

# Soluble Guanylyl Cyclase Activator YC-1 Protects White Matter Axons from Nitric Oxide Toxicity and Metabolic Stress, Probably through Na<sup>+</sup> Channel Inhibition

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## ABSTRACT

In the rat isolated optic nerve, nitric oxide (NO) activates soluble guanylyl cyclase (sGC), resulting in a selective accumulation of cGMP in the axons. The axons are also selectively vulnerable to NO toxicity. The experiments initially aimed to determine any causative link between these two effects. It was shown, using a NONOate donor, that NO-induced axonal damage occurred independently of cGMP. Unexpectedly, however, the compound YC-1, which is an allosteric activator of sGC, potently inhibited NO-induced axonopathy ( $IC_{50} = 3 \mu M$ ). This effect was not attributable to increased cGMP accumulation. YC-1 (30  $\mu M$ ) also protected the axons against damage by simulated ischemia, which (like NO toxicity) is sensitive to Na<sup>+</sup> channel inhibition. Although chemically unrelated to any known Na<sup>+</sup> channel inhibitor, YC-1 was effective in two biochemical assays

for activity on Na<sup>+</sup> channels in synaptosomes. Electrophysiological recording from hippocampal neurons showed that YC-1 inhibited Na<sup>+</sup> currents in a voltage-dependent manner. At a concentration giving maximal protection of optic nerve axons from NO toxicity (30  $\mu M$ ), YC-1 did not affect normal axon conduction. It is concluded that the powerful axonoprotective action of YC-1 is unrelated to its activity on sGC but is explained by a novel action on voltage-dependent Na<sup>+</sup> channels. The unusual ability of YC-1 to protect axons so effectively without interfering with their normal function suggests that the molecule could serve as a prototype for the development of more selective Na<sup>+</sup> channel inhibitors with potential utility in neurological and neurodegenerative disorders.

Guanylyl cyclase enzymes are found throughout the body and catalyze the synthesis of the second messenger cGMP from GTP. They exist in two broad families, membrane-bound (particulate) and soluble. The particulate cyclases serve as peptide (or putative peptide) receptors, whereas soluble guanylyl cyclase (sGC) functions as the major receptor for nitric oxide (NO).

cGMP elicits its many acute physiological effects by influencing the activity of kinases, phosphodiesterases, and ion channels (Lucas et al., 2000). In the longer term, the molecule may also be of relevance to pathophysiological situations, as either a protectant or a mediator of damage. For example, a neuroprotective effect of cGMP has been shown in cerebellar slices (Garthwaite and Garthwaite, 1988), spinal cord (Weill and Greene, 1984; Urushitani et al., 2000), and developing retinal explants (Guimaraes et al., 2001). Conversely, cGMP has been implicated in pathological cascades

in the retina (Tsang et al., 1996), cortical neurons (Frandsen et al., 1992), and elsewhere.

Degenerative disorders in the central nervous system affect both gray and white matter. White matter, containing myelinated axons and glial cells, is selectively affected in several conditions, including stroke, trauma, and multiple sclerosis (Stys, 1998; Trapp et al., 1998). Much less is understood about the mechanisms of damage to white matter relative to neuronal cell bodies residing in the gray matter. A convenient model for white matter pathology is the isolated optic nerve preparation, and studies of the effects of metabolic stress (anoxia and simulated ischemia) have consistently suggested that Na<sup>+</sup> entry through voltage-dependent ion channels leading to lethal Ca<sup>2+</sup> influx is the primary mechanism (Stys, 1998). Evidence from investigations of spinal cord trauma in vivo lends support to this proposal (Rosenberg et al., 1999).

Another putative mediator of damage to numerous tissues, including CNS white and gray matter, is NO, which is produced by constitutive or inducible NO synthases. In the case

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**ABBREVIATIONS:** sGC, soluble guanylyl cyclase; NO, nitric oxide; CNS, central nervous system; YC-1, 3-(5-hydroxymethyl-2-furyl)-1-benzylindazole; aCSF, artificial cerebrospinal fluid; PAPA/NO, 3-(*n*-propylamino)propylamine/NO adduct; OGD, oxygen- and glucose deprivation; IBMX, 3-isobutyl-1-methylxanthine; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; TTX, tetrodotoxin; BTX-B, batrachotoxinin-B; PIPES, piperazine-*N,N'*-bis-(2-ethanesulfonic acid).

of white matter, circumstantial evidence indicates that excessive NO production contributes to damage occurring in multiple sclerosis, glaucoma, acquired immunodeficiency syndrome dementia, and diabetic neuropathy (Bo et al., 1994; Stevens, 1995; Neufeld et al., 1997; Rostasy et al., 1999). However, it remains unclear whether the NO is a cause or an effect of these diseases and whether NO synthase up-regulation or induction in these pathological conditions has a deleterious or ameliorating effect (Willenborg et al., 1999). Hitherto, there have been relatively few studies exploring the potential toxicity of NO toward cells and axons in white matter (Merrill et al., 1993; Redford et al., 1997). In support of this possibility, we have recently found that, when applied to the isolated optic nerve, NO is capable of killing both axons and glial cells, with the former being more vulnerable (Garthwaite et al., 2001). Interestingly, optic nerve axons are also rich in sGC and exposure to NO leads to the apparently exclusive accumulation of cGMP in these elements (Garthwaite et al., 1999b). This raises the question as to whether cGMP plays any role (protective or destructive) in the axon pathology. The initial aim of the present experiments was to examine this possibility by using pharmacological and other tools to manipulate cGMP levels. In the course of these studies, we made the observation that an allosteric activator of sGC, 3-(5-hydroxymethyl-2-furyl)-1-benzyl-indazole [known as YC-1; Fig. 1; Ko et al., 1994; Wu et al., 1995], exerted a powerful protective effect on NO toxicity toward axons and the subsequent experiments were geared toward identifying the underlying mechanism.

## Experimental Procedures

**Optic Nerves.** The method has been described previously (Garthwaite et al., 1999a). Briefly, Wistar rats (220–260 g) were decapitated as approved by the British Home Office and the local ethics committee. The nerves were quickly excised and incubated in 50-ml flasks containing 20 ml of an artificial CSF (aCSF) solution composed of 120 mM NaCl, 2.0 mM KCl, 2.0 mM CaCl<sub>2</sub>, 26 mM NaHCO<sub>3</sub>, 1.18 mM KH<sub>2</sub>PO<sub>4</sub>, 1.19 mM MgSO<sub>4</sub>, and 11 mM glucose, continuously gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub>. The flasks were held in a shaking water bath at 37°C and left to incubate for 1 to 1.5 h before the experiments were started.

NO toxicity was imposed by incubating the nerves with the

NONOate 3-(*n*-propylamino)propylamine/NO adduct (PAPA/NO, 1 mM) for 2 h followed by a recovery period (2 h) in normal incubation medium. For oxygen- and glucose deprivation (OGD), the nerves were transferred into incubation medium lacking glucose and gassed with 5% CO<sub>2</sub> in N<sub>2</sub> for 1 h. They were then given a 2-h recovery period in normal medium. Both these paradigms have previously been shown to result in irreversible damage to the majority of axons (Garthwaite et al., 1999a, 2001). For the Na<sup>+</sup>-free medium, 120 mM choline chloride and 26 mM choline bicarbonate replaced NaCl and NaHCO<sub>3</sub>, respectively. Modified incubation media and test compounds were applied from 15 min before until 15 min after the exposure to PAPA/NO or OGD, except where stated.

**Histology.** The nerves were fixed, embedded in resin, sectioned (1 μm), and stained with toluidine blue by using conventional techniques. Morphometric analysis of the axonal damage was performed using an image analysis system as described previously (Garthwaite et al., 1999a). Briefly, for each nerve, the mean internal Feret diameter of axons was measured in four fields, each having an area of 2500 μm<sup>2</sup>. The numbers of axons with values above 2.5 μm/10<sup>4</sup> μm<sup>2</sup> provided the “axonopathy index”. With this cutoff, the majority of undamaged thicker axons are excluded such that, in control nerves, only about 1.5% of all axons are registered in the index. Mean data were derived from four to eight nerves in two to four separate experiments. For each test condition in a given experiment, there were two to three nerves, each from a different animal.

**cGMP Measurement.** Sister nerves of those used to examine PAPA/NO toxicity were exposed to PAPA/NO for 30 min, a period that does not cause damage (Garthwaite et al., 2001), in the absence or presence of the nonselective phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 1 mM), the inhibitor of sGC 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), or YC-1, as detailed in the text. In some experiments aimed at determining concentration-response curves for PAPA/NO and YC-1 on sGC (Fig. 3B), the exposure period to PAPA/NO was reduced to 5 min to limit possible desensitization of sGC and other associated proteins. At the end of the exposures, the nerves were inactivated by boiling in hypotonic buffer, homogenized by sonication, and the cGMP and protein levels measured by radioimmunoassay and the bicinchoninic method, respectively (Garthwaite et al., 1999b).

**Preparation of Synaptosomes.** This was done essentially as described by Pauwels et al. (1986). Cerebral cortices dissected from Male Wistar rats (200–300 g) were homogenized in 9 volumes of ice-cold 0.25 M sucrose by using a motor-driven, glass-Teflon homogenizer (Potter S; B. Braun, Allentown, PA) using eight up and down strokes at 900 rpm. The homogenate was centrifuged at 1036g at 4°C for 10 min and the supernatant collected. The remaining pellet was resuspended as described above in fresh ice-cold 0.32 M sucrose and the centrifugation step repeated. The supernatant fractions were pooled and centrifuged at 46,000g for 15 min. The resulting pellet was resuspended in assay buffer at a final concentration of 10 to 20 mg of cortex/ml, wet weight.

**Veratrine-Evoked Uptake of [<sup>14</sup>C]Guanidine.** Test compounds, veratrine (100 μg/ml final concentration), and synaptosomes (4 mg/ml, wet weight) were incubated in the absence or presence of tetrodotoxin (TTX; 1 μM) at 37°C for 5 min in polypropylene test tubes. Uptake was initiated by the addition of prewarmed [<sup>14</sup>C]guanidine (final concentration 1 μCi/ml) and stopped 2 min later by the addition of 10 ml of ice-cold wash medium as described by Pauwels et al. (1986). Incubates were immediately filtered under vacuum through GF/C filters by using a Brandel harvester. The incubation tubes were rinsed with 5 ml of ice-cold wash buffer, which was then used to wash the filter. Filters were transferred to minivials (Beckman Coulter, Fullerton, CA) with the use of a Brandel deposit/dispense system and subsequently counted by liquid scintillation spectroscopy with Picofluor<sup>40</sup> liquid scintillator.

**Batrachotoxinin-B (BTX-B) Binding.** This was carried out using the method described by Catterall et al. (1981), except that both bovine serum albumin and TTX were omitted from the incubation

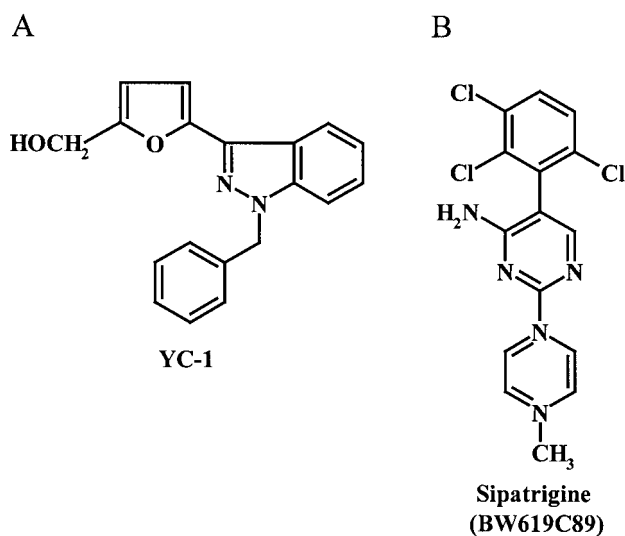


Fig. 1. Chemical structures of YC-1 (A) and sipatrigine (B).

medium. Binding was initiated by the addition of synaptosomes (final concentration 10 mg/ml, wet weight) to a mixture of test compound and 10 nM [ $^3\text{H}$ ]BTX-B in the absence or presence of scorpion venom (25  $\mu\text{g}/\text{ml}$  final concentration). Samples were mixed and incubated for 90 min at 25°C. Ice-cold wash medium (5 ml) was added and then the samples subjected to vacuum filtration through GF/C filters by using a Brandel harvester. Incubation tubes were rinsed with 5 ml of ice-cold wash buffer, which was then used to wash the filter. Radioactivity in the filter was counted as described above.

**Whole-Cell Recording from Dissociated Hippocampal Neurons.** Wistar rats (14–21 days old) were decapitated and the brain was quickly removed and placed into an ice-cold solution containing 119 mM NaCl, 2.5 mM KCl, 1.3 mM  $\text{MgCl}_2$ , 1.0 mM  $\text{NaH}_2\text{PO}_4$ , 26.2 mM  $\text{NaHCO}_3$ , and 11 mM glucose, bubbled with 95%  $\text{O}_2$ /5%  $\text{CO}_2$ . The hippocampi were dissected and 400- $\mu\text{m}$  slices were cut using a vibrotome. Slices were maintained in a holding chamber containing the same solution at room temperature for at least 1 h before use. For preparation of the dissociated neurons, two to three slices were placed in oxygenated PIPES buffer solution that contained 86 mM PIPES, 30 mM NaCl, 3 mM KCl, 2 mM  $\text{MgCl}_2$ , 10 mM glucose at 37°C with added protease XXIII (3 mg/ml). After 10 min, slices were transferred to PIPES buffer at room temperature containing trypsin inhibitor and bovine serum albumin (both at 1 mg/ml). A portion of the CA1 region was removed using a micropunch and cells dissociated by gentle trituration with fire-polished Pasteur pipettes. The cell suspension was then left on a poly(L-lysine)-coated coverslip for about 15 min.

Recording of  $\text{Na}^+$  currents was performed at room temperature ( $\sim 19^\circ\text{C}$ ) under voltage-clamp by using the whole-cell patch-clamp technique. Cells were perfused ( $\sim 1.5$  ml/min) with an oxygenated solution containing 80 mM NaCl, 60 mM tetraethylammonium, 4.7 mM KCl, 1.3 mM  $\text{MgCl}_2$ , 2 mM  $\text{CaCl}_2$ , 0.1 mM  $\text{CdCl}_2$ , 11 mM glucose, and 5 mM HEPES, osmolality 292 to 296 mOsm, pH 7.4. Patch pipettes were fabricated from borosilicate glass (inner diameter, 1.17 mm; outer diameter, 1.5 mm; Harvard Apparatus, Holliston, MA) using a Sutter P-97 electrode puller and filled with a solution containing 120 mM CsF, 10 mM CsEGTA, 10 mM NaCl, and 10 mM HEPES, osmolality 294 mOsm, pH 7.4. The pipette current was set to zero before attempting to form a seal. The quoted membrane potentials are not corrected for liquid junction potentials. On breakthrough, the series resistance was 2.2 to 4.5 M $\Omega$  and this was compensated by 90%. Leak subtraction was performed online by using four hyperpolarizing pulses before the depolarizing steps to 0 mV. Capacitive transients were compensated using the amplifier circuitry (Axopatch 200B; Axon Instruments, Foster City, CA). Currents were filtered at 100 kHz, digitized at 200 kHz, and stored on a personal computer for subsequent analysis (pClamp8; Axon Instruments).

Voltage-dependent block of  $\text{Na}^+$  channels was tested using two protocols that were alternated at 1-min intervals. The first depolarized the cell to 0 mV (10 ms) from a holding potential of  $-90$  mV without any prepulse. In the second, the step to 0 mV was preceded by a 30-s prepulse to  $-60$  mV. Before each step from  $-90$  mV the series resistance was checked and recordings were terminated if any change was noticed. The traces shown are single currents elicited in the same cell. Effects of test compounds were assessed on the amplitude of currents in the presence of the compound compared with those obtained immediately beforehand.

**Optic Nerve Compound Action Potentials.** The grease-gap recording technique described previously was used (Garthwaite et al., 1999a). Briefly, the nerve was placed in a three-compartment recording chamber. The central portion of the nerve was positioned in the middle chamber with the ends passing through greased holes in the partitions. In the first compartment, a bipolar stimulating electrode was positioned on the surface of the nerve. Compound action potentials were elicited at a rate of 0.2 Hz by using square-wave voltage pulses of 60- to 90- $\mu\text{s}$  duration and 10- to 15-V amplitude. To reduce the appearance of biphasic action potentials on the

recording, the length of the nerve entering the third compartment was kept as short as possible. All three chambers were filled with aCSF and were heated to 37°C by a water jacket. The central compartment was perfused with aCSF at a rate of  $\sim 1.5$  ml/min. The outer compartments were not perfused. The YC-1 was applied in the perfusate diluted 3:1000 from a 10 mM stock solution. The compound action potential was monitored differentially using Ag/AgCl electrodes embedded in agar, amplified (Grass P16; Grass Instruments, Quincy, MA), and recorded on a personal computer by using Clampex 8 (Axon Instruments) with a sampling rate of 200 kHz. The traces shown are averages of five sequential responses obtained before drug application and before washout. The area under these averaged waveforms was calculated using Clampfit 8 (Axon Instruments).

**Statistical Analysis.** Results are given as means  $\pm$  S.E.M. and were evaluated using Student's *t* test for unpaired variables (two-tailed), with  $P < 0.05$  being considered significant.

**Materials.** PAPA/NO was from Alexis Corporation (Bingham, Nottingham, UK). TTX was from Latoxan Laboratories (Rosans, France). Sipatrigine was supplied by the Wellcome Research Laboratories (Beckenham, Kent, UK). [ $^{14}\text{C}$ ]Guanidine hydrochloride (2.18 GBq/mmol) was obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK), and batrachotoxinin-A 20- $\alpha$ -benzoate, [benzoyl-2,5- $^3\text{H}$ ] (1258 GBq/mmol) from PerkinElmer Life Sciences (Boston, MA). YC-1 was provided by the chemistry department of the Wolfson Institute for Biomedical Research. Other chemicals were from Sigma-Aldrich (Poole, Dorset, UK), BDH/Merck (Poole, Dorset, UK), or Tocris-Cookson (Bristol, UK).

## Results

**cGMP in NO-Induced Axonopathy.** In agreement with previous findings (Waxman et al., 1992; Garthwaite et al., 1999a), histology of optic nerves incubated under control conditions showed that they contained well preserved axons and glial cells (Fig. 2A). Exposure of the nerves to 1 mM PAPA/NO for 2 h (plus 2-h recovery) resulted in selective axonal degeneration characterized by persistent swelling (Fig. 2B). It has been shown previously that this damage is irreversible, inhibited by the NO scavenger oxyhemoglobin, and is not observed using PAPA/NO depleted of its NO moiety (Garthwaite et al., 2001). Quantitation of the damage was carried out using a morphometric method that gave an "axonopathy index" corresponding to the numbers of axons having an internal Feret diameter of greater than 2.5  $\mu\text{m}/\text{unit area}$  ( $10^4 \mu\text{m}^2$ ). PAPA/NO treatment caused an approximately 10-fold increase in this index (Table 1).

Various experiments were carried out to determine the possible role of cGMP in the axon damage (Table 1). Accumulation of cGMP was measured during the first 30 min of exposure to PAPA/NO, at which time no toxicity was observed (Garthwaite et al., 2001). PAPA/NO itself (1 mM) caused a 2- to 3-fold increase in cGMP in the nerves from the basal value of 5 pmol/mg of protein. The sGC inhibitor ODQ (10  $\mu\text{M}$ ) blocked the cGMP increase but did not affect the axonopathy. The nonselective phosphodiesterase inhibitor IBMX (1 mM) greatly increased cGMP accumulation induced by PAPA/NO (from 13 up to 178 pmol/mg of protein) but also failed to influence the axonopathy. The membrane permeant analog 8-bromo-cGMP (1 mM) did not induce damage on its own and also had no significant protective effect against PAPA/NO-induced toxicity. When the allosteric sGC activator YC-1 (30  $\mu\text{M}$ ) was included with PAPA/NO, the cGMP response was increased 2-fold more than with IBMX; surprisingly, the axonopathy was abolished (Fig. 2C). This powerful



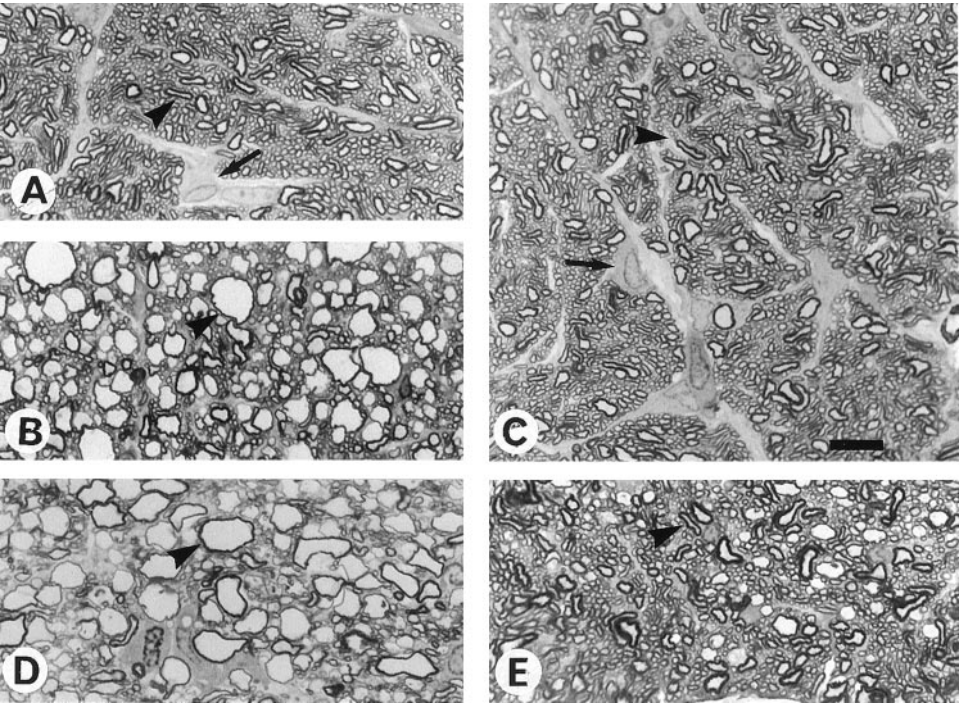
axonoprotective effect of YC-1 persisted in the presence of ODQ, which reduced cGMP accumulation to 70 pmol/mg of protein, or IBMX, which increased the cGMP response to over 800 pmol/mg of protein (Table 1).

These results indicated that there is no relationship between cGMP accumulation and the subsequent axonopathy induced by PAPA/NO, or between cGMP and the protective effect of YC-1 against PAPA/NO-induced axonopathy. Concentration-response curves, however, showed that YC-1 exerted the two effects with overlapping potency. The  $IC_{50}$  for protecting axons against PAPA/NO toxicity was about 3  $\mu$ M, and at 10 and 30  $\mu$ M, the axonopathy index was not significantly different from that of untreated nerves (Fig. 3A). The action of YC-1 on sGC would be expected to be most prominent at low NO concentrations (Friebe et al., 1996). The concentration-response curve for PAPA/NO on optic nerve cGMP levels (in the presence of IBMX) indicated an  $EC_{50}$  value of approximately 30  $\mu$ M (Fig. 3B, inset) and so just a concentration of 1  $\mu$ M, just over the threshold, was selected. Over the range of 0.3 to 30  $\mu$ M (the limit of solubility in

aCSF), YC-1 progressively increased cGMP accumulation in response to 1  $\mu$ M PAPA/NO (Fig. 3B).

**YC-1 against OGD-Induced Axonopathy.** To investigate whether the protective effect of YC-1 extended to other insults, its effects against OGD were tested. Exposure of the nerves to 1 h of OGD followed by 2-h recovery caused severe swelling and distension of the axons, an effect that was qualitatively and quantitatively similar to that produced by PAPA/NO (Figs. 2D and 4). When the nerves were incubated with YC-1 (30  $\mu$ M) during 1-h OGD, axons were largely spared (Figs. 2E and 4). In the same experiments, the  $Na^+$  channel blocker sipatrigine (100  $\mu$ M; Fig. 1; formerly known as BW619C89), which had previously been shown to protect against OGD and NO toxicity in the optic nerve (Garthwaite et al., 1999a, 2001), preserved the axons to a degree not significantly greater than that afforded by YC-1 (Fig. 4).

**Is Axonoprotective Effect of YC-1 Caused by  $Na^+$  Channel Inhibition?** In agreement with other recent findings (Garthwaite et al., 2001) PAPA/NO toxicity toward axons in the present experiments was inhibited by the  $Na^+$



**Fig. 2.** Light micrographs illustrating axonoprotective effect of YC-1 in rat isolated optic nerve subjected to NO toxicity. A, cross section of a control nerve maintained in vitro for 6 h. In A to E, arrowheads indicate axons and arrows indicate glial cells. B, nerve incubated with 1 mM PAPA/NO for 2 h followed by 2-h recovery in normal incubating media, showing swollen axons. C, nerve incubated with 1 mM PAPA/NO for 2 h in the presence of 30  $\mu$ M YC-1, showing no damage. D, nerve subjected to 1 h OGD followed by 2-h recovery showing swollen axons. E, nerve subjected to 1 h of OGD in the presence of YC-1 (30  $\mu$ M), showing significant preservation of axonal morphology. Scale bar (in C, but applies to A–E), 10  $\mu$ m.

TABLE 1

Lack of relationship between cGMP and axon damage in response to NO

Control nerves were incubated for 6 h. The axonopathy index represents the number of axons with Feret diameter above 2.5  $\mu$ m/ $10^4 \mu$ m<sup>2</sup>. Exposure times to PAPA/NO were 2 h (plus 2-h recovery) for assessing the axonopathy and 30 min for the cGMP response. The concentrations of compounds used were 1 mM PAPA/NO, 1 mM IBMX, 10  $\mu$ M ODQ, 30  $\mu$ M YC-1, and 1 mM 8-Br-cGMP. Values are means  $\pm$  S.E.M.

Addition	Axonopathy Index	n	cGMP pmol/mg of protein	n
None	22 $\pm$ 4	6	5 $\pm$ 0.4	4
PAPA/NO	216 $\pm$ 13*	8	13 $\pm$ 1	4
PAPA/NO + ODQ	193 $\pm$ 10* <sup>ns</sup>	4	4 $\pm$ 0.2	4
PAPA/NO + IBMX	193 $\pm$ 13* <sup>ns</sup>	4	178 $\pm$ 4	5
8-Br-cGMP	25 $\pm$ 3 <sup>†</sup>	3	ND	
PAPA/NO + 8-Br-cGMP	187 $\pm$ 11* <sup>ns</sup>	4	ND	
PAPA/NO + YC-1	16 $\pm$ 2 <sup>†</sup>	6	321 $\pm$ 18	5
PAPA/NO + YC-1 + ODQ	18 $\pm$ 1 <sup>†</sup>	4	70 $\pm$ 2	4
PAPA/NO + IBMX + YC-1	13 $\pm$ 2 <sup>†</sup>	4	831 $\pm$ 16	5

\*,  $P < 0.001$  versus control; <sup>†</sup>, not significant versus control; <sup>ns</sup>, not significant versus PAPA/NO alone.

channel blockers TTX ( $1 \mu\text{M}$ ) and sipatrigine ( $100 \mu\text{M}$ ), and by removing extracellular  $\text{Na}^+$  (Fig. 4), confirming the important role played by the entry of  $\text{Na}^+$  through voltage-sensitive channels in NO-induced axonopathy. With all these interventions, the protection was complete and that given by YC-1 ( $30 \mu\text{M}$ ) was not significantly different. The possibility that YC-1 is a  $\text{Na}^+$  channel blocker was therefore examined.

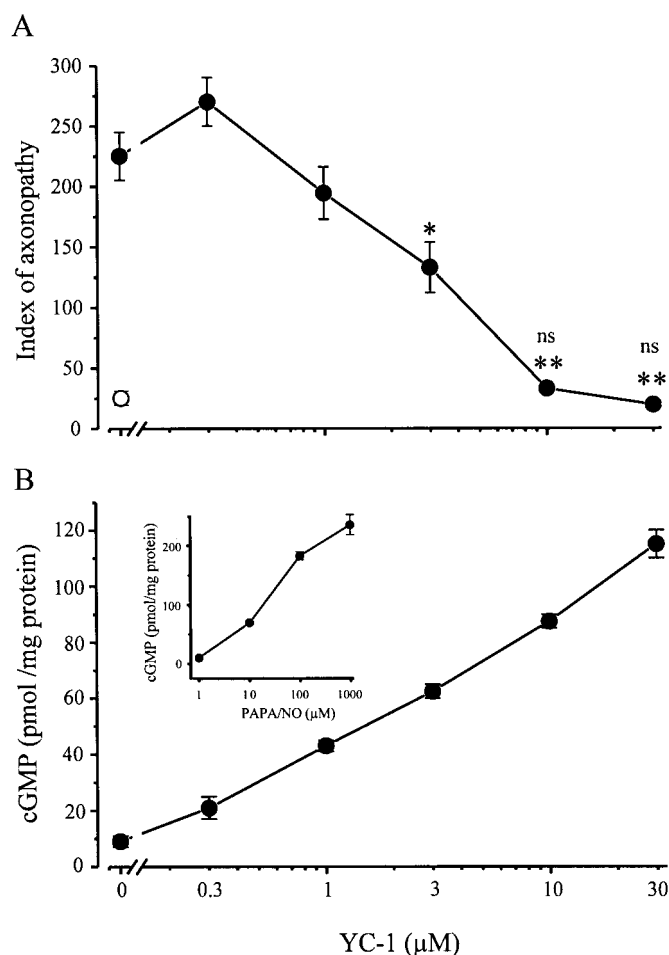
Initially, we tested the ability of YC-1 to inhibit  $\text{Na}^+$  channels in a [ $^{14}\text{C}$ ]guanidine flux assay by using cerebral cortex synaptosomes (Pauwels et al., 1986). In this assay, veratrine holds the  $\text{Na}^+$  channels in an open state and the influx of [ $^{14}\text{C}$ ]guanidine through the channels and into the synaptosomes measured. This uptake of [ $^{14}\text{C}$ ]guanidine shows two components of which the major one is inhibited by  $1 \mu\text{M}$  TTX. Both YC-1 and sipatrigine completely inhibited this TTX-sensitive component of uptake (Fig. 5A), the  $\text{IC}_{50}$  values for the two compounds being very similar at  $23 \pm 3 \mu\text{M}$  ( $n = 3$ ) and  $24 \pm 0.7 \mu\text{M}$  ( $n = 3$ ), respectively.

Next, the effect of YC-1 on the binding of the  $\text{Na}^+$  channel

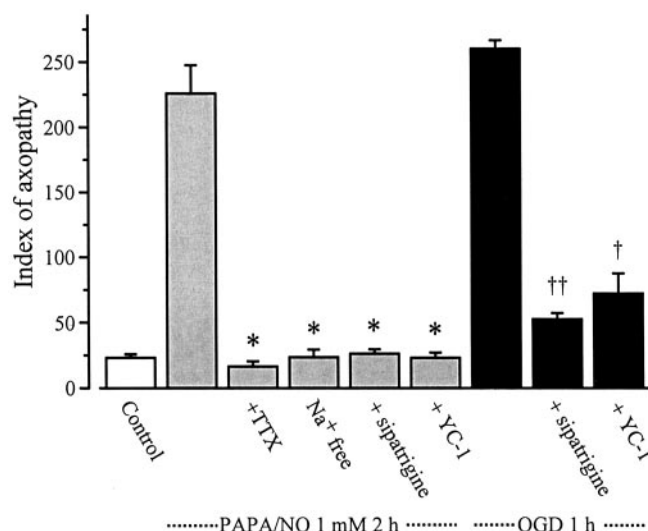
ligand [ $^3\text{H}$ ]BTX-B was assessed (Fig. 5B). Scorpion venom was used to increase the affinity of BTX-B for its binding site (Catterall et al., 1981). YC-1 inhibited [ $^3\text{H}$ ]BTX-B binding completely and concentration dependently, as did sipatrigine. The  $\text{IC}_{50}$  values for YC-1 was similar to that found in the [ $^{14}\text{C}$ ]guanidine flux assay ( $27 \pm 6 \mu\text{M}$ ;  $n = 3$ ), whereas sipatrigine was more potent ( $\text{IC}_{50} = 7 \pm 2 \mu\text{M}$ ;  $n = 3$ ).

In view of these results, the interaction of YC-1 with  $\text{Na}^+$  channels was examined directly using electrophysiological techniques. In dissociated hippocampal neurons in the presence of blockers of other voltage-dependent channels, stepping from  $-90 \text{ mV}$  to  $0 \text{ mV}$  evoked large ( $\sim 10 \text{ nA}$ ), rapidly activating and inactivating currents (Fig. 6A), which were abolished by  $1 \mu\text{M}$  TTX and diminished in amplitude when the extracellular  $\text{Na}^+$  was reduced (results not shown), identifying them as classical  $\text{Na}^+$  currents. In the presence of YC-1 ( $30 \mu\text{M}$ ) currents evoked from a holding potential of  $-90 \text{ mV}$  were not significantly affected ( $94 \pm 2\%$  of control levels; Fig. 6A). In the same cells, the amplitudes of currents evoked from a holding potential of  $-60 \text{ mV}$  were significantly reduced to  $66 \pm 4\%$  of control levels ( $P < 0.01$ ;  $n = 4$ ). After about 2 min of washout, the current amplitudes recovered to be not significantly different from controls ( $86 \pm 6\%$ ;  $P < 0.05$ ;  $n = 4$ ).

The voltage dependence of the block of  $\text{Na}^+$  channels by YC-1 in the hippocampal neurons is similar to that observed with sipatrigine (Xie and Garthwaite, 1996) and with several anticonvulsant drugs (Taylor and Meldrum, 1995) and implies that action potential generation and conduction in healthy tissue might be relatively preserved in the presence of the molecule. This was tested by recording the compound action potential generated in the optic nerve by electrical stimulation of the axons. The typical multicomponent waveform (reflecting groups of axons with different conduction velocities; Stys et al., 1992) was unaffected ( $98 \pm 2\%$  of



**Fig. 3.** Relative potencies of YC-1 for inhibiting NO-induced axonopathy (A) and potentiating NO-stimulated sGC activity (B). In A, the nerves were incubated with PAPA/NO for 2 h followed by 2-h recovery, in the presence or absence of different concentrations of YC-1. \* $P < 0.01$ , \*\* $P < 0.00001$  versus PAPA/NO control; ns, not significant versus control nerves (○);  $n = 4$  to 8. In B, the effect of YC-1 on cGMP levels was measured in nerves ( $n = 4$ ) exposed for 5 min to PAPA/NO ( $1 \mu\text{M}$ ) in the presence of IBMX ( $1 \text{ mM}$  added 15 min beforehand). YC-1 was added 10 min before PAPA/NO. The inset shows the concentration-cGMP response curve for PAPA/NO in the presence of IBMX ( $n = 3$ ). All data represent means  $\pm$  S.E.M.



**Fig. 4.** Protection of axons by YC-1 against PAPA/NO toxicity and OGD in rat optic nerve resembles that of  $\text{Na}^+$  channel inhibitors. Nerves were exposed to PAPA/NO ( $1 \text{ mM}$ ; 2 h) or OGD (1 h) and then allowed to recover in normal incubation medium for 2 h. TTX ( $1 \mu\text{M}$ ), sipatrigine ( $100 \mu\text{M}$ ), YC-1 ( $30 \mu\text{M}$ ), or  $\text{Na}^+$ -free medium were applied 15 min beforehand and remained for the first 15 min of recovery. Data are means  $\pm$  S.E.M.;  $n = 4$  to 8. \* $P < 0.00001$  versus PAPA/NO control; †,  $P < 0.0001$ , ††,  $P < 0.00001$  versus OGD control.

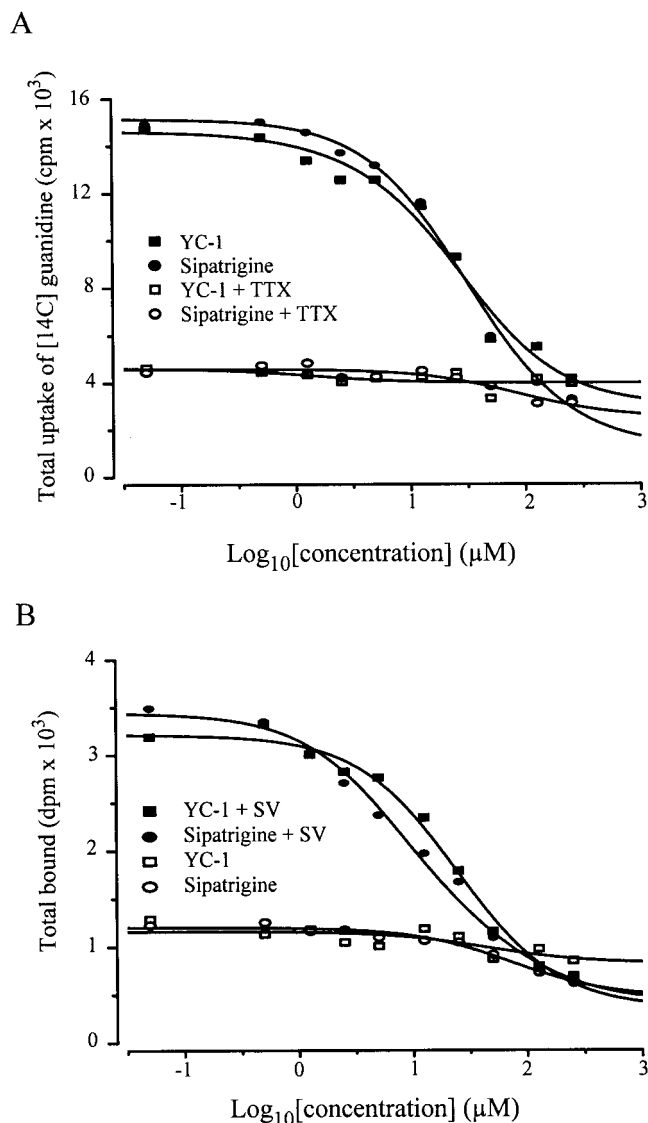
control) by a concentration of YC-1 (30  $\mu\text{M}$ ), giving complete protection against NO toxicity (Fig. 6B).

## Discussion

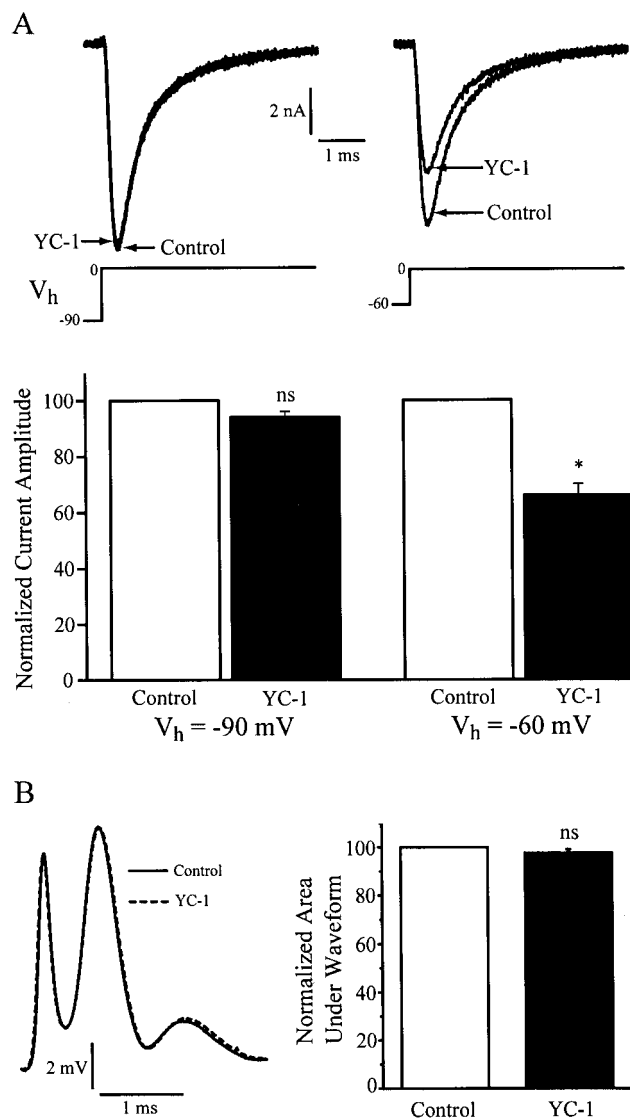
The identification of molecules able to protect CNS tissue at risk in acute and chronic neurodegenerative disorders, with known mechanisms of action, is a goal of potentially great significance for understanding the pathogenesis of those disorders and for the development of new treatments. In the present study, we have serendipitously found a novel chemical species that is able to protect CNS white matter

axons to a remarkable degree, and have attempted to discover how it produces this effect.

**Role of cGMP.** In light of evidence that cGMP can be protective or detrimental to cell survival, together with the finding that NO evoked a large and selective accumulation of cGMP in the principal targets of its toxicity in optic nerve (the axons), the first aim was to investigate whether this response had any relevance to the pathology. Manipulation of cGMP accumulation over a wide range by using inhibitors of sGC and phosphodiesterase enzymes, or addition of an exogenous cGMP derivative, however, had no influence on the PAPA/NO-induced axonopathy. This indicates that the two



**Fig. 5.** Concentration-response curves for YC-1 and sipatrigine in assays for  $\text{Na}^+$  channel inhibition. A, veratrine-evoked uptake of [ $^{14}\text{C}$ ]guanidine into rat cerebral cortex synaptosomes. Compounds under test were pre-incubated with synaptosomes for 5 min in the presence of either veratrine alone or veratrine plus 1  $\mu\text{M}$  TTX before the addition of [ $^{14}\text{C}$ ]guanidine. B, binding of [ $^3\text{H}$ ]BTX-B to rat cerebral cortex synaptosomes. Compounds under test were incubated with rat cerebral cortex synaptosomes and either [ $^3\text{H}$ ]BTX-B or [ $^3\text{H}$ ]BTX-B plus scorpion venom (SV) for 90 min. In both cases, data are from one representative experiment (of three) and are the means of duplicate measurements at each concentration.  $\text{IC}_{50}$  values were computed for the major components of binding and uptake by using the single-site logistic equation, to which the data were fitted (curves).



**Fig. 6.** Electrophysiological analysis of the effect of YC-1 on voltage-dependent  $\text{Na}^+$  channels. A, voltage-dependent inhibition of  $\text{Na}^+$  currents in hippocampal neurons. Currents were elicited using a switched prepulse protocol: one test pulse depolarized from a membrane potential ( $V_h$ ) of -90 mV to 0 mV for 10 ms; the second was preceded by a prepulse to -60 mV for 30 s, as shown beneath each current trace. Sample traces are from the same cell under control conditions and in the presence of YC-1 (30  $\mu\text{M}$ ). Bar graph shows mean data from four cells; ns, not significant; \*,  $P < 0.01$  versus control. B, YC-1 (30  $\mu\text{M}$ ) did not affect compound action potentials in optic nerve. Sample traces are averages of five consecutive sweeps. Bar graph shows mean data from four nerves; ns, not significantly different from control.



effects are not causally related, at least so far as the relatively acute pathology studied here is concerned.

The finding that YC-1 was a powerful protectant against NO toxicity therefore seemed anomalous. The compound was originally described as a selective and NO-independent activator of platelet sGC (Ko et al., 1994; Wu et al., 1995). Subsequently, its most pronounced effect has been found to be to sensitize sGC to NO (and CO), such that the natural ligand becomes a much more potent stimulator of the enzyme (Friebe et al., 1996; Friebe and Koesling, 1998). In accordance with this proposed action, YC-1 augmented cGMP accumulation in optic nerves exposed to low concentration of PAPA/NO (1  $\mu$ M) such that, at the highest concentration at which the compound stayed in solution (30  $\mu$ M), cGMP accumulation was enhanced 10-fold, a level equivalent to that ordinarily requiring a 20-fold higher PAPA/NO concentration. In addition to sensitizing sGC, YC-1 can also inhibit cGMP breakdown by phosphodiesterases (Friebe et al., 1998; Galle et al., 1999) but this is unlikely to explain the potentiation observed because these experiments were carried out in the presence of IBMX. On the other hand, the 25-fold increase in the cGMP response to a near maximally effective PAPA/NO concentration (1 mM) in the absence of IBMX is far greater than would be expected from studies on purified sGC (about 1.5-fold; Friebe et al., 1996). Phosphodiesterases seem to be very active in optic nerve axons, as indicated by the difference of more than 10-fold in cGMP accumulation in response to 1 mM PAPA/NO in the absence and presence of IBMX. Therefore, inhibition of phosphodiesterase activity by YC-1 is likely to contribute to the augmentation of the cGMP response to the high PAPA/NO concentration. The finding that YC-1 still enhanced this response by about 5-fold in the presence of IBMX may be reasonably explained by the fact that competitive phosphodiesterase inhibitors become inherently less effective as cGMP levels rise, enabling additional inhibition to be effective.

**Protection of Axons by YC-1.** The abolition of NO toxicity by YC-1 could not be explained by the attendant increase in cGMP accumulation because inhibition of this response by ODQ or augmenting it with IBMX had no effect. Consequently, an alternative mechanism of action was sought. Several similarities exist between NO toxicity and the injury induced by anoxia or OGD with respect to optic nerve axons, notably the joint dependence on extracellular  $\text{Ca}^{2+}$  and  $\text{Na}^+$  concentration and blockade by  $\text{Na}^+$  channel inhibitors such as TTX (Stys, 1998; Garthwaite et al., 1999a, 2001). This led to the suggestion that NO kills axons by inhibiting mitochondrial respiration, leading to metabolic inhibition (Garthwaite et al., 2001). The common underlying mechanism is likely to be the one first proposed for anoxia-induced axonopathy (Stys et al., 1992). According to this hypothesis, metabolic stress results in excessive influx of  $\text{Na}^+$  through voltage-sensitive  $\text{Na}^+$  channels. Of particular importance here may be the slowly inactivating  $\text{Na}^+$  channels that are prominent in optic nerve axons. Loading of the axoplasm with  $\text{Na}^+$  results in a reversal of the  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger, leading to a secondary influx of  $\text{Ca}^{2+}$ , which causes the irreversible axon damage. That  $\text{Na}^+$  channel inhibitors provide a high degree of protection toward axons in these conditions suggested a plausible mechanism of action of YC-1, a possibility supported by the finding that the compound also protected against OGD-induced axonal damage in

the optic nerve. In accordance with this possibility, in several assays, the compound was found to be a  $\text{Na}^+$  channel inhibitor.

In the [ $^3\text{H}$ ]guanine flux assay, the  $\text{IC}_{50}$  value for YC-1 (23  $\mu$ M) was higher than for protecting axons from NO toxicity (3  $\mu$ M), but the apparent potency of a  $\text{Na}^+$  channel blocker in the flux assay depends on the conditions used (e.g., veratrine concentration). Accordingly, in the flux assay, the apparent potency of sipatrigine (24  $\mu$ M) was the same as that of YC-1 and, when tested against OGD-induced optic nerve damage, the  $\text{IC}_{50}$  for sipatrigine (3  $\mu$ M) was also correspondingly higher (Garthwaite et al., 1999a). Moreover, the degree to which YC-1 (30  $\mu$ M) inhibited  $\text{Na}^+$  currents in hippocampal neurons evoked from  $-60$  mV was similar to that found with sipatrigine (Xie and Garthwaite, 1996). Consequently, although interference with additional processes cannot be excluded, the evidence indicates that inhibition of  $\text{Na}^+$  currents is a sufficient mechanism to account for the protective effect of YC-1.

The type of  $\text{Na}^+$  channel inhibition by YC-1 seemed to be similar to that found with sipatrigine (Xie and Garthwaite, 1996) and various anticonvulsant drugs such as phenytoin, carbamazepine, and lamotrigine (Taylor and Meldrum, 1995; Xie et al., 1995) that also have neuroprotective properties toward optic nerve axons (Fern et al., 1993; Garthwaite et al., 1999a). That is, the  $\text{Na}^+$  current at hyperpolarized membrane potentials was relatively unaffected, whereas after brief depolarization, the inhibition was greatly enhanced. Such a voltage dependence normally indicates an interaction with the slow inactivated state of the channels. During their normal cycle of operation voltage-dependent  $\text{Na}^+$  channels, having opened, pass into a fast-inactivated state from which they can rapidly recover to become available again for activation. If the cell remains depolarized, however, the channels pass into a more enduring state of inactivation that can be stabilized by the above-mentioned drugs. In this way,  $\text{Na}^+$  channel function in depolarized neurons is inhibited and repetitive action potential firing curtailed. Although this mechanism helps explain the anticonvulsant activity of certain drugs, sipatrigine shares a similar action on  $\text{Na}^+$  currents with the structurally related antiepileptic drug lamotrigine, but sipatrigine is a relatively weak anticonvulsant (M. J. Leach, personal communication) and lamotrigine, in contrast, is a relatively poor protectant toward optic nerve axons subjected to OGD (Garthwaite et al., 1999a). Thus, the voltage dependence of the inhibition of  $\text{Na}^+$  currents by YC-1 does not necessarily imply that it is this property that governs its axonoprotective activity. An alternative possibility is that YC-1 inhibits the low-amplitude, noninactivating  $\text{Na}^+$  currents that are prominent in optic nerve axons and that seem to mediate much of the toxic  $\text{Na}^+$  influx under conditions of metabolic inhibition (Stys et al., 1993). Irrespective of the precise mechanism of action of YC-1 on  $\text{Na}^+$  channels, the preservation of  $\text{Na}^+$  channel function at negative membrane potentials in the presence of the compound implies that the normal  $\text{Na}^+$  channel cycle would be relatively unaffected. In accordance, the compound action potential in the optic nerve axons, whose resting membrane potential is estimated to be in the region of  $-80$  mV (Stys et al., 1997), was untouched by a concentration of YC-1 (30  $\mu$ M) that afforded complete protection against NO toxicity. This suggests that, as with anticonvulsant drugs in clinical use, inhibition of

Na<sup>+</sup> channels by YC-1-like compounds in vivo should not produce serious side effects.

**Implications.** In terms of its chemical structure (Fig. 1), YC-1 does not seem to resemble sipatrigine or indeed any other known inhibitor of voltage-dependent Na<sup>+</sup> channels. Although other potential applications of such a molecule (e.g., as an anticonvulsant, analgesic, and protectant for gray matter) need to be explored, compounds able to protect white matter from damage without interfering with normal nerve function are rare. Indeed, only sipatrigine, which has undergone early clinical trials for stroke (Muir et al., 2000), has so far shown such a profile of activity. The other activities of YC-1 on sGC and phosphodiesterases might be undesirable in the setting of neuroprotection in stroke, because they would cause a reduction in blood pressure (Rothermund et al., 2000). Nevertheless, YC-1 could serve as a prototype for the synthesis of more selective Na<sup>+</sup> channel inhibitors that would have potential as therapy for degenerative disorders affecting white matter axons, such as glaucoma and spinal cord injury, and possibly those affecting gray matter as well. Finally, the identification of this additional mechanism of action of YC-1 has implications for the growing use of this agent to probe sGC-related functions, as it now becomes necessary to ensure that any effects seen are not caused, or complicated, by Na<sup>+</sup> channel inhibition. With the development of structural analogs of YC-1 for possible clinical use (Stasch et al., 2001), this issue assumes increasing importance.

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