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Ionic Mechanisms of Anoxic Injury in Mammalian CNS White Matter: Role of Na^+ Channels and Na^+–Ca^{2+} Exchanger

Peter K. Stys, Stephen G. Waxman, and Bruce R. Ransom

Department of Neurology, Yale University School of Medicine, New Haven, Connecticut 06510 and Neuroscience Research Center, Veterans Administration Hospital, West Haven, Connecticut 06516

White matter of the mammalian CNS suffers irreversible injury when subjected to anoxia/ischemia. However, the mechanisms of anoxic injury in central myelinated tracts are not well understood. Although white matter injury depends on the presence of extracellular Ca^{2+}, the mode of entry of Ca^{2+} into cells has not been fully characterized. We studied the mechanisms of anoxic injury using the *in vitro* rat optic nerve, a representative central white matter tract. Functional integrity of the nerves was monitored electrophysiologically by quantitatively measuring the area under the compound action potential, which recovered to 33.5 ± 9.3% of control after a standard 60 min anoxic insult. Reducing Na^+ influx through voltage-gated Na^+ channels during anoxia by applying Na^+ channel blockers (TTX, saxitoxin) substantially improved recovery; TTX was protective even at concentrations that had little effect on the control compound action potential. Conversely, increasing Na^+ channel permeability during anoxia with veratridine resulted in greater injury. Manipulating the transmembrane Na^+ gradient at various times before or during anoxia greatly affected the degree of resulting injury; applying zero-Na^+ solution (choline or Li^+ substituted) before anoxia significantly improved recovery; paradoxically, the same solution applied after the start of anoxia resulted in more injury than control. Thus, ionic conditions that favored reversal of the normal transmembrane Na^+ gradient during anoxia promoted injury, suggesting that Ca^{2+} loading might occur via reverse operation of the Na^+–Ca^{2+} exchanger. Na^+–Ca^{2+} exchanger blockers (bepridil, benzamil, dichlorobenzamil) significantly protected the optic nerve from anoxic injury. Together, these results suggest the following sequence of events leading to anoxic injury in the rat optic nerve: anoxia causes rapid depletion of ATP and membrane depolarization leading to Na^+ influx through incompletely inactivated Na^+ channels. The resulting rise in the intracellular [Na^+], coupled with membrane depolarization, causes damaging levels of Ca^{2+} to be admitted into the intracellular compartment through reverse operation of the Na^+–Ca^{2+} exchanger. These observations emphasize that differences in the pathophysiology of gray and white matter anoxic injury are likely to necessitate multiple strategies for optimal CNS protection.

White matter (WM) of the CNS, a tissue composed exclusively of axons, myelin, and glial cells, is injured by anoxia and ischemia, although it is more resistant to such injury than gray matter (Ransom et al., 1990a). Damage to WM disrupts afferent and efferent axonal connections and can result in severe neurological disability. Clinically, anoxic/ischemic WM injury is commonly caused by focal or global disruption of cerebral blood flow, that is, stroke. In addition, traumatic spinal cord injury prominently involves WM, and damage to spinal tracts is due in part to vascular compromise leading to anoxia/ischemia (Young, 1987; Fehlings et al., 1989). The mechanisms of anoxic/ischemic injury in WM are known to be different from those operating in gray matter (for reviews, see Bengtsson and Siesjö, 1990; Ransom et al., 1990a), but have not yet been fully characterized. An understanding of these fundamental mechanisms in WM may lead the way to developing protective strategies against such injury.

Irreversible anoxic/ischemic injury in gray matter involves influx of Ca^{2+} across the membrane through excitotoxin-gated channels (Choi, 1985), and possibly via voltage-gated Ca^{2+} channels (Krieglstein et al., 1989; Weiss et al., 1990). Using the *in vitro* rat optic nerve model, we showed that influx of extracellular Ca^{2+} is also a critical mediator of anoxic injury in CNS WM (Stys et al., 1990a). In contrast to gray matter, however, this Ca^{2+} influx in WM does not occur via excitotoxin-gated or voltage-gated Ca^{2+} channels (Ransom et al., 1990b; Stys et al., 1990b). Here we report evidence suggesting that WM anoxic injury largely depends on a persistent membrane Na^+ conductance, which in turn allows intracellular [Na^+] to rise sufficiently to promote reverse operation of the Na^+–Ca^{2+} exchanger. We have found that a large part of the damaging Ca^{2+} influx occurs via reverse Na^+–Ca^{2+} exchange; blocking either Na^+ channels or the Na^+–Ca^{2+} exchanger significantly protects CNS WM against anoxic injury.

Materials and Methods

Long–Evans rats aged 50–70 d were anesthetized with an 80% CO_2, 20% O_2 gas mixture and decapitated. The rat optic nerve (RON) has developed mature physiological properties in animals of this age (Connors et al., 1982; Foster et al., 1982; Ransom et al., 1985). The RONs were dissected free, placed in a modified interface perfusion chamber (Medical Systems Corp., Greenvale, NY), and incubated for 60–90 min before measurements were begun. The tissue was maintained at 37°C, oxygenated in a 95% O_2, 5% CO_2 atmosphere (pH 7.45), and perfused with artificial cerebrospinal fluid (CSF). Compositions of normal CSF

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Correspondence should be addressed to Dr. Peter K. Stys, Yale University School of Medicine, Department of Neurology, 710 LCI, 333 Cedar Street, New Haven, CT 06510.

Table 1. Composition of perfusion solutions (in mM)

	Normal CSF	Zero-Na^+ Choline	Zero-Na^+ Li^+
NaCl	126	–	–
KCl	3.0	1.75	1.75
LiCl	–	–	127
Choline Cl	–	127	–
$MgSO_4$	2.0	2.0	2.0
$NaHCO_3$	26	–	–
Choline bicarbonate	–	26	26
NaH_2PO_4	1.25	–	–
KH_2PO_4	–	1.25	1.25
$CaCl_2$	2.0	2.0	2.0
Dextrose	10	10	10

and, choline- and Li^+-substituted zero-Na^+ solutions are shown in Table 1.

Orthodromic stimulation and recording from RONs were accomplished using suction electrodes (Stys et al., 1990a, 1991a). Square constant-voltage stimulus pulses (50 μsec duration) were delivered via an isolation unit at 30 sec intervals. Stimulus strength was set to 25% above the strength that was required to elicit a maximal compound action potential (CAP; typically 70–120 V). Evoked CAPs were digitized (Nicolet 310 digital oscilloscope, 200 kHz sampling rate, 12 bit vertical resolution) and transferred to a microcomputer (Apple Macintosh IIfx), where the records were stored and analyzed using custom software (Stys, 1991).

Functional integrity of the RONs was quantitated by computing the area under the CAP (calculated over the interval from 0.2 to 12 msec with respect to stimulus onset), since activities of individual axons within a myelinated nerve bundle sum linearly to form the compound response (Buchthal and Rosenfalck, 1966; Cummins et al., 1979; Wijesinghe et al., 1991). We believe the CAP area to be the most representative measure of overall functional integrity of the nerve, since it reflects the number of axons that are capable of conducting action potentials. Under some conditions, CAP area recovered almost fully but the shape of the response remained irreversibly altered. This suggests that, in these experiments, most axons recovered the ability to conduct action potentials, but some injury still occurred and resulted in a slowing of the conduction velocities of some constituent fibers. We did not attempt to study conduction velocity systematically. Stable and reproducible measurements of CAP area were obtained after correcting for drift inherent in suction electrode recording (Stys et al., 1991a).

Anoxia was achieved by switching to a 95% N_2, 5% CO_2 atmosphere. Following the switch to 95% N_2, 5% CO_2, the concentration of O_2 in the recording chamber fell from 95% to zero in approximately 2 min, as measured with an O_2 probe (World Precision Instruments, New Haven, CT). A standard 60 min period of anoxia was used. Unless otherwise stated, postanoxic activity was measured 60 min after the end of anoxia, since optic nerves attained their maximal recovery by the end of this 60 min reoxygenation period (Davis and Ransom, 1987; Stys et al., 1990a).

Tetrodotoxin (TTX; Sigma) and saxitoxin (STX; Calbiochem) were diluted from stock solutions in distilled water. Veratridine (Sigma) was first dissolved in ethanol. Benzamil (Research Biochemicals Inc.) and bepridil (Sigma) were dissolved in dimethylsulfoxide (DMSO). 3,4-Dichlorobenzamil (DCB) was a generous gift from Dr. G. Kaczorowski (Merck Sharp & Dohme Research Laboratories) and was prepared as a 20 mM stock solution in DMSO. The final concentration of DMSO never exceeded 0.2% v/v; this concentration of DMSO had no effect on either control or postanoxic CAPs.

Errors are reported as standard deviations, and statistical significance was calculated using the unpaired t test with pooled variance.

Results

The CAP in RON is rapidly attenuated under anoxic conditions. CAP area falls by 50% in approximately 4 min following the switch to a 95% N_2, 5% CO_2 atmosphere, and is virtually completely lost by 8–10 min (Stys et al., 1990a). Following a 60 min anoxic period, and 60 min of reoxygenation, CAP area recovered to a stable level of 33.5 ± 9.3% of control area (Fig. 1*A*).

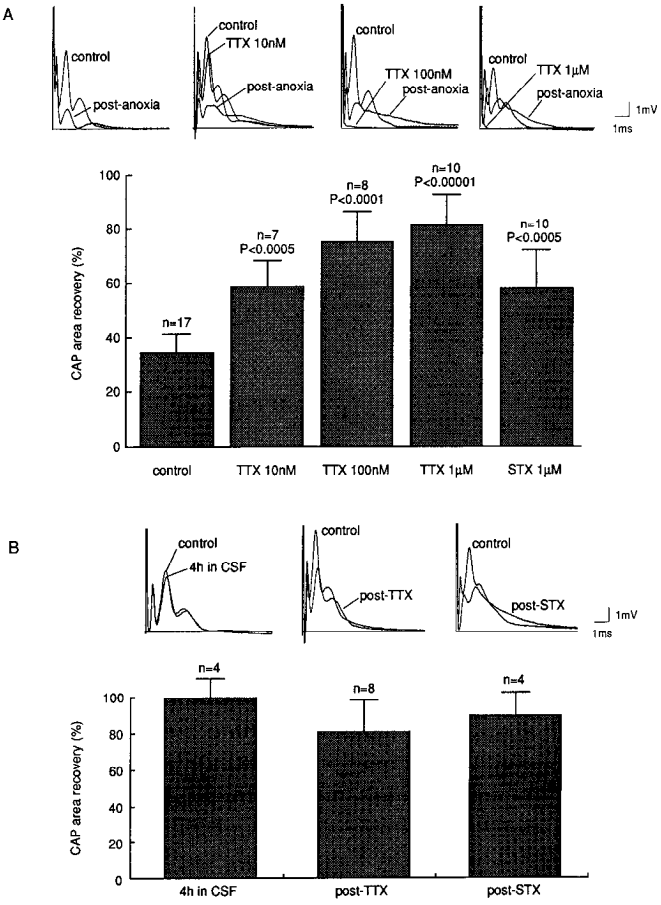

Figure 1. Effect of Na^+ channel blockers on CAP recovery after 60 min of anoxia. *A*, Recovery of CAP area in normal CSF was 33.5% of control after a 60 min anoxic insult. TTX significantly improved recovery in a dose-dependent manner. Note that at 10 nM, TTX had virtually no effect on the area of the preanoxic CAP (*top tracings*) but was significantly protective against anoxic injury. Higher concentrations of TTX abolished the preanoxic CAP. *STX* 1 μM was also markedly protective. The difference in recovery between 1 μM TTX and 1 μM STX was significant ($p < 0.001$). *Top panels* show representative tracings of CAPs before and 1 hr after a 60 min anoxic period in normal CSF, and with exposure to TTX (10 nM to 1 μM). TTX was applied 60 min before the onset of anoxia at 10 and 100 nM, and 20 min before anoxia at 1 μM. TTX was continued until 15 min after reoxygenation when normal CSF was resumed. Up to 3 hr of wash was allowed before postanoxic readings were taken. *B*, Optic nerves were exposed to either 1 μM TTX or STX for 90 min under normoxic conditions and then washed for 3 hr. Neither agent was completely reversible as evidenced by the incomplete recovery of CAP area and persistent alterations in the shape of the waveforms (not significantly different from CAP area after 4 hr in CSF). Nerves incubated for a similar period of time in normal CSF showed no change in shape or CAP area. Therefore, the results shown in *A* likely underestimate the true degree of recovery. *Top panels* show representative CAPs in normal CSF, and 3 hr after a 90 min exposure to 1 μM TTX or STX. *Bars* represent SD in this and all subsequent figures.

Na^+ channel blockade protects against anoxic injury

Na^+ influx during anoxia is responsible for some of the acute injury seen in gray matter (Rothman and Olney, 1986). To test

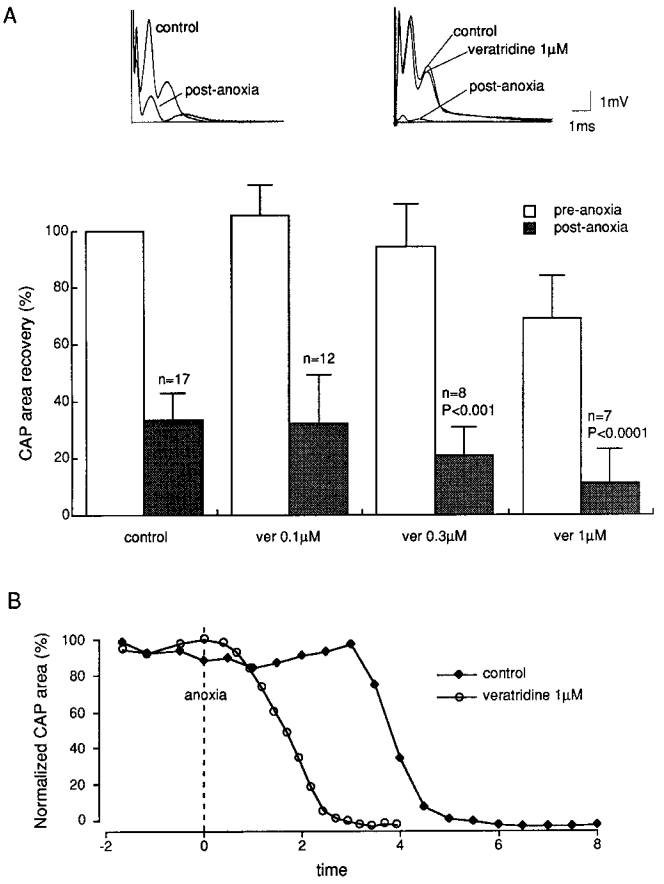

Figure 2. Effect of veratridine on CAP recovery after 60 min of anoxia. *A*, As shown in the bar graph, veratridine had little effect on the pre- or postanoxic CAP at 0.1 μM. At higher concentrations (0.3 and 1 μM), veratridine modestly reduced the preanoxic CAP area in a reversible manner, and significantly increased the degree of anoxic injury. Tracings of CAPs before and 1 hr after 60 min of anoxia in normal CSF and with exposure to 1 μM veratridine. *B*, CAP area diminished more rapidly with the onset of anoxia in the presence of 1 μM veratridine than in normal CSF.

whether voltage-gated Na^+ channels are involved in mediating irreversible anoxic injury in WM, the Na^+ channel blockers TTX and STX were applied at various times before the start of anoxia and continued until 10 min after the end of anoxia; both agents significantly improved postanoxic CAP recovery (Fig. 1*A*). CAP area recovered to 81.5 ± 11% in 1 μM TTX versus 33.5 ± 9.3% in normal CSF. Similar experiments with 1 μM STX also revealed significantly improved postanoxic recovery of CAP area (58.2 ± 14.3%), although not to the same extent as with TTX. Lower concentrations of TTX resulted in progressively less recovery in a dose-dependent fashion.

In order to test whether it was necessary to block electrogenesis with TTX to protect the optic nerve from anoxia, we studied the effects of lower concentrations of TTX on the response of the optic nerve to anoxia. Whereas excitability was completely abolished after 6 min of perfusion with 1 μM TTX, CAP area after 60 min exposure to 10 nM TTX in normal O_2 atmosphere was 107.1 ± 8% of control, although peak latencies in some nerves were prolonged by 10–20%. Such low concentrations of TTX, which had no effect on the control CAP area, were also protective against anoxia (postanoxic CAP area = 56.4 ± 14%).

Control experiments with 1 μM TTX and STX under normoxic conditions revealed that the effects of both agents were not completely reversible in our experimental system, even after 3 hr of wash. Figure 1*B* shows the results of exposing RONs to either TTX or STX at 1 μM for 1 hr, and then washing in normal CSF for 3 hr. Mean CAP area recovered incompletely to 80.8 ± 17% of control at TTX (to a level very near to that seen in anoxia experiments where RONs were exposed to TTX), and to 89.8 ± 13% after STX. Maintaining RONs in normal CSF for the same period of time (4 hr) resulted in a mean CAP area of 99.4 ± 11%, indicating that the incomplete recovery of the CAP following TTX or STX was due to incomplete washout of the blockers and not "rundown" of nerve excitability with prolonged incubation. Observations on the recovery from anoxia after TTX and STX exposure may therefore underestimate the true degree of protection.

If reducing Na^+ channel permeability with TTX or STX is protective during anoxia, increasing Na^+ permeability would be expected to result in more injury. This hypothesis was tested by treating the optic nerves with veratridine, an alkaloid that increases Na^+ permeability (Catterall, 1980). The agent was applied beginning 1 hr before anoxia and continued until 10 min after the end of anoxia. Recovery after a standard 60 min period of anoxia was reduced in the presence of veratridine (Fig. 2). After 1 hr of exposure to 1 μM veratridine, preanoxic CAP area was reduced to 69.0 ± 15% of control, and postanoxic recovery was significantly reduced to 11.0 ± 12% as compared to recovery in normal CSF (33.5 ± 9.3%). The amount of additional injury with veratridine was dose dependent; 0.3 μM veratridine caused a modest increase in injury (21.1 ± 10%), whereas 0.1 μM had little effect (32.0 ± 17%). Control experiments in O_2 showed that the modest depression of the preanoxic CAP magnitude seen with 0.3 and 1 μM veratridine was completely reversible after 1 hr of wash. Veratridine also accelerated the rate of fall of CAP area at the onset of anoxia. At 1 μM, CAP area fell by 50% in about 1.5 min ($n = 2$) versus 3.9 min in normal CSF ($n = 5$; $p < 0.001$) (Fig. 2*B*).

Na^+ influx through Na^+ channels is required for anoxic injury

Because Na^+ channels are known to be permeable to other ions (Hille, 1984), experiments were performed to determine whether influx of Na^+ ions through the Na^+ channel contributes to irreversible anoxic injury, or if the presence of a finite Na^+ permeability (which might admit other ions such as Ca^{2+}) is sufficient. Figure 3*A* shows an experiment where Na^+ in the perfusing solution was replaced with equimolar choline, a cation that does not permeate the Na^+ channel, or with the permeable Li^+ ion. The zero-Na^+ solution was begun 20 min before anoxia and continued until 15 min after the end of anoxia. The rate at which extracellular [Na^+] fell during zero-Na^+ perfusion was estimated by the rate of loss of the CAP (Fig. 3*C*). Recovery of CAP area was significantly improved (88.0 ± 5.4% for choline-substituted solution, and 99.1 ± 20% for Li^+-substituted solution) after a 60 min anoxic challenge in the absence of extracellular Na^+. Perfusion with choline-substituted zero-Na^+ solution for 90 min under normoxic conditions, followed by 60 min in normal CSF, resulted in no diminution of CAP area (102.0 ± 17%; Fig. 3*B*) but subtly altered the shape of the waveform (not shown).

Na^+ influx continues throughout anoxia

The above results suggested that irreversible anoxic injury in WM depends on Na^+ influx through voltage-gated Na^+ channels.

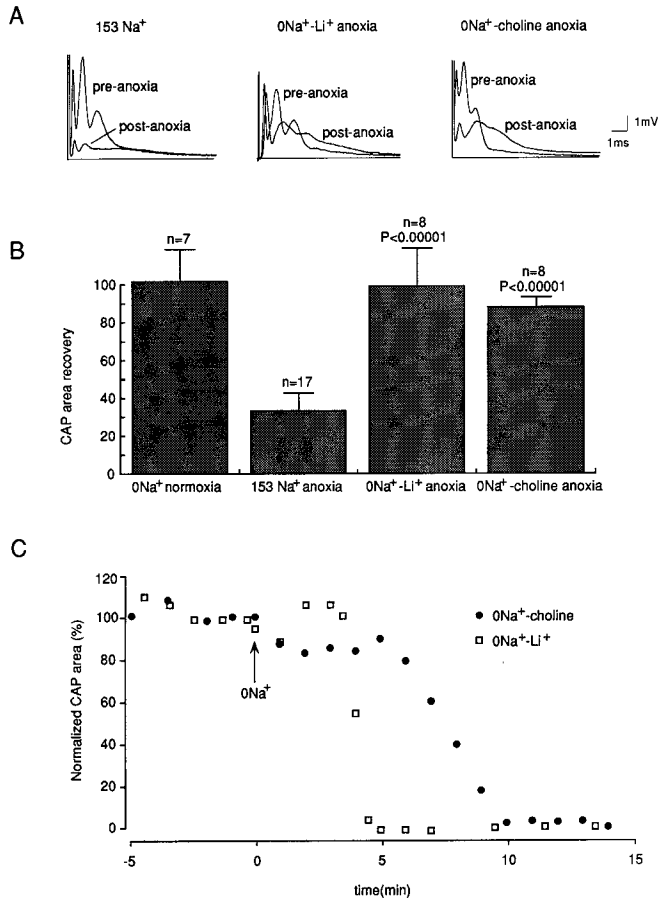

Figure 3. Effect of removing Na^+ from the perfusing solution on CAP recovery after 60 min of anoxia. *A,* Representative CAPs pre- and postanoxia showing the protective effects of zero-Na^+ solution (Li^+ or choline substituted) when applied beginning 20 min before anoxia and continued until 15 min after reoxygenation. Recovery of CAP area after a 60 min anoxic insult was significantly enhanced compared to normal CSF ([Na^+] = 153 mM). *B,* Bar graph quantitatively illustrating the protective effects of zero-Na^+ solutions. Control experiments revealed that exposure of optic nerves to zero-Na^+ solution (choline substituted) under normoxic conditions for 90 min did not cause a reduction in CAP area (measured 1 hr after resuming normal CSF), although the shape of the response was altered. Perfusing the nerves with zero-Na^+ CSF significantly enhanced CAP area recovery in both choline- and Li^+-substituted solutions. Statistical differences were calculated with respect to the postanoxic recovery in normal CSF (*153 Na^+ anoxia*). *C,* Graph of normalized CAP area versus time showing the time course of CAP attenuation with introduction of zero-Na^+ solution into the perfusion chamber. Both solutions completely abolished the evoked response, although Li^+-substituted solution had a more rapid effect.

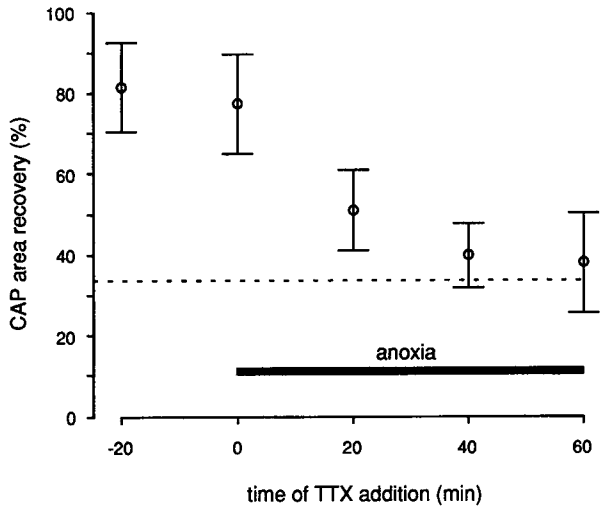

Figure 4. Effects of TTX 1 μM, applied at various times before or after a 60 min anoxic period, on CAP area recovery. Optic nerves were washed for 3 hr in normal CSF after anoxia to remove as much residual TTX as possible, before postanoxic readings were taken. Control experiments showed that TTX was not completely removed even after 3 hr of wash (see Fig. 1). Recovery diminished gradually as the introduction of TTX solution was delayed with respect to the onset of anoxia, suggesting that Na^+ influx begins soon after the onset of anoxia and continues throughout the anoxic period.

Does this damaging Na^+ influx occur rapidly after the onset of anoxia, or more gradually throughout the anoxic period? To answer this question, experiments were performed where TTX (1 μM) was introduced at various times before or after the start of anoxia and continued until 10 min after the end of the anoxic period (Fig. 4). Thus, for example, time $t = -20$ min indicates that TTX was started 20 min before the onset of anoxia, and $t = +20$ min indicates that TTX was introduced 20 min after the start of anoxia. When TTX was started before anoxia (i.e., $t = -20$ min), CAP area recovery was enhanced to 81.5 ± 11%. As the introduction of TTX solution was delayed with respect to the onset of anoxia, progressively less recovery occurred, so that with introduction at 60 min (i.e., at the time of reoxygenation), the degree of recovery (38.1 ± 12%) approached the standard level (33.5 ± 9.3%). Although the precise time course of Na^+ channel blockade with TTX perfusion is unknown, a rough estimate can be obtained from the observation that the CAP was completely abolished about 6 min after exposure to 1 μM TTX. These results suggest that although Na^+ influx probably begins soon after the onset of anoxia, Na^+ movement that contributes to anoxia-induced injury continues throughout the anoxic period.

Reversing the transmembrane Na^+ gradient increases anoxic injury

One way in which changes in [Na^+] can influence anoxic injury is by modulating Ca^{2+} movements across cell membranes by altering the rate and direction of the Na^+–Ca^{2+} exchanger (Stys et al., 1991b). Changes in perfusion [Na^+] will directly alter the transmembrane gradient of Na^+ and indirectly affect it by influencing $[Na^+]_i$. Perfusing the optic nerves with zero-Na^+ solution before anoxia will tend to deplete both the extracellular and the intracellular space of Na^+. Introducing zero-Na^+ after the start of anoxia, when presumably $[Na^+]_i$ has begun to rise above its resting level, will produce a reversed transmembrane Na^+ gradient. If the Na^+–Ca^{2+} exchanger plays a role in mediating Ca^{2+} influx during anoxia, altering the perfusing [Na^+] in this way might influence the degree of anoxic injury. Figure 5 shows an experiment where zero-Na^+ solution (either choline or Li^+ substituted) was begun at various times before or after the start of anoxia. As the introduction of zero-Na^+ CSF was delayed with respect to the onset of anoxia, from $t = -20$ to $t = +20$ min (i.e., 20 min before anoxia and 20 min after the start of anoxia, respectively), progressively more injury occurred. Interestingly, when the choline-substituted zero-Na^+ CSF was introduced well into the anoxic period, at $t = +20$ or $+40$ min, significantly more injury occurred (CAP recovery at $t = +20$ was 16.1 ± 8% and at $t = +40$ was 13.7 ± 6%) than with

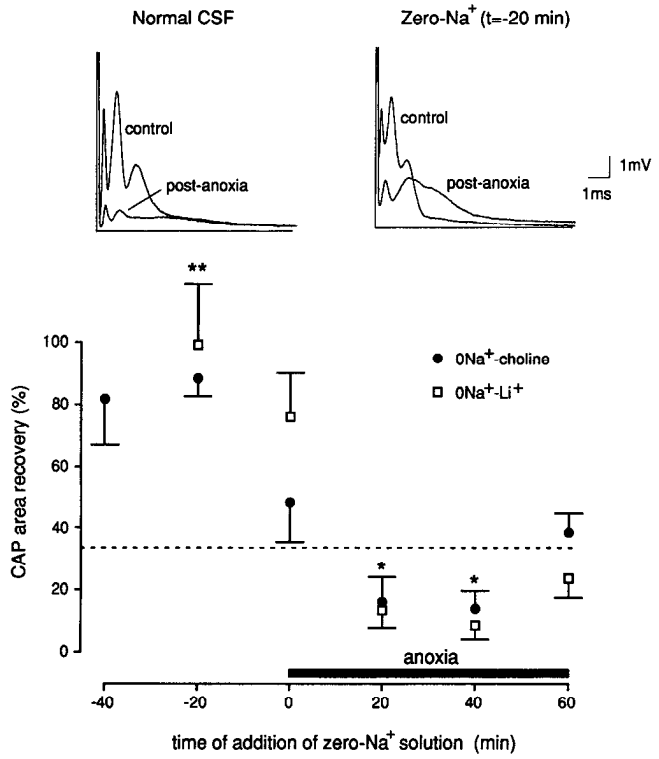

Figure 5. Effects of zero-Na^+ solution, applied at various times before or after anoxia, on CAP area recovery; graph showing CAP area recovery after 60 min of anoxia plotted against the time (in minutes) with respect to the onset of anoxia, when zero-Na^+ CSF (choline or Li^+ substituted) was applied. Normal CSF was resumed 15 min after the end of anoxia, and postanoxic readings were taken 1 hr after reoxygenation. Recovery was computed as the ratio of CAP area postanoxia to area preanoxia in CSF containing normal [Na^+] (153 mM). Recovery was greatly enhanced when zero-Na^+ CSF was started *before* anoxia. As the introduction of zero-Na^+ CSF was delayed with respect to the onset of anoxia, progressively less recovery was seen. When the zero-Na^+ solution was applied *after* the onset of anoxia (t = +20 or +40 min), significantly more injury occurred than with normal CSF ([Na^+] = 153 mM) maintained throughout the anoxic period (*broken line*). When zero-Na^+ CSF was briefly applied for 15 min at t = 60 min, the degree of injury approached the control value. *Top panels* show representative CAPs pre- and postanoxia in normal CSF and in choline-substituted zero-Na^+ CSF that was started 20 min before anoxia. Statistical differences were calculated with respect to the standard recovery in normal CSF (*, p < 0.0001; **, p < 0.00001).

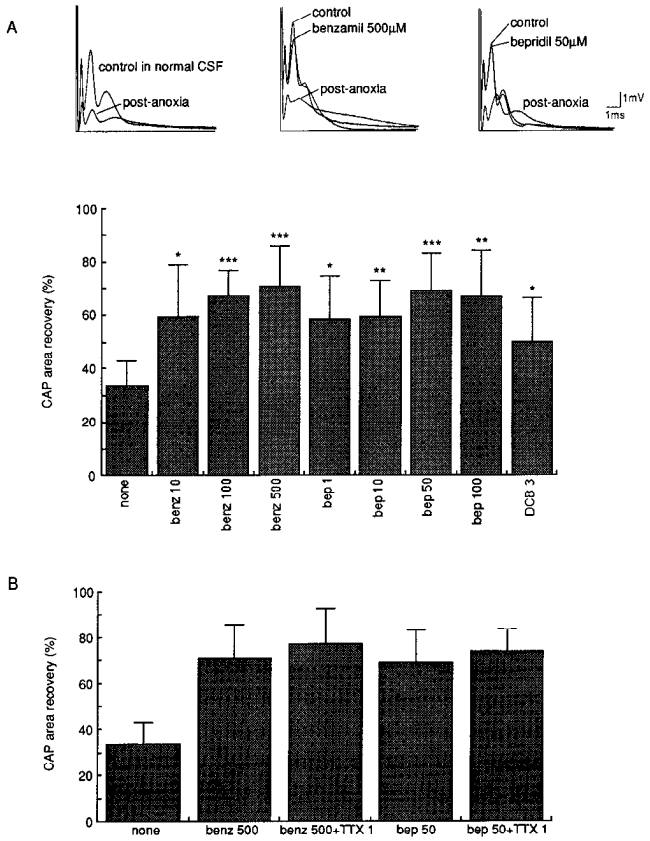

Figure 6. Effects of Na^+–Ca^+ exchange inhibitors on CAP recovery after anoxia. *A*, Benzamil (*benz*), bepridil (*bep*), or *DCB* were applied 60 min before the start of anoxia and continued until 15 min after reoxygenation, when normal CSF was resumed. Postanoxic activity was measured 1 hr after reoxygenation. Benzamil significantly improved recovery of CAP area in a dose-dependent manner over a concentration range of 10–500 μM. Bepridil (1–100 μM) and DCB (3 μM) also significantly improved recovery. Benzamil had no effect on the preanoxic response at 10 and 100 μM, and reduced CAP area modestly to 61.1 ± 20% of control at 500 μM in a reversible manner. Bepridil and DCB had no effect on preanoxic responses at the concentrations shown. *, p < 0.01; **, p < 0.001; ***, p < 0.0001; n = 7–10 for each drug and concentration. *B*, Addition of 1 μM TTX to solutions containing maximally protective concentrations of bepridil or benzamil further improved recovery by 5–10% (not statistically significant; see Results). n = 4–10 for each category.

normal [Na^+] maintained throughout (i.e., 33.5 ± 9.3%). The degree of recovery returned to near normal (38.4 ± 6%) with the introduction of zero-Na^+ for a brief 15 min period at the time of reoxygenation (t = +60 min). Li^+-substituted zero-Na^+ CSF displayed a similar profile.

Na^+–Ca^{2+} exchange blockers protect against anoxic injury

We have previously shown that irreversible anoxic injury in the RON is dependent on extracellular Ca^{2+}, so that the omission of Ca^{2+} from the perfusing solution during 60 min of anoxia results in 100% recovery of CAP area (Stys et al., 1990a). Together with the present results, this suggests that both Na^+ and Ca^{2+} are critical for the production of anoxic injury and that movements of Na^+ and Ca^{2+} are closely interdependent. A major mechanism for coupling the fluxes of Na^+ and Ca^{2+} is via the Na^+–Ca^{2+} exchanger, and a preliminary study suggests that blocking this mechanism is protective in WM (Stys et al., 1991b).

Figure 6*A* shows the effects of three blockers of Na^+–Ca^{2+} exchange on the degree of recovery from anoxia; all three agents block the Na^+–Ca^{2+} exchanger by competitively inhibiting the Na^+ binding site. Bepridil (Kaczorowski et al., 1989), applied 60 min before until 15 min after 60 min of anoxia, was protective at concentrations of 10–100 μM, with optimal protection seen at 50 μM (69.0 ± 14%). Bepridil had no effect on preanoxic CAPs at these concentrations. Two derivatives of amiloride, benzamil and DCB (Kleyman and Cragoe, 1988), also displayed protective effects. Benzamil improved recovery in a dose-dependent manner; at 500 μM it produced 71.0 ± 15% recovery. At this concentration, benzamil reduced the preanoxic CAP area to 61.1 ± 20% of control. However, protective effects were also seen at lower concentrations of benzamil (100 μM) where the preanoxic CAP area was not reduced. DCB (3 μM) also improved recovery (49.9 ± 17%), although to a lesser degree (Fig. 6*A*). No change in the control CAP was seen at this concentration.

Higher concentrations could not be reliably tested due to the limited solubility of this compound.

Because these Na^+–Ca^{2+} exchange blockers did not fully protect the optic nerve from anoxia, we studied the combined effect of TTX plus exchange blockers to determine whether Na^+ conductance played an additive role in mediating anoxic injury. The addition of 1 μM TTX to either bepridil or benzamil further enhanced recovery by 5–10% to 73.7 ± 10% and 77.2 ± 16%, respectively, close to the recovery seen with TTX alone (Fig. 6*B*). It should again be noted that results with TTX probably underestimate the true recovery due to the incomplete washout of this blocker.

Discussion

In the mammalian CNS, both gray and WM regions suffer irreversible injury when subjected to extended periods of anoxia/ischemia, and this injury appears to depend on Ca^{2+} in both tissues. The route of the damaging Ca^{2+} entry into cells in WM during anoxia is not fully understood, but unlike gray matter (Choi, 1990), this is not mediated by voltage-sensitive Ca^{2+} channels (Stys et al., 1990b; Waxman et al., 1991) or by the excitotoxin receptors activated by glutamate and aspartate (Ransom et al., 1990b).

Na^+ channels play a central role in anoxic injury

Could other ion channels, known to be present in myelinated CNS axons, admit Ca^{2+} into the intracellular compartment under anoxic conditions in WM? Blocking Na^+ channels with TTX and STX had a marked protective effect, the magnitude of which is likely to be underestimated in these experiments due to persistent toxin binding. Even at 10 nM TTX, a concentration that had little effect on the control CAP, a significant protective effect was observed. Thus, there exists a "therapeutic window" where action potential electrogenesis need not be abolished in order to protect WM from anoxic injury.

If blocking Na^+ conductance improves recovery from anoxia, it would be expected that increasing Na^+ conductance would worsen outcome. Exposing nerves to veratridine, an alkaloid that increases Na^+ permeability by shifting Na^+ channel activation to more negative potentials and inhibiting inactivation (Catterall, 1980), increased the degree of irreversible anoxic injury in a dose-dependent manner. These results and previous observations (Stys et al., 1990a) indicate that both extracellular Ca^{2+} and a finite Na^+ permeability must be present to cause irreversible anoxic injury. Moreover, the degree of injury is proportional to both the level of Ca^{2+} exposure (Stys et al., 1990a) and magnitude of Na^+ permeability.

Na^+ gradient collapses gradually during anoxia

The CAP is lost within minutes of the onset of anoxia (Stys et al., 1990a) paralleled by a rise in extracellular $[K^+]$ to about 15 mM (Walz et al., 1986), indicating that the optic nerve is heavily dependent on oxidative energy metabolism for the maintenance of ionic gradients. The results shown in Figure 4 suggest that damaging Na^+ influx through Na^+ channels continues throughout the anoxic period. If Na^+ equilibrated completely across membranes within minutes of the onset of anoxia, introducing TTX at +20 min should not have further improved recovery.

Na^+ channels are not perfectly selective and are known to possess a finite permeability to other ions, including Ca^{2+}. The Na^+:Ca^{2+} permeability ratio has been estimated to be as low as 10:1 (Hille, 1984), although higher permeability ratios have been suggested (Lederer et al., 1991). Given that extracellular Ca^{2+} is necessary for anoxic injury in the optic nerve, a critical question is whether the injury related to the finite Na^+ permeability that exists during anoxia is primarily due to Na^+ or Ca^{2+} influx through these channels. Our results indicate that anoxia-induced Ca^{2+} entry does not occur through Na^+ channels, because perfusion with zero-Na^+ solution markedly improved recovery but would not prevent Ca^{2+} influx through this channel (Fig. 3). This result strongly suggests that influx of Na^+, and not Ca^{2+}, through voltage-gated Na^+ channels is an important step in the sequence of events leading to anoxic injury.

Does a persistent Na^+ conductance mediate Na^+ influx?

Under conditions of massive energy failure and membrane depolarization, as suggested by a rapid attenuation of the CAP and rise in $[K^+]_o$, it might be expected that Na^+ channels would be inactivated. Our results suggest, however, that there exists a finite, persistent Na^+ conductance at levels of membrane depolarization attained during anoxia. In some preparations, Na^+ conductance does not inactivate completely even with prolonged membrane depolarization (Stafstrom et al., 1982, 1985; Gilly and Brismar, 1989) and may even start to increase at more positive membrane potentials (Chandler and Meves, 1970; Bezanilla and Armstrong, 1977). In the optic nerve, it is not yet clear if the persistent Na^+ conductance represents incomplete inactivation of a homogeneous population of Na^+ channels, or a subpopulation of noninactivating channels. An intriguing possibility is that the conductance of a subpopulation of the latter channels might be dominant under pathological conditions such as anoxic depolarization. This raises the question whether these channels have distinct pharmacological sensitivity, allowing blockade of the noninactivating component while sparing normally inactivating fast Na^+ channels (those channels responsible for action potential electrogenesis), thus leaving normal axonal conduction relatively unaffected. There is evidence that slowly inactivating or persistent Na^+ channels can be preferentially blocked by certain local anesthetics (Stafstrom et al., 1985; Schneider and Dubois, 1986).

The degree of protection from anoxia by TTX was dose dependent, even over a concentration range where measurable excitability was already completely abolished (Fig. 1*A*). Typically, Na^+ channels are blocked by TTX with high affinity (half-maximal inhibition, <10 nM) (Hille, 1968); at 100 nM TTX, evoked activity was already completely abolished. No difference in the degree of protection against anoxia would be expected between 100 nM and 1 μM TTX, since beyond 100 nM TTX normal Na^+ channels should be fully blocked. This raises the interesting possibility of a subpopulation of Na^+ channels that are partially resistant to TTX (Ransom and Holz, 1977; Roy and Narahashi, 1990), and with a differential sensitivity to TTX and STX. The difference in protection seen with TTX and STX, and the relatively high concentrations of TTX required for optimal protection suggest that the Na^+ channels most relevant to anoxic injury do not follow the pharmacological profile expected for normally inactivating Na^+ channels, and could represent a distinct subpopulation.

Magnitude and direction of Na^+ gradient modulates anoxic injury

Both extracellular Ca^{2+} (Stys et al., 1990a) and Na^+ are required to produce irreversible anoxic injury in WM. One way in which fluxes of these ions could be coupled is via the Na^+–Ca^{2+} ex-

A

$$E_{NaCa} = \frac{n E_{Na} - 2 E_{Ca}}{n - 2} \quad \text{eq.1}$$

$$[Ca]_i = [Ca]_o \, Exp\left[\frac{F\,Vm\,(n-2)}{R\,T}\right]\left[\frac{[Na]_i}{[Na]_o}\right]^n \quad \text{eq.2}$$

B

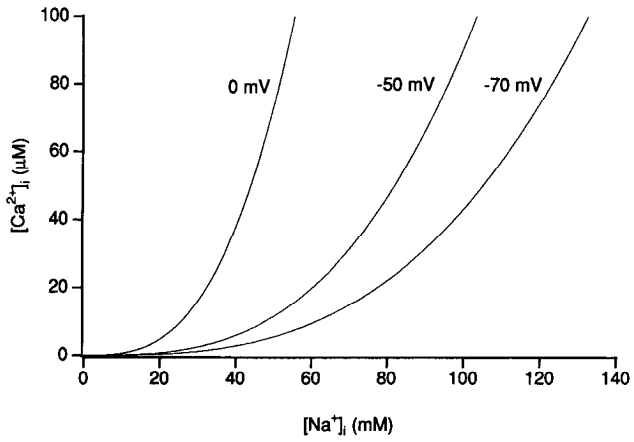

Figure 7. A, Equations describing the behavior of the Na^+–Ca^{2+} exchanger (Blaustein and Santiago, 1977; Sheu and Fozzard, 1982). E_{NaCa}, E_{Na}, and E_{Ca} are reversal potentials of the exchanger, Na^+, and Ca^{2+} ions, respectively, and n represents the exchanger stoichiometry. The exchanger will operate in the direction required to bring its reversal potential closer to membrane potential, V_m. At thermodynamic equilibrium, $E_{NaCa} = V_m$. Substituting V_m into *eq. 1*, expanding the expressions for E_{Na} and E_{Ca}, and rearranging, we obtain an expression (*eq. 2*) for the intracellular $[Ca^{2+}]$ that would be maintained by the Na^+-Ca^{2+} exchanger. *F*, Faraday's constant; *R*, gas constant; *T*, absolute temperature. *B*, Graphical representation of *eq. 2* at three values of membrane potential assuming a stoichiometry of 3 Na^+:1 Ca^{2+}. With increasing $[Na^+]_i$ and/or membrane depolarization, both of which occur during anoxia, the Na^+–Ca^{2+} exchanger will tend to increase $[Ca^{2+}]_i$.

changer. This membrane protein, found in all excitable and many nonexcitable cells, functions to maintain cellular Ca^{2+} homeostasis by extruding Ca^{2+} in exchange for Na^+ (Baker et al., 1969; Allen et al., 1989). The stoichiometry is generally thought to be 3 Na^+:1 Ca^{2+} (Blaustein and Santiago, 1977; Rasgado-Flores and Blaustein, 1987), resulting in a transport process that is electrogenic (one net inward charge per Ca^{2+} ion extruded); consequently, both the rate and direction of Ca^{2+} transport will be influenced by membrane potential. Under normal conditions of membrane polarization and $[Na^+]_i$, the exchanger uses the energy stored in the transmembrane electrochemical gradient of Na^+ to transport Ca^{2+} out of the cell. However, with membrane depolarization and/or increasing $[Na^+]_i$, the Na^+–Ca^{2+} exchanger can operate in reverse, transporting Ca^{2+} into the intracellular compartment in exchange for Na^+ (Baker et al., 1969; Cervetto et al., 1989). Thus, reverse Na^+–Ca^{2+} exchange is a potential route of Ca^{2+} influx during anoxia, a time when membranes depolarize and $[Na^+]_i$ increases as a result of ATP depletion.

The results illustrated in Figure 5 are consistent with the hypothesis that a significant portion of the damaging Ca^{2+} influx that occurs during anoxia is carried by reverse Na^+–Ca^{2+} exchange. When zero-Na^+ solution is introduced before anoxia (Fig. 5, $t = -20$ min), $[Na^+]_i$ will tend to decrease from its resting level of about 25 mM (Ballanyi et al., 1987; Erecińska and Silver, 1989; Erecińska et al., 1991). To operate in the reverse direction, that is, transport of Ca^{2+} into the cell in exchange for Na^+, the exchanger requires intracellular Na^+ [$[Na^+]_i \approx 25$ mM for half-maximal activation in squid giant axon (Requena et al., 1989)]. Reducing $[Na^+]_i$ by perfusing with zero-Na^+ CSF prior to and during anoxia would inhibit reverse operation of the exchanger, reduce Ca^{2+} influx, and protect the tissue from injury.

Interestingly, delaying the introduction of zero-Na^+ until well into the anoxic period results in more injury than seen with normal $[Na^+]$ maintained throughout (Fig. 5, broken line). In normal $[Na^+]_o$ (i.e., 153 mM), $[Na^+]_i$ will increase during anoxia, to above its resting level. Introduction of zero-Na^+ solution after the start of anoxia is deleterious ($t = +20$ and $+40$ min, Fig. 5), because now a large *reverse* Na^+ gradient ($[Na^+]_i > [Na^+]_o$) forces the exchanger to transport more Ca^{2+} into the cell. Under these circumstances, we postulate that even more Ca^{2+} enters the intracellular space than during a normal anoxic period (with $[Na^+] = 153$ mM), where at worst the Na^+ gradient would collapse completely ($[Na^+]_i = [Na^+]_o$) but would never be reversed.

Perfusion with zero-Na^+ solution during normoxia would also result in a reversed Na^+ gradient (e.g., prior to anoxia in Fig. 5, $t = -40$ and -20 min). Nevertheless, in control experiments no decrease in CAP area is observed (though the shape of the CAP is irreversibly altered) following a 90 min exposure to zero-Na^+ CSF in an O_2 atmosphere (Fig. 3*B*). We hypothesize that this does not cause damage to the optic nerve because in a normal O_2 atmosphere the supply of ATP is not limited, and the Ca^{2+}-ATPase would be capable of extruding most of the excess Ca^{2+} admitted through reverse Na^+–Ca^{2+} exchange. Moreover, switching to zero-Na^+ under normoxic conditions (with $[Na^+]_i \approx 25$ mM) would not create a reverse gradient of the same magnitude as when zero-Na^+ is applied during anoxia, when $[Na^+]_i$ has already risen above its resting value.

Although it is clear that introducing zero-Na^+ CSF will, at least transiently, reverse the Na^+ gradient and cause reverse operation of the Na^+–Ca^{2+} exchanger, under realistic conditions of anoxia/ischemia, ion gradients would never reverse but merely collapse. Why then might the exchanger operate to raise $[Ca^{2+}]_i$ without artificial manipulation of ion gradients? Figure 7 shows equations and a graphical representation of the behavior of the Na^+–Ca^{2+} exchanger at thermodynamic equilibrium (Blaustein and Santiago, 1977; Sheu and Fozzard, 1982). Intracellular $[Ca^{2+}]$ at equilibrium is an exponential function of membrane potential (increasing with membrane depolarization) and a function of the cube of the ratio of intracellular to extracellular $[Na^+]$ (assuming a stoichiometry of 3 Na^+:1 Ca^{2+}). Thus, membrane depolarization and/or an increase in $[Na^+]_i$ will result in an increased steady-state $[Ca^{2+}]_i$. This increased $[Ca^{2+}]_i$ could, in theory, occur either by true reversal of the exchanger, or by attenuation of forward exchange, unmasking the effects of an alternate Ca^{2+} influx pathway. If the latter were true, pharmacological inhibition of the exchanger (see below) should result in more anoxic injury, since any beneficial effects of exchanger-mediated Ca^{2+} extrusion would be further attenuated by the

inhibitors. Our results, which demonstrate significantly reduced anoxic injury in nerves treated with Na^+–Ca^{2+} exchanger blockers, are clearly inconsistent with this argument and support the notion that Ca^{2+} is admitted via reversal of the Na^+–Ca^{2+} exchanger. Moreover, the exchanger can operate in reverse without a *reversal* of the Na^+ gradient. If the starting $[Ca^{2+}]_i$ is at or below the exchanger equilibrium, a *diminution* of the Na^+ gradient will be sufficient to cause the exchanger to operate in reverse as it attempts to reach its new, more elevated steady state $[Ca^{2+}]_i$. It should be noted that a combination of membrane depolarization and an increase in $[Na^+]_i$ (both of which occur during anoxia) will have a synergistic effect, raising $[Ca^{2+}]_i$ to high levels if the exchanger is allowed to operate.

Interestingly, inhibition of Na^+–Ca^{2+} exchange activity has been reported to exacerbate glutamate neurotoxicity in cultured neurons (Mattson et al., 1989; Andreeva et al., 1991), suggesting that in model systems containing neuronal cell bodies and synapses, the exchanger functions to extrude rather than to admit Ca^{2+} under pathological conditions (it should be noted that the effects of extracellular K^+-induced depolarization on exchanger function cannot be studied in these systems). The surface-to-volume ratio in neuronal cell bodies is much smaller than in the small (mean diameter, ≈ 0.75 μm; Foster et al., 1982) axons that are present in the RON. When challenged, the rise in $[Na^+]_i$ in a neuron can thus be expected to be much less pronounced than in a small diameter axon, and therefore a favorable exchanger equilibrium (i.e., tending toward a lower $[Ca^{2+}]_i$) would be maintained for a longer period of time. In contrast, a small axon having a limited intracellular volume, a high density of Na^+ channels at the node of Ranvier, and possibly restricted ionic diffusion (see below) may suffer a rapid and pronounced rise in $[Na^+]_i$ with the Na^+–Ca^{2+} exchanger quickly recruited to operate in reverse. If surface-to-volume considerations significantly influence the behavior of the exchanger under pathological conditions, it is possible that this transporter may play opposite roles in different regions of the same neuron, transporting Ca^{2+} out of the soma, while admitting damaging quantities of Ca^{2+} into the axon and dendritic processes.

Pharmacological blockers of Na^+–Ca^{2+} exchange are protective

Further evidence implicating the Na^+–Ca^{2+} exchanger was obtained by demonstrating that direct pharmacological inhibition of this transporter offers significant protection against anoxic injury (Fig. 6*A*). These agents also block voltage-gated Ca^{2+} channels (Galizzi et al., 1986; Kleyman and Cragoe, 1988; Garcia et al., 1990); we have previously shown, however, that such channels do not play a role in WM anoxic injury (Stys et al., 1990b). Benzamil also possesses inhibitory effects on Na^+ channels (Kleyman and Cragoe, 1988); at a concentration of 500 μM in our experiments, benzamil reduced CAP area to 61.1% of control. Nevertheless, protective effects were seen at lower concentrations of 10 and 100 μM, where little if any change in the control CAP was evident, indicating minimal effects of benzamil on Na^+ channels. Similarly, DCB protected the optic nerves at a concentration where the control CAP was unchanged. The reduced efficacy of DCB, compared to the other two inhibitors, may be due to its limited solubility, preventing reliable experiments at higher concentrations. Although the absolute contribution of the Na^+–Ca^{2+} exchanger in isolation must await more specific inhibitors, we conclude that a large part of Ca^{2+} influx during anoxia is mediated by this system. A similar mechanism has been proposed in ischemic myocardium (Renlund et al., 1984), and in reperfusion of heart following exposure to Ca^{2+}-free solution (Chapman and Tunstall, 1987).

Addition of 1 μM TTX to optimal concentrations of both bepridil or benzamil further improved recovery from anoxia by 5–10% (not statistically significant), approximately to the same degree as with TTX alone (Fig. 6*B*). This suggests either that bepridil or benzamil alone failed to block reverse exchange completely (requiring the additional effect of TTX to eliminate the rise in $[Na^+]_i$) or that a small portion of Ca^{2+} influx occurs directly through Na^+ channels. The near-complete recovery seen with Li^+-substituted zero-Na^+ solution introduced 20 min before anoxia suggests that little Ca^{2+} was admitted directly through the Na^+ channels, however.

Protective effects are not due to energy sparing

Although our results are consistent with Ca^{2+} influx via reverse operation of the Na^+–Ca^{2+} exchanger during anoxia in WM, it could be argued that blocking Na^+ channels or eliminating Na^+ from the perfusate is protective because it spares energy reserves. According to this argument, reducing the demand for ATP by preventing or slowing the collapse of the Na^+ gradient would allow residual ATP (generated perhaps from anaerobic metabolism) to fuel other Ca^{2+}-extruding mechanisms, such as the Ca^{2+}-ATPase, sufficiently to prevent significant cellular injury. The data shown in Figure 5 strongly argue against a simple "energy-sparing" effect. If perfusion with zero-Na^+ was protective because of energy sparing, the relationship between recovery and duration of exposure to high $[Na^+]_o$ should be monotonic; that is, the longer the anoxic nerve is exposed to high $[Na^+]_o$, the greater should be the depletion of energy reserves, and the more injury should result. Figure 5 clearly shows this not to be the case; intermediate exposure times ($t = +20$ or $+40$) are more injurious than maintaining the nerves in normal $[Na^+]_o$ solution for the full anoxic period. This observation is inconsistent with an energy-sparing action of zero-Na^+ solution but is readily explained if anoxic injury is primarily caused by Ca^{2+} influx via reverse operation of the Na^+–Ca^{2+} exchanger.

Although it is possible that maneuvers that reduce the consumption of ATP by Na^+-K^+ ATPase may play a protective role, our results suggest that this role is minor and that Ca^{2+} influx via reverse Na^+–Ca^{2+} exchange is a major mechanism of anoxic injury in CNS WM. A diagram of the sequence of events leading to anoxia-induced Ca^{2+} accumulation in WM is shown in Figure 8. According to this hypothetical sequence, anoxic injury begins with ATP depletion and failure of Na^+-K^+ ATPase, resulting in a breakdown of ionic gradients (1). Leakage of Na^+ through Na^+ channels (possibly a subpopulation of noninactivating channels; see above) causes $[Na^+]_i$ to rise (2), resulting in reverse operation of the Na^+–Ca^{2+} exchanger (3). Ca^{2+} admitted through reverse exchange accumulates in the intracellular space, rises to toxic levels, and produces irreversible injury, probably through activation of Ca^{2+}-dependent systems such as calpains, lipases, protein kinase C, and so on (Siesjö and Wieloch, 1985; Nicotera et al., 1986; Orrenius et al., 1988). Due to the serial nature of Na^+ and Ca^{2+} influx in the proposed cascade, blocking either Na^+ channels and/or the Na^+–Ca^{2+} exchanger should protect CNS myelinated axons against anoxic injury. This prediction is supported by the present results.

The very high density of Na^+ channels at the nodes of Ranvier (Waxman and Ritchie, 1985) may render this region especially vulnerable to anoxic injury. Moreover, ultrastructural studies

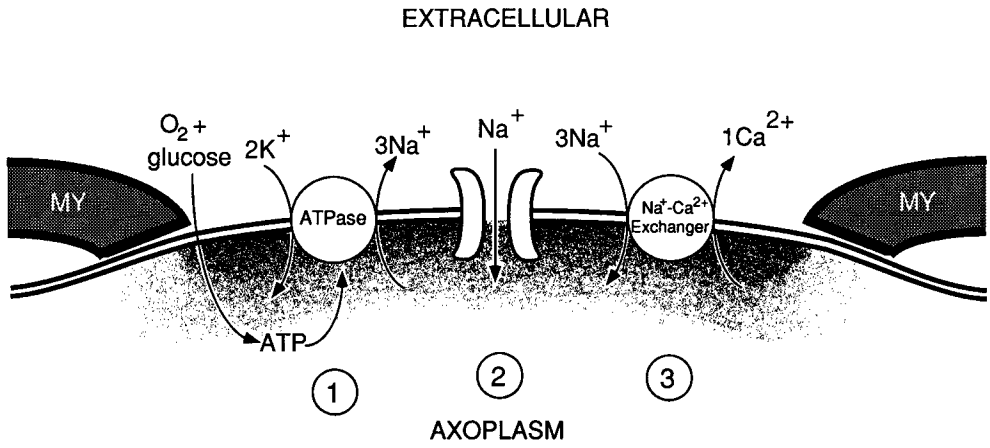

Figure 8. Hypothetical sequence of events leading to anoxia-induced Ca^{2+} accumulation in myelinated (*my*) CNS axons. Under conditions of anoxia/ischemia, reserves of ATP are rapidly depleted, leading to failure of the Na^+-K^+ ATPase and subsequent collapse of ionic gradients (*1*). Na^+ ions enter the axoplasmic space through incompletely inactivated Na^+ channels (*2*). This influx of Na^+ ions leads to a rise in $[Na^+]_i$, which may be exaggerated by restricted diffusion of ions under the nodal membrane. Both membrane depolarization and an increase in intracellular $[Na^+]$ cause reverse operation of the Na^+–Ca^{2+} exchanger, which admits damaging quantities of Ca^{2+} into the cell.

of myelinated axons reveal a dense undercoating subjacent to the nodal membrane (Waxman et al., 1972). It has been suggested that this specialized region of axoplasm may restrict diffusion of ions and focally intensify disturbances in ion concentrations (Bergman, 1970). Localized increases in $[Na^+]_i$ would further drive reverse Na^+–Ca^{2+} exchange, admitting even more Ca^{2+} into a restricted intracellular compartment. Thus, it is possible that the specialized architecture of CNS myelinated axons may serve to enhance their susceptibility to anoxic injury.

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