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N^G-Nitro-L-arginine protects against hypoxia/hypoglycemia-induced decrease in CA1 presynaptic spikes in rat hippocampal slices

Shigenobu Shibata *, Yasuharu Yamamoto, Takeshi Tanaka, Shigenori Watanabe

Department of Pharmacology, Faculty of Pharmaceutical Sciences, Kyushu University 62, Fukuoka 812, Japan

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Abstract

The effects of nitric oxide (NO) synthase inhibitors on the hypoxia/hypoglycemia-induced decrease in CA1 presynaptic fiber spikes elicited by stimulation of the Schaffer collaterals were investigated using rat hippocampal slices. Drugs were added to normal medium for 10 min before incubation under hypoxic/hypoglycemic conditions (15 min), and after a 3-h washout, the CA1 presynaptic potential was measured. Treatment with N^G-nitro-L-arginine methyl ester but not with N^G-nitro-D-arginine methyl ester produced a concentration-dependent attenuation of the hypoxia/hypoglycemia-induced decrease in presynaptic fiber spikes. In contrast, treatment with precursors of NO in the arginine-to-NO pathway, such as sodium nitroprusside, *S*-nitro-*N*-acetylpenicillamine and *N*-morpholino sydnonimine exacerbated the 15-min hypoxia/hypoglycemia-induced decrease in the CA1 presynaptic potential. The neuroprotective effect of N^G-nitro-L-arginine methyl aster was significantly attenuated by co-treatment with L-arginine. The present results suggest a facilitatory role of NO production in hypoxia/hypoglycemia-induced presynaptic dysfunction in CA1 regions of hippocampal slices.

Keywords: Ischemia; Nitric oxide (NO); Hippocampus; Presynaptic potential; Neuroprotection

1. Introduction

Nitric oxide (NO) is a short-lived, highly reactive messenger molecule that is synthesized in a number of tissues, including the brain, by nitric oxide synthase from the terminal guanidino nitrogen atom(s) of L-arginine (Moncada et al., 1991). In addition to its proposed role as a neuronal messenger, NO has been suggested as the underlying factor in ischemic neuronal toxicity (Garthwaite, 1991; Moncada et al., 1991). Most studies have emphasized the detrimental role of an excessive release of glutamate/aspartate from the hippocampus and striatum in producing ischemic injury (Benveniste et al., 1984; Meldrum and Garthwaite, 1990). Microdialysis studies have shown that the ischemia-induced glutamate efflux is reduced after NO synthase inhibitor treatment in a rat model of focal ischemia (Buisson et al., 1993). The involvement *N*-methyl-D-aspartate (NMDA) receptors in ischemic neural damage was demonstrated in studies of the neuroprotective effect of NMDA receptor antagonists (Meldrum, 1990; Simon et al., 1984; Swan and Meldrum, 1990). NO was released in response to elevation of intracellular free Ca^{2+} following NMDA receptor stimulation in rat brain slices (Garthwaite, 1991; Moncada et al., 1991). The increase in NO concentration induced by transient middle cerebral artery occlusion was reported to be reversed to normal levels after the administration of a NO synthase inhibitor (Malinski et al., 1993).

Recent studies have investigated the role of NO synthase inhibitors as neuroprotective agents in animal models of global ischemia (Caldwell et al., 1994; Ohno et al., 1994; Trifiletti, 1992). As in in vivo studies, treatment of primary cortical cultures exposed to high glutamate or NMDA concentrations with inhibitors of NO synthase blocked the toxic insult (Dawson et al., 1991; Reif, 1993; Tamura et al., 1992).

Activation of glutamate receptors and increases in cellular Ca^{2+} have been considered to contribute to ischemic cell damage in vitro (Deshpande et al., 1987). As Ca^{2+} channels are located on the presynaptic nerve terminals, ischemia may impair not only excitatory postsynaptic potentials but also the presynaptic fiber

* Corresponding author. Fax 81-92-632-2752.

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volley. In fact, we demonstrated previously that CA1 presynaptic fiber spikes in hippocampal slices exposed to hypoxia/hypoglycemia medium were decreased, and that this decrease was attenuated by pretreatment with NMDA receptor antagonists and neuroprotective drugs (Minamoto et al., 1994; Shibata et al., 1992; Tanaka et al., 1994). Therefore, we propose that NO, which may be synthesized in the postsynaptic neurons in response to NMDA activation, diffuses back to the presynaptic terminal to inhibit spiking. Thus, the presynaptic fiber volley is suggested as a good marker to evaluate the mechanisms of presynaptic function (Dunwiddie and Miller, 1993).

Blockade of NO synthase with specific inhibitors has been reported to prevent NMDA- or hypoxia-induced excitotoxic damage in rat hippocampal slices (Izumi et al., 1992; Wallis et al., 1992). Recently, Wallis et al. (1993) reported that the neurotoxic effect of NO, assessed directly using NO gas, on the electrophysiological functioning of the CA3-CA1 synapse was predominantly mediated by postsynaptic neurons as NO was without significant effect on presynaptic activity. Therefore, the aim of the present study was to examine the effects of NO synthase inhibitors and NO donors on the hypoxia/hypoglycemia-induced decrease in CA1 presynaptic potential in hippocampal slices.

2. Materials and methods

2.1. Subjects

Male Wistar rats weighing 300–400 g were used. The animals were decapitated and the brain was quickly removed. Parasagittal hippocampal slices (450 μm thick) were then prepared from the dorsal hippocampus of each animal, using a tissue chopper. The composition of the control Krebs-Ringer's solution equilibrated with 95% O_2–5% CO_2 gas mixture, was (in mM): NaCl 129, $MgSO_4$ 1.3, $NaHCO_3$ 22.4, KH_2PO_4 1.2, KCl 4.2, glucose 10.0, $CaCl_2$ 1.5. To induce hypoglycemia, the glucose in the incubation medium was replaced by 10 mM sucrose. The hypoxia solution was equilibrated with 95% N_2–5% CO_2 gas mixture for at least 1 h. The buffer had a pH of 7.3–7.4 and the temperature was kept at 37°C. Preparations were preincubated with normal Krebs-Ringer's solution for 1 h in a recirculation submersion chamber. Our chamber design, slice transfer methods and incubation procedures have been reported previously (Minamoto et al., 1994; Shibata et al., 1992; Tanaka et al., 1994).

2.2. Electrophysiology

Presynaptic potentials were recorded through a glass micropipette filled with normal physiological saline (DC resistance, 0.5–1 MΩ). Stimulation pulses were 0.2 Hz, 0.05 ms in duration and 0.9 mA in intensity and produced a maximal response in the normal non-hypoxic/hypoglycemic group (Minamoto et al., 1994; Tanaka et al., 1994). Extracellular recordings of presynaptic fiber spikes in the stratum radiatum of the CA1 region were made and the latency of the negative portion of the presynaptic fiber spike was fixed at 2.0 ms. Presynaptic fiber spike amplitudes were quantified as a peak-to-peak measurement between the negative peak of the fiber spike and the positivity which immediately preceded the negativity, or between the prestimulus baseline and the negative peak of the fiber spike.

2.3. Drugs

The drugs used in this study were N^G-nitro-L-arginine methyl ester, N^G-nitro-D-arginine methyl ester, L-arginine, sodium nitroprusside, N^2, 2′-O-dibutyrylguanosine 3′ : 5′-cyclic monophosphate (DB-cGMP), *S*-nitro-*N*-acetylpenicillamine (SNAP), *N*-morpholino sydnonimine hydrochloride (SIN-1) and 6,7-dinitroquinoxaline-2,3-dione (DNQX). All drugs were obtained from Sigma Chemicals (USA), Funakoshi Inc. (Tokyo, Japan) or Dojindo (Kumamoto, Japan). The drugs were dissolved in distilled water or dimethylsulfonic acid at concentrations of 100 × the final concentration. There were no solvent effects (Minamoto et al., 1994; Shibata et al., 1992; Tanaka et al., 1994).

2.4. Procedure and data analysis

After a 1-h preincubation, hippocampal slices were exposed to hypoxia/hypoglycemia for 5–15 min. The drugs were added to normal medium 10 min before the incubation under hypoxic/hypoglycemic conditions, and, after a 3-h washout, the CA1 presynaptic potential was measured. To reduce the occurrence of fast excitatory postsynaptic potential and/or population spikes during recording periods, the hippocampal CA1 presynaptic potential evoked by Schaffer collateral stimulation was recorded in a submerged chamber with perfusion of normal Krebs-Ringer's solution (4 ml/min) containing 10 μM DNQX (Dunwiddie and Miller, 1993) at 37°C. DNQX was not applied during hypoxic/hypoglycemic incubation to maintain the activation of NMDA receptor-dependent NO formation.

In previous experiments we found that there was no significant inter-slice variability in the amplitude of presynaptic fiber spikes (Minamoto et al., 1994; Tanaka et al., 1994). To normalize experimental data, the amplitude of presynaptic potential in non-hypoxic/hypoglycemic slices was set at 100%. We averaged the amplitude of this potential across experimental groups and calculated their means and S.E. The significance of differences between groups was determined by Stu-

dent's t-test or ANOVA followed by Duncan's test for individual comparisons.

3. Results

The amplitude of CA1 presynaptic fiber spikes in control hippocampal slices was 1.60 ± 0.03 mV (n = 39), while in slices exposed to 15-min hypoxia/hypoglycemia the amplitude was 0.83 ± 0.03 mV (n = 39) ($P < 0.01$, Student's t-test) after a 3-h washout. Representative examples of the CA1 presynaptic potentials elicited by stimulation of Schaffer collaterals are shown in Fig. 1. The hypoxia/hypoglycemia-induced decrease in amplitude was attenuated by treatment with N^G-nitro-L-arginine methyl ester (Figs. 1D and 2) in a concentration-dependent manner ($F(3, 43) = 6.6$, $P < 0.01$), but not by addition of the inactive

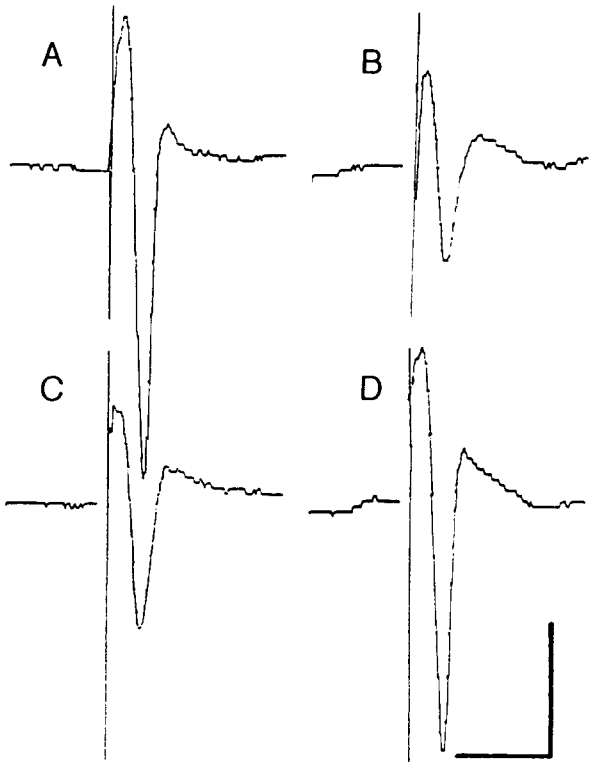

Fig. 1. Effects of N^G-nitro-L-arginine methylester and N^G-nitro-D-arginine methyl ester on the hypoxic/hypoglycemic decreases in CA1 presynaptic potential in rat hippocampal slices. All observations were recorded 3 h after 15 min of combined hypoxia/hypoglycemia. (A) CA1 presynaptic potential evoked by stimulation of Schaffer collaterals (0.05 ms, 0.9 mA) under normal conditions. (B) CA1 presynaptic potential evoked in hippocampal slices at 3 h after 15 min of hypoxia/hypoglycemia. (C) Effect of N^G-nitro-D-arginine methyl ester (100 μM). (D) Effect of N^G-nitro-L-arginine methyl ester (100 μM). Hippocampal slices were exposed to Krebs-Ringer's solution containing each of the drugs for 10 min before the induction of hypoxia/hypoglycemia and during the 15-min ischemic period. Each trace is an average of eight sweeps. Calibrations: 5 ms and 0.5 mV.

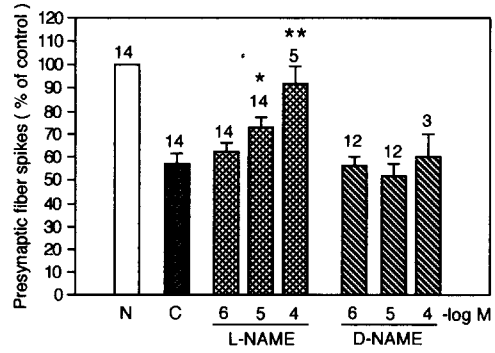

Fig. 2. Effects of N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-L-arginine methyl ester (D-NAME) on the decrease in CA1 presynaptic potential induced by 15 min of hypoxia/hypoglycemia. The amplitude of the CA1 presynaptic potential in control slices subjected to 15 min of non-hypoxic/hypoglycemic solution (N) was regarded as 100%. All observations were recorded at 3 h after hypoxia/hypoglycemia. N and C: vehicle-treated normal and hypoxic/hypoglycemic slices, respectively. Values are shown as means ± S.E.M. Numbers on columns indicate the number of slices. $^*P < 0.05$, $^{**}P < 0.01$ vs. ischemic control group (C) (ANOVA followed by Duncan's test).

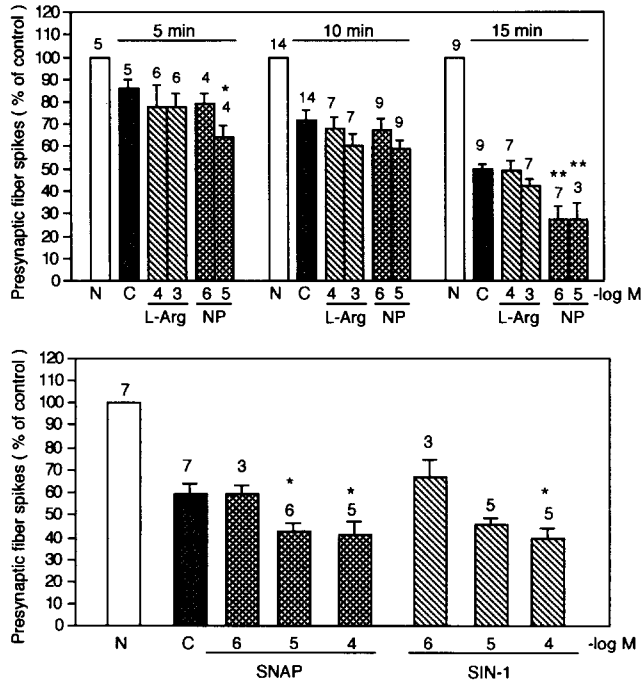

Fig. 3. Effects of L-arginine (L-Arg), sodium nitroprusside (NP), S-nitro-N-acetylpenicillamine (SNAP) and N-morpholino sydnonimine hydrochloride (SIN-1) on the decrease in CA1 presynaptic potential induced by 5–15 min of hypoxia/hypoglycemia. The amplitude of the CA1 presynaptic potential in control slices subjected to 5–15 min (upper panel) or 15 min (lower panel) of non-hypoxia/hypoglycemia solution (N) was regarded as 100%. All observations were recorded at 3 h after hypoxia/hypoglycemia. N and C: vehicle-treated normal and hypoxic/hypoglycemic slices, respectively. Values are shown as means ± S.E.M. Numbers on columns indicate the number of slices. $^*P < 0.05$, $^{**}P < 0.01$ vs. the ischemic control group (C) (ANOVA followed by Duncan's test).

isomer N^G-nitro-D-arginine methyl ester ($F(3, 37) = 0.4$, $P > 0.05$) (Figs. 1C and 2).

We observed the recovery rate of synaptic function in hippocampal slices after 5, 10 and 15 min of hypoxia/hypoglycemia (Fig. 3). The amplitude of CA1 presynaptic potentials decreased markedly, dependent upon the duration of the hypoxic/hypoglycemic conditions ($F(2, 25) = 17.5$, $P < 0.01$). L-Arginine did not affect the 5- ($F(2, 14) = 0.4$, $P > 0.05$), 10- ($F(2, 25) = 1.7$, $P > 0.05$) or 15-min-induced ($F(2, 20) = 1.7$, $P > 0.05$) decreases in CA1 presynaptic potentials. In contrast the 5- or 15-min-induced decline in CA1 presynaptic potentials was potentiated by treatment with sodium nitroprusside ($F(2, 10) = 5.8$, $P < 0.05$) in 5-min hypoxia/hypoglycemia, $F(2, 16) = 10.2$, $P < 0.01$ in 15-min hypoxia/hypoglycemia) (Fig. 3 upper panel). More specific NO donors such as *S*-nitro-*N*-acetyl-penicillamine and *N*-morpholino sydnonimine hydrochloride also significantly protected against the 15-min-induced decline in CA1 presynaptic potentials (Fig. 3, lower panel).

As the N^G-nitro-L-arginine methyl ester showed a protective effect against the presynaptic potential decrease induced by 15 min of hypoxia/hypoglycemia, we investigated whether co-exposure to N^G-nitro-L-arginine methyl ester plus L-arginine would block the N^G-nitro-L-arginine-induced neuroprotection. The protective effect of N^G-nitro-L-arginine methyl ester on the hypoxia/hypoglycemia-induced decrease in the CA1 presynaptic potential was significantly blocked by addition of L-arginine (Fig. 4).

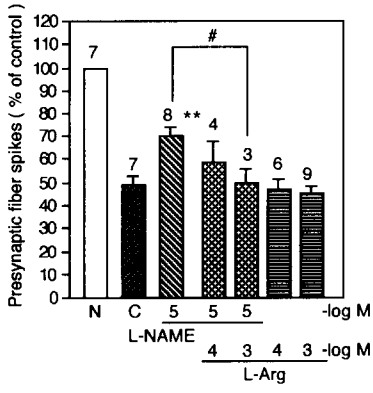

Fig. 4. Effects of L-arginine (L-Arg) on N^G-nitro-L-arginine methyl ester (L-NAME)-induced protection against the decrease in CA1 presynaptic potential induced by 15 min of hypoxia/hypoglycemia. The amplitude of the CA1 presynaptic potential in non-hypoxic/hypoglycemic control slices was regarded as 100%. All observations were recorded at 3 h after hypoxia/hypoglycemia. N and C: vehicle-treated normal and hypoxic/hypoglycemic slices, respectively. Values are shown as means ± S.E.M. Numbers on columns indicate the number of slices. Both agents were applied together from 10 min before the induction of hypoxia/hypoglycemia and during the 15-min ischemic period. $^{**}P < 0.01$ vs. the ischemic control group (C) (ANOVA followed by Duncan's test) and $^{\#}P < 0.05$ vs. N^G-nitro-L-arginine-treated alone (ANOVA followed by Duncan's test).

Not only NMDA but also sodium nitroprusside increases cGMP levels in rat brain cell cultures (East and Garthwaite, 1991; Garthwaite, 1991). Therefore, we investigated the effects of N^2,2′-O-dibutyrylguanosine 3′:5′-cyclic monophosphate (DB-cGMP) on the hypoxia/hypoglycemia-induced reduction in CA1 presynaptic potential. Pretreatment with this agent (10 and 100 μM) failed to attenuate the reduction of presynaptic potential ($F(2, 28) = 1.6$, $P > 0.05$) (52 ± 1.2% (n = 11) for hypoxia/hypoglycemia control, 53 ± 3.8% (n = 10) for 10 μM treatment and 62 ± 4.9% (n = 10) for 100 μM treatment).

Treatment with drugs for 25 min in normal non-hypoxic/hypoglycemic solution did not change the CA1 presynaptic potential after a 3-h washout. The mean amplitudes of CA1 presynaptic potential in hippocampal slices treated with L-NAME (100 μM), L-arginine (1 mM) or sodium nitroprusside (10 μM) for 25 min were 103 ± 13% (n = 7), 105 ± 11% (n = 4), or 92 ± 8.1% (n = 3), respectively.

4. Discussion

The present results demonstrated that the reduction in CA1 presynaptic fiber spikes in hippocampal slices induced by hypoxia/hypoglycemia was attenuated by a NO synthase inhibitor, N^G-nitro-L-arginine methyl ester, but not by an inactive isomer, N^G-nitro-D-arginine methyl ester. Thus, NO synthase inhibitors may provide neuroprotection against hippocampal presynaptic dysfunction. The protective effect of N^G-nitro-L-arginine methyl ester on the deficit in CA1 presynaptic potential was reversed in a concentration-dependent fashion by L-arginine. This indicates that the N^G-nitro-L-arginine methylester-induced neuroprotection was due to inhibition of NO synthase and not to any side effects of this agent. The present results were in accordance with those of a previous report in which the neuroprotective effect of N^G-nitro-L-arginine against cell death elicited by NMDA was shown to be reversed by L-arginine (Dawson et al., 1991).

Recent studies have investigated the role of NO synthase inhibitors as neuroprotective agents in animal models of global ischemia (Caldwell et al., 1994; Ohno et al., 1994; Trifiletti, 1992) and focal ischemia (Buisson et al., 1992; Nagafuji et al., 1992; Nowicki et al., 1991). In primary cell cultures, NO synthase inhibitors have been observed to have protective actions against glutamate- or NMDA-mediated cell damage (Dawson et al., 1991; Tamura et al., 1992). In addition, a recent in vivo study demonstrated that N^G-nitro-L-arginine

protected against CA1 neuronal damage following focal injection of NMDA into the rat hippocampus (Moncada et al., 1992). However, several studies have found NO synthase inhibitors to give no protection or indeed to exacerbate ischemic injury following both focal and global ischemic insults (Dawson et al., 1992; Moncada et al., 1992; Weismann et al., 1992; Yamamoto et al., 1992; Zhang and Iadecola, 1993). Conversely, under certain conditions NO enhancement strategies can also be neuroprotective both in vitro and in vivo (Demerle-Pallardy et al., 1991; Lipton et al., 1993; Morikawa et al., 1992; Zhang and Iadecola, 1993). Thus, there is a great deal of contradictory evidence in the literature. These discrepancies may be explained by the use of different animal models, by dose/timing of administration of NO synthase inhibitors, and by the effects of NO synthase inhibitors on physiological variables know to influence stroke volume (blood pressure, cerebral blood flow, body temperature, and type/use of anesthetic).

The mechanism of the neuroprotective effect of N^G-nitro-L-arginine methyl ester on the reduction of CA1 presynaptic potential induced by hypoxia/hypoglycemia remains to be determined. Most studies have emphasized the detrimental role of an excessive release of glutamate/aspartate from the hippocampus and striatum in producing ischemic injury (Benveniste et al., 1984; Meldrum and Garthwaite, 1990). Microdialysis studies have shown that ischemia-induced glutamate efflux is reduced after NO synthase inhibitor treatment in a rat model of focal ischemia (Buisson et al., 1993). The involvement of NMDA receptors in ischemic neural damage was demonstrated by studies of the neuroprotective effect of NMDA receptor antagonists (Meldrum, 1990; Simon et al., 1984; Swan and Meldrum, 1990). It can be postulated that the stimulation of the NMDA receptor induced by ischemic insult could raise cytosolic calcium, due largely to an increased influx through the receptor-operated ion channels, and this calcium influx could activate NO synthase, leading to an overproduction of NO (Garthwaite, 1991; Moncada et al., 1991). In the present study, we provided evidence that a NO synthase inhibitor has neuroprotective activity against hypoxia/hypoglycemia-induced presynaptic dysfunction in hippocampal slices.

Application of sodium nitroprusside potentiated the reduction of CA1 presynaptic potential induced by 5 min or 15 min of ischemia. Dawson et al. (1991) reported that sodium nitroprusside elicited neurotoxicity in primary cortical cultures. We used sodium nitroprusside as a NO donor to demonstrate that NO can actively potentiate the ischemia-induced decrease in presynaptic potentials. However, it is now well-established that sodium nitroprusside can interact with NMDA receptors independently of NO (Kiedrowski et al., 1992), and more importantly that this agent can also induce CA1 neurotoxicity via a NO-independent mechanism (Izumi et al., 1993). Therefore, we used the more specific NO donors *S*-nitro-*N*-acetylpenicillamine and *N*-morpholino sydnonimine hydrochloride, and showed that these agents significantly potentiated the hypoxia/hypoglycemia-induced decrease in CA1 presynaptic potentials. Recently, Wallis et al. (1993) reported that the neurotoxic effect of NO was predominantly mediated at postsynaptic neurons as NO was without significant effect on presynaptic activity. We observed no significant effect of sodium nitroprusside on CA1 presynaptic potentials when this agent was applied for 25 min under non-ischemic conditions. The present results suggest that NO production may have a facilitatory effect against hypoxia/hypoglycemia-induced deficits in presynaptic activity.

Not only NMDA but also sodium nitroprusside has been shown to increase cGMP levels in rat brain cell cultures (East and Garthwaite, 1991; Garthwaite, 1991). The accumulation of cGMP evoked by L-glutamate was inhibited by an NMDA antagonist, MK-801, and by NO synthase inhibitors (Dawson et al., 1991; Moncada et al., 1991). However, application of cGMP failed to produce a neurotoxic effect on CA1 presynaptic potentials. Thus, the present study suggests that elevation of cGMP levels mediated by NO may not be involved in the presynaptic dysfunction in hippocampal slices.

In summary, the present results demonstrated that stimulation of NO production played a detrimental role in the development of ischemic damage, whereas the blockade of NO production played a neuroprotective role under ischemic conditions. This suggests a facilitatory role for NO formation in ischemia-induced presynaptic dysfunction in CA1 regions of hippocampal slices.

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