained in the physiological range (~2 mM) to estimate the degree of axonal [Ca^{2+}] rise in comparison with that induced in 0 external Ca^{2+} solution. In agreement with studies in ischemic neurons (Lipton, 1999; Sattler and Tymianski, 2000; Erecinska and Silver, 2001; Arundine and Tymianski, 2003; Paschen, 2003), we show that ischemic central axons also suffer large increases in axoplasmic Ca^{2+} concentrations. Although we could not perform absolute Ca^{2+} measurements, the large increase in fluorescence of the low-affinity fluo-4 dextran indicator suggests that free [Ca^{2+}] increased well into the micromolar range. Under conditions when bath Ca^{2+} was removed, a higher-affinity indicator was preferred to show the smaller but still substantial ischemic Ca^{2+} rise originating from internal compartments. Our measurements probably underestimated the contribution of Ca^{2+} release from internal stores because of potential continuous Ca^{2+} washout in 0 Ca^{2+}/BAPTA buffer. This decrease might be related to the proximity of portions of the ER to the axolemma (Berridge, 1998; Ouardouz et al., 2003). In fact, some ER cisternas, including those of axoplasmic reticulum, might approach the plasma membrane to within 20 nm (Lindsey and Ellisman, 1985; Berridge, 1998) and may be depleted in the face of a strongly reversed gradient favoring efflux. Measurements of total axoplasmic Ca^{2+} (which included regions of ER) revealed that exposure of optic nerve to 0 Ca^{2+} perfusate reduced Ca^{2+} to undetectable levels (Stys and Lopachin, 1998), suggesting a robust exchange of the majority of axonal Ca^{2+} with the extracellular space in optic fibers. The 0 Ca^{2+} perfusion was necessary experimentally to dissect out the two components of Ca^{2+} accu-

mulation, but clearly such conditions would not apply during normal physiological or pathophysiological signaling. Curiously, dorsal column fibers are able to maintain levels of internally released Ca^{2+} much more readily than optic fibers under conditions of perfusion with 0 Ca^{2+} solution (Ouardouz et al., 2003). The reasons for this disparity are unclear but may point to differences in how Ca^{2+} handling machinery is organized in different fiber tracts.

We demonstrated previously that in spinal cord dorsal columns, intracellular Ca^{2+} stores caused a robust Ca^{2+} rise in ischemic axons even in Ca^{2+}-free perfusate (Ouardouz et al., 2003). In this tissue, this Ca^{2+} release was triggered to a large extent by axonal depolarization and was reduced significantly by ryanodine. The present study suggests that in optic axons the relative contribution of various intracellular Ca^{2+} stores may be somewhat different, with IP_3-dependent stores and mitochondria being the more important sources, whereas ryanodine receptor-dependent stores play a smaller role. Even in dorsal columns, however, ryanodine reduced, but did not abolish, axoplasmic Ca^{2+} increase caused by ischemia in 0 external Ca^{2+}, suggesting additional parallel sources as in optic nerve. In the present study, CAP recordings indicate that, although extracellular Ca^{2+} plays a major role as a source of deleterious Ca^{2+} into ischemic axons, release of this ion from IP_3-sensitive stores and mitochondria also significantly contributes to functional injury of optic nerve axons.

Na^+ dependence of internal Ca^{2+} release

Na^+ entry is characteristic of ischemic neurons and has been shown to be important in overall cellular injury (Lipton, 1999; Taylor et al., 1999; Erecinska and Silver, 2001; LoPachin et al., 2001; Varadarajan et al., 2001; Tanonaka and Takeo, 2003; Banasiak et al., 2004). This is also true of injured axons, which accumulate Na^+ through TTX-sensitive Na^+ channels, leading to Ca^{2+} influx via reverse Na^+/Ca^{2+} exchange (Stys et al., 1992) and voltage-gated Ca^{2+} channels (Fern et al., 1995).

The ER plays a prominent role in maintaining Ca^{2+} homeostasis in neurons (Berridge, 2002; Verkhratsky and Toescu, 2003). Alterations in Ca^{2+} homeostasis in the ER contribute to neuronal apoptosis and excitotoxicity (Mattson, 1989; Frandsen and Schousboe, 1991; Mody and MacDonald, 1995; Mattson et al., 2000). In white matter, the Ca^{2+} released from ER adds to the injurious effects of Ca^{2+} influx during traumatic compressive injury (Thorell et al., 2002). The unexpected finding in this study was the almost complete dependence of Ca^{2+} release from intracellular sources on Na^+ influx in ischemic optic axons. Although it is known that Na^+_i can promote the release of Ca^{2+} from stores by coupling via the Na^+/Ca^{2+} exchanger, resulting in more Ca^{2+} entry and enhanced Ca^{2+}-induced Ca^{2+} release (Han et al., 2002; Pogwizd and Bers, 2002; Ritter et al., 2003), this would not apply in our experiments because of a lack of external Ca^{2+}; therefore, a direct role for Na^+ is suggested. The link between Na^+ influx and activation of the PLC/IP_3 pathway was described almost 20 years ago (Gusovsky et al., 1986). However, it still remains unclear how Na^+ influences ER Ca^{2+} release other than via the plasmalemmal Na^+/Ca^{2+} exchanger as mentioned above.

Our results suggest that there exist three distinct intracellular sources of Ca^{2+} release in ischemic optic axons: IP_3 receptors (IP_3Rs), RyRs, and mitochondria, with all three being dependent on Na^+ influx. Zhang and Lipton (1999) reported that in ischemic rat hippocampal slices, in addition to influx via NMDA receptors, a substantial proportion of Ca^{2+} originated from mitochondria, being released via the mitochondrial Na^+/Ca^{2+} exchanger. The present study demonstrates that in the ischemic optic nerve mitochondria account for ~50% of the Ca^{2+} rise from intracellular sources. In agreement with Zhang and Lipton (1999), we also found that the increase is Na^+ dependent and is reduced significantly by the application of CGP37157, a blocker of the mitochondrial Na^+/Ca^{2+} exchanger (Chiesi et al., 1988) that mediates most of the efflux of Ca^{2+} from mitochondria in exchange for the influx of Na^+ (Rizzuto, 2003). Blocking the Ca^{2+} uniporter or the permeability transition pore resulted in a statistically insignificant reduction in ischemic Ca^{2+} release; whether this indicates that these two pathways truly do not contribute to Ca^{2+} release from mitochondria in our paradigm or whether our sampling was insufficient to demonstrate a significant effect is unknown and would require additional studies.

Na^+-dependent mitochondrial Ca^{2+} release during ischemia could contribute significantly to free axoplasmic Ca^{2+} because resting mitochondria contain substantial amounts of this cation (Stys et al., 1997; Rizzuto et al., 1999; Yang et al., 2003), mostly in a complex with phosphate, which nevertheless readily can absorb and release Ca^{2+} ions (David, 1999; Yang et al., 2003). Moreover, release of mitochondrial Ca^{2+} also can be damaging because of compromised ATP synthesis; depletion of mitochondrial Ca^{2+} may suppress Ca^{2+}-sensitive matrix dehydrogenases that catalyze key Krebs' cycle reactions (McCormack et al., 1990; Bernardi, 1999). It is curious that previous reports on anoxic axonal mitochondria indicated that these organelles accumulate Ca^{2+} during injury (LoPachin and Stys, 1995), yet the present results suggest the opposite. It is possible that during anoxia, these organelles are able to maintain some electrochemical potential across their inner membrane, likely by hydrolysis of glycolytically derived ATP (Nicholls and Budd, 2000). The residual polarization could be sufficient to support a modest Ca^{2+} accumulation into the matrix, whereas in the face of a profound energy deficit during ischemia the mitochondria would be depolarized more strongly and would thus be driven to release matrix Ca^{2+}.

The Na^+ dependence of IP_3R- and RyR-mediated Ca release is more puzzling. The effects of this ion may be direct on these receptors or indirect, acting at upstream signaling points. In the case of IP_3Rs, there is no convincing evidence of a direct effect of Na^+ ions. Instead, we favor the indirect explanation of ischemic Na^+ influx promoting neurotransmitter release, which may in turn stimulate metabotropic receptors, activate PLC, and generate IP_3. Several observations support this scenario. We had shown that anoxia causes glutamate release from central axons via reverse Na^+-dependent glutamate transport (Li et al., 1999). We also have evidence for the involvement of group I metabotropic glutamate receptors in mediating Ca^{2+} overload in ischemic spinal axons (Stys and Ouardouz, 2002). Moreover, several neurotransmitter uptake mechanisms are Na^+ dependent, and their metabotropic receptors are coupled to PLC (e.g., serotonin, dopamine, norepinephrine); therefore, glutamate may be only one of several neurotransmitters released from ischemic axons that then stimulate metabotropic receptors to generate IP_3. Finally, if Na^+ exerted a direct effect on IP_3Rs, PLC inhibition would not reduce ischemic Ca^{2+} release, yet both U73122 and neomycin reduced the Ca^{2+} response. In the case of RyRs, there is evidence of direct positive modulation by Na^+ ions, which increases the release of Ca^{2+} from the sarcoplasmic reticulum in skeletal muscle even in the absence of Na^+/Ca^{2+} exchange activity (Allard and Rougier, 1992; Hu et al., 2003).

Conclusion

During optic nerve ischemia, axonal intracellular Ca^{2+} stores release Ca^{2+} in a Na^+-dependent manner via IP_3Rs and RyRs from the ER and via the mitochondrial Na^+/Ca^{2+} exchanger (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). This additional contribution to axoplasmic Ca^{2+} increase further exacerbates the deleterious effects of Ca^{2+} overload originating from the extracellular space in the genesis of ischemic and traumatic injury to central myelinated axons.

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