Nitrergic and purinergic regulation of the rat pylorus

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1Division of Gastroenterology, Department of Internal Medicine, University of Michigan Health System, Ann Arbor, Michigan 48109; and 2Second Department of Internal Medicine, Wakayama Medical College, 811-1 Kimiidera Wakayama, 640-0012, Japan

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Ishiguchi, Tadashi, Toku Takahashi, Hidekazu Itoh, and Chung Owyang. Nitrergic and purinergic regulation of the rat pylorus. Am J Physiol Gastrointest Liver Physiol 279: G740–G747, 2000.—The role of nitric oxide (NO) and ATP in the regulation of nonadrenergic, noncholinergic (NANC) inhibitory transmission in the pylorus remains unclear. In the presence of atropine and guanethidine, electric field stimulation induced NANC relaxations in a frequency-dependent manner (1–20 Hz) in the rat pylorus. NANC relaxations were significantly inhibited by $N^\text{G}$-nitro-L-arginine methyl ester (L-NAME; $10^{-4}$ M). $P_{2\text{X}}$ purinoceptor antagonist pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS; $3 \times 10^{-5}$ M) and $P_{2\text{Y}}$ purinoceptor antagonist reactive blue 2 ($2 \times 10^{-5}$ M) had no effect on NANC relaxations. However, the combined administration of L-NAME and PPADS, but not reactive blue 2, evoked greater inhibitory effects on NANC relaxations than that evoked by L-NAME alone. α-Chymotrypsin and vasoactive intestinal polypeptide antagonist did not affect NANC relaxations. ATP ($10^{-5}$–$10^{-3}$ M) and PACAP as NANC neurotransmitters in the rat pylorus. ATP-induced relaxation appears to be mediated by $P_{2\text{X}}$ purinoceptors located on smooth muscle cells.

$P_{2\text{X}}$ purinoceptors; $P_{2\text{Y}}$ purinoceptors

Other putative neurotransmitters, such as purinergic (39) and peptidergic neurotransmitters (1, 33), have also been proposed to be NANC inhibitory neurotransmitters in the pylorus. However, the relative contributions of ATP, vasoactive intestinal polypeptide (VIP), and pituitary adenylate cyclase-activating polypeptide (PACAP) in the mediation of NANC relaxations is not fully understood. It has been demonstrated that NOS colocalizes with ATP or VIP in the myenteric plexus (5, 46), but the nature of the interaction between these inhibitory neurotransmitters remains unclear.

Previous studies demonstrated that NO inhibits ACh release in the gastrointestinal tract (19, 27). We have recently demonstrated that NO inhibits the release of VIP and ACh in the rat gastric myenteric plexus (17). Selemidis and colleagues (38) suggested that NO inhibits the release of an apamin-sensitive neurotransmitter in the guinea pig taenia coli. Because apamin is a $Ca^{2+}$-dependent $K^+$ channel inhibitor and not a selective purinoceptor antagonist, it remains unclear whether there is an interaction between NO and ATP release in the myenteric plexus. In this study, we investigated the possible roles of NO, ATP, VIP, and PACAP as NANC neurotransmitters in the rat pylorus. We also examined the possible interaction between NO and ATP in regulating pyloric relaxations.

MATERIALS AND METHODS

Materials. The following chemicals were used: ATP, atropine, carbachol, α-chymotrypsin, guanethidine, PACAP, sodium nitroprusside (SNP), TTX, and VIP (Sigma Chemical, St. Louis, MO); α,β-methyleneadenosine 5′-triphosphate (α,β-Me-ATP), 2-methylthioadenosine 5′-triphosphate (2-MeS-ATP), $N^6$-nitro-L-arginine methyl ester (L-NAME), L-pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), and reactive blue 2 (Research Biochemicals, Natick, MA); VIP antagonist [p-chloro-D-Phe⁶,Leu¹⁷]VIP (Bachem, Torrance, CA).

Methods. Male Sprague-Dawley rats (body wt 230–250 g) were fasted overnight and euthanized by decapitation under an anesthetic of xylazine and ketamine (13 and 87 mg/kg body wt, respectively). After laparotomy, the stomach and proximal duodenum were removed en bloc and an incision was made along the lesser curvature of the stomach. Circular muscle strips were isolated from the antrum, pylorus, and

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duodenum. The muscle strips from the antrum and duodenum were obtained 0.5 cm proximal and 0.5 cm distal from the pylorus, respectively. As previously described (39), muscle strips (10 × 2 mm) were suspended under a load of 1 g between two platinum electrodes in an organ bath filled with Krebs-Henseleit buffer of the following composition (in mM): 118 NaCl, 4.8 KCl, 2.5 CaCl₂, 25 NaHCO₃, 1.2 KH₂PO₄, 1.2 MgSO₄, and 11 glucose. The Krebs-Henseleit buffer was continuously gassed with 95% O₂-5% CO₂ and maintained at 37°C and pH 7.4. Mechanical activity was recorded on a polygraph by means of isometric transducers.

Electric field stimulation (EFS; 75 V, 1 ms, 1–20 Hz, 30 s) was applied through two platinum wire electrodes. To determine whether EFS acts through neural pathways, the effects of TTX (10⁻⁶ M) on EFS-induced relaxations were studied. To determine the neural pathways responsible for the NANC relaxation induced by EFS, atropine (10⁻⁶ M) and guanethidine (10⁻⁶ M) were added to the organ bath. To investigate the neural pathways responsible for mediating EFS-induced relaxation, we studied the effects of the NO biosynthesis inhibitor (l-NAME; 10⁻⁴ M), P₂X purinoceptor antagonist (PPADS; 3 × 10⁻⁵ M), P₂Y purinoceptor antagonist (reactive blue 2; 2 × 10⁻⁵ M), α-chymotrypsin (2 U/ml), and VIP antagonist (5 × 10⁻⁶ M) on EFS-induced relaxations. The concentrations of these antagonists were determined according to previous studies. α,β-ATP-induced relaxations were antagonized by PPADS (3 × 10⁻⁶–3 × 10⁻⁵ M) in the guinea pig taenia coli (47). Relaxations induced by 2-MeSATP were blocked by reactive blue 2 (2 × 10⁻⁵ M) in the rabbit mesenteric artery (11). Relaxations induced by VIP or PACAP were antagonized by α-chymotrypsin (2 U/ml) in the cat lower esophageal sphincter (29). VIP antagonist (10⁻⁶ M) has been shown to inhibit VIP (5 × 10⁻⁸ M)-induced relaxation by 25% in the guinea pig gallbladder (32). In our previous study, VIP antagonist (10⁻⁶ M) also inhibited VIP (10⁻⁶ M)-induced relaxations by 55% in the rat stomach (40). Muscle strips were preincubated with various antagonists for 15 min before EFS.

To investigate the effects of NO, VIP, and ATP on the rat pylorus, SNP (10⁻⁷–5 × 10⁻⁻⁵ M), VIP (10⁻⁹–10⁻⁶ M), PACAP (10⁻⁹–10⁻⁶ M), ATP (10⁻⁹–10⁻³ M), P₂X purinoceptor antagonist (α,β-ATP; 10⁻⁷–10⁻⁶ M), and P₂Y purinoceptor antagonist (2-MeS-ATP; 10⁻⁷–10⁻⁸ M) were applied in a noncumulative manner. A,β-ATP-induced relaxations were antagonized by PPADS (3 × 10⁻⁶–3 × 10⁻⁵ M) and reactive blue 2 (2 × 10⁻⁵ M) on ATP-induced relaxations or SNP-induced relaxations.

To determine whether NO modulates ATP-induced muscle relaxation of the rat pylorus, the effects of a threshold dose of SNP (10⁻⁷ M) on relaxations induced by ATP (10⁻³ M) were studied. To determine whether ATP modulates NO-induced muscle relaxation, the effects of a threshold dose of ATP (10⁻⁵ M) and α,β-ATP (10⁻⁷ M) on SNP (10⁻⁶ M)-induced relaxations were studied.

Statistical analysis. The reduction of the tone induced by EFS or various agonists was measured and expressed as the relaxation. In the frequency-response studies, the results are expressed as a percentage of the maximal relaxation in response to EFS. In the concentration-response studies, the results are expressed as a percentage of relaxation induced by SNP (10⁻⁸ M). All data are expressed as means ± SE, and the number of preparations are reported. Statistical analysis was performed using ANOVA. Differences were considered significant if the P value was <0.05.
Effect of L-NAME was completely antagonized by L-arginine (10^{-4} M) in the absence (A) and presence (B) of N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME; 10^{-4} M). Effects of L-NAME (10^{-4} M) on NANC relaxations evoked by different frequencies of EFS stimulation are shown in C. NANC relaxations were significantly antagonized by L-NAME (n = 5, df = 1.40, F = 51.6, P < 0.0001). The inhibitory effect of L-NAME was completely antagonized by L-arginine (10^{-9} M) (D). Results were obtained from 5 muscle strips from 5 rats (means ± SE).

Effects of PPADS and reactive blue 2 on NANC relaxations induced by EFS. Neither PPADS (3 × 10^{-5} M) nor reactive blue 2 (2 × 10^{-5} M) affected basal tone or inhibited NANC relaxations induced by EFS (1–20 Hz) (Fig. 3, A and B).

Effect of combined administration of L-NAME and purinoceptor antagonists on NANC relaxations induced by EFS. In the presence of L-NAME (10^{-4} M) and PPADS (3 × 10^{-5} M) together, the relaxant responses to EFS were significantly reduced compared with the inhibitory effects of L-NAME (10^{-4} M) alone (n = 4, df = 1.30, F = 350.4, P < 0.0001) (Fig. 3A). The combined administration of L-NAME (10^{-4} M) and PPADS (3 × 10^{-5} M) reduced NANC relaxations in response to EFS (5 Hz) by 69.0 ± 3.3% (n = 5), a more significant inhibitory effect than that of L-NAME (10^{-4} M) alone, which was 23.7 ± 4.4% (n = 5, P < 0.0001) (Fig. 3, A and C). In contrast, the combined administration of L-NAME (10^{-4} M) and reactive blue 2 (2 × 10^{-5} M) did not further inhibit EFS-induced NANC relaxation compared with the inhibition observed with L-NAME (10^{-4} M) alone (Fig. 3, B and C).

Effect of α-chymotrypsin and VIP antagonist. To determine whether VIP or other neuropeptides are involved in NANC relaxations in response to EFS of the rat pylorus, the effects of VIP antagonist (5 × 10^{-6} M) and α-chymotrypsin (2 U/ml) on NANC relaxations were studied. α-Chymotrypsin (2 U/ml) is a proteolytic enzyme and has been shown to inhibit NANC relaxations as well as VIP-induced and PACAP-induced muscle relaxations in the cat lower esophageal sphincter (29) and rat jejunum (28). We have previously shown (40) that the VIP antagonist [p-chloro-D-Phe\textsuperscript{6},Leu\textsuperscript{7}]VIP (10^{-6} M) inhibits NANC relaxations in the rat stomach. However, the administration of α-chymotrypsin (2 U/ml; data not shown) and VIP antagonist (5 × 10^{-6} M; Fig. 4A) had no inhibitory effect on NANC relaxations induced by EFS in the rat pylorus. Combined administration of L-NAME (10^{-4} M) and VIP antagonist (5 × 10^{-6} M) did not further inhibit EFS-induced NANC relaxation compared with the effect produced by L-NAME (10^{-4} M) alone (Fig. 4B).

Effect of exogenous application of SNP, ATP, α,β-Me-ATP, and 2-MeS-ATP. Exogenously applied SNP (10^{-5}–10^{-6} M), ATP (10^{-5}–10^{-3} M), and α,β-Me-ATP (10^{-5}–10^{-6} M) induced pyloric relaxations in a dose-dependent manner (Fig. 5, A and B). In contrast, 2-MeS-ATP (10^{-7}–10^{-6} M) had no effect (Fig. 5, A and B). TTX (10^{-6} M) did not affect SNP-, ATP- and α,β-Me-ATP-induced muscle relaxations of the rat pylorus, suggesting that these chemicals act directly on smooth muscle cells. PPADS (3 × 10^{-5} M) significantly inhibited the relaxations evoked by ATP (10^{-4} M) and α,β-Me-ATP (10^{-4} M) by 72.8 ± 6.8% and 75.6 ± 4.4% (n = 3), respectively. In contrast, reactive blue 2 (2 × 10^{-5} M) did not affect relaxations induced by ATP (10^{-4} M) and α,β-Me-ATP (10^{-4} M). L-NAME (10^{-4} M) did not inhibit relaxations induced by ATP (10^{-4} M) and α,β-Me-ATP (10^{-4} M). ATP (10^{-5} M) induced relaxations in a dose-dependent manner in various gastrointestinal tissues (20, 29). It has been demonstrated that the VIP antagonist [p-chloro-D-Phe\textsuperscript{6},Leu\textsuperscript{7}]VIP (10^{-6} M) inhibits NANC relaxations induced by EFS in the rat pylorus. Combined administration of L-NAME (10^{-4} M) and VIP antagonist (5 × 10^{-6} M) did not further inhibit EFS-induced NANC relaxation compared with the effect produced by L-NAME (10^{-4} M) alone (Fig. 4B).

Discussion

In the present study, we demonstrated that in the presence of atropine and guanethidine, EFS (1–20 Hz)
caused reproducible relaxations in a frequency-dependent manner. During EFS, the muscle tone was significantly reduced in the rat pylorus. EFS-induced NANC relaxations were abolished by the pretreatment with TTX, suggesting mediation by neural pathways.

\[ \text{\textbf{L-NAME (10}^{-4} \text{M}) \text{ significantly reduced EFS-induced relaxations. This suggests that NO is one of the major inhibitory neurotransmitters in the rat pylorus. Similar results have been shown in the canine pylorus (2). A large number of NOS and NADPH diaphorase-posit} \]
tive nerve elements was found in the myenteric plexus of the cat pylorus (3). In anesthetized rabbits, intraarterial infusion of L\(^{-}\)G-nitro-L-arginine (L-NNA) produced a dose-dependent increase in the frequency of the pyloric contraction (14). Lingenfelser et al. (24) demonstrated that both peripheral and central administration of L-NAME caused enhancement of phasic pyloric activity in urethan-anesthetized ferrets. These results suggest that NO acts as an inhibitory neurotransmitter in the mammalian pylorus. We have previously shown that NANC relaxations evoked by low-frequency stimulation (<2.5 Hz) were almost completely abolished by L-NNAME (>90%) in the rat stomach (17). As shown in Fig. 2, A and C, the inhibitory effect of L-NNAME on NANC relaxations evoked by low frequencies (<2.5 Hz) was smaller (<30%) in the rat pylorus. It is therefore suggested that, in addition to NO, some other inhibitory neurotransmitters may be involved in the mediation of NANC relaxations. It is also conceivable that EFS may release other excitatory neurotransmitters, such as tachykinins or excitatory ATP, which may reduce the effectiveness of NO to mediate relaxations of the pylorus.

ATP has also been demonstrated to be an inhibitory neurotransmitter in the gastrointestinal tract (8, 9). However, it remains unclear whether NO and ATP act independently, exerting their effects directly on smooth muscle. In the canine ileocecal junction and the terminal ileum, relaxations induced by ATP are blocked by TTX and L-NNA (6), suggesting that ATP stimulates the release of NO and that NO is the final neurotransmitter mediating muscle relaxation. In our present study, the relaxations induced by ATP were not affected by TTX or by L-NAME, thus excluding the possibility that ATP stimulates the release of NO. It is also unlikely that NO induces ATP release, because PPADS and reactive blue 2 did not affect SNP-induced relaxations of the rat pylorus.

We also demonstrated that PPADS, a P\(_{2X}\) purinoceptor antagonist, alone had no inhibitory effect on EFS-induced NANC relaxations of the pylorus. Similarly, reactive blue 2, a P\(_{2Y}\) purinoceptor antagonist, did not inhibit NANC relaxation. However, PPADS, but not reactive blue 2, significantly inhibited NANC relaxations in the presence of L-NAME. Because the combined administration of SNP and ATP did not potentiate the relaxations in the rat pylorus, the inhibitory effect of PPADS on NANC relaxations in the presence of L-NAME is not at the muscle level. Conceivably it may involve interaction between neuronal release of ATP and NO.

![image](https://via.placeholder.com/150)

**Fig. 5.** Effects of sodium nitroprusside (SNP; 10\(^{-6}\)–10\(^{-5}\) M) (A), \(\alpha,\beta\)-methyleneadenosine 5\(^{-}\)triphosphate (\(\alpha,\beta\)-Me-ATP; 10\(^{-6}\)–10\(^{-5}\) M) (B), 2-methylthioadenosine 5\(^{-}\)triphosphate (2-MeS-ATP; 10\(^{-6}\)–10\(^{-5}\) M) (C), and ATP (10\(^{-4}\)–10\(^{-3}\) M) (D) on muscle relaxations of the rat pylorus. Concentration-response curves of pyloric relaxation induced by ATP (10\(^{-5}\)–10\(^{-3}\) M), \(\alpha,\beta\)-Me-ATP (10\(^{-7}\)–10\(^{-5}\) M), and 2-MeS-ATP (10\(^{-7}\)–10\(^{-5}\) M) are shown (E). Results were obtained from 3 muscle strips from 3 rats (means ± SE).

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**Fig. 6.** Muscle relaxations of the rat pylorus induced by VIP (10\(^{-7}\)–10\(^{-6}\) M; A) and pituitary adenylate cyclase-activating polypeptide (PACAP; 10\(^{-7}\)–10\(^{-6}\) M; B). Lower concentrations of VIP (10\(^{-9}\)–10\(^{-8}\) M) and PACAP (10\(^{-9}\)–10\(^{-8}\) M) had no effect on the rat pylorus.
There is evidence that NO, in addition to its direct action on smooth muscle cells, has an inhibitory effect on neurotransmission in the myenteric plexus. It has been demonstrated that NO inhibits cholinergic transmission (4, 19, 22, 27). Exogenously applied NO donor inhibits the excitatory response to EFS in the rabbit stomach (17). NO synthesis inhibitors evoke ACh release and enhance EFS-induced contractions in the rabbit stomach (4), guinea pig ileum (22), canine ileum (19), and guinea pig fundus (27). A prejunctional inhibitory effect of NO on substance P neurotransmission has also been demonstrated in the guinea pig ileum (15). We have recently shown that NO inhibits VIP release from the gastric myenteric plexus in rats (17). Furthermore, NO has been suggested to inhibit ATP release from the myenteric plexus (23). Apamin itself has no effect on NANC relaxation, whereas the combination of L-NNA and apamin significantly enhances the inhibitory action of L-NNA on NANC relaxation evoked by EFS in the rabbit internal sphincter (23). We observed a similar phenomenon in the rat pylorus using the P2X purinoceptor antagonist PPADS.

In contrast, Sellemidis proposed that the apamin-sensitive neurotransmitter induces relaxation of the taenia coli as well as inhibition of NO release (38). Although L-NNA itself had no effect on NANC relaxations in the guinea pig taenia coli, the combination of L-NNA and apamin significantly inhibited NANC relaxations compared with the inhibitory effects of apamin alone (38). Holzer-Petsche and Moser (16) demonstrated that L-NNA had no effect on NANC relaxation of the rat gastric corpus, but when combined with apamin, L-NNA significantly inhibited NANC relaxations. We propose that a complex interaction involving a prejunctional mechanism may exist between ATP and NO release. The presence of P2X purinoceptors on the nerve terminal of the vagus (35) suggests that ATP may act through a prejunctional mechanism, in addition to directly acting on smooth muscle. It has been shown that nonselective P2 purinoceptor antagonist suramin increases the release of norepinephrine from the sympathetic axons of the mouse vas deferens (45). This suggests that released ATP inhibits subsequent transmitter release via prejunctional P2 purinoceptors. The histological evidence for the coexistence of ATP and NO in the myenteric neurons (5) further supports the possibility of an interaction between these two neurotransmitters at a neuronal level.

It is conceivable that the neural release of NO in response to EFS may inhibit ATP release, in addition to its direct relaxant effects on smooth muscle. Similarly, the neural release of ATP in response to EFS may inhibit NO release via a P2X purinoceptor located on the NO-producing neurons. Administration of L-NAME inhibits the release of NO, which results in removal of inhibitory effects of NO on ATP release. This may explain the inhibitory actions of PPADS on pyloric relaxation in the presence of L-NAME. On the other hand, administration of PPADS alone may block the smooth muscle relaxation via P2X purinoceptor, and PPADS also removes inhibitory effects of ATP on NO release. This potentially explains the lack of action of PPADS on NANC relaxation when it was given alone. However, direct measurement of ATP release and NO release from the myenteric neurons of the rat pylorus is necessary to prove this hypothesis.

There are other possibilities to explain the lack of inhibition of NANC relaxations by PPADS alone, but the combined administration of L-NAME and PPADS evoked greater inhibitory effects on NANC relaxation than that observed with L-NAME alone. It has been shown that P2X purinoceptors are ligand-gated cationic channels that evoke an increase in intracellular Ca2+ concentration from intra- and extracellular stores (10, 48). A low concentration of ATP may activate P2X purinoceptors, causing an increase in intracellular Ca2+ and muscle contractions (10, 48). On the other hand, high concentrations of ATP may cause relaxation as a result of secondary activation of Ca2+-dependent K+ channels. Thus it is possible that the release of ATP (excitatory input) may have contributed to masking the relaxant effects of ATP (inhibitory input). This may account for the fact that PPADS alone seemingly had no inhibitory effect on NANC relaxation. Once the ATP-dependent excitatory inputs are eliminated by PPADS, L-NAME may become a more effective inhibitor of NANC relaxation.

Classically, P2 purinoceptors are subdivided into two categories, P2X and P2Y. It is generally accepted that P2X purinoceptors mediate muscle contractions and P2Y purinoceptors mediate muscle relaxations and/or suppression of phasic contractions (10). In the blood vessels, ATP exerts a dual action: relaxation is mediated by P2Y purinoceptors and contraction is mediated by P2X purinoceptors (25). It has been demonstrated that the P2X purinoceptor agonist α,β-Me-ATP causes muscle contraction in the guinea pig ileum (26) and guinea pig urinary bladder (18), although recent studies have provided evidence to the contrary. ATP and α,β-Me-ATP induce muscle relaxations that are significantly antagonized by PPADS in the guinea pig taenia coli (7) and proximal colon (48). In contrast, ATP and 2-MeSATP induce muscle contractions that are antagonized by reactive blue 2 or suramin in the guinea pig stomach (31) and in the feline bladder (41). In our current study, ATP and α,β-Me-ATP induced relaxations of the pylorus by a TTX-insensitive mechanism, suggesting that ATP and its analog are acting directly on smooth muscle cells of the rat pylorus. Muscle relaxations induced by ATP and α,β-Me-ATP were antagonized by PPADS, but not by reactive blue 2. Therefore, it appears that ATP induces relaxation through P2X purinoceptors located on smooth muscle cells of the rat pylorus.

Besides NO and ATP, VIP and PACAP have been shown to be inhibitory NANC neurotransmitters in various parts of the gastrointestinal tract (13, 33). However, Soediono and Burnstock (39) previously showed that VIP (up to 3 × 10−7 M) did not induce muscle relaxation in the rat pylorus. Our present study demonstrated that exogenous VIP and PACAP inhibited pyloric contractions only when high concentra-
tions of these peptides (>10^{-7} M) were used. This may indicate the presence of VIP receptors and PACAP receptors in the rat pylorus, as previously reported in the pylorus of dogs (1) and rabbits (33). However, pretreatment with α-chymotrypsin had no effect on EPS-induced relaxations of the rat pylorus. Furthermore, VIP antagonist did not affect EPS-induced relaxations. Similarly, Parkman and colleagues (34) have shown that VIP antagonist was unable to inhibit NANC relaxations of the rabbit pylorus. Thus ATP and NO released in response to EFS may act in concert to relax the pylorus by some complex mechanisms involving interaction between neural release of ATP and NO.

Combined administration of L-NAME and PPADS, which completely abolished NANC relaxations in response to EFS at low frequency (1 Hz), caused only partial reduction of the NANC relaxations in response to EFS at higher frequencies (5–20 Hz; see Fig. 3B). This indicates that other neurotransmitters may be involved in mediating NANC relaxations in the rat pylorus.

In conclusion, our present study demonstrated that the combination of L-NAME and PPADS, but not reactive blue 2, significantly reduced NANC relaxation of the rat pylorus. Thus ATP and NO released in response to EFS may act in concert to relax the pylorus by some complex mechanisms involving interaction between neural release of ATP and NO.

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