Endogenous GABA Attenuates CNS White Matter Dysfunction Following Anoxia

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We studied the effects of GABA on anoxia-induced injury in CNS white matter using optic nerves exposed to 60 min of anoxia. Injury was assessed by recording pre- and postanoxic compound action potentials (CAPs). GABA (1 μ M) significantly increased postanoxic CAP recovery when applied 60 min prior to anoxia. This effect was bicuculline (100 μ M) insensitive, mimicked by baclofen (1 μ M), blocked by GABA-B antagonists, and not mimicked by selective GABA-A agonists. GABA therefore acted at GABA-B receptors. High concentrations of GABA and baclofen did not influence recovery, possibly indicating GABA-B receptor desensitization at high agonist concentrations.

Pertussis toxin (PTX) treatment reduced postanoxic CAP recovery in the presence of 1 μ M GABA to control levels, indicating the recruitment of a G-protein-linked intracellular pathway. Protein kinase C (PKC) activation with 12-myristate 13-acetate (PMA) mimicked the effects of GABA. Inhibition of PKC with 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7) or staurosporine reduced postanoxic recovery in the presence of GABA to lower levels than under control conditions, confirming the involvement of PKC in the protective effect of GABA and indicating that this GABA-B receptor/G-protein/PKC protective pathway might be active under control conditions. This was confirmed by the observation that GABA-B receptor blockade, in the absence of exogenous GABA, significantly reduced postanoxia recovery. Thus, activation of the protective mechanism under control conditions is due to endogenous GABA release. Increasing the level of endogenous extracellular GABA by blocking GABA uptake with 1 mM nipecotic acid also protected against anoxia. We propose a model where release of GABA in white matter helps to limit nerve fiber injury during anoxia via recruitment of a G-protein/PKC pathway with subsequent phosphorylation of an unknown target protein.

[Key words: axon, GABA, GABA-B receptors, glia, G-protein, protein kinase C, nerve fiber]

The nerve fiber tracts of the CNS contain GABA (Balcom et al., 1975; Van De Hayden et al., 1979; Anden et al., 1987), GABA receptors (Bowerly et al., 1987; Chu et al., 1990), and a

high-affinity GABA uptake mechanism (Bull and Blomqvist, 1991). Although the function of GABA in mature CNS white matter is unknown, significant increases in extracellular GABA concentration occur during white matter ischemia (Shimada et al., 1993), and in post-mortem isolated white matter (Anden et al., 1987). Total brain GABA also increases significantly during anoxia (Nilsson and Lutz, 1991). These observations are consistent with a role for GABA in CNS white matter during pathological conditions.

White matter contains no presynaptic terminals and release of neurotransmitters within white matter cannot be synaptic in nature. GABA, however, is found within both the axons and glia which make up white matter (Bull and Blomqvist, 1991; Davanger et al., 1991; Lake, 1992). Conditions are ideal for the release of this GABA during anoxia and ischemia, which favor reverse operation of sodium-dependent GABA uptake (Carvalho et al., 1991; Ransom et al., 1992; Atwell et al., 1993). To examine the possibility that GABA may be involved in the pathophysiology of white matter anoxia, we have employed a model CNS white matter system, the isolated rat optic nerve. Our results indicate that GABA plays a protective role in white matter during anoxia, and we have identified the receptor subtype and intracellular pathway by which GABA acts. Most importantly, it appears that anoxia stimulates release of sufficient endogenous GABA to activate this mechanism *in situ*, thus providing an intrinsic protective system.

Materials and Methods

The *in vitro* rat optic nerve preparation (Davis and Ransom, 1987; Stys et al., 1992) was used to study the role of GABA in anoxia-induced injury of CNS white matter. Female Long-Evans rats 50–70 d old were anesthetized with an 80% CO₂/20% O₂ gas mixture prior to decapitation. The optic nerves were dissected free, placed in a modified interface perfusion chamber (Medical Systems Corp., Greenvale, NY), and incubated for 60–90 min before measurements were initiated. The tissue was maintained at 37°C, oxygenated in a 95% O₂/5% CO₂ atmosphere, and perfused with artificial cerebrospinal fluid (aCSF) with a pH of 7.45. The aCSF contained (in mM): Na⁺, 153; K⁺, 3; Mg²⁺, 2; Ca²⁺, 2; Cl⁻, 133; HCO₃²⁻, 26; H₂PO₄²⁻, 2; dextrose, 10.

The recording arrangement was similar to that reported previously (see Stys et al., 1990). In brief, compound action potentials (CAPs) were evoked by a 125% supramaximal stimulus applied via a suction electrode to the distal nerve end. CAPs were recorded via a second suction electrode from the proximal nerve end employing a method which compensates for instability inherent in the suction electrode technique (Stys et al., 1991). Anoxia was induced by changing to a 5% CO₂/95% N₂ atmosphere, which was maintained for a standard 60 min period. CAP recovery after anoxia was determined by measuring CAP area after 60 min of reoxygenation, a time after which no further recovery occurs (Stys et al., 1992). Changes in the area under the CAP, calculated by computer, were assumed to correspond to changes in the number of

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Figure 1. GABA had no consistent action upon the normoxic CAP. *A*, A plot of CAP area (●, arbitrary units) and the latency between stimulus onset and the peak of the first component of the CAP (+). There are no significant changes in these parameters during 10 min perfusion with either 1 μ M or 1 mM GABA (indicated by the bars). Twelve and 22 mM KCl, however, did influence CAP area and latency, providing a positive control. Similar results were found in five optic nerves. *B* and *C*, Superimposed CAPs recorded prior to (CONTROL) and subsequent to 60 min of perfusion with 1 μ M GABA (*B*) or 1 mM GABA (*C*). The small changes in CAP shape and area were not of a consistent nature.

axons capable of conducting action potentials along the optic nerve (Cummins et al., 1979; Stys et al., 1991; Wijesinghe et al., 1991). Results are presented as means $\pm$ SEM. Each experimental protocol employed was accompanied by an independent set of control experiments, typically interspersed between test experiments, and significance was normally tested for by *t* test. In some cases variations of the same protocol were employed and compared to the same control experiments, in which case significance was tested for by ANOVA (post hoc test).

Experimental protocol. To study the effects of a solution change upon normoxic nerve conduction, comparison was made between the control CAP recorded after 60 min of aCSF perfusion and the CAP 60 min after the initiation of perfusion with test solution. The effect of a test solution on CAP recovery from the standard 60 min anoxic insult was measured by comparing the CAP immediately before anoxia to the CAP recorded 60 min after the end of anoxia. Only one test solution, at one concentration, was examined in any single optic nerve.

Results

Sakatani et al. (1992) reported that the application of GABA to the neonatal rat optic nerve results in axonal depolarization, an effect which is much reduced in rats older than 30 d of age. In the current experiments nerves were taken from 50–70-d-old rats and no significant effect of GABA upon the shape or the area under the normoxic CAP was observed (Fig. 1). The slight changes in the CAP that were observed in the presence of GABA (1 μ M and 1 mM for 60 min) were not of a consistent nature or significant in magnitude (Fig. 1*B,C*; $n = 4$).

Low concentrations of GABA protect against anoxic injury via actions at GABA-B receptors

Low concentrations of GABA significantly reduced the development of irreversible dysfunction after a 60 min period of anoxia in the rat optic nerve. Typical pre- and postanoxic CAPs in Figure 2*A* illustrate the large reduction in CAP area that results from the standard 60 min anoxic period. Perfusion with

Figure 2. One micromolar GABA acted at a site other than the GABA-A receptor to protect rat optic nerves from anoxia. *A–D*, Superimposed pre- and postanoxic CAPs taken under control conditions (*A*) and in the presence of various agents applied prior to anoxia (*B–D*). The larger of each CAP pair is the preanoxic recording and the smaller CAP is the postanoxic recovery. *A*, Under control conditions the mean postanoxic CAP recovery after anoxia was 36.5 $\pm$ 2.9% ($n = 10$). *B*, GABA at 1 μ M significantly increased recovery to a mean of 55.7 $\pm$ 2.5% ($n = 16$, $p < 0.002$). *C*, The selective GABA-A antagonist bicuculline (100 μ M), added 10 min prior to and continually with GABA, did not attenuate the protective effect of 1 μ M GABA. *D*, The selective GABA-A agonist THIP had no protective effect against anoxia. *E*, Data summary showing that mean recovery in 1 μ M GABA + bicuculline was 56.1 $\pm$ 4.4% ($n = 12$), recovery in 1 μ M THIP was 38.5 $\pm$ 2.9% ($n = 8$), and recovery in 1 mM muscimol (also a selective GABA-A agonist) was 38.6 $\pm$ 4.8% ($n = 7$).

1 μ M GABA significantly increased recovery (Fig. 2*B,E*). Mean CAP recovery under control conditions was 36.5 $\pm$ 2.9% ($n = 10$); mean CAP recovery in the presence of 1 μ M GABA was 55.7 $\pm$ 2.5% ($n = 16$; $p < 0.002$).

Previously reported effects of GABA upon nerve fibers have conformed to the pharmacological profile of the GABA-A receptor, being bicuculline sensitive and mimicked by GABA-A agonists (Simmonds, 1983; Liske and Morris, 1989; Sakatani et al., 1991a, 1992). GABA's protective action against anoxic injury was not blocked by 100 μ M bicuculline, applied 10 min before and continually with the application of GABA (Fig. 2*C,E*). Mean CAP recovery in GABA + bicuculline was 56.1 $\pm$ 4.4% compared to 55.7 $\pm$ 2.5% in GABA alone. Furthermore, the selective GABA-A agonist 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-ol hydrochloride (THIP) did not effect the degree of recovery following anoxia. Figure 2, *D* and *E*, shows that postanoxic CAP recovery was not increased by 1 μ M THIP, while perfusion with 1 mM THIP was also without effect ($n = 4$). One millimolar muscimol, another selective GABA-A agonist, also did not effect postanoxic CAP recovery. These data are summarized in Figure 2*E*, confirming that GABA-A receptor activation did not act to reduce the extent of anoxic injury.

The protective effect of 1 μ M GABA was completely abolished by perfusion with 500 μ M phaclofen, a selective GABA-B antagonist. Recovery was slightly lower during perfusion with GABA and phaclofen than under control conditions (Fig. 3*A,C*). A perfusion protocol similar to that used with bicuculline was employed, with phaclofen perfusion initiated 10 min prior to GABA perfusion. The selective GABA-B agonist baclofen (1 μ M) provided protection from anoxia similar to that found with 1 μ M GABA, with postanoxic recovery of 53.1 $\pm$ 3.1% (Fig. 3*B,C*).

Figure 3. GABA acted via GABA-B receptors. *A*, The selective GABA-B antagonist phaclofen (500 μ M) blocked the protective effect of 1 μ M GABA. *B*, The selective GABA-B agonist baclofen (1 μ M) mimicked the protective effect of GABA. *C*, Data summary showing that mean postanoxic recovery in the presence of 1 μ M GABA + 500 μ M phaclofen was similar to that found under control conditions ($31.3 \pm 4.7\%$, $n = 6$ compared to $36.5 \pm 2.9\%$, $n = 12$, respectively), while 1 μ M baclofen significantly increased recovery ($53.1 \pm 3.1\%$, $n = 12$; $p < 0.005$).

High concentrations of GABA and baclofen do not protect against anoxic injury

Optic nerves exposed to increasing GABA concentrations from 10 nM to 1 μ M showed progressively greater protection against anoxic injury, as shown in the dose-response relationship in Figure 4C. However, GABA concentrations higher than 1 μ M were progressively less effective. Specimen records illustrating the lack of effect of 1 mM GABA upon postanoxic CAP recovery are shown in Figure 4A (compare to Fig. 2). That higher concentrations of GABA were less effective at protecting against anoxia may indicate receptor desensitization at high agonist concentrations, over these long application times.

Lee et al. (1993) have reported that GABA may be neurotoxic to spinal cord axons, a toxicity which they suggest is mediated by GABA-A receptors. GABA-A receptor-mediated neurotoxicity at high GABA concentrations could explain the reduced protection found in Figure 4C. However, additional experiments indicated that this was not the case. Figure 4D shows the dose-response relationship for the protective effect of baclofen, and demonstrates that the relationship was similar to that found for GABA, having a negative correlation at higher baclofen concentrations. It appeared, therefore, that high GABA-B receptor occupancy was enough to eliminate the protective effects of GABA-B receptor activation. This kind of relationship is typical of receptor desensitization. To confirm that a deleterious effect of GABA-A receptor activation did not underlie the ineffectiveness of high concentrations of GABA, these receptors were blocked by 100 μ M bicuculline applied 10 min prior to 1 mM GABA. This failed to unmask any recovery produced by isolated GABA-B receptor activation (mean recovery = $35 \pm 4.5\%$, $n = 4$; Fig. 4B).

Figure 4. GABA-B receptors appeared to desensitize at high agonist concentrations. *A*, GABA at 1 mM had no protective effect against anoxia (mean CAP recovery = $34.3 \pm 4.1\%$, $n = 8$). *B*, Block of GABA-A receptors with 100 mM bicuculline did not unmask any protective effect of GABA-B receptor activation with 1 mM GABA. *C*, Dose-response curve for various GABA concentrations used with the standard anoxia protocol. Increasing GABA concentrations from 10 nM to 1 μ M produced increasing degrees of recovery. However, concentrations above 1 μ M had increasingly less effect. Each point is a mean of 6–16 experiments. *D*, Dose-response curve for various baclofen concentrations, showing a similar negative correlation between agonist concentration and protective effect at high concentrations.

Temporal features of GABA-mediated protection

The above experiments were performed with long GABA perfusion times in an attempt to examine the steady-state effect of GABA application. To examine the evolution of GABA-mediated protection, CAP recovery after anoxia was studied in optic nerves perfused with 1 μ M GABA beginning 10, 30, and 60 min before the onset of anoxia (Fig. 5A–C). Some protection was provided by GABA perfusion initiated 30 min prior to anoxia, but 10 min of perfusion had little effect upon postanoxic recovery (Fig. 5D). This requirement for pre-exposure to GABA was probably not due to slow penetration of the neurotransmitter into the nerve. GABA is a relatively small molecule (MW = 103) compared to TTX (MW = 319), and yet TTX has a maximal protective effect against anoxic injury if perfusion is initiated as late as 0 min prior to anoxia (Stys et al., 1992). In

Figure 5. Time course of the development of GABA-mediated protection. One micromolar GABA perfusion was initiated at various times prior to the onset of anoxia. *A*, GABA applied 10 min prior to anoxia produced no protective effect. *B*, Thirty minutes of GABA perfusion prior to anoxia had a significant protective effect. *C*, Typical protection provided by 60 min of GABA perfusion prior to anoxia. *D*, Summary of the data showing mean % CAP recovery from anoxia as a function of the period of preanoxic GABA perfusion. The curve was fitted by eye. *E*, Pretreatment with 1 μ M GABA for 60 min followed by 30 min of washout with aCSF prior to anoxia showed a similar level of recovery after anoxia ($41.6 \pm 2.8\%$, $n = 8$) as untreated control nerves ($36.5 \pm 3.5\%$, $n = 12$).

addition, potassium diffuses very rapidly into our preparation (Fig. 1).

There are a variety of possible mechanisms by which GABA might protect the optic nerve from anoxic injury (see Discussion). To help distinguish whether GABA was acting via a reversible or an irreversible mechanism, we determined whether GABA pretreatment alone was sufficient to provide protection from anoxia. We perfused the rat optic nerve with 1 μ M GABA for 60 min and then washed GABA from the optic nerve with aCSF for 30 min prior to a 60 min anoxic period. No significant protection from anoxic injury was afforded by this period of pretreatment (Fig. 5E).

Endogenous GABA release limits anoxic injury

As experiments described above indicated that activation of GABA-B receptors protected optic nerve axons from anoxic injury, we examined whether these receptors were activated by GABA *in situ* during anoxia. Endogenous GABA release can be enhanced by nipecotic acid (Sakatani et al., 1992), which inhibits GABA uptake and stimulates GABA release (Brown and Marsh, 1978). If releasable GABA stores are present in the adult optic nerve, nipecotic acid would be expected to cause a rise in ex-

Figure 6. GABA released from endogenous stores protected against anoxia. *A*, Typical control recovery from the standard anoxic insult. *B*, Ninety minutes of perfusion prior to anoxia with the GABA reuptake blocker nipecotic acid provided protection from anoxia (mean recovery = $52.6 \pm 6.4\%$, $n = 8$; $p < 0.02$). *C*, Relationship between % CAP recovery and the period of nipecotic acid preperfusion. Preperfusion for 60 and 90 min periods provided significant protection ($p < 0.05$ and $p < 0.02$, respectively). Each point represents a mean of 6–14 experiments.

tracellular GABA concentration and provide protection against anoxic injury. One millimolar nipecotic acid (applied 90 min prior to the onset of anoxia and continually over the anoxic period) increased postanoxic CAP recovery compared to the control level (Fig. 6A,B). The mean % CAP recovery for various periods of nipecotic acid perfusion are shown in Figure 6C, confirming the protective effect of nipecotic acid when applied for 90 min ($p < 0.02$).

An additional set of experiments was performed to examine whether endogenous GABA was present in high enough concentration in the rat optic nerve to moderate nerve fiber injury during anoxia when GABA uptake has not been interrupted. Optic nerves were perfused with the GABA-B receptor antagonists phaclofen or 5-aminovaleric acid (AVA) (Schwarz et al., 1988) to block any GABA-B receptor activation resulting from endogenous GABA release. Figure 3 shows that 500 μ M phaclofen blocked the protective action of 1 μ M GABA, and Figure 7 shows that GABA-B receptor blockade by phaclofen in the absence of exogenous GABA reduced the recovery found after anoxia, from a mean of $39.5 \pm 3.8\%$ down to $23.6 \pm 3.8\%$ ($p < 0.005$; Fig. 7A–C). Presumably the same low level of recovery was not also found in Figure 3 due to the presence of 1 μ M GABA in the bath, which will partially displace phaclofen, a competitive antagonist of low potency. AVA also reduced recovery to below control value (mean CAP recovery = $26.5 \pm 3.5\%$, $p < 0.03$; Fig. 7C).

To test whether the GABA-B receptor antagonists might be neurotoxic to rat optic nerve axons, we employed a protocol similar to the above experiments but maintained the nerves in a normoxic environment. Although changes in CAP shape could follow prolonged GABA-B receptor blockade, neither 500 μ M

Figure 7. Endogenous GABA acted at GABA-B receptors to protect against anoxia. *A*, Typical control recovery from anoxia. *B*, Block of endogenous GABA-B receptor activation with 500 μ M phaclofen reduced recovery following anoxia from that found under control conditions. *C*, Summary of the data showing control CAP recovery of $39.5 \pm 3.1\%$ ($n = 13$) compared to $23.6 \pm 3.8\%$ ($n = 11$) recovery in 500 μ M phaclofen ($P < 0.005$) and recovery of $26.5 \pm 3.5\%$ ($n = 8$) in 1 mM AVA ($p < 0.03$), another GABA-B receptor antagonist. *D* and *E*, Superimposed CAPs recorded prior to and subsequent to 120 min periods of perfusion with either 500 μ M phaclofen (*D*) or 1 mM AVA (*E*), in a normoxic environment. While changes in CAP shape were seen, there was no significant change in CAP area following GABA-B receptor block, compared to control. Mean CAP area after 120 min of phaclofen perfusion was $97.2 \pm 11.9\%$ of the initial area ($n = 6$) and after 120 min AVA CAP area was $86.5 \pm 8.4\%$ ($n = 6$); these changes in CAP area were not significantly different from that found after 120 min of perfusion with aCSF ($78.4 \pm 9.5\%$ of the initial CAP area, $n = 6$).

phaclofen nor 1 mM AVA caused significant changes in CAP area (Fig. 7*D–F*). Mean CAP area was $97.2 \pm 11.9\%$ ($n = 6$) of the initial area after 120 min of phaclofen perfusion, and $86.5 \pm 8.4\%$ ($n = 6$) of initial area after 120 min of 1 mM AVA perfusion (compared to $78.4 \pm 9.5\%$ after 120 min of perfusion with aCSF). These control experiments indicated that endogenous GABA indeed acted to protect the optic nerve during anoxia.

GABA-B receptors recruit a G-protein/PKC-mediated intracellular pathway

The involvement of a G-protein in GABA-mediated protection from anoxia was assessed using pertussis toxin (PTX), which is known to irreversibly block the biological activity of some G-proteins (Ui, 1984). Rat optic nerves were perfused for 120 min with 200 ng PTX prior to a 60 min period of 1 μ M GABA perfusion and subsequent anoxia. Following PTX pretreatment, there was significantly less recovery than in control experiments performed using the same protocol but in the absence of PTX. Mean CAP recovery following PTX pretreatment was $38.7 \pm 3.9\%$ compared to $53.6 \pm 4.0\%$ when PTX was omitted ($P < 0.02$; Fig. 8*A,D*). CAPs recorded following 120 min of PTX treatment and 60 min perfusion with aCSF were of normal shape and area compared to CAPs perfused in aCSF alone. This suggests that PTX was not toxic to the optic nerves and was not acting in a nonspecific fashion to reduce CAP recovery during anoxia.

Cyclic AMP is an important second messenger which regulates a number of neuronal conductances. We examined whether cAMP was involved in GABA-mediated protection from anoxia by employing the membrane-permeable cAMP analogue di-

Figure 8. Pertussis toxin (PTX) blocked and PKC activation mimicked the protective action of GABA. *A* and *D*, Mean CAP recovery in 1 μ M GABA, applied 60 min prior to anoxia and following an initial 120 min period of perfusion with aCSF, was $53.6 \pm 4.0\%$ ($n = 7$). When aCSF was substituted with aCSF containing 200 ng/ml PTX, the protection produced by 1 μ M GABA was reduced (mean CAP recovery = $38.7 \pm 3.9\%$, $n = 8$). *B*, CAP recovery in dibutyl cAMP, a membrane-permeable form of cAMP, was not different from control CAP recovery ($42.6 \pm 4.9\%$, $n = 8$ compared to $41.6 \pm 3.3\%$, $n = 8$). *C* and *E*, Perfusion with 1 μ M PMA (an activator of PKC) increased recovery from anoxia from $34.3 \pm 3.6\%$ ($n = 13$) to $53.9 \pm 4.7\%$ ($n = 12$, $p < 0.003$). One micromolar of PMA applied simultaneously with 1 μ M GABA occluded the protection normally provided by GABA (mean CAP recovery = $34.5 \pm 1.4\%$, $n = 8$).

butyl cAMP. One millimolar dibutyl cAMP was applied to the nerves 60 min prior to the onset of anoxia and mean CAP recovery was not significantly altered from control recovery ($42.6 \pm 4.9\%$ compared to $41.6 \pm 3.3\%$, respectively; $p > 0.8$; Fig. 8*B*). It appeared, therefore, that an intracellular pathway involving elevation of cAMP was not an important element in GABA-mediated protection from anoxia.

Neuronal voltage-gated ion channels and ion exchangers in various cell types can be modulated by PKC (Hidaka et al., 1984; Vasilets et al., 1990; Mené et al., 1991; West et al., 1991; Dascal and Lotan, 1991; Gomez et al., 1993), and PKC is known to be activated by GABA-B receptors (see Borman, 1989). To test for the involvement of a PKC-linked intracellular pathway, we employed 12-myristate 13-acetate (PMA), a selective PKC activator. Perfusion with 1 μ M PMA for 60 min prior to anoxia resulted in a significant increase in the degree of postanoxic recovery ($53.9 \pm 4.7\%$ compared to $34.1 \pm 4.7\%$, $p < 0.01$; Fig. 8*C,E*). If GABA does recruit a PKC-mediated intracellular pathway during anoxia, addition of PMA with 1 μ M GABA should affect the degree of protection provided by the GABA. We found that coapplication of PMA with 1 μ M GABA occluded

Figure 9. Block of PKC with H7 removed the protective effect of GABA. *A*, Pre- and postanoxic CAPs recorded in the presence of 1 μ M GABA. *B*, Recordings from a similar experiment but in the presence of 20 μ M H7, a PKC inhibitor (applied 10 min prior to GABA). *C*, Data summary showing that inhibition of PKC (20 μ M H7) removed the protective effect of GABA. Recovery in the absence of H7 was $47.7 \pm 3.7\%$ ($n = 7$) compared to $24.4 \pm 2.7\%$ ($n = 11$) in H7 ($p < 0.0001$). Note that recovery in the presence of H7 was lower than that normally found under control conditions. *D*, Dose-response relationship for H7 inhibition of GABA-mediated protection ($n = 7$ to 11) demonstrating that H7 was effective over a concentration range consistent with selective inhibition of PKC.

the protection normally produced by GABA (mean CAP recovery in 1 μ M GABA + 1 μ M PMA = $34.5 \pm 1.4\%$, $n = 8$; Fig. 8C). These results indicate that any additional recruitment of the PKC-mediated pathway beyond that produced by 1 μ M GABA alone resulted in a reduction in the degree of postanoxic CAP recovery, as predicted by Figure 4.

To further test the role of PKC, we employed the widely used PKC antagonist 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7). Specimen records of representative pre- and postanoxic CAPs recorded in the presence of 1 μ M GABA $\pm$ 20 μ M H7 (added 10 min prior to the addition of GABA) illustrate that H7 abolished the protective effect of 1 μ M GABA (Fig. 9*A,B*). CAP recovery with 1 μ M GABA alone was $47.8 \pm 3.7\%$ and with both H7 and GABA present was reduced to $24.4 \pm 2.7\%$ ($p < 0.0001$; Fig. 9C).

Inhibition of PKC with H7 occurs over a 2–50 μ M concentration range (Hidaka et al., 1984; Kawamoto and Hidaka, 1984; Nakadate et al., 1990; Chakrabarty et al., 1992), with a reported K_i of 6 μ M (Hidaka et al., 1984). The dose-response relationship for H7 inhibition of GABA protection from anoxic injury (Fig. 9D) showed that H7 inhibited GABA protection over a similar concentration range. However, H7 has inhibitory effects upon cAMP-dependent protein kinases as well upon PKC. We therefore employed the more specific and potent PKC inhibitor staurosporine (Tamaoki et al., 1986) to confirm the role of PKC during GABA-mediated protection. Control, 1 μ M GABA, and 1 μ M GABA + 50 nM staurosporine experiments are shown in Figure 10*A–E*, showing mean recovery of $33.2 \pm 3.9\%$, $49.2 \pm 4.9\%$, and $24.6 \pm 3.4\%$, respectively. Recovery in the presence of GABA + staurosporine was significantly lower than in the presence of GABA alone ($p = 0.001$). Indeed, recovery in the presence of GABA during PKC inhibition with either H7 or staurosporine was lower than under control conditions and corresponded to that observed during GABA-B receptor blockade.

Figure 10. PKC inhibition with staurosporine removed the protective effect of GABA. *A*, CAP recovery from anoxia in the absence of GABA. *B*, Increased CAP recovery found in the presence of GABA. *C*, Perfusion of 50 nM staurosporine, initiated 10 min prior to perfusion with 1 μ M GABA, reduced CAP recovery to below control levels. *D*, Staurosporine alone reduced the level of postanoxic CAP recovery, compared to control recovery. *E*, Data summary showing the abolition of GABA protection by staurosporine: mean CAP recovery under control conditions was $33.2 \pm 3.9\%$ ($n = 11$), $49.2 \pm 4.9\%$ ($n = 8$) in the presence of GABA, and $24.6 \pm 3.4\%$ ($n = 7$) in GABA + staurosporine. CAP recovery in staurosporine + GABA was significantly different from in GABA alone ($p < 0.001$). The effect of staurosporine could not be accounted for by a nonspecific toxic effect. Coadministration of staurosporine and 500 μ M phaclofen produced a level of CAP recovery similar to that found in the presence of staurosporine alone (mean CAP recovery = $27.4 \pm 2.5\%$, $n = 7$; $P < 0.05$). Staurosporine, administered alone, reduced the level of CAP recovery from that found under control conditions (mean CAP recovery = $26.8 \pm 2.6\%$, compared to control recovery of $39.8 \pm 2.9\%$, $p < 0.05$). This suggests that an endogenous, PKC-dependent process helped to protect rat optic nerves against anoxia.

rosoporine (Tamaoki et al., 1986) to confirm the role of PKC during GABA-mediated protection. Control, 1 μ M GABA, and 1 μ M GABA + 50 nM staurosporine experiments are shown in Figure 10*A–E*, showing mean recovery of $33.2 \pm 3.9\%$, $49.2 \pm 4.9\%$, and $24.6 \pm 3.4\%$, respectively. Recovery in the presence of GABA + staurosporine was significantly lower than in the presence of GABA alone ($p = 0.001$). Indeed, recovery in the presence of GABA during PKC inhibition with either H7 or staurosporine was lower than under control conditions and corresponded to that observed during GABA-B receptor blockade.

The low recovery found with staurosporine cannot be accounted for by a toxic effect of the drug during anoxia. Thus, coapplication of 50 μ M staurosporine with 500 μ M phaclofen, where GABA-B receptor-mediated protection will be inhibited by phaclofen and any toxic effect of staurosporine would be expected to result in a lower level of CAP recovery compared to that found in phaclofen alone, produced CAP recovery similar to the application of either staurosporine alone or phaclofen alone ($27.4 \pm 2.5\%$, $n = 7$; Fig. 10E; $23.6 \pm 3.5\%$, Fig. 7, respectively). To assess whether endogenous GABA protected white matter during anoxia via recruitment of the PKC-mediated pathway that underlies the protective effect of exogenous GABA, we perfused optic nerves with staurosporine alone. Mean CAP recovery in 50 nM staurosporine was $26.8 \pm 2.6\%$, which

was significantly lower than the control recovery of $39.8 \pm 2.9\%$ ($p < 0.05$; Fig. 10D,E).

PKC activation does not influence normoxic nerve conduction in rat optic nerve axons

PKC activation is known to inhibit voltage-gated sodium channels (Hidaka et al., 1984; Dascal and Lotan, 1991; West et al., 1991; Gomez et al., 1993). Since voltage-gated sodium channels are responsible for the generation and conduction of action potentials in axons, PKC activation may influence nerve conduction (Rang and Ritchie, 1988; Meiri and Gross, 1989). However, the effect of PKC activation upon the optic nerve CAP area and peak latency was found to be negligible (Fig. 11A–E). Similar results were found in six other optic nerves. Data from 12 optic nerves from a separate series of experiments were pooled in Figure 11F, which shows the mean CAP area following initiation of 60 min perfusion with $1 \mu\text{M}$ PMA. No change in CAP area was found, indicating that in the rat optic nerve PKC activation did not influence nerve conduction.

Discussion

Our results support a novel functional role for the ubiquitous inhibitory neurotransmitter GABA in CNS white matter. GABA released from endogenous stores acted on GABA-B receptors to significantly improve CAP recovery in the optic nerve following anoxia. The evidence also implies that GABA's protective effect was mediated via a G-protein/PKC intracellular pathway. These findings were unexpected for many reasons. Prior studies indicate that GABA can influence peripheral axons (Brown and Marsh, 1978; Arenson and Nistri, 1983; Morris et al., 1983a,b; Liske and Morris, 1989) and neonatal CNS axons (Sakatani et al., 1992; Lee et al., 1993), but actions upon mature myelinated CNS axons are either small or nonexistent (Simmonds, 1983; Sakatani et al., 1991a, 1992). Observed GABA effects on axons have thus far always been mediated by GABA-A receptors; that is, they have depended on chloride ions, have been blocked by bicuculline, and have been activated by selective GABA-A agonists such as THIP and muscimol. The effect of GABA on white matter anoxic injury reported here was robust and mediated by GABA-B receptors. Given the low concentration of GABA that was necessary for protection against anoxic injury, there is reason to think that GABA might act in the same protective manner *in vivo*. These observations, therefore, suggest the possibility that enhancement of brain GABA levels may improve neurologic outcome following anoxic insults such as strokes, nearly a quarter of which are located solely in the white matter of the brain (Fisher, 1982; Bamford et al., 1987).

GABA has previously been implicated in protection from anoxia in a number of anoxia-resistant species, such as freshwater turtles and crucian carp (Hitzig et al., 1985; Lutz et al., 1985; Nilsson et al., 1990; Nilsson and Lutz, 1991; Lutz, 1992). In these species, both total brain GABA concentration (Hitzig et al., 1985; Lutz et al., 1985) and extracellular GABA concentration (Nisson and Lutz, 1991) rise significantly during anoxia. It has been suggested that in these species GABA release inhibits neuronal activity to limit energy use (Lutz, 1992), but the current data suggest a different and more complex action of GABA in the anoxic mammalian brain.

We have found that low concentrations of GABA protect rat optic nerve axons against anoxic injury via a sequence of steps, as shown in Figure 12. Development of anoxic damage in white matter is dependent upon the presence of extracellular sodium

Figure 11. PKC activation does not significantly effect the normoxic CAP in the rat optic nerve. A–C, CAPs recorded from the same optic nerve prior to, during, and following a 35 min period of $1 \mu\text{M}$ PMA treatment. D, A plot of CAP area showing the complete data set from which A–C were drawn (as indicated by the appropriate letters). E, A plot similar to D showing the conduction latency (time between stimulation and the first and second peaks of the CAP) for the same nerve as in D. F, Mean change in CAP area in 12 nerves following the initiation of $1 \mu\text{M}$ PMA perfusion (different experiments from A–E). There is no significant effect of PMA upon any of the conduction parameters.

and calcium ions (Stys et al., 1990, 1992). Sodium ions enter the intracellular compartment during anoxia through “persistent” or noninactivating sodium channels (Stys et al., 1992, 1993) following the membrane depolarization which results from the efflux and accumulation of potassium ions (Ransom et al., 1992; Ransom and Philben, 1993). This degrades the normal transmembrane sodium ion gradient, promoting calcium ion entry via reverse operation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger leading to calcium-mediated injury (Waxman et al., 1992, 1993) with concomitant loss of CAP recovery (Stys et al., 1990, 1992).

The pharmacology of GABA protection indicated that GABA acted at the GABA-B receptor. Thus, GABA's action was insensitive to bicuculline and was not mimicked by selective GABA-A agonists, while being sensitive to GABA-B antagonists and mimicked by the selective GABA-B agonist baclofen. Blocking GABA uptake with nipecotic acid, which presumably increases the concentration of endogenous GABA in the extracellular space (Sakatani et al., 1992), acted to increase post-

Figure 12. A model for the protective effect of GABA during anoxia in rat optic nerve. During anoxia, K^+ efflux and Na^+ influx lead to reverse operation of the Na^+/Ca^{2+} exchanger and Ca^{2+} influx. Anoxia also induces release of GABA from a site endogenous to the nerve ("GABA store"). The resulting increase in extracellular GABA concentration triggers the activation of GABA-B receptors and initiates an intracellular pathway involving a G-protein/PCK cascade. The optic nerve axons are made more resistant to anoxia as a result. The target of PKC phosphorylation is unknown but could be the Na^+/Ca^{2+} exchanger, phosphorylation and subsequent downregulation of which would limit Ca^{2+} influx (see text).

anoxic recovery. In addition, blocking the GABA-B receptor in the absence of exogenous GABA reduced the degree of recovery seen following anoxia. These results lead us to conclude that (1) GABA-B receptor activation protected optic nerve axons from anoxic injury, (2) mature optic nerves contained stores of GABA capable of release, and (3) optic nerve GABA stores are capable of raising extracellular GABA concentration to a level which protected optic nerve axons from anoxic injury. This intrinsic, GABA-mediated cascade may constitute an autoprotective mechanism directed against the development of irreversible dysfunction.

The mechanism underlying the protective effect of GABA is only partially understood. GABA-B receptor activation was apparently linked to the recruitment of an intracellular second messenger pathway involving a PTX-sensitive G-protein. Protection was removed by H7 over a concentration range where this compound inhibits PKC, and by the more specific PKC inhibitor staurosporine. In addition, activation of PKC with PMA mimicked the effects of GABA, while coadministration of PMA with GABA occluded the protection normally provided by GABA. Thus we can conclude that GABA protected white matter from anoxia by activating a GABA-B receptor/G-protein/PCK cascade which presumably phosphorylated a target protein to increase resistance to anoxia. Phosphorylation of the target protein was apparently reversible as removal of GABA 30 min prior to anoxia failed to produce any increase in resistance to anoxia.

PKC activation does not influence CAP conduction in the rat optic nerve

PKC activation results in rapid modification of voltage-gated ion channels in neurons (Hidaka et al., 1984; Dascal and Lotan, 1991; Gomez et al., 1993). Such changes can have significant consequences for action potential conduction, as demonstrated in peripheral nerves (Rang and Ritchie, 1988; Meiri and Gross, 1989). We found no significant change in the rat optic nerve CAP following activation of PKC. It is possible that axonal sodium channels were fully phosphorylated in the isolated rat optic nerve.

GABA in CNS white matter

GABA is widely distributed throughout the CNS, being present not only in gray matter but also in white matter (Balcom et al., 1975; Van De Hayden et al., 1979; Anden et al., 1987; Lake, 1992; Ochi et al., 1993; Shimada et al., 1993). Both GABA-A and GABA-B receptors are also found in white matter (Bowery et al., 1987; Chu et al., 1990), although GABA and its receptors occur in lower concentrations in white matter than in gray matter. However, the smaller amount of excitable membrane in white matter may be an important factor in determining the functional significance of differences in receptor density between brain regions. Thus, only approximately 0.5% of the length of optic nerve fibers is excitable (Butt and Ransom, 1989), compared to a much larger fraction of the neuronal somatic, dendritic, and preterminal axon membranes which are present in gray matter. Even if the functional density of GABA receptors was similar in excitable membranes of gray and white matter, a smaller total number of white matter GABA receptors would be expected.

GABA stores in mature CNS white matter are probably localized in glial cells. White matter astrocytes possess the capability to take up extracellular GABA (Siemers et al., 1982) and to accumulate GABA when degradation enzymes are inhibited (Bull and Blomqvist, 1991), and GABA-positive glia have been demonstrated in mature optic nerve (Lake, 1992; but see also Ochi et al., 1993). In addition, GABA-positive axons have been identified in a number of sites in the CNS, including the optic nerve (Mosinger et al., 1986; Koontz et al., 1989; Radian et al., 1990; Todd and Sullivan, 1990; Davanger et al., 1991), suggesting that axons may represent another possible source of GABA in white matter.

The protective effect of GABA increased over a 10 nM–1 μ M concentration range. Concentrations higher than 1 μ M had progressively less effect, possibly due to receptor desensitization. Block of GABA-B receptors reduced recovery from anoxia, indicating that the endogenous GABA concentration was high enough to protect optic nerves during anoxia. Mean CAP recovery under control conditions was approximately 36%, which lies between the mean CAP recovery found during GABA-B receptor block (presumably the recovery seen in zero extracellular GABA) and perfusion with 10 nM GABA. These data are therefore consistent with a GABA concentration close to the value of 20 nM reported in normoxic white matter by Shimada et al. (1993).

Conditions during anoxia are ideal for release of GABA following reversal of uptake transport (Blaustein and King, 1976; Carvalho et al., 1991; Ransom et al., 1992; Stys et al., 1992). Indeed, Shimada et al. (1993) have reported that GABA concentration in white matter increases during ischemia, reaching levels as high as 0.1 μ M 120 min after the blood supply to the brain had been partially interrupted. Furthermore, Anden et al. (1989) reported that GABA concentration increased by 200% in isolated white matter post mortem, an effect thought to be due to anoxia (Baxter, 1976).

The mechanism of action of GABA recruitment of an intracellular pathway

GABA-B receptor activation could interrupt the cascade of events which leads to irreversible anoxic injury at a number of points. For example, GABA-B receptor activation influences ionic conductances in a variety of neuronal cell types (for review see

Borman, 1989), and ion flux through channels is essential to the development of anoxic injury in white matter (Stys et al., 1992). Alternatively, recruitment of the GABA-B receptor/G-protein/PKC second messenger system could result in downregulation of membrane transporters, such as the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Men et al., 1991), that participate in the development of anoxic injury (Stys et al., 1992). An effect of GABA upon ATP metabolism during the early stages of anoxia could also underlie the protection provided by GABA. It is unclear, however, how the intracellular pathway we have described could produce a direct modulation of ATP breakdown.

Downregulation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger or inactivation of persistent sodium channels would produce a protective effect consistent with our data. GABA-mediated protection was found to be relatively slow to develop and was fully reversible. Downregulation and internalization of membrane transporters have similar characteristics (Schmalzing et al., 1990; Vasilets et al., 1990), while inactivation of axonal ion channels by phosphorylation is both rapid and irreversible (Rang and Ritchie, 1988). The results are therefore more consistent with the idea that downregulation of the $\text{Na}^+/\text{Ca}^{2+}$ transporter underlies GABA-mediated protection in white matter (see Fig. 12), although effects upon ion channels cannot be excluded.

It is interesting to consider these possible actions of GABA in relation to results that have been reported in chronic experiments using the GABA degradation inhibitor vigabatrin. Vigabatrin increases the levels of GABA in the brain and is used clinically in the treatment of epilepsy. In animal studies it has been shown that while vigabatrin has no pathological actions upon gray matter, it causes microvacuolization in white matter (Butler et al., 1987; Butler, 1989; Sabers and Gram, 1992). These ultrastructural changes are similar to those seen in white matter following anoxia (Waxman et al., 1992). Conceivably, vigabatrin's pathological effects on white matter might result from a sustained extracellular GABA increase which induces a downregulation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. During anoxia, GABA elevation would be beneficial since temporary downregulation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger would block or reduce Ca^{2+} loading by reverse exchange. In contrast, under normoxic conditions sustained GABA elevation would compromise the ability of white matter to regulate intracellular Ca^{2+} in a normal manner.

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