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The influence of nitric oxide donors on the responses to nitrergic nerve stimulation in the mouse duodenum

Nuran Öğülener *, Yusuf Ergün, Naciye Döndaş, Atilla Dikmen

Department of Pharmacology, Medical Faculty, Çukurova University, 01330 Adana, Turkey

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Abstract

We investigated whether exogenous nitric oxide (NO) donors have a prejunctional and/or postjunctional inhibitory effect on the nitrergic responses and whether this inhibitory effect was mediated by NO itself and in part, by cyclic GMP in mouse duodenal strips. N^{ω}-nitro-L-arginine inhibited relaxations induced by electrical field stimulation of nitrergic nerves, but not those with acidified $NaNO_2$. Furthermore, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) inhibited both types of relaxations while 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine (AMT) and *N*-ethylmaleimide were ineffective. NO donors, nitroglycerin and sodium nitroprusside, inhibited relaxations induced by nitrergic nerve stimulation, but not those with acidified $NaNO_2$. Hemoglobin, exogenous Cu^{2+}/Zn^{2+} superoxide dismutase, diethyldithiocarbamic acid and pyrogallol did not influence the relaxation with nitrergic nerve stimulation. However, hemoglobin, diethyldithiocarbamic acid, pyrogallol and diethyldithiocarbamic acid plus pyrogallol attenuated the inhibitory effect of NO donors on relaxation with nitrergic nerve stimulation, and exogenous superoxide dismutase potentiated this inhibitory effect. Moreover, nitrergic nerve-mediated relaxations were inhibited by 8-bromo-cyclic GMP, but not by 8-bromo-cyclic AMP. These results suggest that exogenous NO donors have a prejunctional inhibitory effect on the nerve-mediated nitrergic relaxation and that the inhibitory effects of nitroglycerin and sodium nitroprusside are NO-dependent, but not related to NO metabolites such as peroxynitrite or a nitrosothiol intermediate. However, a contribution of *S*-nitrosothiol formed intracellularly cannot be entirely ruled out. Also, this prejunctional inhibition is mediated, at least in part, by the cyclic GMP, but not the cyclic AMP, pathway.

Keywords: Duodenum, mouse; Nitrergic neurotransmission; Nitric oxide (NO); Nitric oxide donor; Hemoglobin; Cu^{2+}/Zn^{2+} superoxide dismutase, endogenous/exogenous; Pyrogallol

1. Introduction

Nitric oxide (NO) is formed via the enzymatic activity of NO synthase (NOS), which catalyzes the oxidation of L-arginine to NO plus L-citrulline. There are several different molecular forms of NOS, including neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) (Moncada et al., 1991; Brookes, 1993; Rand and Li, 1995; Kröncke et al., 1995). At the smooth muscle neuromuscular junction in the gut, nNOS has primarily been localized to nerve endings (Murthy et al., 1993; Jarvinen et al., 1999). Recently, experimental results from molecular biological studies have indicated that NO may have an autoregulatory role via negative feedback on NOS activity (Griscavage et al., 1995). It was suggested that NO inhibits the activity of the constitutive isoforms of NOS from rat and bovine cerebellum or bovine aortic endothelial cells, as well as the inducible isoform of NOS from activated murine macrophages (Rogers and Ignarro, 1992; Rengasamy and Johns, 1993; Buga et al., 1993; Assreuy et al., 1993; Griscavage et al., 1994) In addition, it has been shown that exogenous NO inhibited basal NO release from vascular endothelium both in vivo and in vitro (Ma et al., 1996). However, it is not completely established whether NO regulates its own synthase and release in the nerve terminals. De Man et al. (1995) demonstrated that prolonged exposure to NO donors inhibited electrically induced nerve-mediated nitrergic relaxations without affecting the postjunctional response to NO or vasoactive intestinal polypeptide, pointing to a prejunctional inhibitory effect on the nerve-mediated nitrergic responses in the rat gastric fundus. Similar findings were reported by Hosoda et al. (1998) for the rat gastric myenteric plexus, and it was proposed that NO synthesis and release were prejunctionaly inhibited by NO and that this inhibition was mediated, at least in part, through the cyclic GMP path-

* Corresponding author. Tel.: +90-322-338-6060/3239; fax: +90-322-338-6572/6847.

E-mail address: ogulener@mail.cu.edu.tr (N. Öğülener).

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way. However, Lefèbvre and Vandekerckhove (1998) observed that nitroglycerin did inhibit both electrical field stimulation- and exogenous NO-induced relaxations and suggested that this effect was due to postjunctional tolerance to nitrergic stimuli in the pig gastric fundus. On the other hand, a recent study provided evidence that inhibition of enteric NOS by exogenous NO was mediated via the intermediate formation of nitrosothiol (Kurjak et al., 1999). However, the precise mechanism(s) by which NO inhibits NOS activity is still unknown. The first aim of the present study was to elucidate the role of NO in non-adrenergic non-cholinergic relaxations induced by electrical field stimulation in mouse duodenum. Secondly, we aimed to investigate whether exogenous NO donors have a prejunctional and/or postjunctional inhibitory effect on the nitrergic responses and, if this was the case, to investigate whether this effect was due to NO itself. It is known that superoxide anion generation is unavoidable in a variety of cell lines and NO reacts with superoxide anion to form peroxynitrite, therefore inhibition of NOS in response to NO donors may be mediated by peroxynitrite. Finally, we further studied whether cyclic GMP is involved in this inhibition.

2. Materials and methods

2.1. Tissue preparation

Swiss albino mice of either sex, weighing 20–25 g, were used in these experiments. They were fasted for 24 h with free access to water. They were killed by stunning and cervical dislocation. Duodenal segments were rapidly removed and the proximal portion of duodenum (approximately 12–15-mm long) was mounted as a tube under 0.2-g tension in a 20-ml organ bath filled with Krebs solution (in mM: NaCl 117.9, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 0.89, $NaHCO_3$ 25, glucose 10.1, Na_2EDTA 0.05 and ascorbic acid 0.02). Throughout the experiments, atropine (1 μM) and guanethidine (1 μM) were present in the bathing medium to obtain non-adrenergic non-cholinergic (NANC) conditions. In preliminary experiments, we tested different temperatures, i.e. 32°C and 25°C, since strips had excess spontaneous activity at 37°C. In conclusion, the only temperature in which the strips reached stability was 25°C. Therefore, the solution was maintained at 25°C and aerated with a mixture of 95% O_2 and 5% CO_2. The tissues were equilibrated for 60 min with rinsing at 15-min intervals. Changes in muscle length were recorded isotonically via an isotonic transducer (Ugo Basile 7006) connected to an ink-writing recorder (Ugo Basile, Gemini 7070).

2.2. Experimental protocols

In cumulative concentration–response curves on mouse duodenal strips, 0.1 μM serotonin produced a contraction, which represented 81.5 ± 2.7% of the maximum attainable contraction with serotonin ($n = 8$). All strips were contracted with 0.1 μM serotonin. This resulted in an active tone that reached a stable level within 5 min; at the end of this period, electrical field stimulation (1, 5 and 10 Hz; 25 V, 1 ms) was applied to the tissue for 10 s at 5-min intervals. To study the relaxant action, acidified $NaNO_2$ (1, 10 and 100 μM), nitroglycerin (500 μM), sodium nitroprusside (500 μM), and isoproterenol (10 nM) were added to the pre-contracted tissue. Only one type of relaxant stimulus (train stimulation or relaxant chemical bolus) was studied per tissue, and after the relaxant response had been obtained, the tissues were rinsed at 10-min intervals for at least 30 min and the second series of responses were recorded in the same manner. These second series of relaxations were assessed as control groups for further experiments.

In a first series of experiments, the effects of N^{ω}-nitro-L-arginine (10–50 μM), an NOS inhibitor, 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine (AMT; 10 μM), an inducible NOS inhibitor, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ; 100 μM), selective inhibitor of guanylate cyclase and *N*-ethylmaleimide (10 μM), an inhibitor of adenylate cyclase, were investigated on relaxations in response to nitrergic nerve stimulation and chemicals. After the first responses were recorded, the above-mentioned substances were added to the medium, and at the end of 30 min incubation, the second series of relaxations due to nitrergic nerve stimulation or chemicals was examined.

In a second series of experiments, the inhibitory effects of NO donors, nitroglycerin (0.1–500 μM) or sodium nitroprusside (0.1–500 μM), were studied on the relaxant responses to nitrergic nerve stimulation or acidified $NaNO_2$. To study inhibitory effects of NO donors, we used two different protocols. In the first, after the responses to nitrergic nerve stimulation or acidified $NaNO_2$ were obtained, the strips were incubated for 30 min with NO donors, and the relaxant stimuli were studied in the presence of NO donors. In the second protocol, after the first control responses to nitrergic nerve stimulation had been recorded, the tissue was incubated with nitroglycerin (500 μM) or sodium nitroprusside (500 μM) for 30 min, and nitrergic nerve stimulation was studied for the second time; then followed by the incubation with NO donors for 30 min, and nitrergic nerve stimulation was applied for the third time. We investigated the time point at which the NO donors achieved their inhibition on nitrergic nerve-induced relaxation by testing several incubation periods. The inhibitory effects of NO donors on nitrergic nerve-mediated relaxations were not observed after 1 or 10 min, but only at the end of 30 min, which was chosen as the incubation time. The basal tone of the tissue was not altered by NO donors before the next administration of serotonin, so that the contraction level at which relaxant stimuli were tested after incubation with NO donors was similar to that of

before. Further, the effects of NO donor pretreatment were investigated on the relaxant response to isoproterenol (10 nM).

In a third series of experiments, the effects of hemoglobin (20 μM), NO scavenger, exogenous Cu^{2+}/Zn^{2+} superoxide dismutase (200 U/ml), diethyldithiocarbamic acid (8 mM), an inhibitor of the endogenous Cu^{2+}/Zn^{2+} superoxide dismutase or pyrogallol (50 μM), a superoxide anion generator per se were studied on nitrergic nerve stimulation-induced relaxations as follows. After the first control responses to nitrergic nerve stimulation were obtained, the above-mentioned substances were added to the medium, and nitrergic stimulation was applied for the second time. The tissue was incubated with hemoglobin, diethyldithiocarbamic acid or pyrogallol for 30 min; exogenous Cu^{2+}/Zn^{2+} superoxide dismutase was added on top of the contraction 2 min before nitrergic stimulation was applied. Furthermore, to clarify whether the inhibitory effects of NO donors on nitrergic nerve-mediated relaxation were NO-dependent, effects of hemoglobin, exogenous Cu^{2+}/Zn^{2+} superoxide dismutase, diethyldithiocarbamic acid or pyrogallol on the inhibitory action of NO donors were studied. In some experiments, these substances were added at the end of the second series of relaxation due to nitrergic nerve stimulation and were incubated in the presence of NO donors.

In other experimental groups, the effect of 8-bromo-cyclic GMP (30 μM) or 8-bromo-cyclic AMP (30 μM) was investigated on relaxations in response to nitrergic nerve stimulation or acidified $NaNO_2$.

At the end of each experiment, papaverine (50 μM) was added to the organ bath to confirm the responsiveness of the tissue to relaxant stimuli.

2.3. Drugs and solutions

Atropine sulphate, guanethidine sulfate, serotonin hydrochloride (5-hydroxytryptamine), sodium nitroprusside dihydrate, N^{ω}-nitro-L-arginine, 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine, *N*-ethylmaleimide, 8-bromo-guanosine 3′,5′-cyclic monophosphate sodium salt, 8-bromo-adenosine 3′,5′-cyclic monophosphate sodium salt, human hemoglobin, superoxide dismutase (from bovine erythrocytes), diethyldithiocarbamic acid sodium salt, pyrogallol and isoproterenol hydrochloride were obtained from Sigma (St. Louis, USA). Nitroglycerin and 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one were kindly provided by Adeka (Adeka Drug, Samsun, Turkey; Schwarz Farma, Monheim, Germany) and The Wolfson Institute for Biomedical Research, University College London, as gifts, respectively. Acidified sodium nitrite was obtained by diluting sodium nitrite in de-aerated water acidified to pH 2 with HCl, was stored at −4°C and used at its original concentrations as 1, 10 and 100 μM. (Cocks and Angus, 1990). Drugs were dissolved in distilled water, except 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one (in dimethylsulphoxide) and nitroglycerin (1 mg ml^{-1} solution in dextrose 5%, polyethylene glycol 0.1 and water); the final concentrations of these solvents showed no significant biological effects.

2.4. Presentation of results and statistical analysis

Relaxations were calculated as percentage peak reductions of the serotonin contraction. Results were expressed as means ± S.E.M., and *n* refers to strips from different animals. Results between tissues were compared using an unpaired Student's *t*-test, and when more than two groups were tested, the analysis of variance (ANOVA) and the *t*-test corrected for multiple comparisons (Bonferroni correction) were applied. *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. Relaxant effects of nitrergic nerve stimulation, acidified $NaNO_2$, nitroglycerin and sodium nitroprusside in mouse duodenal strips

Short-term electrical field stimulation (1, 5 and 10 Hz, 25 V, 1 ms, pulse trains of 10 s) of nitrergic nerves elicited frequency-dependent transient and reproducible relaxations which were fast in onset in 0.1 μM serotonin-contracted tissues treated with atropine and guanethidine ($n = 8$). Bolus injection of acidified $NaNO_2$ (1, 10 and 100 μM) caused fast and transient relaxations in a concentration-dependent manner ($n = 8$). Also, nitroglycerin (500 μM) and sodium nitroprusside (500 μM) induced fast but sustained and slow relaxations, respectively ($n = 6$). Data not shown.

3.2. Effects of N^{ω}-nitro-L-arginine, AMT, ODQ and N-ethylmaleimide on nitrergic relaxations

The nitrergic nerve-induced relaxations were antagonized by N^{ω}-nitro-L-arginine (10 and 50 μM). The effect of N^{ω}-nitro-L-arginine on nitrergic nerve-induced relaxations was concentration-dependent; nitrergic relaxations were completely abolished by 50 μM N^{ω}-nitro-L-arginine ($n = 6$, Fig. 1A). However, N^{ω}-nitro-L-arginine (50 μM) did not affect relaxations with acidified $NaNO_2$ (1, 10 and 100 μM, $n = 6$; Fig. 1A), nitroglycerin (500 μM) and sodium nitroprusside (500 μM) ($n = 6$, data not shown). On the other hand, AMT (10 μM), an iNOS inhibitor, did not inhibit nitrergic nerve-induced relaxations ($n = 7$, Fig. 1A). Pretreatment with 100 μM ODQ, a specific inhibitor of soluble guanylate cyclase, completely inhibited the relaxations induced by nitrergic nerve stimulation, acidified $NaNO_2$ (100 μM), nitroglycerin (500 μM) or sodium nitroprusside (500 μM) ($n = 6$, Fig. 1B). However, *N*-ethylmaleimide (10 μM), an adenylate cyclase inhibitor, did not inhibit relaxations with nitrergic nerve stimulation ($n = 6$, data not shown).

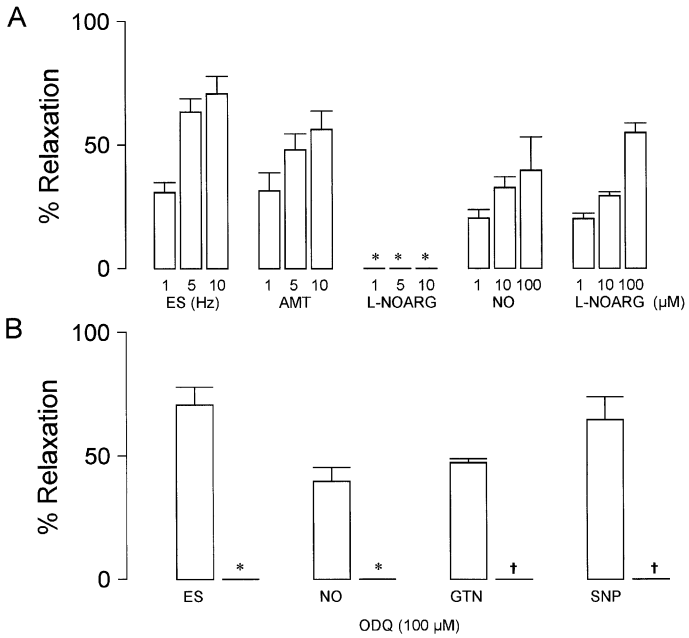

Fig. 1. (A) Effect of amino-5-6-dihidyro-6-methyl-4*H*-1,3-thiazine (AMT; 10 μM) on the nitrergic relaxations in response to nitrergic nerve stimulation (ES; 1, 5 and 10 Hz; 25 V, 1 ms) and effect of N^{ω}-nitro-L-arginine (L-NOARG; 50 μM) on the nitrergic relaxations in response to ES and acidified $NaNO_2$ (NO; 1, 10 and 100 μM) in mouse duodenal strips precontracted with 0.1 μM serotonin. (B) Effect of ODQ (100 μM) on the nitrergic relaxations in response to ES (10 Hz), acidified $NaNO_2$ (100 μM), nitroglycerin (500 μM) and sodium nitroprusside (500 μM). Data are expressed as means ± S.E.M. ($n = 6$). $^{*}P < 0.05$ significantly different from the control, one-way ANOVA followed by Bonferroni multiple comparison *t*-test. $^{*}P < 0.05$ significantly different from the control, unpaired Student's *t*-test. GTN: nitroglycerin, SNP: sodium nitroprusside.

3.3. Effect of nitroglycerin and sodium nitroprusside pretreatment on relaxations in response to nitrergic nerve stimulation or acidified $NaNO_2$

Pretreatment with nitroglycerin at 0.1, 1, 10 and 100 μM concentrations did not inhibit the amplitude of the nitrergic nerve stimulation to 1, 5 and 10 Hz. However, a higher concentration of nitroglycerin (500 μM) significantly inhibited relaxations caused by nitrergic nerve stimulation ($n = 6$, Fig. 2A). A similar inhibitory effect was observed on pretreatment with another NO donor, sodium nitroprusside. Incubation for 30 min with sodium nitro-

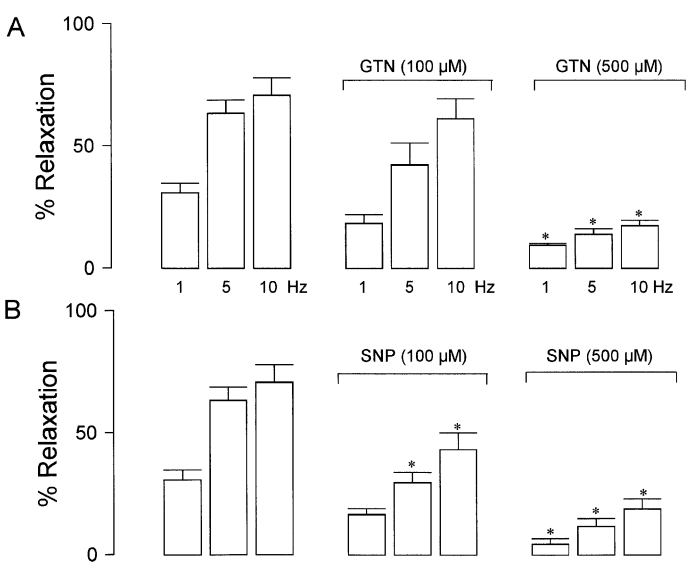

Fig. 2. Effects of nitroglycerin (100 and 500 μM) (A) and sodium nitroprusside (100 and 500 μM) (B) on the nitrergic relaxation in response to nitrergic nerve stimulation (ES 1, 5 and 10 Hz, 25 V, 1 ms) in the mouse duodenal strips precontracted with 0.1 μM serotonin. Data are expressed as means ± S.E.M. ($n = 6$). $^{*}P < 0.05$ is considered as significantly different from the control, one-way ANOVA followed by Bonferroni multiple comparison *t*-test. GTN: nitroglycerin, SNP: sodium nitroprusside.

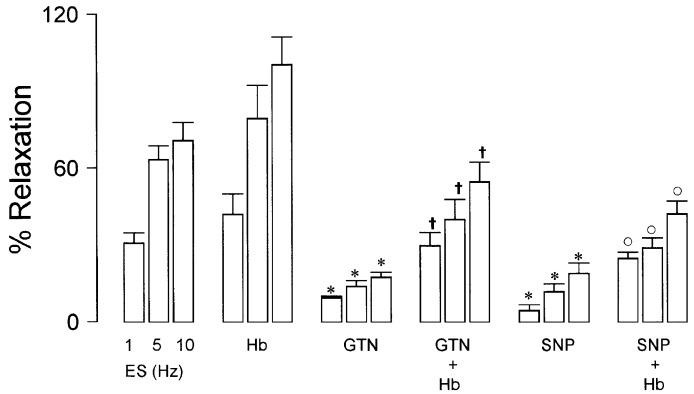

Fig. 3. Effect of hemoglobin (Hb; 20 μM) on the nitrergic relaxation in response to nitrergic nerve stimulation (ES 1, 5 and 10 Hz, 25 V, 1 ms) and on the inhibitory effects of nitroglycerin (500 μM) and sodium nitroprusside (500 μM) in mouse duodenal strips precontracted with 0.1 μM serotonin. Data are expressed as means ± S.E.M. ($n = 5$–6). $^{*}P < 0.05$: significantly different versus control; $^{*}P < 0.05$: significantly different versus nitroglycerin; $^{\circ}P < 0.05$: significantly different versus sodium nitroprusside, one-way ANOVA followed by Bonferroni multiple comparison *t*-test. GTN: nitroglycerin, SNP: sodium nitroprusside.

prusside, 0.1, 1 and 10 μM did not significantly inhibit the amplitude of the nitrergic nerve-induced relaxations, whereas pretreatment with 100 μM sodium nitroprusside significantly reduced nitrergic relaxation with 5 and 10 Hz ($n = 6$, Fig. 2B). At the concentration of 500 μM, nitrergic relaxations with 1, 5 and 10 Hz were significantly inhibited ($n = 6$, Figs. 2B and 5B). Nitroglycerin and sodium nitroprusside (0.1–500 μM) did not affect relaxations induced by acidified $NaNO_2$ (1–100 μM) ($n = 6$, data not shown). In addition, inhibitory effects of NO donors were not changed while the incubation period of NO donors was continued for a further 30 min and nitrergic nerve stimulation was applied for the third time ($n = 5$–6, data not shown). Duodenal strips recovered their responsiveness to nitrergic nerve stimulation within 30 min after withdrawal of the NO donors, ruling out a neurotoxic effect.

3.4. Effect of hemoglobin on inhibitory effects of NO donors

In order to determine whether the inhibitory effects of nitroglycerin and sodium nitroprusside on nitrergic nerve-induced relaxations were NO-dependent, we investigated

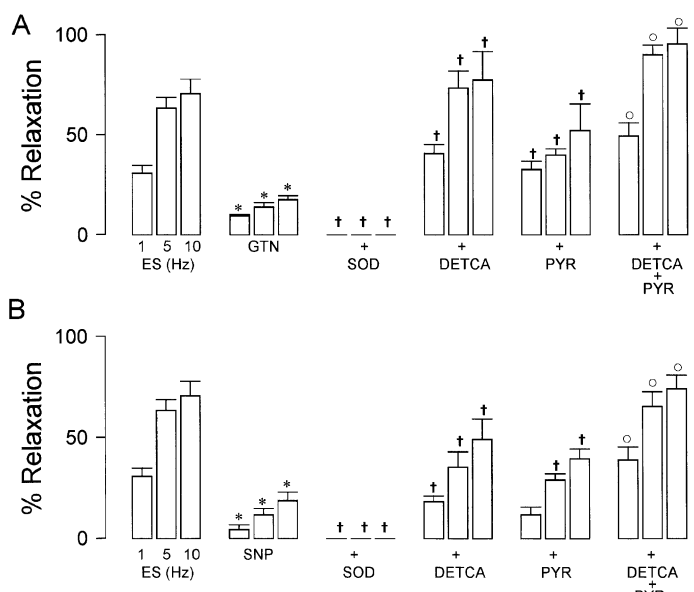

Fig. 4. Effects of exogenous Cu^{2+}/Zn^{2+} superoxide dismutase (SOD; 200 U/ml), diethyldithiocarbamic acid (DETCA; 8 mM), pyrogallol (PYR; 50 μM) and diethyldithiocarbamic acid plus pyrogallol (DETCA + PYR; 8 mM and 50 μM) on the inhibitory effects of nitroglycerin (500 μM) (A) and sodium nitroprusside (500 μM) (B) in mouse duodenal strips precontracted with 0.1 μM serotonin. Data are expressed as means ± S.E.M. ($n = 5$–6). $^{*}P < 0.05$: significantly different versus control; $^{*}P < 0.05$: significantly different versus nitroglycerin and sodium nitroprusside; $^{\circ}P < 0.05$: significantly different versus DETCA, one-way ANOVA followed by Bonferroni multiple comparison *t*-test. GTN: nitroglycerin, SNP: sodium nitroprusside.

the effect of the NO scavenger, hemoglobin, on the inhibition mediated by NO donors. The administration of hemoglobin (20 μM) alone did not significantly alter the relaxation in response to nitrergic nerve stimulation ($n = 6$, Fig. 3). On the other hand, 20 μM hemoglobin, administered simultaneously with nitroglycerin (500 μM) and sodium nitroprusside (100 and 500 μM) reduced the inhibitory effects of nitroglycerin and sodium nitroprusside on nitrergic nerve-mediated relaxations, but certainly did not prevent it ($n = 6$, Figs. 3 and 5C). In contrast, when it was administered after the inhibition of nitrergic relaxations in response to NO donors, hemoglobin failed to reverse the inhibitory action of NO donors ($n = 6$, data not shown).

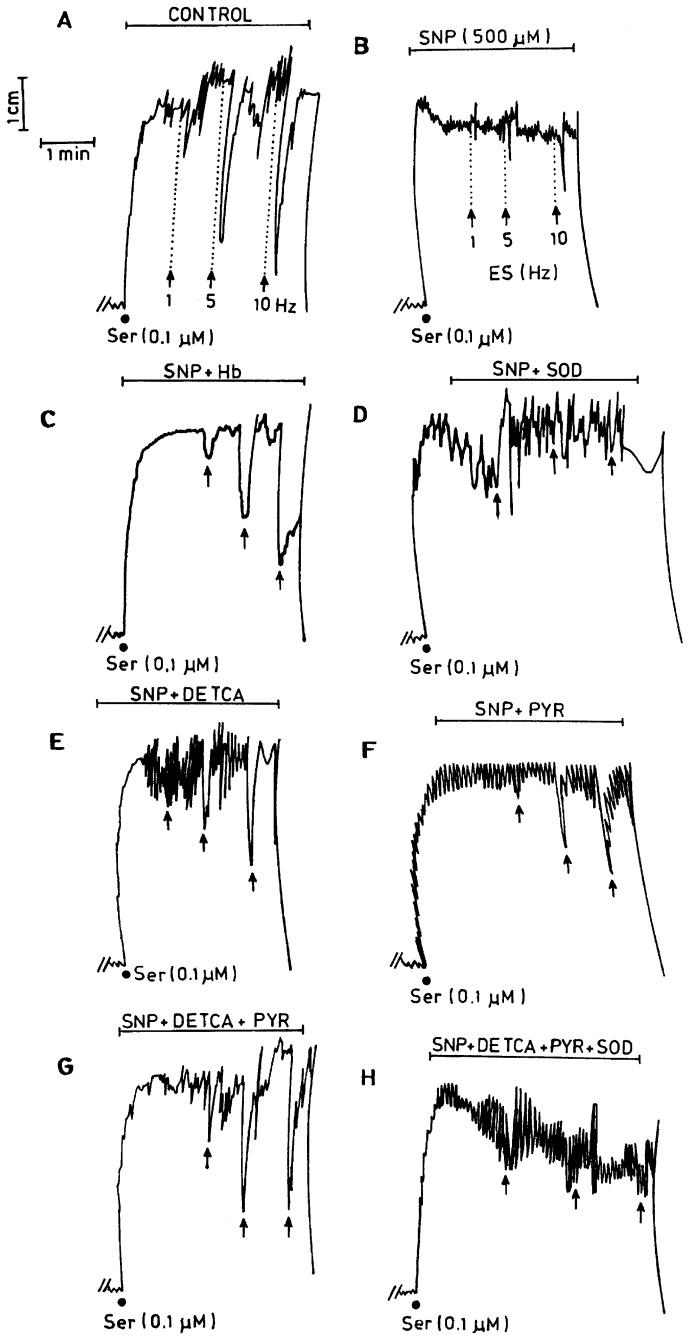

Fig. 5. Representative traces showing the relaxations induced by nitrergic nerve stimulation (ES 1, 5 and 10 Hz, 25 V, 1 ms) under control conditions (A) and in the presence of sodium nitroprusside (500 μM) (B). The effects of hemoglobin (Hb; 20 μM) (C), exogenous Cu^{2+}/Zn^{2+} superoxide dismutase (SOD; 200 U/ml) (D), diethyldithiocarbamic acid (DETCA; 8 mM) (E), pyrogallol (PYR; 50 μM) (F), diethyldithiocarbamic acid plus pyrogallol (DETCA + PYR; 8 mM and 50 μM) (G) and diethyldithiocarbamic acid plus pyrogallol plus exogenous superoxide dismutase (DETCA + PYR + SOD; 8 mM and 50 μM and 200 U/ml) (H) on the inhibitory effect of sodium nitroprusside (500 μM) in mouse duodenal strips precontracted with 0.1 μM serotonin (Ser). GTN: nitroglycerin, SNP: sodium nitroprusside.

3.5. Effects of exogenous Cu^{2+}/Zn^{2+} superoxide dismutase, diethyldithiocarbamic acid or pyrogallol on inhibitory effects of NO donors

To ascertain if the inhibitory effects of nitroglycerin and sodium nitroprusside on nitrergic relaxations result from free NO, but are not related to NO metabolites, such as peroxynitrite, the effect of the removal of superoxide anions by exogenous Cu^{2+}/Zn^{2+} superoxide dismutase was investigated. Both the exogenous supply of Cu^{2+}/Zn^{2+} superoxide dismutase and the inhibition of endogenous Cu^{2+}/Zn^{2+} superoxide dismutase by the specific inhibitor, diethyldithiocarbamic acid, were studied. Exogenous Cu^{2+}/Zn^{2+} superoxide dismutase (200 U/ml) per se did not significantly influence the relaxations induced by nitrergic nerve stimulation ($n = 4$, data not shown), but when superoxide dismutase (200 U/ml) was administered in the presence of NO donors (500 μM), the inhibitory effects of NO donors on nitrergic relaxations were further augmented and relaxations turned into contractions ($n = 6$, Figs. 4A,B and 5D). However, when it was added after the inhibition of nitrergic relaxations in responses to NO donors, exogenous Cu^{2+}/Zn^{2+} superoxide dismutase (200 U/ml) was ineffective ($n = 3$, data not shown).

Diethyldithiocarbamic acid (8 mM), an inhibitor of the endogenous Cu^{2+}/Zn^{2+} superoxide dismutase, did not affect the relaxations in response to nitrergic nerve stimulation ($n = 6$, data not shown), but the inhibitory effects of NO donors were significantly antagonized in the presence of diethyldithiocarbamic acid (8 mM) ($n = 5–6$, Figs. 4A,B and 5E). Diethyldithiocarbamic acid (8 mM) did not reverse the inhibitory effects of the NO donor when it was added after the inhibitory effect of the NO donors on nitrergic nerve-mediated relaxations was observed ($n = 5–6$, data not shown).

Because the involvement of peroxynitrite in the inhibitory effect of NO donors could not be eliminated, the effect of pyrogallol, a superoxide anion generator, was investigated. Pyrogallol (50 μM) per se did not significantly influence either nitrergic nerve-mediated relaxation ($n = 6$, data not shown). However, the inhibitory effect of nitroglycerin (500 μM) on the nitrergic relaxation to 1, 5 and 10 Hz was significantly reversed by pyrogallol ($n = 5$, Fig. 4A). In addition, pyrogallol (50 μM) did not reverse the inhibitory effect of sodium nitroprusside (500 μM) on the nitrergic relaxation with 1 Hz, but significantly reversed the inhibitory effect of sodium nitroprusside on the nitrergic relaxation in response to 5 and 10 Hz ($n = 6$, Figs. 4B and 5F). Furthermore, the inhibitory effects of NO donors on nitrergic relaxations were not affected when pyrogallol was added after inhibitory responses of nitrergic relaxations to NO donors were obtained ($n = 6$, data not shown). Treatment with diethyldithiocarbamic acid (8 mM) plus pyrogallol prevented the inhibitory effect of NO donors and this prevention was more pronounced than that by diethyldithiocarbamic acid alone ($n = 6$, Figs. 4A,B and 5G). Exogenous Cu^{2+}/Zn^{2+} superoxide dismutase (200 U/ml) almost completely blocked the ability of pyrogallol plus diethyldithiocarbamic acid to inhibit the inhibitory effects of sodium nitroprusside (500 μM) on nitrergic relaxations ($n = 4$, Fig. 5H).

3.6. Effects of 8-bromo-cyclic GMP and 8-bromo-cyclic AMP pretreatment on nitrergic relaxations in response to nitrergic nerve stimulation and acidified $NaNO_2$

Incubation with 50 and 100 μM 8-bromo-cyclic GMP caused significant reduction of the serotonin-induced contraction, while the low concentration of 8-bromo-cyclic GMP (30 μM) did not affect the serotonin-induced contraction level. Pretreatment with 30 μM 8-bromo-cyclic GMP significantly reduced nitrergic nerve-mediated relaxations, but did not affect relaxations induced by acidified $NaNO_2$ ($n = 6$, Fig. 6). The inhibitory effect of 8-bromo-cyclic GMP on nitrergic relaxation was reversed by washing. On the other hand, 8-bromo-cyclic AMP (30 μM) did not inhibit relaxations induced by nitrergic nerve stimulation ($n = 6$, Fig. 6).

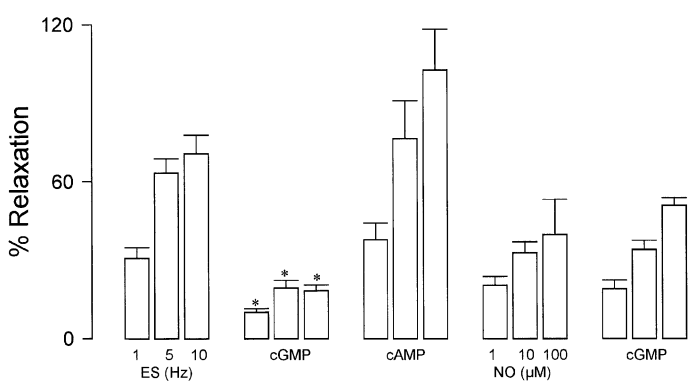

Fig. 6. Effects of 8-bromo-cyclic GMP (cGMP; 30 μM) and 8-bromo-cyclic AMP (cAMP; 30 μM) on the nitrergic relaxation in response to nitrergic nerve stimulation (ES 1, 5 and 10 Hz, 25 V, 1 ms) and acidified $NaNO_2$ (1, 10 and 100 μM) in mouse duodenal strips precontracted with 0.1 μM serotonin. Data are expressed as means ± S.E.M. ($n = 6$). $^{*}P < 0.05$ significantly different from the control, one-way ANOVA followed by Bonferroni multiple comparison *t*-test.

3.7. Effects of NO donor pretreatment on isoproterenol-induced relaxations

Isoproterenol (10 nM) induced sustained relaxations in the duodenal muscle strips. Pretreatment with nitroglycerin (500 μM) and sodium nitroprusside (500 μM) had no effect on isoproterenol-induced relaxations (control: 58.57 ± 4.29, isoproterenol + nitroglycerine: 63.07 ± 2.89; isoproterenol + sodium nitroprusside: 54.77 ± 4.23; $n = 6$).

4. Discussion

Results of recent studies suggest that NO may have a prejunctional and/or postjunctional inhibitory effect on nitrergic relaxations and point to the presence of an autoregulatory mechanism for the nitrergic innervation. In the rat duodenum, NO has been shown to mediate non-adrenergic non-cholinergic relaxations in response to electrical field stimulation (Martins et al., 1993; Postorino et al., 1995). Also, in the present study, relaxations induced by nitrergic nerve stimulation were completely abolished by N^{ω}-nitro-L-arginine and ODQ, an NOS inhibitor and a selective inhibitor of guanylate cyclase, respectively, which indicates that non-adrenergic non-cholinergic relaxations of mouse duodenum evoked by electrical field stimulation were mediated by NO release from nitrergic nerves. Furthermore, AMT, an inducible NO synthase inhibitor (Nakane et al.,1995; Tracey et al., 1995), did not inhibit relaxations with nitrergic nerve stimulation, suggesting that the constitutive type of NOS (Ca^{2+}/calmodulin-dependent), but not the inducible type of NOS (Ca^{2+}/calmodulin-independent), is activated by nitrergic nerve stimulation. Additionally, the presence of a cyclic AMP pathway was ruled out, since *N*-ethylmaleimide, an adenylate cyclase inhibitor, did not inhibit these relaxations.

To investigate the effect of NO-pretreatment on the nitrergic component in the mouse duodenum, we studied the effects of NO-releasing compounds, nitroglycerin and sodium nitroprusside, which enzymatically and non-enzymatically generate NO, respectively, on the NO-mediated relaxations with nitrergic nerve stimulation and acidified $NaNO_2$. We observed inhibition of nitrergic relaxations at higher concentrations of NO donors (100–500 μM). Similar concentrations of nitroglycerin and sodium nitroprusside were required in studies where the inhibitory effect of NO donors on the nitrergic relaxation was determined (De Man et al., 1995; Lefèbvre and Vandekerckhove, 1998). The requirement of a high concentration is most likely due to incomplete metabolism of these agents to NO. Pretreatment with the NO donors, nitroglycerin and sodium nitroprusside, significantly inhibited the relaxations with nitrergic nerve stimulation but not with acidified $NaNO_2$, pointing to a prejunctional site of inhibition rather than a postjunctional effect. These observations are in parallel with those of De Man et al. (1995), who showed that pretreatment with NO donors inhibited the non-adrenergic non-cholinergic nerve-mediated relaxations without altering the postjunctional response to NO and vasoactive intestinal polypeptide and suggests the presence of an autoregulatory mechanism for the nitrergic innervation. Also, Hosoda et al. (1998) showed that non-adrenergic non-cholinergic relaxations and [^{3}H]citrulline formation in response to transmural electrical stimulation were markedly antagonized by pretreatment with various NO donors, including 3-morpholino-sydnoninime, *S*-nitroso-*N*-acetylpenicillamine and sodium nitroprusside. Moreover, Kurjak et al. (1999) provided direct evidence that exogenous NO can inhibit the endogenous synthesis of the putative neurotransmitter NO within enteric synaptosomes by nNOS. In previous studies, it was shown that NO donors inhibited NOS activity in several tissues (Buga et al., 1993; Rengasamy and Johns, 1993; Assreuy et al., 1993; Griscavage et al., 1994). The observations in the present report are consistent with the hypothesis that exogenous NO has a prejunctional inhibitory effect on the nitrergic component of the non-adrenergic non-cholinergic responses. In contrast to our results, Lefèbvre and Vandekerckhove (1998) demonstrated that nitroglycerin inhibited relaxations in response to both nitrergic nerve stimulation and exogenous NO and illustrated that nitroglycerin can induce postjunctional tolerance to nitrergic stimuli, but they do not provide evidence for the prejunctional inhibition of nNOS by NO in the pig gastric fundus. However, in the present study, nitroglycerin and sodium nitroprusside did not affect the relaxations induced by acidified $NaNO_2$. This controversy may have resulted from differences in experimental conditions or in species used.

In order to address the question of whether the inhibitory effect of NO donors on nitrergic nerve-mediated relaxations results from free NO released from nitroglycerin and sodium nitroprusside, but not related to NO metabolites, such as peroxynitrite or a nitrosothiol intermediate, additional experiments were conducted. The NO-induced inhibition was reversed in the presence of the NO scavenger, hemoglobin, suggesting that the inhibitory effect was mediated by free NO rather than a nitrosothiol intermediate, which contrasts with the finding in enteric synaptosomes of the rat (Kurjak et al., 1999). However, this finding does not entirely exclude the possibility that an *S*-nitrosothiol is formed intracellularly after NO enters the tissue. It is well known that endogenous Cu^{2+}/Zn^{2+} superoxide dismutase inhibits the inactivation of NO by superoxide anions, thereby prolonging the half-life of NO and potentiating its biological effects. The administration of exogenous Cu^{2+}/Zn^{2+} superoxide dismutase further potentiated the inhibitory effect of NO donors, and relaxations turned into contractions. Also, diethyldithiocarbamic acid, an inhibitor of the endogenous Cu^{2+}/Zn^{2+} superoxide dismutase, antagonized NO donor-induced inhibition of nitrergic relaxations in our experiments. Further evidence supporting the proposal that NO itself, but not peroxyni-

trite, exerts an inhibitory effect on nitrergic relaxations comes from experiments using pyrogallol, a superoxide anion generator. Also, under conditions of expected high concentrations of superoxide anions in the presence of pyrogallol, the inhibition of nitrergic nerve-mediated relaxations induced by NO donors was decreased. In addition, treatment with diethyldithiocarbamic acid plus pyrogallol completely prevented the inhibitory effect of NO donors on nitrergic nerve-mediated relaxations. It is conceivable that tissue superoxide dismutase protects NO against superoxide anions, and inhibition of this enzyme increases its susceptibility to destruction by superoxide anions, since this effect of pyrogallol plus diethyldithiocarbamic acid was abolished following addition of exogenous Cu^{2+}/Zn^{2+} superoxide dismutase. In contrast, the above-mentioned substances did not affect relaxations in response to nitrergic nerve stimulation, suggesting that the nitrergic NANC neurotransmitter is not free NO but a superoxide-resistant, NO-containing, molecule. Early studies on the nature of the nitrergic NANC neurotransmitter showed that relaxations induced by nitrergic stimulation of different tissues were resistant to hydroquinone, free radical scavenger and/or superoxide anion generator, 6-anilino-5,8-quinolinedione (LY83583), superoxide anion generator, and hemoglobin and hydroxocobalamin, NO binding substances, whereas relaxations with NO were fully blocked (Hobss et al., 1991; Gibson et al., 1992; Barbier and Lefèbvre, 1992; Jenkinson et al., 1995; Lefèbvre, 1996). It was suggested that the endogenous neurotransmitter is not free NO but NO-linked to a carrier, protecting it from scavengers and superoxide radical generators, *S*-nitrosothiols being candidates (Gibson et al., 1992; Rand and Li, 1993, Barbier and Lefèbvre 1994) and alternatively that the nitrergic neurotransmitter is free NO, which is protected from breakdown by the tissue antioxidants such as superoxide dismutase, which scavenges superoxide radicals (Lilley and Gibson, 1996; De Man et al., 1996). Further studies are needed to clarify the nature of the nitrergic NANC neurotransmitter in mouse duodenum. Additionally, hemoglobin, superoxide dismutase, diethyldithiocarbamic acid and pyrogallol did not affect the inhibitory effect of NO donors when added after inhibitory responses to NO donors were obtained, emphasising the importance of the administration time of drugs. These data suggest that free NO itself, but not its biradical reaction product with superoxide, peroxynitrite or a nitrosothiol intermediate, exerts the feedback inhibitory effect. This finding is in disagreement with a recent report demonstrating that the NO-donor-induced inhibition of NOS was not influenced in the presence of oxyhemoglobin, exogenous Cu^{2+}/Zn^{2+} superoxide dismutase and pyrogallol in rat enteric synaptosomes, suggesting that the effect was not mediated by free NO, but by a nitrosothiol intermediate (Kurjak et al., 1999). On the other hand, it was shown that oxyhemoglobin prevented the inhibition of NOS activity by NO, and exogenous superoxide dismutase potentiated the inhibitory effect of the NO donor, suggesting that NO itself causes inhibition of NOS in the bovine cerebellum, rat cerebellum, bovine aortic endothelial cells and rat thoracic aorta (Rengasamy and Johns, 1993; Griscavage et al., 1994; Buga et al., 1993; Ma et al., 1996).

It is not completely understood whether cyclic GMP has a role in mediating the inhibition of NOS activity by exogenous NO. Hosoda et al. (1998) and Vaziri and Wang (1999) showed the inhibitory effect of cyclic GMP on [^{3}H]citrulline formation in response to transmural electrical stimulation in rat gastric myenteric plexus and on eNOS expression in human coronary endothelial cells, respectively, suggesting that the inhibitory effect of NO on NOS activity is mediated in part by cyclic GMP. On the other hand, it was shown that the membrane permeable cyclic GMP analogs, 8-bromo-cyclic GMP and CPT-cyclic GMP, had no effect on NOS activity in the murine macrophage cell line and rat enteric synaptosomes, suggesting that cyclic GMP did not seem to be involved in the inhibition of NOS by exogenous NO (Assreuy et al., 1993; Kurjak et al., 1999). In the present study, nitrergic nerve-mediated relaxations were attenuated by 8-bromo-cyclic GMP, but not by 8-bromo-cyclic AMP, cell permeable analogs of cyclic GMP and cyclic AMP, respectively, indicating that prejunctional inhibition of nitrergic nerve-induced relaxation is mediated, at least in part, by the cyclic GMP pathway. Also, relaxation with isoproterenol, which acts by stimulation of postjunctional β-adrenoreceptors, was not inhibited by NO donors, indicating the specific inhibitory action of NO donors on cyclic GMP pathway, but not on cyclic AMP pathway.

It has been reported that neuronal NOS is localized to nerve endings in the rat proximal duodenum (Jarvinen et al., 1999) and the release of NO, as a nitrergic neurotransmitter, is Ca^{2+}-dependent via N-type Ca^{2+} channels (Boeckxstaens et al., 1993). Cyclic GMP-dependent mechanisms appear to regulate Ca^{2+} transport both directly and indirectly, and cyclic GMP has a dual effect on Ca^{2+} entry; low cyclic GMP activates Ca^{2+} entry, while high cyclic GMP inhibits Ca^{2+} entry (Xu et al., 1994). It was reported that NO modulates retinal ganglion cell N-type Ca^{2+} channels by facilitating their voltage-dependent activation via a mechanism involving guanylyl cyclase/protein kinase G-dependent phosphorylation (Hirooka et al., 2000). On the other hand, Desole et al. (1994) showed that sodium nitroprusside and 8-bromo-cyclic GMP significantly inhibits KCl-stimulated increase in $[Ca^{2+}]_i$, suggesting that NO selectively inhibits voltage-dependent calcium influx in neuronal cells through a cyclic GMP-dependent mechanism. Inhibition of Ca^{2+} channels by NO through the cyclic GMP pathway or additional mechanisms would reduce calcium entry and thereby reduce calcium-dependent NO synthesis.

In conclusion, these results suggest that exogenous NO has a prejunctional inhibitory effect on the nerve-mediated nitrergic relaxation and the inhibitory effects of nitroglyc-

erin and sodium nitroprusside are NO-dependent, but not dependent on related NO metabolites, such as peroxynitrite, or a nitrosothiol intermediate, which are generated extracellularly in the mouse duodenum. However, a contribution of *S*-nitrosothiol formed intracellularly cannot be entirely ruled out. And also, this prejunctional inhibition is mediated, at least in part, by the cyclic GMP, but not the cyclic AMP, pathway. Further studies are needed to explain the mechanism(s) by which NO inhibits its own synthesis.

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