Nitroxergic nerves mediate vagally induced relaxation in the isolated stomach of the guinea pig
(nitric oxide/nonadrenergic, noncholinergic neurons/adaptive relaxation/receptive relaxation)

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Contributed by John R. Vane, September 27, 1991

ABSTRACT Here we show that the relaxation induced by stimulation of the vagus nerve in the presence of cholinergic (muscarinic) and adrenergic blockade in the isolated stomach of the guinea pig is mediated by nitric oxide (NO). This is substantiated by inhibition of vagal relaxation by N⁰-monomethyl-L-arginine, an inhibitor of NO synthesis. The effect of N⁰-monomethyl-L-arginine was partially reversed by coinubcation with L-arginine but not with D-arginine. NO activates soluble guanylate cyclase, and relaxation of the stomach induced by vagal stimulation was prevented by an inhibitor of soluble guanylate cyclase, methylene blue, further supporting our conclusions. The relaxant effect of vagal stimulation was also ablated by hexamethionum, an inhibitor of ganglionic nicotinic receptors, thereby showing that ganglionic transmission did not rely on NO, through its release from preganglionic neurons. However, hexamethionum did not inhibit the gastric relaxation brought about by increasing the intragastric pressure, which is also mediated by NO as previously described by us. The selective inhibition by hexamethionum of only the vagally mediated relaxation but not of the pressure-induced relaxation of the stomach indicates the existence of at least two separate neuronal pathways able to generate NO and bring about gastric accommodation of food or fluid.

Endothelium-derived relaxing factor, or nitric oxide (NO; ref. 1), plays important roles in the cardiovascular and immune systems (2, 3). Demonstration of the biosynthesis of NO by neuronal cells in the central nervous system (4) suggested a role for NO in intercellular communication in the brain. NO synthase, an NADPH-dependent dioxygenase that catalyzes formation of NO from one of the guanidino nitrogen of L-arginine, has been purified from the rat brain (5), sequenced, and expressed from its cDNA (6). The enzyme has been immunolocalized in nerve fibers and cell bodies of neurons in the brain and in the myenteric plexus in the gastrointestinal tract (7). Moreover, the myenteric plexus is an abundant source of neurons showing NADPH diaphorase activity (8), and NADPH diaphorase is a NO synthase (9). All these findings suggest the existence of nerves that release NO as a transmitter (“nitroergic” nerves) in the central and peripheral nervous system. Inhibitory nerves were described in the stomach (10) and nonadrenergic, noncholinergic (NANC) inhibitory nerves have now been recognized in many parts of the gastrointestinal tract (11). Recent evidence demonstrates that these nerves are nitroergic at some sites. Thus, the generation and release of NO has been demonstrated on electrical stimulation of NANC nerves in the canine ileocolic junction (12) and rat stomach fundus (13). Also, the inhibition of NO formation prevents the relaxation of gastrointestinal smooth muscle, induced by electrical field stimulation of NANC nerves (13-15) or by other putative NANC neurotransmitters such as vasoactive intestinal peptide (VIP) (15), adenosine 3'-triphosphate (ATP), or γ-amino butyric acid (16).

Recently we showed that NO mediates adaptive relaxation in the isolated stomach of the guinea pig (17). Adaptive relaxation is a reflex in which the fundus of the stomach dilates in response to small increases in intragastric pressure (18). In receptive relaxation the gastric fundus dilates when food passes down the pharynx and the esophagus (19) or during early stages of vomiting (20, 21). This reflex depends on extragastric innervation provided by the vagus nerve (22) and is mediated by NANC nerves (23). VIP (24), ATP (25), and calcitonin gene-related peptide (CGRP) (26) have all been proposed as NANC mediators of gastric relaxation. Here, as with adaptive relaxation (17), we demonstrate that NO is an essential mediator of relaxation of the isolated stomach of the guinea pig, induced by stimulation of the vagus nerve.

MATERIALS AND METHODS

Materials. N⁰-Monomethyl-L-arginine (MeArg) acetate was purchased from Calbiochem. L-Arginine hydrochloride, D-arginine hydrochloride, atropine sulfate, guanethidine sulfate, hexamethionum bromide, and methylene blue were purchased from Sigma. All other reagents used were of the highest commercially available purity and were purchased from either BDH or Sigma.

Methods. Male Hartley guinea pigs (300-350 g) were used. The guinea pig was killed by cervical dislocation followed by exsanguination. The abdomen and the thorax were cut open by a midline incision. The esophagus along with the vagus nerve was exposed by gentle dissection. They were cut at the proximal end, removed with the stomach after the proximal duodenum was cut off, and transferred to oxygenated (95% O₂/5% CO₂) Krebs solution (118 mM NaCl/4.7 mM KCl/1.17 mM KH₂PO₄/2.5 mM MgSO₄/25 mM NaHCO₃/2.5 mM CaCl₂/5.6 mM glucose, pH 7.4). The stomach was then cannulated by a wide-bore plastic cannula (i.d., 5 mm) through the duodenal end and ligated. The esophagus was ligated at its proximal end so as not to damage the vagus. The contents of the stomach were flushed out gently through the cannula. The stomach then was mounted in a warmed (38°C) organ bath (450 ml) filled with 200 ml of oxygenated Krebs solution. The esophagus with the vagus nerve intact was passed through a narrow tube with ring electrodes, connected to a Harvard Grass S 88 stimulator, and immersed in Krebs solution to prevent drying of the tissue. The cannula in the

Abbreviations: MeArg, N⁰-monomethyl-L-arginine; VIP, vasoactive intestinal peptide; CGRP, calcitonin gene-related peptide; NANC, nonadrenergic, noncholinergic.

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RESULTS

MeArg Inhibits Relaxation of the Isolated Stomach in Response to Vagal Stimulation. Increasing gastric pressure increased the gastric volume until a point was reached where the volume increased sharply (e.g., at 6 cm H2O; Fig. 1). This increased filling of the stomach represents a response, which was independent of extragastric innervation, NANC in nature, not susceptible to nicotinic receptor blockade, but blocked by NO synthesis inhibitors (17). After the reservoir supplying Krebs solution to the stomach was raised to a point 1–2 cm H2O below that at which pressure-induced adaptive relaxation occurred, the vagus nerve was stimulated, which caused a strong relaxation. The mean relaxation induced by vagal stimulation was 61.2 ± 5.4% (n = 9) of the total gastric volume (see Fig. 4). Preincubation of the stomach with MeArg (300 μM) for 30 min inhibited vagally induced relaxation of the stomach to 16.8 ± 6.8% of the total gastric volume (n = 5, P < 0.001). This inhibition was not reversed by coincubation (25 min) of MeArg with d-arginine (2 mM; 19.5 ± 9.7%, n = 5) but was partially reversed by L-arginine (2 mM; 44.3 ± 6.5%, n = 5, P < 0.01) coincubated for the same time. MeArg did not influence the pressure-volume relationship of the stomach determined at pressures from 1 to 4 cm H2O (n = 7–11, P > 0.05). Furthermore, incubation of the stomach with D- or L-arginine (2 mM) alone for 25 min did not influence the relaxation induced by vagal stimulation. In these experiments, control responses to vagal stimulation were 31.7 ± 6.9% (n = 3), whereas responses were 52.1 ± 5.8% or 54.4 ± 0.9% of the total gastric volume in the presence of L-arginine or D-arginine (2 mM), respectively (n = 3).

Methylene Blue Inhibits Relaxation of the Isolated Stomach in Response to Vagal Stimulation. Incubation of the stomach (20–45 min) with methylene blue (10–40 μM) inhibited responses to vagal stimulation (Fig. 2). In the presence of methylene blue, vagal stimulation elicited relaxation of the stomach that was 12.5 ± 6.3% of the total gastric volume (see Fig. 4; n = 3, P < 0.001).

Hexamethonium Inhibits Relaxation of the Isolated Stomach in Response to Vagal Stimulation. As previously reported (10, 17), hexamethonium did not block the adaptive relaxation response to increases in intragastric pressure. However, it inhibited gastric relaxation induced by vagal stimulation (Fig. 3). Preincubation of the stomach with hexamethonium (0.5 mM) for 15 min strongly inhibited the vagally mediated relaxation of the stomach, to 7.2 ± 3.0% of the total gastric volume (see Fig. 4; n = 4, P < 0.001).

MeArg and Hexamethonium Do Not Inhibit the Relaxation of the Isolated Stomach Induced by Glyceryl Trinitrate. To

![Fig. 1](attachment:image.png)

Fig. 1. Inhibition of NO synthesis attenuates gastric relaxation in response to vagal stimulation. This trace depicts changes in gastric volume at different intragastric pressures (control) and after vagal stimulation (VS) at a fixed intragastric pressure in the isolated guinea pig stomach. The control record was obtained by stepwise increments (1 cm H2O each) of intragastric pressure up to a reproducible point (6 cm H2O) where the fundus suddenly relaxed and the volume increased sharply with no increase in the pressure. Subsequently, the intragastric pressure was raised 2 cm H2O below the threshold for reflex relaxation and the vagus nerve was stimulated. Each of three periods of stimulation induced gastric relaxation that was inhibited by incubation with MeArg (300 μM). This inhibition was partially reversed by coincubation with L-arginine (2 mM) but not D-arginine (2 mM). E denotes emptying of the stomach after a response and arrowheads indicate vagal stimulation (VS). This trace is representative of five experiments.
confirm that MeArg and hexamethonium inhibited the vagally induced generation of NO but not its action within the gastric wall, the effects of these inhibitors on the relaxant effect of glyceryl trinitrate, an exogenous NO donor, were tested. At intragastric pressures below the point at which adaptive relaxation occurred, glyceryl trinitrate (0.7-1.1 μM) increased the gastric volume by 64.0 ± 0.9% of the total gastric volume (n = 3). This effect was not inhibited by MeArg (300 μM) alone (58.6 ± 1.6%, n = 3, P > 0.05) or in combination with L-arginine (2 mM; 50 ± 4.7%, n = 3, P > 0.05); nor was it inhibited by hexamethonium (0.5 mM; 60.5 ± 0.5%, n = 3, P > 0.05).

**DISCUSSION**

Our results demonstrate that NO mediates relaxation of the isolated stomach of the guinea pig induced by stimulation of the vagus nerve, for the relaxation was blocked by MeArg, an inhibitor of NO synthase (27). Moreover, the inhibitory effect of MeArg was reversed by L-arginine, the physiological substrate for NO synthase, but not by D-arginine. MeArg did not inhibit gastric relaxation induced by glyceryl trinitrate, which is metabolized by smooth muscle to NO independently of NO synthase (28), indicating the specificity of action of MeArg on the endogenous pathway of NO formation.

NO relaxes vascular and nonvascular smooth muscle through the activation of soluble guanylate cyclase and the subsequent increase in cGMP levels (29). Gastric relaxation induced by vagal stimulation was inhibited by methylene blue, an inhibitor of NO-induced activation of guanylate cyclase (30), further demonstrating that this effect depends on the generation of NO within the gastric wall. Moreover, they mediate relaxation of the stomach during vomiting (36), affent vagal stimulation (37), and distension of the pharynx or esophagus (38) or of the stomach antrum (39). In 1957 Paton and Vane (10), studying the relationship between intragastric pressure and volume in the isolated stomach, observed that when the intragastric pressure was raised to about 4–7 cm H2O the fundus of the stomach suddenly dilated, allowing for a significant increase in intragastric volume without further increases in pressure and indicating the existence of a local, inhibitory reflex.

The studies summarized above showed that the reservoir function of the stomach is regulated by a reflex activated by distension of the stomach, as well as by vago-vagal reflexes responsible for receptive relaxation during deglutition or vomiting. Similar mechanisms operate in man, for vagotomy causes symptoms of epigastric fullness and early satiety that correspond to increased intragastric pressure and a shift of the pressure-volume curve to the right (40). The neurotrans-
mitters responsible for these important physiological reflexes were not identified over a century of investigations.

Recently we showed (17) that l-arginine-derived NO mediates adaptive relaxation in the isolated stomach of the guinea pig. Our present results demonstrate the involvement of NO in the vagally induced gastric relaxation and strongly suggest that NO also mediates gastric relaxation caused by vago-vagal inhibitory reflexes. Moreover, in our previous study (17) we further showed that, in l-dimethyl-4-phenylpiperazinium (DMPP), a nicotinic receptor agonist, elicited concentration-dependent relaxations of the stomach that were also mediated by NO. Hexamethonium antagonized the effects of DMPP but it did not block the pressure-induced adaptive relaxation. Therefore, we postulated the existence of a second neuronal pathway, different from the reflex activated by changes in intragastric pressure, which involves ganglionic nicotinic transmission and leads to NO-mediated relaxation of the stomach. Our present results explain the physiological role of this second NO-dependent pathway, which is mediation of the vagal inhibitory reflexes.

Complete blockade of both pressure-induced and vagally induced relaxation of the stomach by inhibitors of NO synthesis demonstrates that NO is an essential mediator of these responses. In contrast, NANC relaxations of isolated strips of rat gastric fundus elicited by electrical field stimulation were only partially inhibited by MeArg and the residual responses were inhibited by VIP antiserum (15). This may reflect species differences or perhaps a more diffuse activation of all neurons by electrical field stimulation.

We cannot completely exclude some involvement of other putative NANC neurotransmitters in adaptive and receptive relaxation. Thus, electrical stimulation of the vagus nerve or distension of the esophagus increased concentrations of immunoreactive VIP in the venous effluent of the stomach in the cat (24), and other studies demonstrated inhibition by VIP antiserum or VIP-cleaving proteases of the relaxations of isolated fundal strips from the rat (15, 41), guinea pig (42), and cat (43) induced by electrical field stimulation of NANC nerves. Desensitization of P2 receptors (44) or their blockade (45) caused inhibition of vagally induced relaxations of the stomach of the cat. Moreover, substance P-, VIP-, CGRP-, serotonin-, tyrosine hydroxylase (dopamine)-, and neuropeptide Y-containing neurons have been immunolocalized in the myenteric plexus of the guinea pig fundus (46) and, therefore, have also been considered as possible transmitters of neuronal reflex pathways in the stomach.

However, our results do not support a role for any of these substances as a final common mediator of physiological reflexes regulating intragastric pressure. As depicted in Fig. 5, where we propose the neuronal pathways involved in reflex relaxation of the stomach, a role of NANC transmitters other than NO, if any, can only be limited to interneuronal transmission. Another conclusion is that NO is not a mediator of ganglionic transmission involving preganglionic fibers, as indicated by complete inhibition of vagally induced gastric relaxation by hexamethonium. The resistance of pressure-induced gastric relaxation to hexamethonium can also be explained by an axon reflex (47) leading to NO release from sensory nerves, though experimental proof for this is required. Local release, as with some other mediators (48), of NO from the same sensory nerve terminal that is activated by a stimulus is not supported by the observed tetrodotoxin sensitivity of pressure-induced gastric relaxation (17).

We conclude that NO is an essential NO diatomic of both intrinsic and extrinsic inhibitory reflexes regulating the storage function of the stomach and that nitroergic neurons form a common final pathway in these reflexes. Our results thus present a function for NO release that is associated with adaptive and vagally induced relaxation of the stomach.

**FIG. 5.** A schematic diagram showing the intrinsic and the extrinsic vagally mediated reflex pathways in the stomach wall that mediate adaptive and receptive relaxation of the stomach. Adaptive relaxation is an intragastric pressure-induced reflex. Stretch of the stomach wall activates mechanoreceptors (MR) in the mucosa (Mu), which generate impulses carried by the sensory neuron (SN), leading to the release of one or more putative neurotransmitters (e.g., substance P, VIP, CGRP, ATP, dopamine). The SN can synapse on the inhibitory efferent neuron (EN) directly or activate it via interneurons (IN) of the myenteric plexus (MP). This leads to the release of nitric oxide (NO) from the nitroergic EN, which activates guanylate cyclase (GC) and causes relaxation of the circular muscle (CM) and hence of the fundus. Alternatively, an axon reflex causes NO release from the SN, resulting in hexamethonium (Hm)-resistant gastric relaxation. The neurons involved in the extrinsic reflex responsible for receptive relaxation of the stomach enter the stomach wall as vagal motor fibers (M). The impulses carried by these fibers activate the nitroergic EN, directly or through IN, and lead to the generation of NO. However, in contrast to the pressure-activated reflex arc, ganglionic nicotinic transmission, inhibited by Hm, is essential in vagally mediated relaxation. Whether the same efferent neuron is involved in the pressure-activated reflex and in the vagal pathway remains to be established. (Se, serosa; LM, longitudinal muscle; S, sensory fiber).

This work was supported by a grant from Glaxo Group Research Ltd.