Suppression by $N^G$-Nitro-L-Arginine of Relaxations Induced by Non-Adrenergic, Non-Cholinergic Nerve Stimulation in Dog Duodenal Longitudinal Muscle

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ABSTRACT — In dog duodenal longitudinal muscle strips, transmural electrical stimulation (10 Hz, 15 sec) elicited a transient contraction, which was abolished by tetrodotoxin and atropine but potentiated by treatment with $N^G$-nitro-L-arginine (L-NA), a nitric oxide (NO) synthesis inhibitor. The potentiation was reversed by L-arginine but not by its D-enantiomer. Acetylcholine-induced contractions were not influenced by L-NA. After treatment with atropine, the electrical neural stimulation relaxed the muscle strips partially contracted with bradykinin, the relaxation being abolished by tetrodotoxin and suppressed by L-, but not D-, NA. L-arginine reversed the L-NA-induced inhibition. Oxyhemoglobin abolished the relaxation caused by nerve stimulation and NO. The neurally-induced relaxation was not attenuated by adrenoceptor antagonists and indomethacin. It is concluded that electrical stimulation of non-adrenergic, non-cholinergic nerves relaxes dog duodenal smooth muscle, due possibly to NO produced upon neural excitation, and potentiation by L-NA of the contractile response to cholinergic nerve stimulation would be derived from elimination of the neurally-induced relaxation.

Autonomic efferent innervation plays quite an important role in regulating functions of smooth muscle in the gastrointestinal tract, gall bladder, urinary bladder, vas deferens, uterus, iris, blood vessel, etc. In addition to classical adrenergic and cholinergic innervation, histochemical studies have demonstrated abundant nerves innervating the gastrointestinal and cerebroarterial wall that contain a variety of peptides including substance P, vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP) and galanin (1, 2). However, information concerning the functional roles of the peptides released by nerve stimulation is lacking.

Very recently, we have determined that relaxations of endothelium-denuded cerebral arteries caused by electrical and chemical stimulation of non-adrenergic, non-cholinergic nerves are abolished by treatment with nitric oxide (NO) synthesis inhibitors such as $N^G$-monomethyl-L-arginine (L-NMMA; 3) and $N^G$-nitro-L-arginine (L-NA; 4), although the D-enantiomers are ineffective; and the inhibitory effect is prevented and reversed by L-, but not D-, arginine (4–7). Therefore, NO appears to play a crucial role in transmitting information from non-adrenergic, non-cholinergic vasodilator nerves to cerebral artery smooth muscle. Our preliminary study showed that similar inhibition by L-NA was observed in the relaxant response to non-adrenergic nerve stimulation of isolated dog duodenal longitudinal muscle strips (8). Bult
et al. (9) and Desai et al. (10) have also reported a possible involvement of NO in the neurally-induced relaxation in isolated dog ileocolonic junction and isolated guinea pig stomach.

We performed the present study to analyze the mechanism of relaxation caused by nerve stimulation in dog duodenal longitudinal muscle strips with special reference to NO synthesis inhibition, and to determine the functional relationship between the inhibitory and the excitatory cholinergic nerves.

MATERIALS AND METHODS

Mongrel dogs of either sex, weighing 7 to 15 kg, were anesthetized with intraperitoneal injections of sodium pentobarbital (50 mg/kg) and killed by bleeding from the carotid arteries. The duodenum was rapidly removed, and longitudinal muscle strips, approximately 20-mm long, were prepared. The specimens were vertically fixed between hooks in a muscle bath (20-ml capacity) containing the nutrient solution, which was aerated with a mixture of 95% O2 and 5% CO2 and maintained at 37 ± 0.3°C. The hook anchoring the upper end of the strips was connected to the lever of a force-displacement transducer (Nihon-Kohden Kogyo Co., Tokyo, Japan). The resting tension was adjusted to 1.5 g, which is optimal for inducing the maximal contraction. Constituents of the solution were as follows: 120 mM NaCl, 5.4 mM KCl, 2.2 mM CaCl2, 1.0 mM MgCl2, 25.0 mM NaHCO3, and 5.6 mM dextrose. The pH of the solution was 7.35 to 7.42. Before the start of the experiments, all of the strips were allowed to equilibrate in the bathing media for 60 to 90 min, during which time the media were replaced every 10 to 15 min.

The duodenal strips were placed between stimulating electrodes. The gaps between the strip and the electrodes were wide enough to allow undisturbed contraction and relaxation, and yet sufficiently narrow to stimulate intramural nerve terminals effectively. A train of 0.2 msec square pulses of supramaximal intensity were transmurally applied at frequencies of 2, 5, 10 and 20 Hz for a period of 15 sec. The stimulus pulses were delivered by an electronic stimulator (Nihon-Kohden Kogyo Co.).

Isometric contractions and relaxations were displayed on an ink-writing oscillograph. The contractile response to 5 mM Ba++ was first obtained, and then the strips were washed three times with fresh media and equilibrated for 30 to 40 min. Transmural electrical stimulation was applied repeatedly at intervals of 10 min until steady responses were obtained, and then blocking agents were applied. To examine the relaxant responses, the strips were precontracted partially with bradykinin (1-3 × 10^-8 M); the contractions ranged between 55 and 70% of the contraction induced by 5 mM Ba++. At the end of each series of experiments, papaverine (10^-4 M) was added to attain the maximal relaxation; relaxations induced by transmural stimulation or chemical agents relative to those induced by papaverine are shown in this report.

The results shown in the text, table and figures are expressed as mean values ± S.E. Statistical analyses were made by Student's paired or unpaired t-test and Tukey's method after one-way analysis of variance. Drugs used were N^G-nitro-L- and D-arginine (L- and D-NA), bradykinin, vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP), substance P (Peptide Research Foundation, Minoh, Japan); galanin (Peninsula Lab., San Carlos, CA, USA); L- and D-arginine (Nacalai Tesque, Kyoto, Japan); adenosine 5'-triphosphate (ATP, Boehringer Mannheim, FRG); acetylicholine chloride (Daiichi Seiyaku Co., Tokyo); atropine sulfate (Tanabe Co., Tokyo); dl-propranolol hydrochloride (Sumitomo Co., Osaka, Japan); phenotamine mesylate (Ciba-Geigy, Japan, Takarazuka, Japan); aminophylline, indomethacin (Sigma Chemical Co., Ltd., St. Louis, MO, USA); tetrodotoxin (Sankyo Co., Tokyo); prostaglandin (PG) E2, PGE_2 (Ono Co., Osaka); beraprost sodium, a stable analog of PGI_2 (Toray Industries Inc., Tokyo); and
papaverine hydrochloride (Dainippon Co., Osaka). Responses to nitric oxide (NO) were obtained by adding NaNO₂ solution (NO) adjusted to pH 2 (11). Oxyhemoglobin (HbO₂) was prepared from dog Hb (Sigma) with the method described by Martin et al. (12).

RESULTS

Contractile response to transmural electrical stimulation

Transmural electrical stimulation at 2 to 20 Hz produced a frequency-dependent contraction in dog duodenal longitudinal muscle strips under resting conditions. The contractions induced at a submaximal frequency of 10 Hz for 15 sec applied every 10 min were consistent and reproducible; therefore, the experiments in the remainder of this paper were carried out with this frequency of stimulation. The stimulation-induced contraction was potentiated by treatment with 10⁻⁶ and 10⁻⁵ M L-NA in a dose-dependent manner (Fig. 1, upper panel). Typical responses are shown in Fig. 2. The addition of L-arginine (10⁻³ M) reversed the potentiation (Figs. 1 and 2). Treatment of the control strips with 10⁻³ M L-arginine did not significantly alter the response to electrical stimulation (96.8 ± 2.0% of the contraction obtained in control media, n = 4); and in the presence of this amino acid, L-NA (10⁻⁵ M) did not increase the response (97.1 ± 1.8% of the control before the addition of L-NA, n = 4). The contractile response was abolished by 10⁻⁷ M atropine (Fig. 2) and 3 × 10⁻⁷ M tetrodotoxin.

Acetylcholine in concentrations ranging from 10⁻⁸ to 10⁻⁴ M contracted the duodenal strips dose-dependently. The concentration-response curve was not influenced by treatment with L-NA in concentrations of 10⁻⁶ and 10⁻⁵ M. The maximal contractions were 97.6 ± 3.7 and 102.3 ± 6.6% of that in the control strips (n = 6), and the apparent median effective concentrations of acetylcholine in the control and L-NA-treated strips were [3.1 ± 0.86], [4.3 ± 1.4] and [3.6 ± 0.86] × 10⁻⁶ M (n = 6), respectively.

Relaxant response to transmural electrical stimulation

In the duodenal muscle strips partially contracted with bradykinin, transmural electrical stimulation elicited a transient contraction followed by a relaxation. Atropine abolished the
contraction, and only relaxations were obtained, which were abolished by $3 \times 10^{-7}$ M tetrodotoxin. Treatment with L-NA ($10^{-6}$ and $10^{-5}$ M) reduced the relaxant response in a concentration-related manner (Fig. 1, lower panel, and Fig. 3), the inhibition being reversed by the addition of L-arginine ($3 \times 10^{-4}$ and $10^{-3}$ M) but not by $10^{-3}$ M D-arginine. On the other hand, D-NA ($10^{-5}$ M) failed to attenuate the relaxation. In control media, L-arginine ($10^{-3}$ M) did not significantly alter the response (Table 1). Under the treatment with L-arginine, the stimulation-induced response was not inhibited by $10^{-5}$ M L-NA.

**Fig. 2.** Contractile responses to transmural electrical stimulation ($10$ Hz, $15$ sec) of a duodenal longitudinal muscle strip before and after treatment with L-NA, L-arginine (L-arg.) and atropine ($10^{-7}$ M). The cross under the tracing represents electrical stimulation in the presence of atropine.

**Fig. 3.** Relaxant responses to transmural electrical stimulation ($10$ Hz, $15$ sec) of a duodenal longitudinal muscle strip contracted with $3 \times 10^{-8}$ M bradykinin before and after treatment with D- and L-NA, D- and L-arginine (arg.), oxyhemoglobin (OxyHb, $1.6 \times 10^{-5}$ M) and tetrodotoxin (TTX, $3 \times 10^{-7}$ M). The recording continues from the upper right to the lower left. The strip was treated with $10^{-7}$ M atropine. PA = $10^{-4}$ M papaverine to attain the maximal relaxation.
Treatment with \(1.6 \times 10^{-5}\) M \(\text{HbO}_2\) suppressed the relaxation caused by electrical stimulation, whereas propranolol \((10^{-6}\) M\), phentolamine \((10^{-6}\) M\) and indomethacin \((10^{-6}\) M\) did not alter the response (Table 1).

The addition of NO \((10^{-5}\) to \(10^{-4}\) M\) produced a transient relaxation in the bradykinin-contracted strips. The maximal relaxation averaged 77.8 ± 4.4\% (n = 8), and the apparent median effective concentration was \([2.8 ± 0.48] \times 10^{-5}\) M (n = 8). The NO-induced relaxation was not influenced by \(10^{-5}\) M L-NA (45.8 ± 3.5 vs. 39.3 ± 6.3\% at \(3 \times 10^{-5}\) M NO, n = 5) but was abolished by treatment with \(1.6 \times 10^{-5}\) M \(\text{HbO}_2\) (from 40.5 ± 4.3 to 1.5 ± 0.62\%, n = 5).

Response to peptides, ATP, prostaglandins and L-arginine

Galanin \((3 \times 10^{-8}\) M\) elicited a moderate, transient relaxation (38.6 ± 5.3\%, n = 8); however, the second dose of the peptide did not alter the muscle tone. Under such a condition in which the strips were made tachyphylactic to galanin, relaxations caused by transmural stimulation were not reduced, as compared to those before the addition of galanin (Table 1). VIP and CGRP \((10^{-8}\) M\) did not relax the strips contracted with bradykinin or acetylcholine (n = 5). Substance P \((10^{-8}\) M\) contracted the strips (n = 3). ATP in concentrations up to \(10^{-6}\) M did not relax the strips; and at \(10^{-5}\) M, it produced a slight, slowly-developing relaxation (24.4 ± 0.9\%, n = 5). Aminophylline \((2 \times 10^{-3}\) M\) markedly suppressed the ATP-induced relaxation (to 2.0 ± 1.3\%, n = 5, P < 0.001 vs. control), but did not alter the relaxant response to transmural stimulation (42.8 ± 6.0 vs. 44.5 ± 5.2\%, n = 5) in the strips treated with atropine. PGE2, PGF\(_2\alpha\), and beraprost, a stable analog of PGI\(_2\) (13), in concentrations of \(10^{-8}\) to \(10^{-6}\) M did not relax the bradykinin-contracted strips (n = 5).

The muscle strips contracted with bradykinin did not appreciably respond to L-arginine \((10^{-3}\) M\) in 3 trials (0.63 ± 0.39, 0.68 ± 0.40 and 0.68 ± 0.45\% contraction, respectively, relative to that caused by 5 mM \(\text{Ba}^{++}\), n = 5). After 15 to 20 min of observations in each trial, the strips were repeatedly washed with the fresh media and then equilibrated for about 60 min. After the series of experiments was over, the strips were incubated in the bathing media overnight, and L-arginine was applied again on the next day. About 24 hr after the first trial on the first day, L-arginine did not relax the strips at all (0.75 ± 0.45\% contraction, n = 5).

DISCUSSION

Transmural electrical stimulation produced a contraction of duodenal longitudinal muscle that was abolished by tetrodotoxin and atropine, suggesting the involvement of acetylcholine released from cholinergic nerves. The induced contraction was potentiated by treatment with L-NA, a NO synthesis inhibitor (4,
14), which however did not alter the response to exogenously applied acetylcholine. Therefore, the increased response to nerve stimulation does not seem to be associated with increased responsiveness to acetylcholine or inhibition of acetylcholinesterase. Although the effect of L-NA on the release of acetylcholine from the nerve was not determined, the release of \(^{3}\)H-norepinephrine from adrenergic nerves is not inhibited in isolated dog temporal arteries (15), in which the contraction caused by nerve stimulation is potentiated by L-NA. NO relaxes duodenal longitudinal muscle (in the present study and in ref. 8). Elimination of the inhibitory effect of NO synthesized in association with transmural electrical stimulation may therefore be involved in the potentiation by L-NA of the neurally-induced contraction.

Only a relaxation was induced by transmural electrical stimulation in the duodenal strips treated with atropine and partially contracted with bradykinin. Substance P contracted the duodenum, and VIP, CGRP and PGs (PGE\(_2\), PGF\(_{2\alpha}\) and beraprost) did not produce relaxation. Galanin has been histochemically detected in enteric neurons (16, 17). Galanin relaxed the duodenal strips; however, even after the relaxing action of exogenous galanin was abolished by tachyphylaxis, relaxant responses to nerve stimulation were not attenuated. Indomethacin, phentolamine, propranolol and aminophylline in concentrations sufficient to suppress the responses to PG-releasing substances, norepinephrine, isoproterenol and ATP, respectively (in refs. 18, 19; in the present study), did not significantly alter the relaxation caused by inhibitory nerve stimulation. It is thus concluded that the polypeptides used in the present study, PGs, adrenergic agonists and ATP are not involved in the neurally-induced relaxation.

The duodenal muscle relaxation caused by transmural neural stimulation was inhibited dose-dependently by treatment with L-NA, the inhibition being reversed by L-arginine but not by D-arginine. D-NA did not inhibit the response. Similar results were also obtained in rat anococcygeus muscle (20), dog ileocolic junction (9) and dog and monkey cerebral arteries (4–7). HbO\(_2\) abolishes the action of NO or EDRF by binding it and inactivates guanylate cyclase in smooth muscle (12). In this and previous (8) studies, HbO\(_2\) abolished the relaxant response to nerve stimulation. The possibility of NO synthesis in smooth muscle is suggested from the finding that L-arginine elicits relaxations and cyclic GMP increments, sensitive to HbO\(_2\), methylene blue and L-NA, in isolated, endothelium-denuded bovine pulmonary artery rings after deprivation of cellular L-arginine during exposure of the preparations for 24 hr to the bathing media (21). However, duodenal muscle strips immersed in the same way in the bathing media did not respond to L-arginine with relaxations, indicating that there is no evidence for the synthesis of muscle-derived NO in the dog duodenum. In addition, an immunohistochemical study (22) has demonstrated that NO synthase-containing nerve cells and fibers are present in the myenteric plexus but not in the duodenal smooth muscle in rats. Therefore, we hypothesized that NO synthesized upon the electrical stimulation possibly in inhibitory nerves is released to transmit information to smooth muscle.

Inhibition by L-NA of the relaxant response to electrical neural stimulation correlates well with potentiation of the stimulation-induced contraction (Fig. 1). These findings may indicate that the tone and motility of duodenal longitudinal muscle are regulated by a balance of the excitatory and inhibitory nerves shown in the present study. In addition to adrenergic prejunctional inhibition in the release of excitatory transmitter from cholinergic nerves (23, 24), direct, inhibitory influences on smooth muscle possibly via the release of NO upon nerve activation appear to play an important role in the regulation of duodenal muscle function and the pathogenesis of muscular dysfunction.
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