Mediators of nonadrenergic, noncholinergic inhibition in the proximal, middle and distal regions of rat colon

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1 The mediators of non-adrenergic non-cholinergic (NANC) relaxation of the longitudinal muscle of rat proximal, middle and distal colon were examined *in vitro*.

2 Electrical transmural stimulation (TMS) of proximal, middle and distal segments of rat colon induced NANC relaxations which were inhibited by tetrodotoxin (1 μM), but not by atropine (1 μM) or guanethidine (4 μM).

3 In the proximal colon, L-nitro-arginine (N^5 -nitroamidino-L-2,5-diaminopentanoic acid) inhibited the TMS-induced NANC relaxation and L-arginine (1 mM) reversed this inhibition. Nitric oxide (0.3–10 μM) induced relaxation of the proximal segment.

4 NANC relaxation of the proximal segments was still evident after desensitization to vasoactive intestinal peptide (VIP). A VIP antagonist (VIP 10-28, 10 μM) had no effect on the TMS-induced NANC relaxation, which was also resistant to α -chymotrypsin (2 units ml^{-1}) and a substance P antagonist ([D-Pro², D-Trp^{7,9}]substance P, 1 μM).

5 In the middle colon, L-nitro-arginine did not inhibit the TMS-induced NANC relaxation in 6 of 9 preparations tested and partially inhibited the relaxation in the other 3 preparations. L-Arginine did not reverse the partial inhibition.

6 Complete desensitization to VIP was not achieved in the middle colon. The VIP antagonist had no effect on the TMS-induced NANC relaxation. After α -chymotrypsin treatment of the segment, desensitization of the segments to substance P, or in the presence of the substance P antagonist, the TMS-induced NANC relaxation was augmented.

7 In the distal colon, L-nitro-arginine did not have any significant effect on the TMS-induced relaxation and nitric oxide did not induce relaxation. The VIP antagonist significantly inhibited TMS-induced NANC relaxation. α -Chymotrypsin-treatment of the distal segments resulted in significant inhibition of NANC relaxation. No desensitization to substance P was achieved. Treatment with the substance P antagonist had no effect.

8 These results suggest that nitric oxide is the mediator of the NANC inhibitory response in the proximal region of rat colon; in the middle colon, substance P acts as an excitatory neurotransmitter, antagonizing the NANC relaxation caused by the mediator of the response, which is still uncertain. Our results also suggest that VIP is the most likely candidate as a NANC transmitter in the distal colon.

Keywords: Nitric oxide; vasoactive intestinal peptide; NANC inhibition; colonic motility; substance P

Introduction

Since Burnstock (1986) first reported a non-adrenergic, non-cholinergic (NANC) inhibitory neural system in the gastrointestinal tract, several possible neurotransmitters have been studied. The distribution and combinations of NANC neurones in the myenteric and submucous plexuses are extremely complex. Investigations on neurotransmission of the enteric inhibitory neurones throughout the gut have indicated that inhibitory neural control varies from one region of the gut to another (Wood, 1987).

Recent reports have indicated a role of nitric oxide in NANC neuromuscular transmission in many tissues other than blood vessels, such as anococcygeus muscle in the rat (Gillespie *et al.*, 1989; Hobbs & Gibson, 1990) and mouse (Gibson *et al.*, 1990), the ileocolonic junction (Bult *et al.*, 1990) and duodenum (Toda *et al.*, 1990) of the dog, the gastric fundus in the rat (Boeckxstaens *et al.*, 1991), the lower oesophageal sphincter in the opossum (Tottrup *et al.*, 1991) and the dog (De Man *et al.*, 1991), and guinea-pig trachea (Tucker *et al.*, 1990; Li *et al.*, 1991). We have also suggested that, in rats, nitric oxide is important in inducing descending relaxation in the circular muscle of the proximal colon (Hata *et al.*, 1990) and relaxation of both the longi-

tudinal and circular muscles of the ileum (Kanada *et al.*, 1992). Furthermore, nitric oxide synthase, an enzyme that produces nitric oxide from L-arginine, has been demonstrated in rats in the brain (Knowles *et al.*, 1990) and nerve fibres in the intestine, retina, adrenal medulla and blood vessels (Bredt *et al.*, 1990). Thus, nitric oxide seems to act as a mediator of the NANC inhibitory response in various tissues.

Another putative neurotransmitter of the enteric nervous system, vasoactive intestinal peptide (VIP), has also been studied extensively. There are numerous reports of VIP-induced relaxation of gastrointestinal muscle (Fahrenkrug *et al.*, 1978; Goyal *et al.*, 1980; Bitar & Makhoul, 1982; Furness & Costa, 1982; Grider *et al.*, 1985a,b) and inhibition by VIP antiserum of neurally-induced muscle relaxation (Grider & Makhoul, 1986; Grider & Rivier, 1990). In the present study, we examined the effects of nitric oxide and VIP as possible mediators of the NANC inhibitory responses in longitudinal muscles of the proximal, middle and distal regions of rat colon. We also studied the role of substance P neurones, which have been identified in the gastrointestinal tract of many species of mammals (Pernow, 1951; Pearse & Polak, 1975; Brodin & Nilsson, 1981; Brodin *et al.*, 1983; Bartho & Holzer, 1985), in relation to NANC relaxation in the three regions of rat colon. The results indicated that the mediators of the NANC inhibitory response, and the parti-

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cipation of excitatory and inhibitory peptide neurones, in the motility of the colon differed in the proximal, middle and distal regions of rat colon. A preliminary report of these results has been published (Suthamnatpong *et al.*, 1991).

Methods

Preparations of proximal, middle and distal segments of rat colon

Male Wistar rats (250–380 g) were lightly anaesthetized with ether and then stunned by a blow on the head and bled via the carotid arteries. Proximal, middle and distal segments of the colon were removed and placed in Tyrode solution consisting of (in mM): NaCl 137, KCl 2.7, CaCl₂ 1.8, MgCl₂ 1.1, NaH₂PO₄ 0.42, NaHCO₃ 11.9, and glucose 5.6.

The narrow part formed by sphincter in the ascending colon defined the boundary between proximal and middle regions. The portion which is attached by mesentery with the small intestine was defined as the distal region. The contents of the excised segments were gently flushed out with Tyrode solution, and lengths of 2.0–3.0 cm were used for experiments.

Recording of responses of longitudinal muscle to electrical transmural stimulation

Colonic segments were suspended in an organ bath filled with Tyrode solution aerated with 5% CO₂ in O₂ and maintained at 37°C. The oral end of each segment was attached to a transducer and the anal end was mounted on an anodal electrode placed at the bottom of the bath. Responses of the longitudinal muscle to transmural stimulation with trains of 100 pulses of 0.1 ms width at 30 V, and 10 Hz frequency, were recorded isotonicity with a 10 min interval between tests. The longitudinal muscle was subjected to a resting load of 1.0 g. The preparations were equilibrated for at least 30 min before experiments. Drugs were added to the organ bath, when responses to TMS became constant, in volumes of less than 1.0% of the bathing solution. These volumes of vehicle of the drugs, redistilled water, did not affect the spontaneous contractile activity or the muscle tone.

Treatment of the colonic segments with α -chymotrypsin

Colonic segments were treated with α -chymotrypsin by adding the agent to the organ bath at a final concentration of 2 units ml⁻¹, which is known to reduce peptide response (De Beurme & Lefebvre, 1987). Also in our preliminary experiments, substance P and VIP did not affect the segments at all after a preincubation of them with this concentration of α -chymotrypsin for 20 min at 37°C.

Drugs

L-Nitro-arginine (N⁵-nitroamidino-L-2,5-diaminopentanoic acid), L-arginine, α -chymotrypsin and D-arginine hydrochloride were purchased from Sigma Chemical Co., St. Louis, U.S.A. Atropine sulphate, guanethidine, vasoactive intestinal peptide and tetrodotoxin were from Wako Pure Chemical, Osaka, Japan. Substance P and [D-Pro², D-Trp^{7,9}]substance P were from the Peptide Institute, Osaka, Japan. Vasoactive intestinal peptide (10–28) was from Peninsula Laboratories, Calif., U.S.A. All other chemicals were of analytical grade. Gaseous nitric oxide was dissolved in Tyrode solution freshly before experiments as described by Gillespie & Sheng (1988). The nitric oxide solution was added to the organ bath in volumes of 0.3–300 μ l. These volumes of Tyrode solution alone did not affect the spontaneous contractile activity or the muscle tone.

Statistical analysis

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

Results

Responses of longitudinal muscle of the proximal, middle and distal colon to electrical transmural stimulation

Longitudinal muscle of the proximal segment of rat colon exhibited spontaneous contractile activity with wider amplitude and higher frequency than muscles of the middle and distal segments. TMS of the proximal segment at 1–50 Hz induced rapid, transient relaxations and then rebound contraction, both in the absence and presence of atropine (1 μ M) and guanethidine (4 μ M). The amplitude of the relaxation increased with an increase in frequency. In subsequent studies, the segments were stimulated at 10 Hz, because this stimulation gave the most reproducible responses (Figure 1). The frequency of the spontaneous contractile activity of the middle colon was less than that of the proximal and distal segments, and transient rapid relaxation followed by rebound contraction on TMS at 10 Hz was seen only in the presence of atropine and guanethidine (Figure 2). On the other hand, the distal colon exhibited moderate spontaneous contractile activity and very small relaxation on TMS. However, the resting tone of the longitudinal muscle gradually increased during successive trains of TMS at 10 Hz at 10 min intervals. With progressive increase in the tone in this way, the segments began to exhibit clear relaxation followed by a large rebound contraction on TMS over the range of frequencies tested (1–50 Hz), but only in the presence of atropine and guanethidine. In subsequent studies, the segments were stimulated at 10 Hz with good reproducibility of response (Figure 3).

Thus the longitudinal muscles of the proximal, middle and distal colon exhibited different patterns of spontaneous contractile activities, but all three segments exhibited rapid clear transient relaxation on TMS in the presence of atropine and guanethidine. Tetrodotoxin (1 μ M) inhibited the TMS-induced responses of the preparations.

Effects of L-nitro-arginine and L-arginine on non-adrenergic, non-cholinergic inhibitory responses of longitudinal muscles obtained from three different regions of the rat colon

Neither L-nitro-arginine (N⁵-nitroamidino-L-2,5-diaminopentanoic acid) at concentrations up to 10 μ M nor L-arginine at concentrations of up to 1 mM had any significant effect on the spontaneous contractile activity or on the tone of the longitudinal muscle of the rat proximal colon. However, L-nitro-arginine (10 μ M) markedly inhibited TMS-induced NANC relaxation observed over the range of frequencies tested (1–50 Hz), causing almost complete inhibition within 20–40 min (Figure 1 and Table 1). Addition of L-arginine (1 mM) to the bathing fluid gradually reversed the effect of L-nitro-arginine, causing complete reversal in 20–30 min (Figure 1 and Table 1). D-Arginine (1 mM) had no effect (data not shown).

In the middle colon, TMS-induced NANC relaxation was partially inhibited by L-nitro-arginine in only 3 of 9 preparations (Figure 2a) and L-arginine did not reverse the effect of L-nitro-arginine. In the other 6 preparations, however, L-nitro-arginine did not have any significant effect (Figure 2b and Table 1).

In contrast to its effect in the proximal colon, L-nitro-arginine did not have any significant effect on TMS-induced NANC relaxation in any of 9 preparations of the distal colon (Figure 3 and Table 1).

Figure 1 Effects of L-nitro-arginine and L-arginine on transmural stimulation (TMS)-induced relaxation of segments of proximal colon. L-Nitro-arginine ($10 \mu\text{M}$) and L-arginine (1 mM) were added at the times indicated by the arrows. The continuous lines indicate the presence of L-nitro-arginine and L-arginine in the bathing fluid. Times noted on the lines indicate the time after addition of L-nitro-arginine. Small bars indicate 10 s TMS at 10 Hz. Arrowhead indicates the beginning of the third train of TMS. Atropine ($1 \mu\text{M}$) and guanethidine ($4 \mu\text{M}$) were present throughout.

Figure 2 Effects of L-nitro-arginine and L-arginine on transmural stimulation (TMS)-induced relaxation of segments of middle colon. (a) Type A, an example of 3 out of 9 preparations in which responses were reduced by L-nitro-arginine; (b) Type B, an example of 6 out of 9 preparations in which L-nitro-arginine had no effect. The lines indicate the presence of L-nitro-arginine ($10 \mu\text{M}$) and L-arginine (1 mM). Times noted on the lines indicate time after addition of L-nitro-arginine. Small bars indicate 10 s TMS at 10 Hz.

Figure 3 Transmural stimulation (TMS)-induced relaxation of segments of distal colon before and after treatment with L-nitro-arginine. The lines indicate the presence of L-nitro-arginine ($10 \mu\text{M}$). Times noted on the line indicate time after addition of L-nitro-arginine. Small bars indicate 10 s TMS at 10 Hz.

Effects of nitric oxide on the longitudinal muscles of different regions of rat colon

Nitric oxide ($0.3\text{--}10 \mu\text{M}$) induced relaxation of the proximal colon in a concentration-dependent manner (Figure 4). L-nitro-arginine had no effect on the relaxation induced by nitric oxide (not shown). Nitric oxide slightly inhibited spontaneous contractile activity, but did not induce relaxation of either the middle or distal colon (not shown).

Effects of α -chymotrypsin on TMS-induced NANC relaxation of colonic segments

Next, we examined the effect of α -chymotrypsin treatment to determine the possible contribution of peptidergic neurones to NANC inhibition in the different regions of the colon. Treatment of the proximal colon with α -chymotrypsin (2 units ml^{-1}) for 30 min had no effect on the muscle tone, spontaneous contractile activity, or TMS-induced NANC relaxation (Table 1). In the middle colon, TMS-induced NANC relaxation increased progressively during treatment

with α -chymotrypsin for 10–20 min (Table 1) in all 5 preparations tested. In some preparations, however, this treatment moderately inhibited the spontaneous contractile activity and slightly decreased the muscle tone. In the distal colon, treatment with α -chymotrypsin for 20 min resulted in significant decrease of TMS-induced NANC relaxation (Table 1). In some preparations it slightly decreased the muscle tone and inhibited the spontaneous contractile activity.

These data suggested that some excitatory or inhibitory peptide neurones affect NANC inhibition, so we next tested the effects of various peptides.

Effects of substance P and a substance P antagonist on the motility and TMS-induced NANC relaxation of colonic segments

Substance P ($1 \mu\text{M}$) significantly increased the muscle tone and spontaneous activity of segments of all three regions. Three consecutive applications of substance P to middle colonic segments, without washing, resulted in desensitization to substance P. In contrast, repetitive applications of substance

Figure 4 Relaxations of segments of proximal colon to nitric oxide (NO). Various concentrations of nitric oxide were added at the times indicated by the triangles. The bathing fluid was not changed during the time period shown. This record is typical of 10 preparations.

Table 1 Effects of various treatments on electrical transmural stimulation (TMS)-induced relaxation of longitudinal muscle obtained from three regions of the rat colon

Treatment	Relaxation after treatment (% of control)		
	Proximal	Middle	Distal
L-Nitro-arginine (10 μ M)	12.7 $\pm$ 6.7 (11)*	Type A 28.3 $\pm$ 15.2 (3)	96.7 $\pm$ 6.3 (9)
L-Arginine (1 mM) after L-nitro-arginine	92.6 $\pm$ 4.3 (8)†	Type B 103.0 $\pm$ 4.3 (6)	—
		Type A 38.0 $\pm$ 7.6 (3)	
α -Chymotrypsin (2 u ml ⁻¹)	98.8 $\pm$ 2.9 (5)	174.0 $\pm$ 26.5 (5)**	69.4 $\pm$ 6.4 (9)*
Substance-P desensitization	—	184.0 (167, 200)	—
Substance-P antagonist (1 μ M)			
	100 (93, 106)	200.0 $\pm$ 22.0 (7)*	91.3 $\pm$ 17.0 (3)
VIP desensitization	98.3 $\pm$ 3.8 (3)	—	69.5 $\pm$ 11.6 (4)*,***
VIP antagonist (10 μ M)	109 (118, 100)	97 (100, 94)	64.8 $\pm$ 7.5 (5)*

Segments were treated with drugs for the durations noted in the text. Responses induced by TMS at 10 Hz after the treatments were expressed as a percentage of those before the treatments (Control). Relaxation in middle colon was partially inhibited (Type A) or not affected (Type B) by L-nitro-arginine. *The preparations were incompletely desensitized to VIP (see text). Values are means $\pm$ s.e. mean 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 corresponding control by Student's paired *t* test: **P* < 0.01; ***P* < 0.02; ****P* < 0.05; and from the value with L-nitro-arginine: †*P* < 0.01.

P to proximal and distal colonic segments did not result in desensitization. In two desensitized preparations of the middle colon, TMS-induced relaxation was somewhat augmented (Table 1). This result suggested that substance P counteracts the NANC inhibitory response, so we tested the effect of a substance P antagonist in the following experiment.

A substance P antagonist ([D-Pro², D-Trp^{7,9}]substance P, 1 μ M) had no effect on the basal tone, spontaneous contractile activity or TMS-induced NANC relaxation of segments of proximal and distal colon, but it gradually increased the muscle tone and spontaneous contractile activity of middle colonic segments and significantly increased TMS-induced relaxation in all 7 preparations tested (Table 1). The antagonist (1 μ M) completely inhibited the response induced by exogenously added substance P.

Effects of VIP and a VIP antagonist on motility and TMS-induced NANC inhibition in colonic segments

VIP (0.1 μ M) caused transient relaxation of longitudinal muscle of the proximal colon and inhibited the spontaneous contractile activity. However, within 10 min, the spontaneous contractile activity and muscle tone were restored, even in the presence of VIP. Repetitive application of 0.1 μ M VIP, every

10 min without washing, markedly decreased the sensitivity of the segments to VIP, and complete desensitization was consistently achieved after 2 or 3 consecutive applications of VIP. TMS-induced NANC relaxation remained unchanged in all 3 desensitized preparations (Figure 5 and Table 1).

In the segments of the middle and distal colon, 0.1 μ M VIP inhibited spontaneous contractile activity without having any significant effect on muscle tone. The inhibitory effects of VIP in the middle and distal colon persisted for about 15–20 min and more than 1 h, respectively. Repeated application of the same concentration of VIP after recovery of spontaneous contraction resulted in similar prolonged inhibition, although the duration of inhibition became progressively shorter. Thus, segments of the middle colon were not desensitized to VIP even by 5 to 6 applications of VIP in this way. Moreover, TMS-induced NANC relaxation was not significantly altered after 5–6 applications of VIP in all 5 preparations tested (not shown).

The distal colon was not totally desensitized to VIP even by 3–4 applications of VIP in a period of 2–3 h. However, repeated applications of VIP resulted in partial inhibition of TMS-induced NANC relaxation in all 4 preparations tested (Table 1).

As the middle or distal colon showed no or incomplete desensitization to VIP, respectively, we further examined the

Figure 5 Transmural stimulation (TMS)-induced relaxation of segments of proximal colon before and after desensitization with vasoactive intestinal peptide (VIP). VIP (100 nM) was added at the times indicated by the arrows. The line indicates the presence of VIP. Small bars indicate 10 s TMS at 10 Hz.

Figure 6 Effect of the vasoactive intestinal peptide (VIP) antagonist, VIP (10–28) on transmural stimulation-induced relaxation of segments of proximal (Δ), middle ($\bullet$) and distal ($\circ$) colon. Relaxations in the presence of the antagonist are expressed as a percentage of those obtained before addition of the antagonist (control). Values are mean $\pm$ s.e.mean (vertical bars) of the number of experiments shown in parentheses. * $P < 0.01$; ** $P < 0.05$: significantly different from the value of the corresponding control.

effect of a VIP antagonist (VIP 10–28) to study the participation of VIP neurones in these regions. In proximal and middle colonic segments, the relaxation induced by TMS was not altered by this VIP antagonist at concentrations up to $10 \mu\text{M}$ (Figure 6 and Table 1). The VIP antagonist also had no effect on the basal tone or spontaneous contractile activity. In the distal colon, the VIP antagonist had no effect on the basal tone or spontaneous contractile activity but inhibited the TMS-induced NANC relaxation in a concentration-dependent manner: it inhibited the TMS-induced NANC relaxation slightly at $1 \mu\text{M}$, significantly at concentrations of over $2 \mu\text{M}$, and by about 40% at $30 \mu\text{M}$ (Figure 6 and Table 1). The inhibitory effect was pronounced 5–10 min after its application. The antagonist ($10 \mu\text{M}$) completely inhibited the response induced by exogenously added VIP.

Discussion

The rat colon can be divided into three regions morphologically (Christensen *et al.*, 1984) and functionally (Ferre & Ruckebusch, 1985). In the present study, we found several clear differences in the motilities of these three regions, proximal, middle and distal. First, their frequencies and amplitude of spontaneous contraction were different, being highest in the proximal region, and lowest in the middle region. Second, development of TMS-induced relaxation was different in the distal colon from that in the other two regions: TMS initially induced very little relaxation in distal colon, but on successive TMS at 10 min intervals, relaxation of the distal colon gradually increased and became constant after 4–5 stimulations, whereas the relaxations of the other two regions usually remained constant from the beginning of the experiment. A third difference was the excitatory control by cholinergic neurones: in middle and distal regions, TMS-induced NANC relaxation occurred only in the presence of atropine, whereas, in the proximal colon it occurred in both the presence and absence of atropine. The absence of effect of atropine on NANC inhibition in the proximal colon suggests dominant NANC control or inferior cholinergic control in this region. The inhibitory effect of tetrodotoxin on the TMS-induced relaxation in all three regions of rat colon in the presence of atropine and guanethidine confirmed that the TMS-induced relaxation is mediated by NANC inhibitory neurones.

There were clear differences in NANC inhibitory neurotransmission in the proximal, middle and distal colon. L-Nitro-arginine is a guanidino-substituted arginine derivative that inhibits the synthesis of nitric oxide from L-arginine (Gibson *et al.*, 1990). L-Nitro-arginine inhibited TMS-induced NANC relaxation appreciably in the proximal colon, and this inhibition was reversed by L-arginine. However, L-nitro-arginine inhibited TMS-induced NANC relaxation only slightly in the middle colon and was not inhibitory in the distal colon. The reversibility of the inhibition by L-arginine in the proximal colon suggests that L-nitro-arginine acts competitively, and the absence of effect of the D-isomer indicates that the effect is stereo-specific. In a previous study, we found that nitric oxide mediates relaxation of circular muscle in the proximal colon of rats (Hata *et al.*, 1990). The present results on the effect of nitric oxide on the longitudinal muscle of this region are parallel to this finding.

A tonic inhibitory control mediated by nitric oxide has been suggested in rat anococcygeus muscle (Gillespie *et al.*, 1989) and rat gastric fundus (Grider *et al.*, 1985b): in these tissues, blockade of biosynthesis of nitric oxide raised the basal tension or elicited contraction. However, the inhibitory effect of L-nitro-arginine was selective for TMS-induced NANC relaxation in the proximal colon: this drug had no effect on the spontaneous contractile activity or muscle tone in segments of any of the three regions, in the presence or absence of atropine and guanethidine.

Interestingly, L-nitro-arginine did not inhibit the TMS-induced NANC relaxation in 6 of 9 preparations of middle colon. Furthermore, in the other 3 preparations whose TMS-induced NANC relaxation was partially inhibited by L-nitro-arginine, L-arginine at concentrations of up to 10 mM did not reverse the inhibitory effect of L-nitro-arginine. The reason for this absence of effect of L-arginine is unknown, but the results suggest that nitric oxide has only a minor role, if any, in inducing NANC relaxation in the middle region. The ineffectiveness of nitric oxide in inducing relaxation in this region supports this idea. Similarly, in the distal region, it seems unlikely that nitric oxide mediates NANC relaxation, because L-nitro-arginine did not inhibit NANC relaxation and nitric oxide did not induce relaxation.

It has been suggested that VIP is a neurotransmitter of NANC nerves in the gut (Goyal *et al.*, 1980; Furness & Costa, 1982; Grider & Makhlof, 1986; Grider & Rivier, 1990). To investigate this possibility, we examined the influence of α -chymotrypsin treatment of the colonic segments, and the effects of a VIP antagonist, and exogenous VIP, on the TMS-induced NANC relaxation of the colonic segments. α -Chymotrypsin is known to cleave peptides at tyrosine, lysine and arginine residues, and these three amino acids are present in the VIP sequence. Therefore, the influence of α -chymotrypsin treatment on VIP-induced or NANC relaxation has been studied in many tissues, such as duodenum (Manzini *et al.*, 1990), ileum (Manzini *et al.*, 1986) and gastric fundus (De Beurme & Lefebvre, 1987) of rats. A VIP antagonist, the C-terminal partial sequence of VIP (VIP 10–28), was reported to be a stronger antagonist than [4-Cl-D-Phe⁶, Leu¹⁷]VIP or [Ac-Tyr¹, D-Phe²]GRF1–29 (Grider & Rivier, 1990).

In the proximal colon, TMS-induced NANC relaxation was still observed after desensitization to VIP, and α -chymotrypsin treatment had no effect on TMS-induced NANC relaxation. Thus, VIP is unlikely to be a NANC neurotransmitter in the proximal colon. In the middle colon also, the VIP antagonist and α -chymotrypsin-treatment did not decrease the TMS-induced relaxation. On the other hand, both α -chymotrypsin-treatment and the VIP antagonist inhibited TMS-induced NANC relaxation in the distal colon. Complete desensitization to VIP was not observed, but TMS-induced NANC relaxation was partially inhibited by successive administrations of exogenous VIP. Thus, VIP seems to have a role as a neurotransmitter in TMS-induced NANC

relaxation in the distal colon.

In contrast to the present findings, Grider & Rivier (1990) reported that a VIP antagonist and VIP antiserum abolished descending NANC relaxation in the middle colon of rats. They used segments of 4–6 cm length of the midcolon, while we used segments of 2–3 cm length. Thus, their segments may have included part of the region that we defined as distal colon. Moreover, they induced descending relaxation by mechanical stretch of the circular muscle on the oral side of the segment. Thus, their observed response might have been affected by the response of the distal region.

Interestingly, treatment of only the middle colon with α -chymotrypsin augmented the TMS-induced NANC relaxation and inhibited spontaneous contractile activity. These findings suggest that the activity of some excitatory peptidergic neurones normally masks the NANC inhibitory response to some extent. Substance P is known to cause contraction of smooth muscle of the gut (Pernow, 1983; Bárho & Holzer, 1985). Hou *et al.* (1989) suggested that there are two sites of action of substance P to induce smooth muscle contraction depending on whether the nerves function normally or not. Substance P releases acetylcholine by acting preferentially on cholinergic neurones when the nerves are intact, but acts directly on the smooth muscle when conduction of the nerves is blocked. Therefore, we studied the effect of substance P and a substance P antagonist, [D-Pro², D-Trp^{7,9}]substance P on the NANC inhibitory responses of the colonic segments.

Substance P stimulated colonic contractile activity, resulting in prolonged, large-amplitude contraction. As our experiments were carried out in the presence of atropine, the site of action of substance P was its receptor on the smooth muscle. Desensitization of the middle colonic segments to substance P was achieved by successive applications of substance P without washing. In this state the TMS-induced NANC relaxation was significantly augmented. This result is consistent with the finding that the substance P antagonist augmented TMS-induced NANC relaxation of the middle colon. On the other hand, no desensitization to substance P was observed in the proximal or distal colonic segments, and the substance P antagonist had no effect on TMS-induced NANC relaxation in these two regions. Thus, substance P neurones seem to participate mainly in rat middle colon and normally mask the NANC inhibitory response to some extent in this region.

The present results suggest that the mediators of the NANC inhibitory response in longitudinal muscle differ in different regions of rat colon. Our results indicate a role of nitric oxide, but not of VIP, as the mediator in the proximal region, and the participation of VIP neurones but not of nitric oxide in the NANC inhibitory response in the distal region. Our results did not identify the mediator of the response in the middle region, but suggested that activation of substance P neurones had a negative effect on the NANC inhibitory response in this region.

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