Nonadrenergic, Noncholinergic Relaxation in Longitudinal Muscle of Rat Jejunum

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ABSTRACT—The mediators of nonadrenergic, noncholinergic (NANC) relaxation in the longitudinal muscle of rat jejunum were studied in vitro. Electrical field stimulation (EFS) of segments of rat jejunum induced a rapid transient relaxation followed by a subsequent contraction in the presence of atropine and guanethidine. N^G-Nitro-L-arginine (L-NOARG, 10 μM) inhibited the EFS-induced NANC relaxation by about 25%, and L-arginine (1 mM) completely reversed this inhibition. Exogenously added nitric oxide (0.1–10 μM) induced relaxation of the segment. Treatment of the segment with α-chymotrypsin resulted in about 50% inhibition of the EFS-induced relaxation. Several peptide candidates for the mediator of NANC relaxation were examined by using selective antagonists of their receptors or by a receptor-desensitization method. Results indicated that vasoactive intestinal peptide, pituitary adenylate cyclase activating peptide, peptide histidine isoleucine, atrial natriuretic peptide and neurotensin are not associated with NANC relaxation of the segments. On the other hand, apamin at 1 μM inhibited the EFS-induced relaxation by 74%. Inhibitory effects of L-NOARG and, apamin or α-chymotrypsin treatment on the EFS-induced relaxation were additive and almost complete. Exogenous nitric oxide-induced relaxation was not affected by apamin. Inhibitory junction potentials (i.j.p.'s) were recorded from longitudinal muscle cells of rat jejunum. Apamin at 200 nM abolished i.j.p.'s induced by two pulses of EFS. These results suggest that NANC relaxation in longitudinal muscle of rat jejunum involves two independent components: one is a nitric oxide-mediated minor component, and the other is an unknown substance-mediated apamin-sensitve major component that is inhibited by α-chymotrypsin treatment.

Keywords: Nonadrenergic, noncholinergic (NANC) relaxation, Jejunum (rat), Nitric oxide, Apamin-sensitive K^+ channel

Studies of the enteric nonadrenergic, noncholinergic (NANC) inhibitory neurons throughout the gut have indicated that the characteristics of inhibitory neuronal control vary from one region of the gut to another (1). There are numerous reports on the mediator of NANC relaxation in the gastrointestinal tract of rats. Nitric oxide was suggested to mediate NANC relaxation in the gastric fundus (2) and proximal colon (3) of rats. Subsequent studies revealed that nitric oxide also mediates the relaxation in the longitudinal and circular muscle of the ileum (4), longitudinal muscle of the duodenum (5–7), and circular muscle of the rectum (our unpublished data) of rats. Another putative mediator of NANC relaxation, vasoactive intestinal peptide (VIP), has also been suggested to mediate the relaxation in the gastric fundus (8–10) and mid (11, 12) and distal (13) colon of rats. A role of pituitary adenylate cyclase activating peptide (PACAP) was also suggested in descending relaxation in the mid colon (14) and in NANC inhibition in the longitudinal muscle of the distal colon (15) of rats. Thus nitric oxide is only one known mediator of NANC relaxation in the rat small intestine, although only the duodenum (5–7) and ileum (4) were studied. On the other hand, there are clear differences in the mediator of the relaxation among different regions of the large intestine as we showed (13, 15, 16). Since the mediator of NANC relaxation in the rat

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jejunum has not been studied yet, it would be interesting to examine the mediator in the jejunum, in comparison to other regions of the small intestine.

Another interesting unclarified problem is the action mechanism of the mediators of NANC relaxation. Nitric oxide was believed to mediate the inhibitory response via generation of cyclic GMP, but we have suggested that nitric oxide-mediated relaxation is not associated with changes in membrane potentials (17) or cyclic GMP level (18) in the rat proximal colon. VIP was suggested to mediate NANC relaxation by generating nitric oxide in dispersed gastric muscle cells obtained from rabbits (19), while activation of charybdotoxin-sensitive K^+ channels by VIP was suggested in longitudinal muscle of the rat distal colon (15). Furthermore, apamin, an antagonist of small conductance Ca^{2+}-activated K^+ channel, was suggested to inhibit NANC relaxation in the distal but not proximal colon of rats (15, 17). These findings suggest that the action mechanism of the mediator of NANC relaxation is different in different regions of the rat gastrointestinal tract. Therefore, we studied the mediators of NANC relaxation in the rat jejunum with special interest in the mechanism of its action in the present study.

MATERIALS AND METHODS

Male Wistar ST rats obtained from Nippon SLC, Shizuoka (8- to 9-weeks-old) were used. They were lightly anesthetized with ether and then stunned by a blow on the head and bled via the carotid arteries. Jejunal segment was removed and placed in Tyrode solution containing of: 137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.1 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 11.9 mM $NaHCO_3$ and 5.6 mM glucose. The portion that is adjacent to the duodenum and is attached by mesentery with the distal colon was defined as the jejunum. The contents of the excised whole segments, 2–3 cm in length, were gently flushed out with Tyrode solution.

Recording of responses of longitudinal muscle to electrical field stimulation (EFS)

Jejunal segments were suspended in an organ bath filled with Tyrode solution aerated with 5% CO_2 in O_2 and maintained at 37℃. Atropine (1 μM) and guanethidine (5 μM) were present throughout the experiment to block cholinergic and adrenergic responses, respectively. Responses of the longitudinal muscle to electrical field stimulation with trains of 100 pulses of 0.5-msec width at 30 V, 10-Hz frequency, were recorded isotonically with a 10-min interval between tests. The longitudinal muscle was subjected to a resting load of 0.75 g. The preparations were equilibrated for at least 30 min before the experiments. Drugs were added to the organ bath, when responses to EFS became constant, in volumes of less than 1.0% of the bathing solution. These volumes of the vehicle of the drugs, redistilled water, did not affect the spontaneous contractile activity or muscle tone.

Recording of inhibitory junction potentials (i.j.p.'s) in longitudinal muscles of rat jejunum induced by EFS

The segments of the jejunum were mounted in a 1.5-ml organ bath maintained at 30℃ and perfused continuously with Tyrode solution containing 1 μM nifedipine, which prevents contractions of the muscle, at a rate of 3 ml/min. Atropine (1 μM) and guanethidine (5 μM) were added to the bathing solution throughout the experiment. Membrane potentials were recorded with a conventional glass microelectrode filled with 3 M KCl with a resistance of 50–80 MΩ. The electrode impalement was made into the longitudinal muscle cells of the superficial layer. Intramural nerves within the segment were stimulated by a pair of Ag wire electrodes.

Drugs

VIP, PACAP and its fragment $PACAP_{6-38}$, neurotensin, atrial natriuretic peptide (ANP) and charybdotoxin were purchased from Peptide Institute, Osaka. N^G-Nitro-L-arginine (L-NOARG), L-arginine hydrochloride, D-arginine hydrochloride, apamin, α-chymotrypsin, peptide histidine isoleucine and VIP_{10-28} were from Sigma Chemical Co., St. Louis, MO, USA. HS-142-1, an antagonist of ANP receptors, was a generous gift from Dr. Yuzuru Matsuda (Kyowa Hakko Kogyo Co., Ltd., Tokyo). All other chemicals were of analytical grade. Gaseous nitric oxide was dissolved in Tyrode solution freshly before the experiments as described by Gillespie and Sheng (20).

Statistical analyses

Results were analysed by Student's *t*-test and a P value of <0.05 regarded as significant.

RESULTS

Spontaneous contractile activity and EFS-induced relaxation of longitudinal muscle of rat jejunum

The longitudinal muscle of the rat jejunum exhibited spontaneous frequent contraction with an amplitude similar to that in the ileum or duodenum. EFS induced a rapid transient relaxation followed by a subsequent contraction or only a contraction, the responses being variable from segment to segment. In the presence of 1 μM atropine and 5 μM guanethidine, however, EFS induced clear relaxation with subsequent contraction in every segment. Therefore, atropine and guanethidine were added to the bathing fluid in subsequent experiments.

Effects of L-NOARG and L-arginine on EFS-induced relaxation of longitudinal muscle of rat jejunum

We first examined the effect of L-NOARG on EFS-induced NANC relaxation of longitudinal muscle of the rat jejunum, because the role of nitric oxide in the relaxation was suggested in both adjoining regions of the jejunum, duodenum (5–7) and ileum (4). Treatment of the segments of the rat jejunum with 10 μM L-NOARG did not affect spontaneous contractile activity or tone of the longitudinal muscle, but it resulted in a decrease in the EFS-induced NANC relaxation. The effect of L-NOARG reached the maximum within 20–40 min, but the maximal effect was only about 25% inhibition. L-Arginine at 1 mM, but not D-arginine, completely reversed the inhibitory effect of L-NOARG within 10–30 min (Fig. 1, Table 1).

Exogenous nitric oxide at concentrations ranging from 0.1 to 10 μM induced concentration-dependent relaxation of the longitudinal muscle of the rat jejunum (Fig. 1B).

Examination of roles of putative peptide mediators of NANC relaxation in rat jejunum

Since VIP and PACAP were suggested to mediate NANC relaxation in the longitudinal muscle of the rat distal colon (15), we next examined whether peptides were responsible for the NANC relaxation that persisted after L-NOARG treatment.

α-Chymotrypsin treatment of the segments resulted in decrease in EFS-induced relaxation: treatment for 20–30 min with 3 U/ml slightly decreased it, and treatment with 10 U/ml maximally decreased it by about 50% (Table 1). However, α-chymotrypsin at these concentrations did not have any significant effects on exogenous noradrenaline-induced relaxation (data not shown).

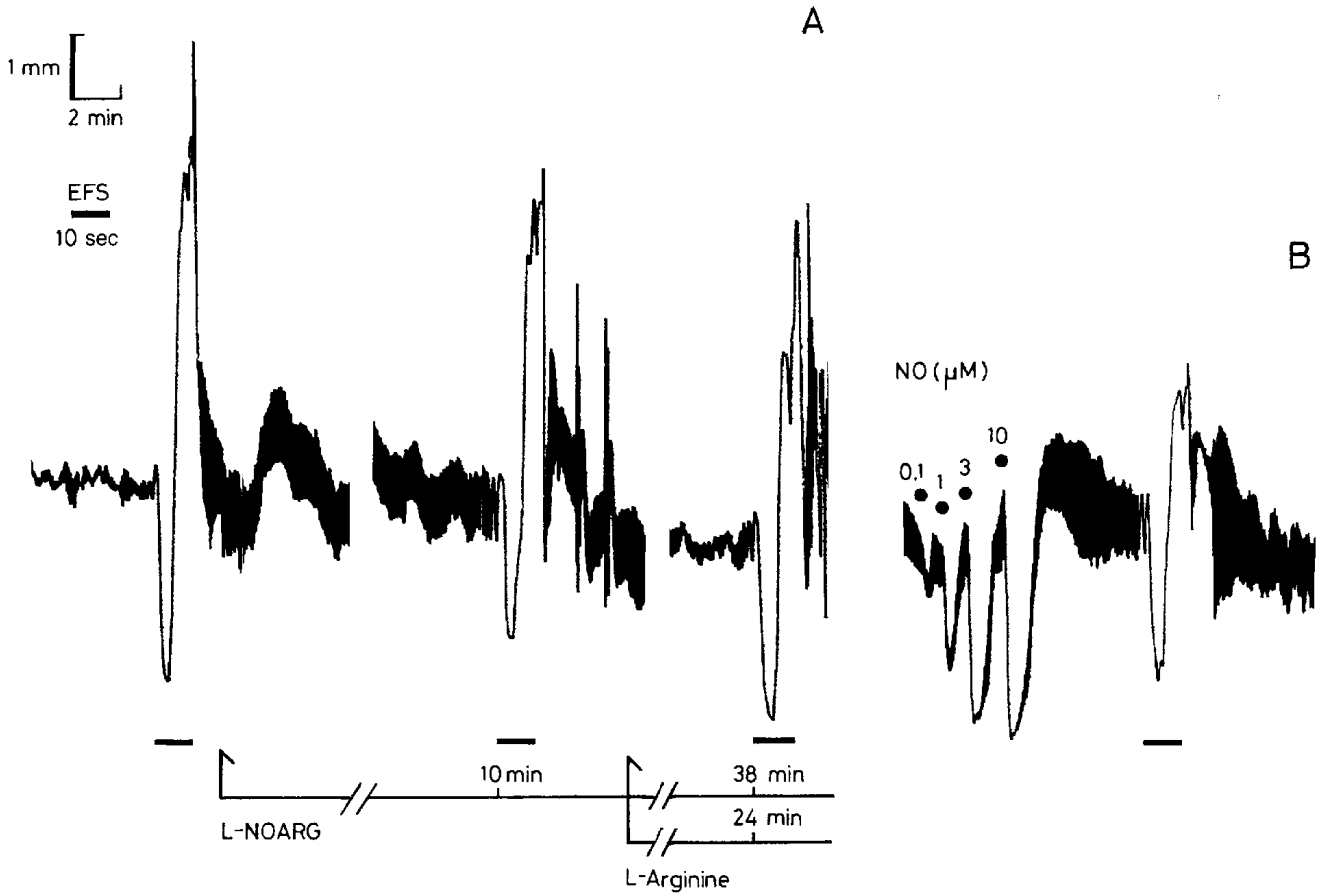

Fig. 1. Effects of L-NOARG and L-arginine on electrical field stimulation (EFS)-induced relaxation of longitudinal muscle of rat jejunum and effect of nitric oxide (NO) on the muscle. A: L-NOARG (10 μM) and L-arginine (1 mM) were added at the times indicated by the arrows. The continuous lines indicate the presence of L-NOARG and L-arginine in the bathing fluid. Times noted on the lines indicate the time after addition of the drugs. Bold black lines indicate the duration of EFS for 10 sec. After recording normal spontaneous movements, the chart was run at a fast speed immediately before the stimulation to make the relaxant response clear. B: Various concentrations of NO were added at the times indicated by the dots. The bathing fluid was not changed during the time period shown. This record is typical of 4 preparations.

Table 1. Effects of L-NOARG, apamin and α-chymotrypsin on EFS-induced NANC relaxation of the longitudinal muscle of rat jejunum

Treatment	Relaxation after treatment (% of control)
L-NOARG (10 μM)	74.5±4.5 (5)*
L-Arginine (1 mM) after L-NOARG	93.1±3.7 (3)#
D-Arginine (1 mM) after L-NOARG	77.2 (78.7, 75.7)
Apamin (1 μM)	26.3±6.8 (8)*
Charybdotoxin (100 nM)	104.5 (103.3, 105.6)
α-Chymotrypsin (10 U/ml)	47.1±3.1 (7)*
L-NOARG + Apamin	2.5 (0, 5.0)
L-NOARG + α-Chymotrypsin	0 (0, 0)
Apamin + α-Chymotrypsin	21.8±2.8 (3)
VIP_{10-28} (3 μM)	98.5±7.6 (5)
$PACAP_{6-38}$ (3 μM)	100.0 (100.0, 100.0)
HS-142-1 (3 μg/ml)	95.1 (97.8, 92.3)

Segments were treated with drugs for the durations noted in the text. Responses induced by EFS after treatments were expressed as a percentage of those before the treatments (control). Values are means±S.E.M. for the numbers of experiments shown in parentheses. Where only 2 observations were obtained, the numbers in parentheses give the individual values. Significantly different from the value of the corresponding control by Student's *t*-test: *$P<0.05$ and from the value with L-NOARG: #$P<0.05$.

VIP at concentrations up to 100 nM induced no significant relaxation but at 300 nM only, there was a slight relaxation of the longitudinal muscle. VIP_{10-28} (3 μM), an antagonist of VIP, did not affect spontaneous contractile activity, muscle tone and EFS-induced relaxation (Table 1).

PACAP (≤100 nM) showed a weak relaxant effect, and at 300 nM, it induced moderate relaxation. However, $PACAP_{6-38}$ (3 μM), an antagonist of PACAP receptors, did not show any significant effect on the EFS-induced relaxation (Table 1). The concentration of the antagonist employed was the maximal effective concentration to inhibit EFS-induced relaxation in rat distal colon (15).

ANP (100 nM) induced moderate relaxation of the longitudinal muscle of the rat jejunum. Repetitive application of 100 nM ANP every 10 min without washing decreased the sensitivity of the muscle to the drug, and three consecutive applications of ANP resulted in complete desensitization to the drug. In both of the two desensitized preparations examined, EFS-induced relaxation remained unchanged. Moreover, an antagonist of ANP receptors, HS-142-1 (up to 3 μg/ml, a concentration 2.5 times higher than its K_i value in the receptor binding study) (21, 22) did not affect the EFS-induced relaxation (Table 1).

Neurotensin (up to 1 μM) and peptide histidine isoleucine (PHI, up to 1 μM) did not induce any appreciable relaxation of the longitudinal muscle.

Effects of apamin and charybdotoxin on EFS-induced relaxation

Apamin slightly increased muscle tone and spontaneous contractile activity. Apamin at concentrations ranging from 1 nM to 1 μM concentration-dependently inhibited EFS-induced relaxation and at 1 μM, maximally inhibited it by 74% (Fig. 2 and Table 1). Charybdotoxin, an antagonist of the large conductance Ca^{2+}-activated K^+ channel, at concentrations up to 100 nM did not have any significant effect on the spontaneous contractility and EFS-induced relaxation (Table 1).

Effects of L-NOARG in combination with apamin on EFS-induced relaxation

Because L-NOARG and apamin inhibited EFS-induced relaxation by 25% and 74%, respectively, we studied the relationship between L-NOARG- and apamin-sensitive components of the relaxation. L-NOARG (10 μM) in the presence of apamin (1 μM) almost completely inhibited the EFS-induced relaxation (Table 1). Similarly, L-NOARG and α-chymotrypsin treatment also resulted in complete inhibition. However, inhibition by apamin and α-chymotrypsin treatment was similar in magnitude to that by apamin alone (Table 1). Exogenous nitric oxide (0.1−10 μM)-induced relaxations were not affected by apamin at 100 nM (Fig. 3). These results suggest that the inhibitory effects of both antagonists are brought about by their independent blocking effects.

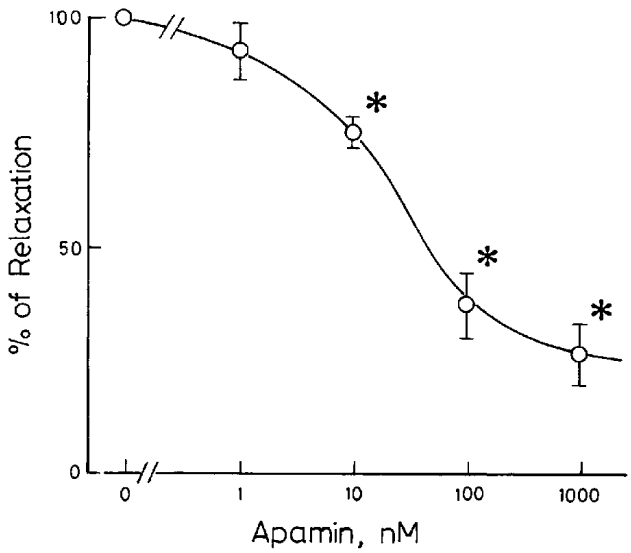

Fig. 2. Effect of apamin on EFS-induced relaxation of longitudinal muscle of rat jejunum. Relaxations in the presence of apamin are expressed as a percentage of those obtained before the addition of apamin (control). Values are means±S.E.M. (vertical bars) for 6−9 experiments. Significantly different from the value of the corresponding control: *$P<0.05$.

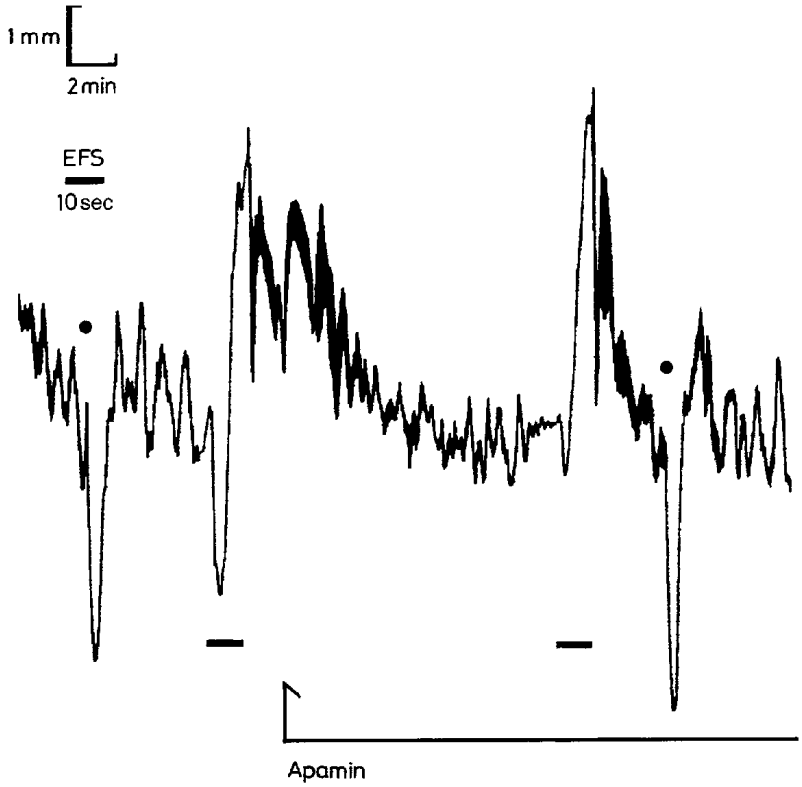

Fig. 3. Effect of apamin on exogenous nitric oxide- and EFS-induced relaxation of longitudinal muscle of rat jejunum. Relaxations were induced by 10 μM nitric oxide (dots) or EFS (bold black lines) before and after apamin (100 nM) treatment. This record is typical of 4 preparations. For further details, see the legend of Fig. 1.

Effect of apamin on i.j.p.'s of longitudinal muscle cells by EFS

The resting membrane potentials of longitudinal muscle cells of the rat jejunum were 52.1±2.9 mV (n=63). The muscle cells spontaneously exhibited slow potential changes, a slow wave (Fig. 4). In response to two pulses at 10 Hz, of sixty muscle cells, fourty-three cells generated hyperpolarization. The hyperporalization induced by EFS ceased following tetrodotoxin (0.1 μM) treatment. These results indicate that the potential changes produced by EFS are due to generation of i.j.p.'s. Apamin did not have any significant effect on the resting membrane potential at 20−200 nM, but it concentration-dependently reduced the amplitude of i.j.p.'s induced by two pulses and abolished them at 200 nM in all longitudinal muscle cells tested (n=7, Fig. 4).

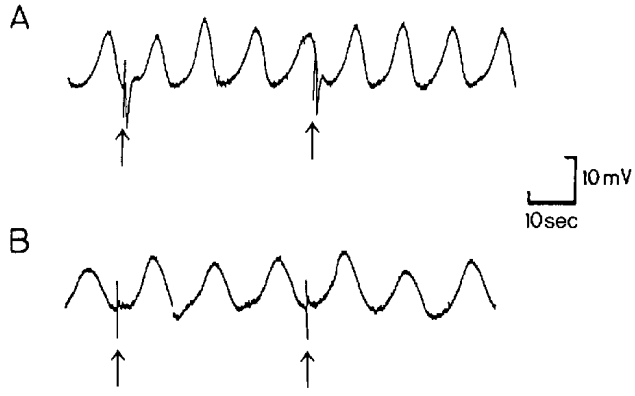

Fig. 4. Effects of apamin on EFS-induced i.j.p.'s in longitudinal muscle cell of rat jejunum. I.j.p.'s were induced by two pulses at 10 Hz in the absence (A) or presence (B) of 200 nM apamin. Atropine (1 μM) and guanethidine (5 μM) were added to the bathing fluid throughout the experiment. EFS was applied at the points indicated by the arrows. Both records were from the same longitudinal muscle cell.

DISCUSSION

Treatment of segments of the rat jejunum with 10 μM L-NOARG resulted in only a 25% decrease in EFS-induced NANC relaxation. A higher concentration, 100 μM, did not further inhibit it. Thus, nitric oxide partially participates in NANC relaxation of the longitudinal muscle of the rat jejunum. We previously reported that L-NOARG inhibited EFS-induced NANC relaxation of longitudinal muscle in the proximal colon by more than

80%, indicating an essential role of nitric oxide in the response. Such significant participation of nitric oxide was also suggested in the rat ileum (4). However, partial association was suggested in the duodenum (5, 6) and lack of association in the distal colon (13) and rectum (our unpublised data). Taken together, these previous results and the present results indicate that the significance of the participation of nitric oxide in NANC relaxation of longitudinal muscle varies among different regions of the rat intestinal tract: participation is maximum in the ileum and the proximal colon, and it decreases in regions toward the oral and anal sides.

Tissue segments were treated with α-chymotrypsin in vitro to examine the association of the peptide mediator with mechanical responses of the gastrointestinal tract (13, 23–25). In rat colon, α-chymotrypsin treatment selectively inhibited NANC relaxation in the distal region (13) in which VIP and PACAP were suggested to mediate the response (15), but not in the proximal region in which nitric oxide was suggested as the mediator of the response (13). Also in the present study, α-chymotrypsin at concentrations up to 10 U/ml did not have any significant effect on exogenous noradrenaline-induced relaxation of the segment, but it inhibited EFS-induced relaxation by about 50%. Thus, association of the peptide mediator with the relaxant response was strongly suggested in the longitudinal muscle of rat jejunum. As the mediator of the NANC relaxation, VIP and PACAP were suggested in descending relaxation of the mid or distal colon (11, 12, 14, 26) and longitudinal muscle of the distal colon (13, 15) of rats. PHI was suggested to be cosynthesized and co-released with VIP in guinea pig taenia coli and gastric fundus (27) and reported to induce relaxation of the rat colon (12). Neurotensin was reported to cause relaxation of the ileum (28) and duodenum (29) of rats and the ileum of guinea pigs (30). ANP was reported to cause relaxation of guinea pig ileum (31), chick rectum (32) and rat proximal colon (16). All of these peptides mentioned were denied as a possible mediator of NANC relaxation in the rat jejunum: VIP, PACAP and ANP were eliminated by the results obtained by selective antagonists for each receptor; ANP was also ruled out by a desensitization method; and neurotensin and PHI did not induce relaxation under the present experimental conditions. Thus, the mediator of α-chymotrypsin-sensitive component of the NANC relaxation in the rat jejunum was not identified in the present study.

We have reported that VIP and PACAP mediate NANC relaxation via activation of charybdotoxin- and apamin-sensitive K^+ channels, respectively, in the longitudinal muscle of rat distal colon (15). In the present study, EFS-induced relaxation of the longitudinal muscle of rat jejunum was significantly inhibited by apamin, but not by charybdotoxin. Although we were unable to record i.j.p.'s induced by the same conditions of EFS as those for the mechanical response due to a technical reason, two pulses-induced i.j.p.'s were completely abolished by apamin. A stimulatory effect of PACAP on apamin-sensitive K^+ channel was also suggested in the guinea pig tenia coli (33), stomach (34) and human colon (35). Apamin- and L-NOARG-sensitive NANC relaxation was also reported in the duodenum of rats (7). So, it seems likely that an unknown mediator(s) causes NANC relaxation via activation of apamin-sensitive K^+ channels in rat jejunum, representing a clear difference in the mediator of apamin-sensitive NANC relaxation among different intestinal regions.

EFS-induced relaxation of the jejunum in the present study was inhibited by L-NOARG and apamin by about 25% and 75%, respectively. Interestingly, L-NOARG in the presence of apamin almost completely inhibited the relaxation. Exogenous nitric oxide-induced relaxation was not inhibited by apamin. Therefore, it seems likely that L-NOARG and apamin inhibited a different component of NANC relaxation in the tissue. α-Chymotrypsin further inhibited the reduced relaxation by L-NOARG, but not that by apamin. Thus, the present results suggest that an unknown α-chymotrypsin sensitive mediator, which activates apamin-sensitive K^+ channels, and nitric oxide independently mediate the NANC relaxant response of the longitudinal muscle of rat jejunum, although participation of the apamin-sensitive component has a dominant role.

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