응용약물학회지, The Journal of Applied Pharmacology 3, 149-153(1995)

Another Evidence for Nitric Oxide as One of the Mediators of the Rat Gastric Fundus in Response to NANC-Mediated Relaxation

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(Received June 3, 1995; accepted June 17, 1995)

Abstract—Nitric oxide (NO) has been regarded as one of the neurotransmitters of nonadrenergic, noncholinergic (NANC) nerve stimulation in rabbit corpus cavernosum, rat gastric fundus and human intestine. PIANO (photo-induced adequate nitric oxide) is a very useful tool to investige the role of NO in various smooth muscles where NO is a mediator. The present study was undertaken to compare the physiological responses of the rat gastric smooth muscle in response to NANC nerve stimulation and to PIANO. Photolysis of L-NAME, D-NAME and streptozotocin (STZ) by UV light in the bathing medium caused relaxation of rat gastric fundus that contracted with carbachol, but was resistant to tetrodotoxin (TTX, 1 μM). Electrical stimulation (20 V, 2~32 Hz, 0.2 msec, 10s) of the gastric fundus, in the presence of atropine and guanethidine, induced frequency-dependent, TTX-sensitive relaxation. Sodium nitroprusside (1 nM-10 μM), a NO donor, mimicked the relaxations observed after NANC-stimulation or PIANO. Furthermore, PIANO caused UV light exposure time-dependent increase of cGMP in rat gastric fundus strips. These results provide another evidence indirectly that NO is one of the mediators of the NANC inhibitory nerve stimulation in the rat gastric fundus.

Keywords □ rat gastric fundus; nitric oxide; photorelaxation; nonadrenergic noncholinergic neurotransmission.

Since Burnstock (1986) first reported a nonadrenergic noncholinergic (NANC) inhibitory neuronal system in the gastrointestinal tract, several possible neurotransmitters have been studied. The exact nature of the neurotransmitter released by NANC neurons is still unknown. Vasoactive intestinal peptide (VIP) has been proposed as the possible candidate. VIP is present in the intrinsic neurons of the stomach (Larsson *et al.*, 1976), directly relaxes the smooth muscle and is released during neural stimulation (Grider *et al.*, 1985). Furthermore, VIP antiserum inhibits the neurally induced relaxation of the rat, cat and guinea pig gastric fundus (Grider *et al.*, 1985; D'Amato *et al.*, 1988; De Beurme and Lefebvre, 1988; Kamata *et al.*, 1988). ATP (Burnstock, 1972) have also been proposed as another mediator of NANC neurotransmission in gastric relaxation. On the other hand, growing lines of evidence indicate that nitric oxide (NO) is most likely candidate as inhibitory NANC neurotransmitter in rat and guinea pig gastric fundus (Li and Rand, 1991; Desai *et al.*, 1990; Boeckxstaens *et al.*, 1991). Recently, Chang *et al.*, (1993 a, 1993b) and Chung and Chang (1994) reported so called a photo-induced adequate nitric oxide (PIANO) generating system in which a NO- or NO_2-carrying molecule is photoactivated to release NO. Thus, the PIANO system can be exploited to investigate the role of NO in various physiological processes in which NO is a mediator. So far, PIANO is effective in vascular (Chen and Gillis, 1992; Chang *et al.*, 1993b), trachea (Chang *et al.*, 1993a), corpus cavernosum (Chung and Chang, 1994), gall bladder (Chang and Hong, 1994) and human uterus (Lee and Chang, 1995). However, the effect of PIANO has not been investigated in gastric smooth muscles, where NO is regarded considerably an important neuronal messenger. Furthermore, L-NAME and L-NOARG have been widely used as inhibitors of NO synthase, but paradoxically these chemicals can be used as a source of NO by PIANO (Chen and Gillis, 1992; Chang *et al.*, 1993a; Bauer *et al.*, 1993). The aim of the present study was to investigate whe-

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ther 1) PIANO is also applicable to gastric smooth muscle and 2) to know NO is a common mediator of both NANC nerve stimulation and the PIANO system, hence 3) provide another evidence that NO is one of the inhibitory neurotransmitters in response to NANC nerve stimulation in rat gastric fundus.

Materials and Methods

Muscle preparation

Wistar rats of either sex (150~200 g) were fasted for 24 h but allowed drinking water. Under ketamine anesthesia (75 mg kg^{-1}, i.p.), the intact stomach was removed and cleaned. Incision was made in upper fundus region and longitudinal muscle strips of 1 to 1.5 cm long and 0.3 cm wide were prepared.

Tension recording

In organ bath (10 m*l*) filled with Krebs-Ringer bicarbonate solution (composition in millimolar: NaCl, 136.9; KCl, 5.4; $MgCl_2$, 1.0; $CaCl_2$, 1.5; $NaHCO_3$, 23.8, glucose 5.5 and EDTA 0.03), the tissues were mounted as described by Chang *et al.*, (1993a). The solution was maintained as 36℃ and aerated with a mixture of 95% O_2 and 5% CO_2. One end of the muscle strip was connected to a metal rod whereas the other end was attached to a force transducer (Grass model, FT-03C) for recording of the isometric tension. The strips were equilibrated at 1 g resting tension for 90 min prior to the drug addition. Isometric tension was recorded on a Grass physiograph (model 79E, Grass Instrument, Quincy, MA).

NANC-mediated relaxation

To investigate if NO is the mediator of NANC induced relaxation, two platinum electrodes were placed parallel to the muscle strip. The tissues were contracted with 5-hydroxytryptamine (5-HT, 1 μM) in the presence of atropine (1 μM) and guanethidine (1 μM). After reaching plateau of contraction, electrical field stimulation (EFS, 20 V, 2-32 HZ, 0.2 msec, 10s) were delivered by a Narco SI-10 stimulator. The effects of L-NAME, L-arginine and TTX were studied on the relaxations induced by electrical field stimulation.

PIANO-mediated relaxation

The tissues contracted with either 5-HT in the presence of atropine and guanethidine or carbachol were exposed to UV light (5~60s) using a UV lamp (Waldmann, West Germany, range: 315~395 nm, peak at 355~365 nm) in the presence or absence of STZ, D-NAME, L-NAME and TTX.

NO donor-mediated relaxation

The effects of sodium nitroprusside (SNP, 1 nM~10 μM) was investigated on carbachol (1 μM)-induced contraction of rat gastric fundus.

Nitrite and cGMP determination

Tissues were incubated (30 min, 36℃, under 95% O_2~5% CO_2) in Krebs solution with or without STZ (1~10 mM), which were exposed to UV light for 10 min. Nitrite content of medium was quantified by the method of Stuehr et al (1992), using the Griess reagent. Nitrite concentrations were calculated from a standard curve using $NaNO_3$ as the standard. Tissue cGMP levels were measured by radioimmuno assay according to the manufacturer's instruction (Amersham, U.K).

Drugs

Guanethidine sulphate, tetrodotoxin (TTX), streptozotocin (STZ), N^w-nitro-L-arginine methyl ester (L-NAME), N^w-nitro-D-arginine methyl ester (D-NAME), carbachol, 5-hydroxytryptamine (5-HT), atropine sulphate, sodium nitroprusside (SNP) were purchased from Sigma Co. Ltd.

Results

NANC neuron-mediated relaxation

In functional blockade of adrenergic and cholinergic neurons by guanethidine and atropine, respectively, EFS (2~32 Hz) caused fast but transient frequency-

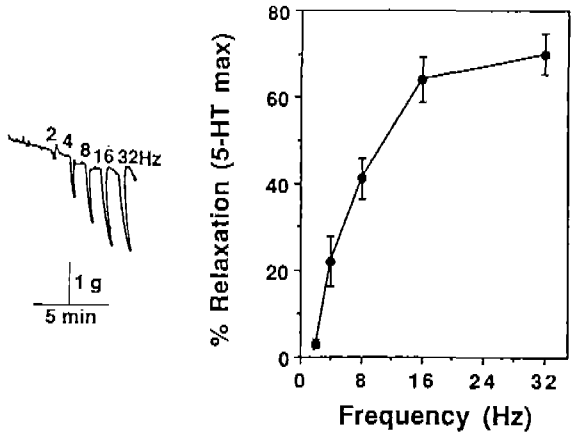

Fig. 1. Typical tracing of the NANC responses to electrical stimulation (2~32 Hz, 0.2 msec) in the rat gastric fundus (left) and the frequency-dependent relaxation response curve (right). The experiments were performed during a 5-HT (1 μM)-induced contraction and in the presence of atropine (1 μM) and guanethidine (1 μM). Values are the means± SEM of 6 experiments.

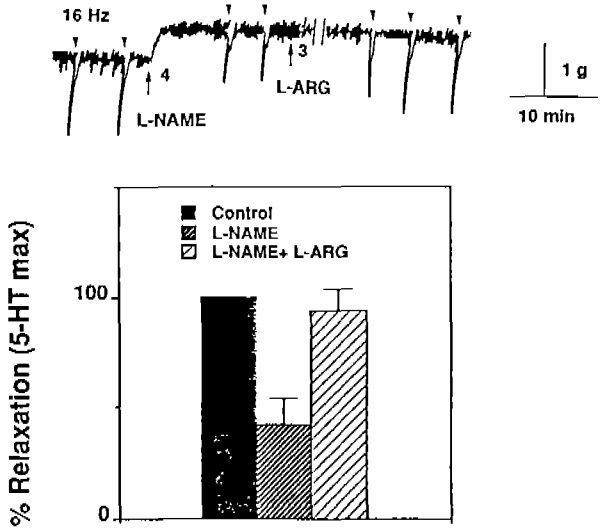

Fig. 2. Representative chart recording showing partial inhibition of NANC responses to electrical stimulation (16 Hz, 0.2 msec) by L-NAME, NO synthase inhibitor and restoration of relaxation by L-ARG (top panel). represents time laps for 15 min. Drug concentrations are represented as negative log molar concentration. Percent response of L-NAME on NANC-mediated relaxation and its reversal by L-ARG (bottom panel). Values are the means± SEM of 6 experiments.

dependent relaxation of the longitudinal muscle strips of the rat gastric fundus that had been contracted with 5-HT (n=6, Fig. 1). EFS at 16 Hz of the strips of gastric fundus of rat treated with guanethidine and atropine caused transient and reproducible relaxation, in which L-NAME, nitric oxide synthase inhibitor, inhibited the EFS-induced NANC relaxation and L-arginine (1 mM) reversed this inhibition (n=6, Fig. 2). TTX (1 μM), a neuronal sodium channel blocker, abolished the electrically elicited relaxation (Fig. 3).

PIANO-mediated relaxation

Irradiation of UV light alone to the tissues caused neither contraction nor relaxation. In contrast, in the presence of L-NAME, D-NAME and STZ, UV light irradiation caused rapid and transient exposure time dependent relaxation, respectively (n=7, Fig. 3). TTX failed to prevent the relaxation induced by photolysis of D-NAME (n=7, Fig. 3). In case of L-NAME (0.5 mM) and D-NAME (0.5 mM), even though 5 times higher concentration than STZ (0.1 mM) was used, the magnitude of relaxation by photolysis was much greater in the presence of STZ than that of the L-NAME and D-NAME. However, no differences were observed between the same concentration of L-NAME (0.5 mM) and D-NAME (0.5 mM). In some preparations, the photolysis of STZ produced rapid and transient relaxation that was followed by a rebound contraction (Fig. 4).

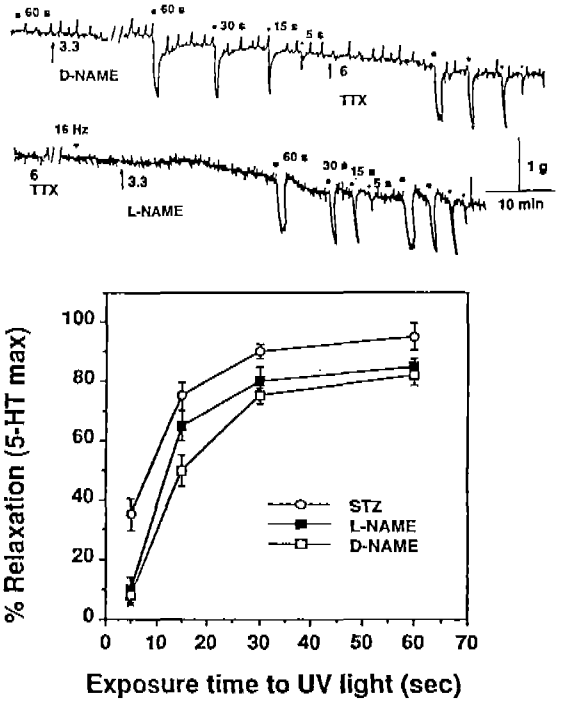

Fig. 3. Typical tracings of the PIANO-mediated relaxation (top panel). Arrow indicates administration of drugs. Drug concentrations are represented as negative log molar concentration. Note that TTX completely blocked the relaxation induced by electrical stimulation (16 Hz) but PIANO-mediated relaxation was unaffected. Exposure time-dependent PIANO-mediated relaxation (bottom panel). Values are the means ± SEM of 7 experiments.

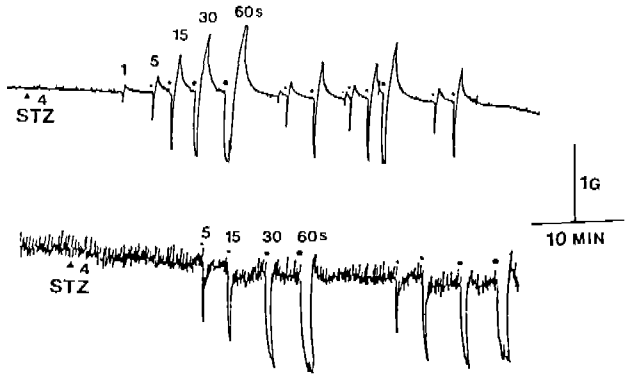

Fig. 4. Representative chart recording showing two different type of PIANO-mediated relaxation. STZ was utilized as photosensitizer. In this preparation, the gastric longitudinal muscle was contracted with carbachol (1 μM). Drug concentrations are represented as negative log molar concentration.

NO donor-mediated relaxation

As shown in figure 5, SNP, NO donor, concentration dependently relaxed the strip (n=3) precontracted

Table I. Accumulation of nitrite and cyclic GMP due to photolysis of STZ in isolated rat gastric fundus. Tissues were exposed to UV light for 3 min in the presence or absence of STZ.

Treatment	nitrite (mM)	cGMP (fmol/mg tissue)	n
STZ (0 mM)	4± 0.7	203.1± 18.3	9
STZ (0.1 mM)	12± 2	432.6± 35.2	9
STZ (1 mM)	46± 5	764.8± 125.6	6

Values are the means± SEM of 6 to 9 assays from 3 different experiments.

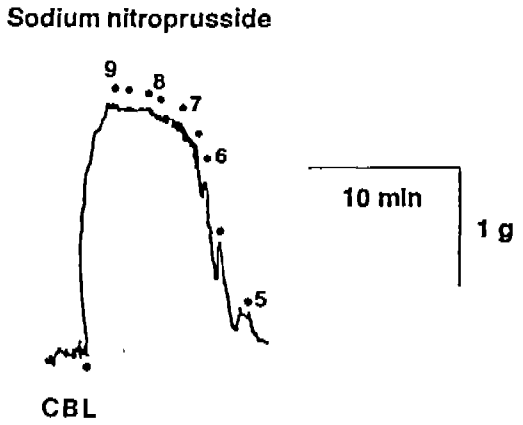

Fig. 5. Typical tracing of concentration dependent relaxation effect of sodium nitroprusside, a NO donor, in the rat gastric fundus longitudinal strip that had been contracted with carbachol(CBL). Drug concentrations are represented as negative log molar concentration.

with carbachol (1 μM) which was mimicked by NANC- or PIANO-mediated relaxation.

Nitrite and cGMP accumulation

As shown in Table 1 nitrite, spontaneous oxidation product of NO, was accumulated in the medium as compared to control and tissue cGMP also increased by photoactivation of STZ. Much greater nitrite was accumulated by increasing time of UV exposure (data not shown).

Discussion

The purpose of the present study was 1) to confirm that NO is a mediator of NANC-mediated relaxation by comparing physiological parameters such as, relaxation pattern and onset of action, with PIANO-mediated relaxation, another NO releasing method. Thus, 2) to provide further evidence indirectly that NO is the common mediator of the NANC inhibitory response and the PIANO in the rat gastric fundus, and 3) PIANO generating system may serve as an additional tool for investigating NO-mediated physiological processes in gastrointestinal smooth muscles. The present results show that isolated rat gastric fundus releases a labile factor upon electrical stimulation. This release was completely inhibited by TTX and partially by L-NAME, NO synthase inhibitor, but not by atropine or guanethidine, whereas it was increased by L-arginine. This confirms earlier report of others (Boeckxstaens *et al.*, 1991) that NO is formed and released upon brief stimulation of NANC nerves in the rat gastric fundus.

If NO is a mediator of NANC neurotransmission by EFS in rat gastric fundus, the NANC-mediated physiological responses (relaxation) should be identical to or at least mimic to those that NO is administered exogenously or any procedure utilized to generate NO like PIANO. Previous investigations have shown that NO or NO_2 carrying molecule is photoactivated to release a potent relaxing substance, which is rapidly inactivated by O_2 and inhibited by methylene blue. Thus, the substance is probably NO and may be the same as EDRF (Chang *et al.*, 1993a). If this hypothesis is correct, D-NAME lacking of NO synthase inhibition should also release NO by UV irradiation. Upon UV light irradiation, as expected, the isolated rat stomach fundus that was treated with D-NAME caused exposure time dependent relaxation which was indistinguishable that made by L-NAME. Photolysis of STZ also induced UV exposure time-dependent rapid transient relaxation. It shoud be noted that STZ was much more potent than D- or L-NAME even the same molar concentration used. At the present time, the reason why STZ shows much stronger effect than D- or L-NAME is not certain. However, it can be speculated that photolysis of STZ may release solely NO but L-NAME or D-NAME release NO and other radicals that may inactivate NO, possibly $\cdot O_2^-$, which warrants further study. A positive Griess reaction can be detected by irradiation of STZ in the medium. Furthermore, tissue cGMP levels were elevated by PIANO method, indicating that NO is released from STZ upon UV light irradiation which may stimulate guanylate cyclase, which was confirmed also in rat bladder (Chang and Chung, 1995) and human uterus (Lee and Chang, 1995). The fact that TTX failed to prevent the relaxation induced by the PIANO generating system is quite natural in that exogenous administration of NO induced TTX-resistant relaxations which were similar to those obtained by NANC nerve stimulation (Boeckxstaens *et al.*, 1991). In addition, SNP, NO donor, concentration de-

pendently relaxed carbachol induced gastric fundus strip. At the present time, rebound contraction in some preparations can not be exactly explained. The rebound contraction is reported to be neurogenic in nature with activation of cholinergic and tachykininergic neurones in guinea pig ileum (Bartho and Lefebve, 1994). While it seems attractive to speculate that a response characterized by rapid relaxation followed by abrupt contraction would be ideally suited to participate in the normal voiding cycle of the stomach.

In conclusion, evidence is presented that NO release seems to be essential for the transient NANC relaxation in rat gastric fundus and relaxations induced by PIANO generating system mimicked those of NANC nerve stimulation, suggesting NO as inhibitory NANC neurotransmitter. Even though the physiological significance of PIANO generating system is not known, however, this method may serve as an additional valuable tool for investigating NO-mediated physiological processes.

Acknowledgment

This work was supported by a grant from the Ministry of Education for Basic Medical Research (1994).

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