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The Role of Nitric Oxide in Non-Adrenergic Non-Cholinergic Relaxation in the Guinea-Pig Gastric Fundus

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The role of nitric oxide (NO) in non-adrenergic non-cholinergic (NANC) neurotransmission was studied on circular muscle strips of the dorsal part of the guinea-pig gastric fundus. In the presence of atropine and guanethidine, a low frequency of electrical stimulation (1~10 Hz) induced frequency-dependent relaxations which were not affected by adrenergic and cholinergic blockage but abolished by tetrodotoxin. N^G-nitro-L-arginine (L-NNA), a stereospecific inhibitor of NO-biosynthesis, inhibited the relaxations induced by electrical stimulations but not the relaxations to exogenous nitric oxide. The effect of L-NNA was prevented by L-arginine, the precursor of the NO biosynthesis but not by its enantiomer, D-arginine. Exogenous administration of NO caused concentration-dependent relaxations which showed a similarity to those obtained with electrical stimulation. Hemoglobin, a NO scavenger, abolished the NO-induced relaxations and also markedly reduced those induced by electrical stimulation. The inhibitory effect of hemoglobin was similar to that of L-NNA. Application of ATP caused weak relaxations compared with those to electrical stimulation, which were unaffected by L-NNA. Exogenously applied vasoactive intestinal polypeptide (VIP) induced concentration-dependent relaxation which was not affected by L-NNA. These results suggest that NO is produced and released mainly as a neurotransmitter from enteric neurons during NANC relaxation induced by low frequencies and short trains of electrical stimulation and has a main role in NANC neurotransmission at relaxation induced by these electrical stimulations in the guinea-pig gastric fundus.

Key words : Non-adrenergic non-cholinergic neurotransmission, N^G-nitro-L-arginine, Hemoglobin, Nitric oxide, Vasoactive intestinal polypeptide, Adenosine triphosphate, Guinea-pig gastric fundus.

INTRODUCTION

Non-adrenergic non-cholinergic (NANC) nerves play an important role in functional regulation of the stomach in respect that they mediate the receptive relaxation when a large volume of foods is taken (Abrahamsson, 1986). ATP (Burnstock, 1972; 1981; Fujiwara *et al.*, 1982; Hong and Kim, 1985) or vasoactive intestinal polypeptide (Fahrenkrug, 1982; Grider *et al.*, 1985; Grider and Makhlouf, 1987; Makhlouf, 1985; Grider, 1990) was proposed as putative mediator(s) of NANC nerve in different parts of the gastrointestinal (GI) tract. Recently, there are many evidences indicating that nitric oxide (NO) is also a neurotransmitter of NANC nerves (Lefebvre, 1993; Rand, 1992; Sanders and Ward, 1992). In various GI tissues such as the canine (De Man, *et al.*, 1991) and opossum (Murray *et al.*, 1991; Tøttrup *et al.*, 1991) lower oesophageal sphincter, the rat (Boeckxstaens *et al.*, 1991a; Shimamura *et al.*, 1993), guinea-pig (Grider *et al.*, 1992; Lefebvre *et al.*, 1992) and rabbit (Jin and Grider, 1993; Hong *et al.*, 1994) gastric fundus, the canine duodenum (Toda *et al.*, 1991), the human (Maggi *et al.*, 1991) and guinea-pig (Osthaus and Galligan, 1992) ileum, the canine ileocolonic junction (Boeckxstaens *et al.*, 1990; 1991b; Bult *et al.*, 1990) and the human colon (Boeckxstaens *et al.*, 1993), inhibitors of NO biosynthesis reduced relaxations induced by electrical stimulation. Furthermore, the release of an unstable vasorlaxant factor with the properties of NO has been shown on stimulation of the NANC nerves in the canine ileocolonic

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junction (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1991b) and the rat gastric fundus (Boeckxstaens *et al.*, 1991a), and NO-synthase has been immunohistochemically detected in the myenteric plexus of the rat intestine (Bredt *et al.*, 1990). Moreover, hemoglobin, known to trap NO (Martin, 1985), also reduced NANC relaxations (Boeckxstaens *et al.*, 1990; Bult *et al.*, 1990; Osthaus and Galligan, 1992). These data suggest that NO has a transmitter role of NANC nerve in the GI tract. Since the NANC relaxation induced by lower frequencies of electrical stimulation in the guinea-pig gastric fundus was almost abolished in the presence of VIP antiserum (Grider *et al.*, 1985), vasoactive intestinal polypeptide (VIP) has been proposed as neurotransmitter of the NANC relaxation in this tissue (Grider *et al.*, 1985; Grider and Makhlouf, 1987). However, the incomplete blockade of NANC relaxations by VIP antiserum indicates that a non-VIP component may be involved (De Beurme and Lefebvre, 1988; D'Amato *et al.*, 1988). It was shown that NANC relaxation induced by electrical stimulation is reduced by VIP antibody, and further reduced by NO biosynthesis inhibitor N^G-monomethyl-L-arginine (L-NMMA) in the presence of VIP antibody (Li and Rand, 1990). Thereby, it was suggested that both NO and VIP contribute to NANC relaxation. Recently, Grider and colleagues proposed that VIP released from enteric neurons, NO released from the neurons and NO regenerated from muscle cells by the action of VIP cooperatively involve to NANC relaxation in the guinea-pig (Grider *et al.*, 1992) and rabbit (Jin and Grider, 1993) gastric fundus and that NO is mainly derived from muscle cells during nerve stimulation as a result of the action of VIP (Grider *et al.*, 1992; Jin and Grider, 1993; Makhlouf and Grider, 1993).

The present study was undertaken to investigate whether NO induced NANC relaxation of guinea-pig gastric fundus is released mainly as a transmitter from enteric neuron(s) of guinea-pig gastric fundus or is also produced by the action of putative transmitter(s), ATP or VIP.

MATERIALS AND METHODS

Tissue preparation and experimental protocols

Guinea-pigs of either sex (400-700 g) were fasted for 24 hr and were stunned and exsanguinated from the common carotid arteries. The stomach was removed and after careful removal of the mucosa circular muscle strips (15 to 20 mm long and 2 to 3 mm wide) were prepared from the dorsal part of the fundus. The strips were mounted vertically in a 20 or 5 ml organ bath containing the nutrient solution. Initial tension of 0.5 g was loaded and 90 min was allowed for equilibration before initiation of the experiment. Changes in isometric tension were recorded through a force-displacement transducer (Narco F60) on a Narco physiograph (Narco MK IV). The bathing media were maintained at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2. The composition of nutrient solution was as follows (mM): NaCl, 118.3; KCl 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; Ca-EDTA, 0.026 and glucose, 11.1 (pH 7.3). It was not necessary to raise tone in order to observe relaxation response since the preparation of the guinea-pig gastric fundus raised tone spontaneously (Sahyoun *et al.*, 1982). Electrical stimulation was applied transmurally through a pair of parallel platinum wire electrodes in low frequencies (1~10 Hz, 9 V) by 0.5 ms width square wave pulses for periods of 5 s with a Bio Science stimulator 200. NO, ATP or VIP was added into the bath medium. All experiments were performed in the presence of atropine (1 μM) and guanethidine (3 μM). The effects of hexamethonium (100 μM), phentolamine (10 μM) plus propranolol (1 μM), hemoglobin (10 μM) and tetrodotoxin (0.3 μM) were studied on the relaxations induced by electrical stimulation (1~10 Hz, 0.5 ms). The effect of L-NNA (10 μM) was examined on the relaxations induced by electrical stimulation, NO (0.3~3 μM), ATP (3~30 μM) and VIP (1~100 nM), respectively. To prevent VIP adhesion to glassware, it was added in the presence of 0.01% bovine serum albumin. L-NNA, hemoglobin and other antagonists were added at least 10 min prior to the electrical stimulation with the exception that hemoglobin was added 10 min before administration of NO. L-arginine (5 mM) and D-arginine (5 mM) were added 5 min before L-NNA.

Preparation of hemoglobin

Heparinized blood, 10~15 ml, taken from rabbit was centrifuged at 1200 g for 20 min at 4°C, and plasma and buffy coat were removed by aspiration. The remaining erythrocytes were washed three times with isotonic phosphate-buffered saline (pH 7.4). Hemolysis was effected by pipetting 2 ml of the washed erythrocytes into 8 ml of hypotonic phosphate buffer (20 m osmoles, pH 7.4). The contents were mixed and centrifuged at 20,000 g at 4°C for 40 min. The supernatant from this procedure constituted the hemolysate. This method was based on that described by Bowman and Gillespie (1982). Hemoglobin concentration of the hemolysate was estimated by the cyanmethemoglobin method (Simmons, 1976). The approximate final concentration of hemoglobin in each experiment was about 20 μM.

Drug and solutions

The following drugs were used: N^G-nitro-L-arginine,

L-arginine hydrochloride, D-arginine hydrochloride, VIP, bovine serum albumin and ATP (Sigma Chemical Co., St. Louis, MO, U.S.A.), tetrodotoxin (TTX; Sankyo, Tokyo, Japan), atropine sulfate (Wako, Osaka, Japan), guanethidine sulfate (Tokyo-Kasei, Tokyo, Japan), phentolamine hydrochloride (gifted by Ciba-Geigy, Switzerland), propranolol (Nakarai Chemical, Japan). NO solution was prepared just before use according to the method described by Furchgott (1988). Drugs were dissolved and diluted with distilled water. Stock solutions of tetrodotoxin (0.1 mM) and hemoglobin (1.3 mM) were stored at -20°C. Phosphate buffers were made in the way described by Dodge *et al.*(1963): stock solution was sodium phosphate, monobasic (NaH_2PO_4), 0.155 M and sodium phosphate, dibasic (Na2HPO4), 0.103 M. Isotonic phosphate buffer was made by mixing appropriate volumes of the above solutions to give pH 7.4. Hypotonic phosphate buffer (20 m osmole, pH 7.4) was made by diluting isotonic phosphate buffer 1 in 15.5. Isotonic phosphate-buffered saline was made by mixing four volumes of 0.9% NaCl and one volume of isotonic phosphate buffer (pH 7.4).

Statistical analysis

Relaxations were expressed as percentage of the relaxation induced by electrical stimulation of 10 Hz (0.5 ms) in the beginning of experimentation. Results were shown as mean±S.E.M. for the number of experiments indicated. All data were analyzed by Student's *t* test for paired and unpaired observations. *P* values of less than 0.05 were considered to be significant.

RESULTS

NANC relaxation induced by electrical stimulation

In the presence of atropine (1μM) and guanethidine (3μM), low frequencies of electrical stimulation (ES, 1~10 Hz, 0.5 ms, 9 V) induced frequency-dependent relaxations of circular muscle strips of the dorsal part of the guinea-pig gastric fundus (Fig. 1 to 5). When the strip was stimulated electrically, tone was very quickly decreased during stimulation and a biphasic recovery of tone was followed after cessation of the 5 s stimulus train, an initial rapid phasic being followed by a second slower phase (Fig. 1 to 5). These relaxations were not affected by hexamethonium (100 μM) or propranolol (1 μM) and phentolamine (10 μM), but were abolished by tetrodotoxin (0.3 μM) (Fig. 1).

Effects of L-NNA, D-arginine, L-arginine, or hemoglobin on NANC relaxation

After exposure of the gastric fundus strips for 10 min to L-NNA (10 μM), NANC relaxation induced by electrical stimulation at 1 Hz was abolished and those at 5 and 10 Hz were markedly reduced (Fig. 2, Table 1), the mean relaxation being 14.41±2.99 and 22.69±3.62% compared with relaxation (89.15±1.96 and 100%) induced by electrical stimulation at 5 and

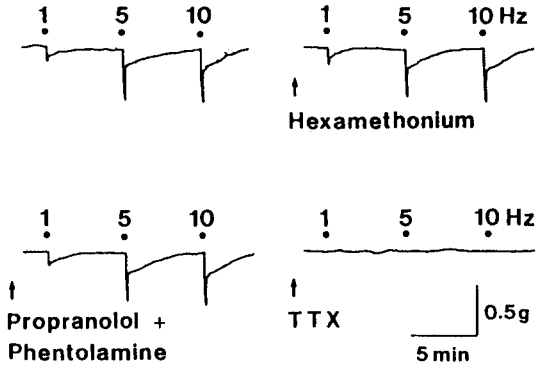

Fig. 1. Effects of hexamethonium, propranolol plus phentolamine and tetrodotoxin on the relaxations to electrical stimulation in a circular muscle strip of the dorsal part of the guinea-pig gastric fundus. The experiments were performed in the presence of atropine (1 μM) and guanethidine (3 μM). Hexamethonium (100 μM), propranolol (1 μM) plus phentolamine (10 μM) and tetrodotoxin (TTX; 0.3 μM) were added at arrow. Electrical stimulations (1~10 Hz, 0.5 ms for 5 s) were applied at dot. Tracing-breaks represent periods of tissue equilibration. Similar results were obtained from seven other experiments.

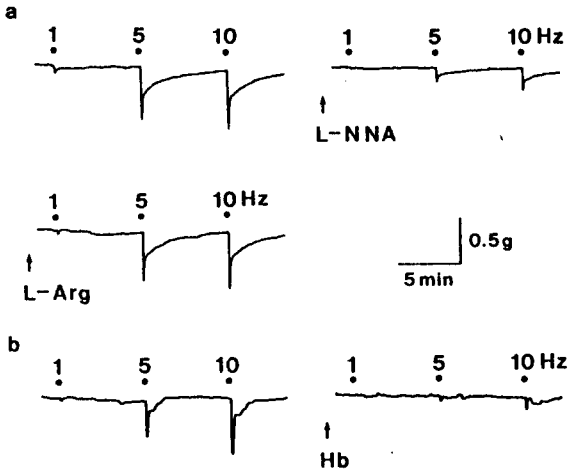

Fig. 2. Representative tracing showing the effects of N^G-nitro-L-arginine, L-arginine and hemoglobin on the relaxation to electrical stimulation in circular muscle strips of the dorsal part of the guinea-pig gastric fundus. N^G-nitro-L-arginine (L-NNA, 10 μM), L-arginine (L-Arg, 5 mM) and hemoglobin (Hb, 20 μM) were added at arrow. a and b were different preparations. Other experimental conditions were the same as those described in Fig. 1. Similar results were obtained from three to ten other experiments.

Table 1. Effects of L-NNA, D-arginine, L-arginine and hemoglobin (Hb) on NANC relaxations to electrical stimulation in circular muscle strips of the dorsal part of the guinea-pig gastric fundus. Results represent as the means±S.E.M. for the number of experiments indicated in parentheses and are expressed as percentage of the relaxation induced by electrical stimulation of 10 Hz. The experiments were performed in the presence of atropine (1 μM) and guanethidine (3 μM).

	Electrical stimulation (Hz)					
	1		5		10	
Control	29.51±5.44	(11)	89.15±1.96	(11)	100	(11)
L-NNA, 10 μM	0[b]	(11)	14.41±2.99[b]	(11)	22.69±3.62[b]	(11)
L-NNA, 10 μM+L-arginine, 5	13.02±4.84[a, c]	(7)	79.13±6.80[c]	(7)	97.06±4.36[c]	(7)
L-NNA, 10 μM+D-arginine, 5	0[b]	(4)	16.73±5.35b	(4)	20.95±7.13[b]	(4)
Control	29.79±6.73	(7)	88.18±2.05	(7)	100	(7)
Hb, 20μM	0[b]	(7)	11.49±1.89[b]	(7)	19.98±3.92[b]	(7)

[a]$P < 0.05$ and [b]$P < 0.01$ different from value in control induced by electrical stimulation (1~10 Hz), student's t test for paired and unpaired observations, respectively. [c]$P < 0.005$, different from value in L-NNA-treated muscle strip for unpaired observations

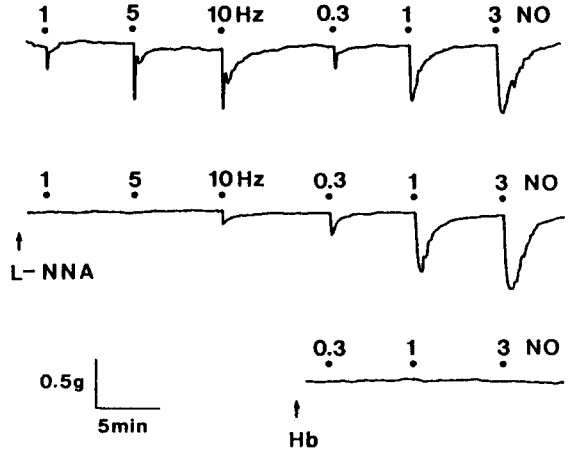

Fig. 3. Effects of N^G-nitro-L-arginine and hemoglobin on the relaxations to electrical stimulation and exogenous nitric oxide in a circular muscle strip of the dorsal part of the guinea-pig gastric fundus. Nitric oxide (NO, 0.3~3 μM) was added at dot. L-NNA (10 μM) and hemoglobin (Hb, 20 μM) were added at arrow, respectively. Other experimental conditions were the same as those described in Fig. 1. Similar results were obtained from three other experiments.

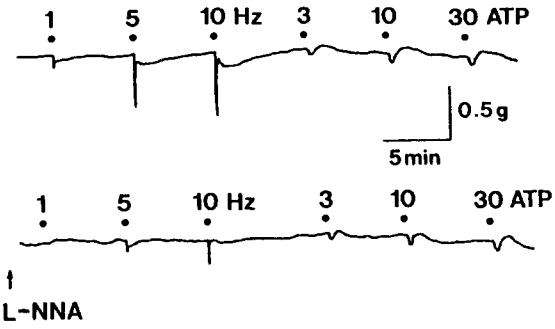

Fig. 4. Comparison of NANC relaxation with ATP-induced relaxation in a circular muscle strip of the dorsal part of the guinea-pig gastric fundus. ATP (3~30 μM) was added at dot. L-NNA (10 μM) was added at arrow. Other experimental conditions were the same as those described in Fig. 1. Similar results were obtained from eight other experiments.

10 Hz in the absence of L-NNA, respectively (Table 1). L-NNA did not consistently increase the basal tone of the strips. Administration of excess L-arginine (5 mM), but not D-arginine (5 mM), prevented the inhibitory effect of L-NNA (10 μM), though partially prevented the effect of L-NNA at 1 Hz of electrical stimulation. Neither L-arginine nor D-arginine influenced the NANC relaxations induced by electrical stimulation per se, nor did they influenced basal tone. In addition, hemoglobin (20 μM) also markedly reduced the relaxations induced by electrical stimulation (1~10 Hz, 0.5 ms) (Fig. 2, Table 1), and the inhibitory effects of hemoglobin (20 μM) were similar to those of L-NNA (10μM) (Table 1).

Effects of L-NNA and hemoglobin on NO-induced relaxation

Exogenously applied NO (0.3~3 μM) caused concentration-dependent relaxations similar to the NANC relaxations obtained with electrical stimulations (1~10 Hz, 0.5 ms), while the effects of NO were slightly persistent compared to those of the electrical stimulations. The NO-induced relaxations were not influenced by L-NNA (10 μM), but abolished by hemoglobin (20 μM) (Fig. 3).

Comparison of NANC relaxation with ATP-induced relaxation

Administration of ATP (3~30 μM) caused concentration-dependent relaxations. The relaxations induced by ATP (3, 10 and 30 μM) were weak (Fig. 4, Table 2), the mean relaxation being only 13.18±2.31, 26.58±8.03 and 34.22±8.05% compared with re-

Table II. Comparison of NANC relaxation with ATP-induced relaxation in circular muscle strips of the dorsal part of the guinea-pig gastric fundus. Results represent the means±S.E.M. for nine experiments and are expressed as percentage of relaxation to electrical stimulation of 10 Hz. ATP-induced relaxations were unaffected by L-NNA. Experiments were performed in the presence of atropine (1 μM) and guanethidine (3 μM).

	Electrical stimulation (Hz)			ATP (μM)		
	1	5	10	3	10	30
Control	27.07±4.71	88.74±1.82	100	13.18±2.31[a]	26.58±8.03[b]	34.22±8.05[b]
L-NNA, 10 μM	0[b]	18.02±5.12[b]	30.15±4.14[b]	9.62±1.57[b]	21.99±4.80[b]	31.73±6.56[b]

[a]$P < 0.025$ and [b]$P < 0.005$, different from value in control induced by electrical field stimulation (1~10 Hz), student's t test for paired observations

Table III. Comparision of NANC relaxation with VIP-induced relaxation in circular muscle strips of the dorsal part of the guinea-pig gastric fundus. Results represent the means±S.E.M. of ten experiments and are expressed as percentage of relaxation to electrical stimulation of 10 Hz. VIP-induced relaxations were unaffected by L-NNA. Experiments were performed in the presence of atropine (1 μM) and guanethidine (3 μM).

	Electrical stimulation (Hz)			VIP (nM)		
	1	5	10	1	10	100
Control	33.22±3.75	80.57±2.63	100	11.91±3.64	70.21±7.03	122.41±9.92
L-NNA, 10 μM	0[a]	13.67±3.47[a]	23.2±3.99[a]	20.15±4.68	57.18±9.59	125.77±18.16

[a]$P < 0.005$, different from value in control induced by electrical stimulation (1~10 Hz), student's *t*-test for paired observations

laxation (27.07±4.71, 88.74±1.82 and 100%) induced by electrical stimulation at 1, 5 and 10 Hz, respectively (Table 2). The ATP-induced relaxations were not significantly influenced by L-NNA (10 μM) (Table 2).

Comparison of NANC relaxation with VIP-induced relaxation

Exogenous administration of VIP (1~100 nM) caused the concentration-dependent relaxation, while its effects were slower in onset and more sustained relaxation compared to those induced by electrical stimulation (Fig. 5). VIP-induced relaxations were not significantly affected by L-NNA (10 μM) (Table 3).

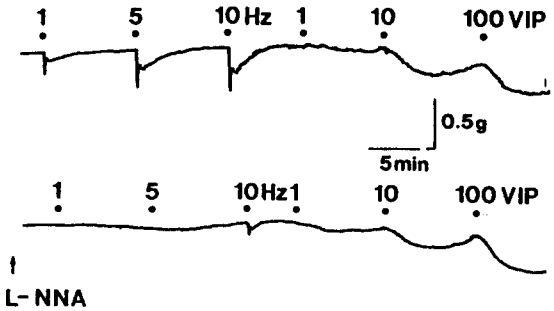

Fig. 5. Comparison of NANC relaxation with VIP-induced relaxation in a circular muscle strip of the dorsal part of the guinea-pig gastric fundus. VIP (1~100 nM) was added at dot. L-NNA (10 μM) was added at arrow. Other experimental conditions were the same as those of described in Fig. 1. Similar results were obtained from nine other experiments.

DISCUSSION

In the presence of adrenergic and cholinergic blockage, low frequencies of electrical stimulation of circular muscle strips of the dorsal part of the guinea-pig gastric fundus induced frequency-dependent relaxations which were abolished by tetrodotoxin, a nerve conductance blocker. This result suggests that NANC relaxations have been resulted from NANC nerve stimulation. Since this NANC relaxation was markedly inhibited by L-NNA, an inhibitor of NO biosynthesis (Ishii *et al.*, 1990; Moore *et al.*, 1990; Mulsh and Busse, 1990) and the effect of L-NNA was prevented by L-arginine, the precursor of the NO biosynthesis (Palmer *et al.*, 1988; Schmidt *et al.*, 1988), but not by its enantiomer, D-arginine, suggesting that these NANC relaxations were mediated by NO and L-arginine was a stereospecific substrate of NO. In addition, exogenous administration of NO caused concentration-dependent relaxations similar to those obtained by electrical stimulation (Fig. 3). Since the relaxation to exogenous NO was not affected by L-NNA, the inhibitory effect of L-NNA on NANC relaxations induced by electrical stimulation was on the NO biosynthesis system rather than on the postjunctional effector cells. Hemoglobin which neutralized extracellular NO (Martin *et al.*, 1985) abolished the NO-induced relaxations (Fig. 3) and

not completely but markedly reduced those responses induced by electrical stimulation (Fig. 2, Table 1). This difference in efficacy of inhibition between the relaxation to exogenous NO and that to electrical stimulation may be explained by the large molecular size of hemoglobin so that a small portion may reach the neuromuscular junction (De Man *et al.*, 1991). On the other hand, it is a possibility that the other transmitter(s) may have been involved in the relaxation by more higher frequencies (5 and 10 Hz) of electrical stimulation since NO-independent NANC relaxations appeared similarly in the presence of L-NNA and hemoglobin, respectively (Table 1). NO-independent NANC relaxations induced by low frequencies of electrical stimulation were shown in other tissues such as the canine lower oesophageal sphincter (De Man *et al.*, 1991), the rat (Boeckxstaens *et al.*, 1991a) and rabbit (Hong *et al.*, 1994) gastric fundus. Therefore, identification of transmitter(s) that mediate these NO-independent NANC relaxation will be very an important subject for future work.

ATP has been proposed as a NANC neurotransmitter in different regions of the gut (Burnstock, 1972, 1978, 1981; Fujiwara et al., 1982; Hong and Kim, 1985). In the present study, it was found that the relaxations induced by exogenous administration of ATP (3 ~ 30μM) were weak compared with those induced by electrical stimulation (1 ~ 10 Hz) (Table 2). ATP-induced relaxation was not significantly influenced by L-NNA (Table 2), meaning that NO is not produced by ATP. Therefore, it is suggested that ATP is not a main neurotransmitter of NANC nerve in the guinea-pig gastric fundus.

There are considerable evidences that VIP is the most likely candidate of NANC neurotransmitter in various regions of the gut (Fahrenkrug, 1982; Grider *et al.*, 1985; Grider and Makhlouf, 1987; Grider, 1990). Recently, Grider and his colleagues showed that during electrical stimulation of muscle strips VIP release and NO production were increased in the guinea-pig and rabbit gastric fundus (Grider *et al.*, 1992; Jin and Grider, 1993) and the NO biosynthesis inhibitor L-NNA abolished NO production while partly inhibited VIP release and relaxation. Moreover, large amounts of hemoglobin (100 μM) partly inhibited VIP release and relaxation but to a lesser extent than L-NNA. From these results, they proposed that VIP released from enteric neurons, NO released from the neurons and NO regenerated from target muscle cells by the action of VIP involve NANC relaxation in guinea-pig (Grider *et al.*, 1992), rabbit (Jin and Grider, 1993) gastric fundus and rat colon (Grider, 1993), and that NO is mainly derived from the muscle cells during nerve stimulation as a result of the action of VIP.

In the present study, exogenously applied VIP caused the concentration-dependent relaxation, while the relaxation was not significantly inhibited by L-NNA (Table 3), implying that NO is not produced by the action of a putative polypeptide transmitter, VIP. Furthermore, its pattern of relaxation was not similar to that induced by low frequencies and short trains of electrical stimulation (Fig. 5). On the other hand, Li and Rand (1990) proposed that both NO and VIP contribute to NANC neurotransmission, NO being mainly involved at low frequencies, but also in the initial part of relaxation induced by high frequencies of electrical stimulation.

In this study, we observed that the NO biosynthesis inhibitor, L-NNA markedly decreases NANC relaxation as similar extent as NO scavenger hemoglobin (Table 1) and exogenous ATP or VIP-induced relaxation was not significantly inhibited by L-NNA (Table 2 and 3). These results suggest that NO is produced and released mainly as a neurotransmitter from enteric neurons during NANC relaxation induced by low frequencies and short trains of electrical stimulation and has a main role in NANC neurotransmission at relaxation induced by these electrical stimulations in the guinea-pig gastric fundus.

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