Pharmacological Features of Non-adrenergic Non-cholinergic (NANC) Relaxation Induced by Electrical Vagal Stimulation in Isolated Mouse Stomach

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ABSTRACT—The non-adrenergic non-cholinergic (NANC) relaxatory response in mouse isolated whole stomach was investigated by electrical vagal stimulation (EVS) to clarify whether nitric oxide (NO) mediates vagal NANC transmission. The stomach was mounted in an organ bath, and the intragastric pressure was measured. Dual electrodes were placed on the esophagus. In the presence of atropine, propranolol and phentolamine, EVS induced a marked gastric relaxation. The response was frequency-dependent, and reproducible by repeated stimulation. The response was blocked by hexamethonium and N°-nitro-L-arginine (L-NNA), a NO synthase inhibitor, and significantly depressed by methylene blue, a soluble guanylate cyclase inhibitor, but not by hemoglobin, a radical trapping agent. The inhibitory effect of L-NNA was reversed by L-arginine, a substrate for NO synthase, but not by D-arginine. Exogenous NO caused a relaxation that was inhibited by hemoglobin and methylene blue, but not by L-NNA. The electrical field stimulation also elicited a gastric relaxation that was inhibited by L-NNA and methylene blue, but not by hexamethonium and hemoglobin. These results suggest that the inhibitory NANC response to EVS in the mouse stomach is largely mediated by release of NO, and it is exclusively due to stimulation of vagal preganglionic neurons.

Keywords: Non-adrenergic non-cholinergic (NANC), Stomach (mouse isolated), Gastric relaxation, Vagal stimulation, Nitric oxide (NO)

Inhibitory non-adrenergic non-cholinergic (NANC) neurons are present throughout the gastrointestinal tract (1, 2). The stomach actively dilates to accommodate food. Mechanical stimulation of the pharynx and esophagus through the passage of nutrients induces a gastric receptive relaxation via vagal NANC nerves in the stomach of cats (3). Distension by gastric contents elicits vago-vagal reflex responses that keep the stomach relaxed via the NANC neurons (4).

Martinson and Muren (5) showed that vagal efferent stimulation induced a long-lasting NANC relaxation of the stomach in vivo. Thereafter, vagally induced relaxations have been demonstrated in many parts of the gastrointestinal tract and have been considered to be mediated by release of unknown transmitters from the NANC nerves on electrical vagal stimulation. In subsequent studies, purinergic and peptidergic neurotransmitters such as ATP (6) and vasoactive intestinal polypeptide (VIP) (7, 8) were proposed as possible neurotransmitters.

More recently, the endothelium-derived relaxing factor (EDRF) has been identified as nitric oxide (NO) or a NO-related substance (9, 10) that is synthesized from L-arginine. NO has been proposed to play many roles in the regulation of biological responses (11), and a definite role of NO in neural transmission has been strongly suggested (12, 13). There is now evidence to support the idea that NO is a mediator of NANC relaxation in many parts of the gastrointestinal tract such as canine ileocolonic junction (13) and lower esophageal sphincter (14), rat gastric fundus (15, 16), guinea pig stomach (17–19), and rat or mouse anococcygeus muscle (20, 21). These NANC responses induced by electrical stimulation were definitely reduced by the NO synthase (NOS) inhibitor N°-nitro-L-arginine (L-NNA), which indicates that NO is responsible for the inhibitory NANC transmission.

Some studies suggest that there exists a species difference in the importance of NO as an inhibitory neurotransmitter (15, 19). In the literature, however, no reports
concerned the inhibitory NANC relaxatory response to vagal stimulation in the mouse stomach. Recently, we have established a reliable method for electrical vagal stimulation (EVS) in isolated mouse stomach (22). The present study investigated whether or not NO is responsible for the relaxation of the isolated stomach by EVS in mice. In addition, the EVS responses have been compared with those induced by electrical field stimulation (EFS) in order to verify the characteristics of vagal stimulation.

MATERIALS AND METHODS

Measurement of intragastric pressure in the isolated mouse whole stomach
Male mice of the ddY strain (25–35 g; Takasugi Exp. Animals, Kasukabe) were killed by a blow on the head and exsanguination. After ligation of the esophagus, the stomach was isolated and washed by Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25.0 mM NaHCO3, 10 mM glucose). The pylorus was cannulated and connected to a pressure transducer (Biophysiograpgh 180 system; San-Ei, Tokyo) to record changes in intragastric pressure. The preparation was placed in a 20-ml organ bath filled with Krebs-Henseleit solution, which was kept at 37°C and gassed with 95% O2 plus 5% CO2. Atropine (10⁻⁶M), phentolamine (3 nM) and propranolol (10⁻⁶M) were contained in the solution to block cholinergic and adrenergic receptors. The intragastric pressure was monitored with a recorder (Model 561; Hitachi, Tokyo) under a water pressure of 10 cm. Gastric relaxation was assessed as pressure change from the control level (net pressure change) or percentage of the control response. The preparation was allowed to stabilize for at least 20 min.

EVS and experimental designs
EVS was applied via a pair of platinum electrodes at the position of the lower esophagus, according to our previously reported method (22). Square-wave pulses were delivered from an electronic stimulator (SEN-2201; Nihon Kohden, Tokyo). The vagal stimulation was applied with the stimulation parameters of 5 Hz, 0.5 msec, 100 V (supramaximal voltage) and 10 sec trains, unless otherwise described. The subsequent stimulations were repeated at intervals of 10 min. The response to the 2nd stimulation was used as a control response for the following treatments. Test drugs were applied at 5 or 40 min before the onset of the 3rd stimulation. In some experiments, another series of drugs was applied after termination of the response to the 3rd stimulation.

At the end of each experiment, a maximal relaxation was induced by administration of papaverine (30 μM). In some experiments the relaxation of the stomach was expressed as a percentage of the papaverine induced relaxation.

EFS
EFS was applied via two parallel platinum electrodes around the isolated whole stomach. The stimulation conditions were 5 Hz, 3.0 msec, 100 V (supramaximal voltage) and 10 sec trains with an interval of 10 min. Drug treatments were conducted similarly to those of the EVS experiments.

Drugs
Drugs used were as follows: tetrodotoxin (Sankyo, Tokyo); atropine sulfate monohydrate, propranolol hydrochloride, sodium nitrite (Wako Pure Chemical Industries, Osaka); phentolamine mesylate (Funakoshi, Tokyo); hexamethonium dichloride (Tokyo Kasei, Tokyo); trimetaphan camsilate (Japan Roche Co., Tokyo); L-NAME, N⁵-nitro-L-arginine methylester (L-NAME), L-arginine, D-arginine, bovine hemoglobin (Sigma Chemical Co., St. Louis, MO, USA); papaverine hydrochloride (Iwaki Pharmaceuticals, Tokyo); and methylene blue trihydrate (Nacalai Tesque, Inc., Kyoto). All drugs were dissolved in saline.

NO was made by adding 1 N hydrochloride solution to sodium nitrite (NaNO2) solution under cooling with ice. The concentration of NO in the solution was regarded to be the same as that of sodium nitrite.

Statistical analyses of data
All data are expressed as the means ± S.E.M. Statistical analyses were performed by the unpaired Student’s t-test, and P values of ≤0.05 were considered to indicate a significant difference.

RESULTS

EVS-induced relaxatory response in the isolated mouse whole stomach
EVS (5 Hz, 0.5 msec, 100 V with 10 sec trains) induced a maximal relaxation in the isolated mouse whole stomach (Figs. 1A and 2). The magnitude of this relaxatory response amounted to 45.0±16.6% (the mean ± S.E.M. of 4 experiments) of the relaxation induced by 30 μM papaverine. The relaxatory response to EVS quickly occurred upon stimulation. After cessation of stimulation, the decreased tone returned to the basal level with both rapid and slow recovery phases. The relaxatory response was reproducible by repeated stimulation with intervals of 10 min (data not shown). In most cases, the response to the 2nd EVS was very similar to the subsequent EVS responses in the magnitude and pattern of relaxation, so that the 2nd EVS response was referred...
to as the control in the subsequent experiments with drugs.

The magnitude of the relaxation was dependent on the frequency of vagal stimulation from 1 Hz to 5 Hz (Figs. 1A and 2). The frequencies of 10 and 20 Hz apparently caused a decrease in relaxatory response. Accordingly, the optimal frequency for vagal stimulation in eliciting relaxation was considered to be 5 Hz. In contrast, changes in duration of stimulation (0.5, 2.0 and 3.0 msec) had no significant influence on the magnitude of relaxation (data not shown).

**Effect of autonomic ganglionic blockers and tetrodotoxin on the EVS-induced NANC relaxation**

Experiments were carried out to determine if autonomic ganglionic blockers and tetrodotoxin inhibit the EVS-induced responses, because the EVS was applied at the position of the esophagus (Table 1). The relaxatory response to EVS was markedly inhibited by 300 μM hexamethonium and completely suppressed by 300 μM trimetaphan. The relaxation was also abolished by 0.3 μM tetrodotoxin.
Effect of NOS inhibitors, hemoglobin and methylene blue on the EVS-induced NANC relaxation

Application of 100 μM L-NNA in itself caused no significant change in the original luminal tone (Fig. 1, B and C). In the presence of L-NNA, the EVS relaxatory responses were markedly reduced, regardless of the stimulation frequency (Figs. 1, B and C; and 2). The inhibition of the EVS responses by L-NNA was markedly reversed by 2 mM L-arginine, but not by 2 mM D-arginine (Table 1). L-Arginine alone did not have any significant influence on the luminal tone, but D-arginine frequently caused a transient rise in the luminal tone. The EVS-induced relaxations were also blocked by pretreatment with 100 μM L-NAME, another NOS inhibitor, and this blockade was significantly reversed by 2 mM L-arginine (data not shown).

The experiments were conducted to determine if hemoglobin, a radical trapping agent, and methylene blue, a soluble guanylate cyclase inhibitor, could block the EVS relaxatory responses (Table 1). The EVS relaxatory responses were not significantly changed by pretreatment with either of the drugs for 5 min. When the stomach was treated with the drug for 40 min before the EVS, the relaxatory response was markedly inhibited by 30 μM methylene blue, but not by 10 μM methylene blue and 10 μM hemoglobin. Hemoglobin caused no significant change in the basal tone throughout a period of 40 min, but methylene blue caused a transient rise in the basal tone, with spontaneous spike contractions (data not shown). In all cases, however, the increased luminal tone returned to the original level.

Table 1. Effect of several autonomic drugs and NO related agents on NANC relaxations induced by EVS (5 Hz, 0.5 msec, 100 V, 10 sec trains) in the mouse whole stomach

| Drug            | Concentration (μM) | Relaxation (% of 2nd EVS response) |
|-----------------|--------------------|------------------------------------|
| Pretreatment for 5 min |                    |                                    |
| Control         |                    | 100.4± 3.9                         |
| Tetrodotoxin    | 0.3                | 0.0± 0.0***                        |
| Hexamethonium   | 300                | 2.0± 1.3***                        |
| Trimetaphan     | 300                | 0.0± 0.0***                        |
| L-NNA           | 100                | 1.8± 1.8***                        |
| + L-Arginine    | 2000               | 78.4± 41.1**                       |
| L-NNA           | 100                | 3.1± 3.1***                        |
| + D-Arginine    | 2000               | 3.3± 3.3***                        |
| Hemoglobin      | 10                 | 83.2± 12.1                         |
| Methylene blue  | 10                 | 102.5± 9.7                         |
| Pretreatment for 40 min |                |                                    |
| Control         |                    | 79.1± 6.5                          |
| Hemoglobin      | 10                 | 59.6± 8.7                          |
| Methylene blue  | 10                 | 102.1± 14.1                        |

Values represent the mean ± S.E.M. of 4 experiments. **P<0.01, ***P<0.001 vs control response (unpaired comparison). 

Fig. 3. Original recording of relaxing effect of exogenous NO in the mouse stomach and the effect of NO related agents on the relaxations induced by exogenous NO. This is typical of 4 experiments. A: Concentration-dependent effect of exogenous NO (addition of acidified NaNO2). The magnitude of NO relaxation was compared with that of 30 μM papaverine (Pap). B: Effect of 100 μM L-NNA. C: Effect of 10 μM hemoglobin (Hb). D: Effect of 10 μM methylene blue (MB).
Effect of exogenous NO on the luminal tone

Addition of acidified NaNO₂ (NO; 1–300 μM) produced a decrease in the luminal tone in a concentration-dependent fashion (Fig. 3A). More than 100 μM NO evoked long-lasting relaxations. Repeated addition of acidified NaNO₂ produced a sustained relaxation without desensitization to NO (data not shown). The relaxatory response to NO (10 μM) was not influenced by 0.3 μM tetrodotoxin, 300 μM hexamethonium (data not shown) or 100 μM L-NNA (Fig. 3B). In contrast, the relaxatory response to NO was markedly inhibited by pretreatment with 10 μM hemoglobin (Fig. 3C) or with 10 μM methylene blue for 5 min (Fig. 3D).

Table 2. Effect of several autonomic drugs and NO related agents on NANC relaxations induced by EFS (5 Hz, 3.0 msec, 100 V, 10 sec trains) in the mouse whole stomach

| Drug                | Concentration (μM) | Relaxation (% of 2nd EFS response) |
|---------------------|-------------------|------------------------------------|
| Pretreatment for 5 min |                    |                                    |
| Control             | 100.4±10.2        |                                    |
| Tetrodotoxin        | 0.3               | 0.0±0.0**                          |
| Hexamethonium       | 300               | 100.7±8.1                          |
| L-NNA + L-Arginine  | 100               | 18.0±1.8**                         |
|                     | 2000              | 89.5±5.9**                         |
| Pretreatment for 40 min |                  |                                    |
| Control             | 67.7±5.1          |                                    |
| Hemoglobin          | 10                | 56.8±7.3                           |
| Methylene blue      | 30                | 24.7±8.4**                         |

Values represent the mean±S.E.M. of 4 experiments. **P<0.01 vs control response (unpaired comparison). ***P<0.001 vs L-NNA (unpaired comparison).

Effect of exogenous NO on the luminal tone

Addition of acidified NaNO₂ (NO; 1–300 μM) produced a decrease in the luminal tone in a concentration-dependent fashion (Fig. 3A). More than 100 μM NO evoked long-lasting relaxations. Repeated addition of acidified NaNO₂ produced a sustained relaxation without desensitization to NO (data not shown). The relaxatory response to NO (10 μM) was not influenced by 0.3 μM tetrodotoxin, 300 μM hexamethonium (data not shown) or 100 μM L-NNA (Fig. 3B). In contrast, the relaxatory response to NO was markedly inhibited by pretreatment with 10 μM hemoglobin (Fig. 3C) or with 10 μM methylene blue for 5 min (Fig. 3D).

EFS-induced relaxatory response in the isolated mouse whole stomach

EFS (5 Hz, 3.0 msec, 100 V with 10 sec trains) induced a relaxation in the isolated mouse whole stomach (Fig. 1D). The magnitude of this response amounted to 41.3±6.9% of the relaxation induced by 30 μM papaverine. The relaxatory response was reproducible by repeated stimulation at intervals of 10 min and was frequency-dependent (data not shown). This relaxation was abolished by 0.3 μM tetrodotoxin, but not affected by 300 μM hexamethonium (Table 2). On the other hand, the relaxation was abolished by 100 μM L-NNA, and this blockade was reversed by 2 mM L-arginine. Pretreatment with 30 μM methylene blue, but not with 10 μM hemoglobin, for 40 min produced a marked reduction in the EFS relaxatory responses (Table 2).

DISCUSSION

Many reports have so-far concerned the role of NO in mediating NANC relaxation of isolated gastric smooth muscle in animal species such as rats (15, 16), cats (23), dogs (24), guinea pigs (17–19) and ferrets (25). At present, however, no knowledge is available about the NANC inhibitory response in the mouse stomach. In addition, most of these studies involve EFS in the isolated gastric tissues. As for stimulation of the vagus nerve, several studies on the isolated guinea pig stomach (17, 19, 26) have suggested that the NANC relaxation induced by the EVS is mediated by NO. In the present study, the NANC response has been induced by EVS as well as by EFS in the isolated mouse whole stomach. The most important findings of our study were that the EVS also elicits an inhibitory NANC response in the mouse stomach and that the response is markedly inhibited by L-NNA, with an apparent antagonism by L-arginine, and is also depressed by methylene blue.

In the present study, the optimal conditions of EVS for producing relaxatory responses were 5 Hz, 0.5 msec, and a supramaximal voltage of 100 V with 10 sec trains. The EVS response was reproducible by repeated stimulation at intervals of 10 min. Since the relaxatory response was observed in the presence of atropine, propranolol and phentolamine, the NANC neurons of the vagus nerve may be involved in the inhibitory neurotransmission. The relaxatory response to the EVS was blocked by tetrodotoxin, suggesting that the response may be of neurogenic origin. The two kinds of autonomic ganglionic blockers, hexamethonium and trimetaphan, strongly inhibited the relaxatory response to EVS. These findings indicate that the EVS elicits a relaxation of the stomach exclusively via the preganglionic neurons. The same neural transmission induced by the EVS has been observed in the acid secretory response of the isolated mouse whole stomach (22).

As for the NANC neurotransmitter in the relaxatory response of the gastrointestinal tract, NO is one of the most important candidates (17, 18). This has been strongly supported by recent studies which immunocytochemically demonstrate that NO synthase is localized to nerve cell bodies and nerve fiber varicosities of the myenteric plexus in the guinea pig stomach (19). The present study showed that the EVS relaxatory response was blocked by the NOS inhibitor, regardless of the stimulation frequency. In addition, the blockade by the NOS inhibitor was greatly reversed by L-arginine, a substrate for NOS, but not by D-arginine. It is well known that there is a stereospecific requirement for arginine as the NOS substrate. These findings suggest that the EVS-induced relaxatory response is elicited via the re-
lease of NO in the NANC nerves.

Effect of exogenous NO on the luminal tone was investigated by addition of acidified NaNO₂. Exogenous NO induced a transient relaxation in the gastric basal tone in a concentration-dependent fashion. Repeated application of NO did not produce any desensitization of the relaxatory response. The short-lasting action of exogenous NO might be due to its disappearance from the organ bath. The relaxatory response to exogenous NO was not affected by tetrodotoxin, hexamethonium and L-NNA. However, the relaxation induced by NO was abolished by pretreatment with 10 µM hemoglobin, a NO radical trapping agent, or with 10 µM methylene blue, an inhibitor of soluble guanylate cyclase for 5 min. On the other hand, the EVS-induced relaxation was seemingly resistant to the same application of hemoglobin or methylene blue. However, the relaxation was apparently inhibited by pretreatment with 30 µM methylene blue for 40 min. Differences in the response to methylene blue and, particularly, to hemoglobin, would be due to their delayed diffusion into the synaptic cleft where NO is released. Our findings are consistent with those reported in the isolated guinea pig stomach (17, 26) and suggest a primary role of NO in the NANC relaxatory transmission.

The relaxation induced by EFS was also investigated in the mouse whole stomach. The relaxatory response to EFS was very similar to that of the EVS response in its magnitude and pattern. However, the relaxation induced by EFS was not affected by hexamethonium. This finding indicates that the relaxatory response to EFS may be induced by stimulation of the vagal postganglionic neurons or the myenteric plexus nerve. Generally speaking, however, the EFS may stimulate all kinds of neurons including the vagus nerves and sympathetic nerves, regardless of the pre- or postganglionic fibers. Therefore, as compared with the EFS, the EVS is characterized with selective stimulation of the preganglionic neurons of the vagus nerve. On the other hand, the relaxation induced by the EFS was greatly inhibited by L-NNA, with an apparent antagonism by L-arginine and methylene blue, suggesting a primary involvement of NO in the NANC relaxatory response.

In the EVS relaxatory responses of the mouse stomach, the release of NO may account for much of the magnitude of the relaxation. This finding is consistent with the results obtained for the isolated guinea pig stomach (17, 19, 26). In the rat gastric fundus, however, there is already a good deal of evidence to suggest that VIP contributes to the NANC relaxation induced by EFS, especially with stimulation of higher frequency (15, 27). At a frequency lower than 8 Hz, relaxation is reported to be mediated by NO (27) and to be partially blocked by the L-arginine analog L-N⁵G-monomethyl arginine (15). Accordingly, both NO and VIP may be inhibitory NANC transmitters in the rat gastric fundus.

In conclusion, the present results clearly show that the EVS, which selectively excites the preganglionic neurons of the vagus nerve, elicits NANC relaxation in the isolated mouse whole stomach. The EFS, which excites several kinds of myenteric plexus nerves, also induces the NANC relaxation. NO or some NO related substance(s) may be an essential transmitter in the vagal NANC transmission in the mouse stomach.

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