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VIP- and PACAP-mediated nonadrenergic, noncholinergic inhibition in longitudinal muscle of rat distal colon: involvement of activation of charybdotoxin- and apamin-sensitive K^+ channels

*Masami Kishi, *,†Tadayoshi Takeuchi, *Naowarat Suthamnatpong, *,†Toshiaki Ishii, *Hideaki Nishio, [1],*,†Fumiaki Hata & **Tadashi Takewaki

*Department of Veterinary Pharmacology, College of Agriculture and †Department of Molecular Physiology and Biochemistry, Research Institute for Advanced Science and Technology, Osaka Prefecture University, Sakai 593 and **United Graduate School of Veterinary Sciences, Gifu University, Gifu 501-11 Japan

1 The mediators of nonadrenergic, noncholinergic (NANC) inhibitory responses in longitudinal muscle of rat distal colon were studied.

2 An antagonist of pituitary adenylate cyclase activating peptide (PACAP) receptors, $PACAP_{6-38}$, concentration-dependently inhibited the rapid relaxation of the longitudinal muscle induced by electrical field stimulation (EFS), resulting in a maximal inhibition of 47% at 3 μM.

3 $PACAP_{6-38}$ inhibited the relaxation by 75% in the presence of the vasoactive intestinal peptide (VIP) receptor antagonist, VIP_{10-28} at 3 μM, which inhibited the relaxation by 44%.

4 An antagonist of large conductance Ca^{2+}-activated K^+ channels, charybdotoxin, concentration-dependently inhibited the rapid relaxation of the longitudinal muscle, resulting in a maximal inhibition of 58% at 100 nM.

5 An antagonist of small conductance Ca^{2+}-activated K^+ channels, apamin, concentration-dependently inhibited the relaxation (58% at 1 μM).

6 Treatment with both K^+ channel antagonists resulted in 84% inhibition of the EFS-induced relaxation, which is comparable to the extent of inhibition induced by $PACAP_{6-38}$ plus VIP_{10-28}.

7 The inhibitory effect of VIP_{10-28} and of apamin, but not of charybdotoxin was additive: the same applied to $PACAP_{6-38}$ and charybdotoxin, but not apamin.

8 Exogenously added VIP (100 nM – 1 μM) induced a slow gradual relaxation of the longitudinal muscle. Charybdotoxin, but not apamin significantly inhibited the VIP-induced relaxation. VIP_{10-28}, but not $PACAP_{6-38}$ selectively inhibited the VIP-induced relaxation.

9 Exogenously added PACAP (10–100 nM) also induced slow relaxation. Apamin and to a lesser extent, charybdotoxin, inhibited the PACAP-induced relaxation. $PACAP_{6-38}$, but not VIP_{10-28} selectively inhibited the PACAP-induced relaxation.

10 Apamin at 100 nM inhibited inhibitory junction potentials (i.j.ps) induced by a single pulse of EFS. Apamin also inhibited a rapid phase, but not a delayed phase of i.j.ps induced by two pulses at 10 Hz. VIP_{10-28} did not inhibit i.j.ps induced by a single pulse, but significantly inhibited the delayed phase at two pulses. A combination of apamin and VIP_{10-28} abolished the i.j.ps induced by two pulses.

11 Both VIP and PACAP induced slow hyperpolarization of the cell membrane of the longitudinal muscle. Apamin inhibited the PACAP-, but not VIP-induced hyperpolarization.

12 From these findings it is suggested that VIP and PACAP are involved in NANC inhibitory responses of longitudinal muscle of the rat distal colon via activation of charybdotoxin- and apamin-sensitive K^+ channels, respectively.

Keywords: Rat distal colon; NANC inhibition; VIP; PACAP; charybdotoxin; apamin

Introduction

We previously reported the difference in mediators of nonadrenergic, noncholinergic (NANC) inhibitory responses among different regions of rat colon. In the proximal region, nitric oxide mediated NANC inhibitory responses of circular (Hata *et al.*, 1990) and longitudinal muscle (Suthamnatpong *et al.*, 1993a) by a mechanism independent of changes in membrane potentials of the cell membrane (Suthamnatpong *et al.*, 1994) and changes in cyclic GMP content (Takeuchi *et al.*, 1996). However, there was no evidence of participation of nitric oxide in relaxant responses of the distal colon: N^G-nitro-L-arginine (L-NOARG) did not inhibit NANC relaxation induced by electrical field stimulation (EFS) and exogenous nitric oxide did not induce any appreciable relaxation (Suthamnatpong *et al.*, 1993a). In the distal region, vasoactive intestinal peptide (VIP) was the most likely candidate as an NANC transmitter, but participation of VIP in the relaxation was estimated as about 40% of the total response (Suthamnatpong *et al.*, 1993a). The mediator(s) of the remaining component of the relaxation is(are) still unknown.

Although NANC relaxation was not related to changes in membrane potentials of smooth muscle cells in the rat proximal colon (Suthamnatpong *et al.*, 1994), many reports suggest an association of hyperpolarization with nitric oxide-mediated relaxation of the smooth muscle in the gastrointestinal tract. Thus, exogenous nitric oxide evoked hyperpolarization in circular muscle of the canine proximal colon (Thornbury *et al.*, 1991) and jejunum (Stark *et al.*, 1991). The nitric oxide synthase inhibitors, N^G-nitro-L-arginine methyl ester (L-NAME)

[1] Author for correspondence.

and N^G-monomethyl-L-arginine inhibited the inhibitory junction potentials (i.j.ps) evoked by EFS in the canine proximal colon (Dalziel *et al.*, 1991) and jejunum (Stark *et al.*, 1991), respectively. Oxyhaemoglobin, which binds and inactivates nitric oxide, reduced the amplitude of i.j.ps and blocked hyperpolarization induced by exogenous nitric oxide (Stark *et al.*, 1991; Thornbury *et al.*, 1991). L-NAME also abolished the i.j.ps recorded by the double sucrose gap method from circular muscle of opossum oesophagus and dog intestine (Christinck *et al.*, 1991). Nitric oxide was suggested to enhance the open probability of Ca^{2+}-activated K^+ channels which mediate the hyperpolarization response to inhibitory neurotransmission in dog colonic muscle cells (Thornbury *et al.*, 1991), and to mediate NANC hyperpolarization of opossum oesophageal smooth muscle (Du *et al.*, 1991). Recently, it was shown that nitric oxide mediates i.j.ps, which depend on an increase in the cyclic GMP level and activation of apamin-sensitive K^+ channels in the opossum oesophagus (Cayabyab & Daniel, 1995) and that nitric oxide mediates apamin-sensitive relaxation in the rat proximal duodenum (Martins *et al.*, 1995).

Pituitary adenylate cyclase activating peptide (PACAP) was found in the digestive tract as well as in central and other peripheral tissues in rats (Arimura *et al.*, 1991). Exogenous PACAP was recently shown to induce relaxation via apamin-sensitive potassium channels in the guinea-pig tenia caeci (Schworer *et al.*, 1992) and stomach (Katsoulis *et al.*, 1994), and the human colon (Schworer *et al.*, 1993). More recently, PACAP was shown to be released by nerve stimulation and to be responsible for the apamin-sensitive component of relaxation in the guinea-pig tenia coli (Jin *et al.*, 1994). PACAP was also shown to be released by orad stretch of segments and to be responsible for the apamin-insensitive descending relaxation in the rat mid colon (Grider *et al.*, 1994).

Thus it was of interest to study whether NANC inhibitory responses of the rat distal colon relate to changes in membrane potentials. In the present work, we further studied the mechanism of VIP-induced relaxation of the rat distal colon in relation to membrane potentials of the smooth muscle cells. We also studied mediator(s) of NANC inhibition other than VIP, with special interest for the possible role of PACAP in NANC inhibition in this tissue. The data strongly suggest that VIP and PACAP mediate relaxation of longitudinal muscle of the rat distal colon, by activating charybdotoxin- and apamin-sensitive K^+ channels, respectively.

Methods

Male Wistar rats (250–350 g) were lightly anaesthetized with ether and then stunned by a blow on the head and bled via the carotid arteries. The colon was then removed and placed in Tyrode solution consisting of (in mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.1, NaH_2PO_4 0.42, $NaHCO_3$ 11.9 and glucose 5.6. The contents of the excised segment were gently flushed out with Tyrode solution. The descending colon which is attached by a mesentery to the small intestine was defined as the distal region.

Recording of responses of longitudinal muscle of rat distal colon to EFS

Entire segments of the distal colon (2.5–3.0 cm in length) were suspended in an organ bath containing 5 ml of Tyrode solution maintained at 37°C and bubbled with 95% O_2: 5% CO_2. 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. After an equilibration period of 30 min, responses of the longitudinal muscle to EFS with a single pulse or trains of 100 pulses of 0.5 ms width and supramaximal voltage (50 V) at a frequency of 10 Hz (Electronic stimulator, SEN-3301, Nihonkohden, Tokyo, Japan) were recorded isotonically, with 10 min intervals between tests. The longitudinal muscle was subjected to a resting load of 1.0 g. Longitudinal muscle of the distal segment of rat colon exhibited moderate spontaneous contractile activity compared to the proximal segment. The resting tone of the longitudinal muscle gradually increased during successive trains of EFS at 10 Hz at 10 min intervals. With progressive increase in the tone, the segments began to exhibit clear relaxation on EFS only in the presence of atropine (1 μM) and guanethidine (5 μM), as shown previously (Suthamnatpong *et al.*, 1993a). Therefore, atropine (1 μM) and guanethidine (5 μM) were added to the bathing solution throughout the experiment.

Recording of i.j.ps in longitudinal muscle of distal colon induced by EFS

The segments of the distal colon were mounted in a 1.5 ml organ bath maintained at 30°C and perfused continuously with Tyrode solution at a rate of 3 ml min^{-1}. This temperature allowed stable recording of the membrane potentials, since the spontaneous and evoked mechanical responses were reduced. 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. Inhibitory junction potentials were elicited by EFS to intramural nerves within the segment with square-wave pulses of 0.5 ms duration at an appropriate intensity (10–30 V). The electrode impalement was made into the longitudinal muscle cells of the superficial layer from the serosal side (Takewaki & Ohashi, 1977). The stimulus pulses were delivered with a pair of Ag-AgCl wire electrodes, one on the serosal surface 1–2 mm away from the impaled glass microelectrode and the other in the solution. The distance between the two electrodes was about 20 mm.

Drugs

Vasoactive intestinal peptide (VIP), charybdotoxin, pituitary adenylate cyclase activating peptide (PACAP) and its fragment $PACAP_{6-38}$ were purchased from the Peptide Institute, Osaka, Japan. Apamin, VIP_{10-28}, N^G-nitro-L-arginine (N^5-nitro-amidino-L-2,5,-diamino-pentanoic acid), L-arginine hydrochloride and D-arginine hydrochloride were from Sigma Chemical Co., St. Louis, U.S.A. Tetrodotoxin was from Wako Pure Chemical, Osaka, Japan. All other chemicals were of analytical grade. Durgs were added to the organ bath as solution in redistilled water in volumes of less than 1.0% of the bathing solution. A similar volume of redistilled water alone had no affect on the muscle.

Statistical analysis

Data are expressed as means ± s.e.mean. Results were analysed by Student's t test and a P value of <0.05 was regarded as significant.

Results

Responses of longitudinal muscle of the distal colon to electrical field stimulation (EFS)

A single pulse of EFS induced rapid transient relaxation only, but pulse trains at 1–50 Hz for 10 s induced rapid transient relaxation (rapid relaxation), with a subsequent rebound contraction and delayed sustained relaxation (delayed relaxation) of the longitudinal muscle of the distal segments (Figure 1). In the following studies the segments were stimulated by a single pulse or pulse trains at 10 Hz for 10 s in the presence of atropine and guanethidine. Tetrodotoxin (TTX, 1 μM) inhibited the EFS-induced responses of the preparations.

Effects of VIP_{10-28} and $PACAP_{6-38}$ on EFS-induced relaxation of longitudinal muscle of the rat distal colon

VIP_{10-28}, a VIP antagonist (Grider & Rivier, 1990), had no effect on the basal tone or spontaneous contractile activity but partially inhibited rapid relaxation induced by pulse trains at 10 Hz for 10 s as shown previously (Suthamnatpong *et al.*, 1993a; Figure 1a, Table 1). Rapid relaxation induced by a single pulse was also inhibited by the antagonist (44.9±7.1% inhibition at 3 μM VIP_{10-28}, $n=6$). $PACAP_{6-38}$, a PACAP antagonist (Shuttleworth & Keef, 1995), at concentrations up to 3 μM did not have any significant effect on the spontaneous contractile activity or on the tone of the longitudinal muscle of rat distal colon. However, $PACAP_{6-38}$ inhibited the pulse train-induced rapid relaxation in a concentration-dependent manner: it inhibited the relaxation at concentrations of over 0.1 μM, and by about 50% at 3 μM (Figures 1b and 2). A single pulse-induced rapid relaxation was also inhibited (52.6±3.3% inhibition at 3 μM $PACAP_{6-38}$, $n=4$). The inhibitory effect was most pronounced 20 min after its application. However, $PACAP_{6-38}$ did not have any significant effect on EFS-induced contraction or delayed relaxation. The antagonist (3 μM) completely inhibited the relaxant reponse induced by exogenously added PACAP (data not shown). A combination of the maximal effective concentration of VIP_{10-28} (3 μM) and that of $PACAP_{6-38}$ (3 μM) further inhibited the EFS-induced rapid relaxation to about 25% (Figure 1a, Table 1).

Effects of charybdotoxin and apamin on EFS-induced relaxation of longitudinal muscle of the rat distal colon

Charybdotoxin, an antagonist of large conductance Ca^{2+}-activated K^+ channels (Castle *et al.*, 1989), at 100 nM slightly increased the muscle tone, and frequency and amplitude of spontaneous contractile activity of the colonic segments. Charybdotoxin at concentrations over 0.1 nM concentration-dependently inhibited rapid relaxation induced by pulse trains at 10 Hz and maximally inhibited it by about 60% at 300 nM within 10 min (Figure 3), but did not show any effect on EFS-induced contraction or delayed relaxation (Figure 4). Rapid

Table 1 Effects of VIP_{10-28}, $PACAP_{6-38}$ and K^+ channel antagonists on EFS-induced relaxation of longitudinal muscle of the distal colon of rats

A *Drug treated*	*% inhibition*
VIP_{10-28} (3 μM)	44.4±2.4 (6)
$PACAP_{6-38}$ (3 μM)	47.5±3.8 (9)
VIP_{10-28} (3 μM) + $PACAP_{6-38}$ (3 μM)	74.5±2.1 (3)*
Charybdotoxin (100 nM)	58.3±3.4 (7)
Apamin (1 μM)	58.3±5.0 (14)
Charybdotoxin (100 nM) + apamin (1 μM)	84.3±4.3 (8)**
B	
VIP_{10-28} (3 μM) + charybdotoxin (100 nM)	58.0±8.5 (6)
VIP_{10-28} (3 μM) + apamin (1 μM)	75.3±4.3 (7)$^{\#}$
$PACAP_{6-38}$ (3 μM) + charybdotoxin (100 nM)	86.3±1.6 (3)$^{\#\#}$
$PACAP_{6-38}$ (3 μM) + apamin (1 μM)	59.6±7.5 (8)

Relaxations induced by EFS (200 pulses) in the presence of indicated antagonists are expressed as a percentage of those obtained before addition of antagonists (control). Values are means±s.e.mean for the numbers of experiments shown in parentheses. *Significantly different from the value for VIP_{10-28} or $PACAP_{6-38}$ alone, $P<0.05$; and **from the value for apamin or charybdotoxin alone, $P<0.05$; and $^{\#}$from the value for VIP_{10-28} alone, $P<0.05$; and $^{\#\#}$from the value for $PACAP_{6-38}$ alone, $P<0.05$.

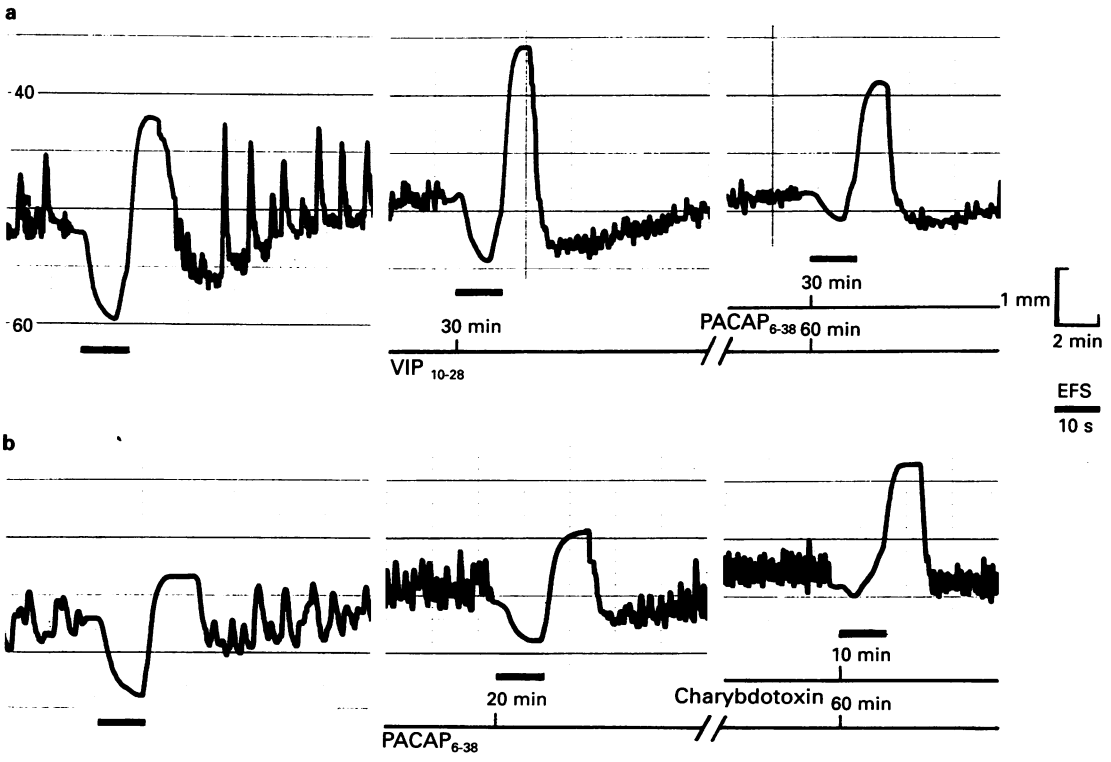

Figure 1 Effects of VIP_{10-28}, $PACAP_{6-38}$ and charybdotoxin on EFS-induced relaxation of longitudinal muscle in the rat distal colon. (a) Relaxation was induced by EFS (100 pulses at 10 Hz, 50 V) in the absence or presence of 3 μM VIP_{10-28} without or with 3 μM $PACAP_{6-38}$. (b) Relaxation was induced by EFS in the absence or presence of 3 μM $PACAP_{6-38}$ without or with 100 nM charybdotoxin. The continuous lines indicate the presence of VIP_{10-28}, $PACAP_{6-38}$ and charybdotoxin in the bathing fluid. Times noted on the lines are those after addition of the drugs. Bold black lines indicate duration of EFS for 10 s. After recording normal spontaneous movements, the chart was run fast immediately before the stimulation to make the relaxant response clear.

relaxation induced by a single pulse was also inhibited by the antagonist (41.3±6.4% inhibition at 100 nM charybdotoxin, $n=3$). Apamin, an antagonist of small conductance Ca^{2+}-activated K^+ channels (Castle *et al.*, 1989), had slight stimulatory effects on the muscle tone and spontaneous contractile activity and concentration-dependently inhibited rapid relaxation induced by pulse trains within 10 min (Figure 5) or a single pulse (56.0±7.0% inhibition at 1 μM, $n=5$). A combination of the maximal effective concentration of charybdotoxin (100 nM) and that of apamin (1 μM) further inhibited the rapid relaxation to about 85% inhibition, suggesting that charybdotoxin- and apamin-sensitive K^+ channels mediate different components of the relaxant response (Figure 4, Table 1).

Effects of K^+ channel antagonists on VIP- and PACAP-induced relaxations

VIP (100 nM–1 μM) induced slow gradual relaxation of longitudinal muscle of the segments and inhibited the spontaneous contractile activity. VIP-induced relaxation was abolished by VIP_{10-28} (data not shown), but not significantly inhibited by $PACAP_{6-38}$ (Table 2). Although apamin at concentrations up to 1 μM did not have any significant effect on the relaxation, charybdotoxin concentration-dependently inhibited the relaxation and at 100 nM significantly inhibited it within 10 min (Table 2).

PACAP (10–100 nM) also induced slow gradual relaxation of the muscle. The PACAP-induced relaxation was abolished by $PACAP_{6-38}$, but not inhibited by VIP_{10-28} (Table 2). In contrast to the effects on VIP-induced relaxation, apamin at 1 μM significantly inhibited PACAP-induced relaxation, while charybdotoxin at 100 nM slightly inhibited it (Table 2). These data suggested that VIP and PACAP activate charybdotoxin- and apamin-sensitive K^+ channels, respectively.

The relaxations induced by exogenous VIP and PACAP were not inhibited by L-NOARG at up to 100 μM (data not shown), suggesting that nitric oxide is not involved in these relaxations as shown before for EFS-induced relaxation (Suthamnatpong *et al.*, 1993a).

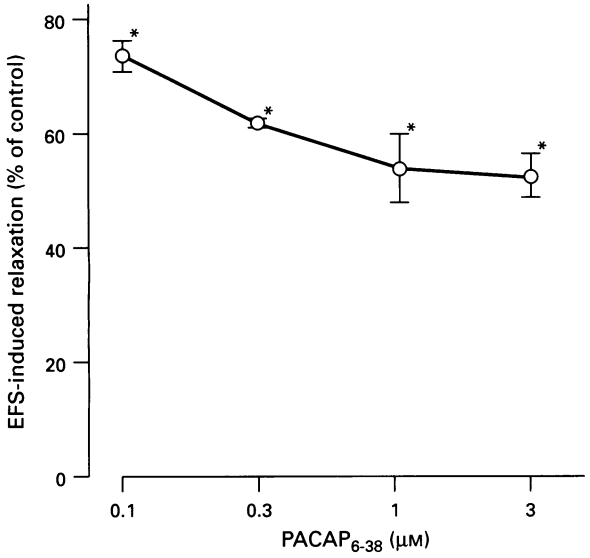

Figure 2 Effect of $PACAP_{6-38}$ on EFS-induced relaxation: relaxation was induced by EFS (100 pulses) every 10 min. After the relaxation had reached a constant level, effects of various concentrations of $PACAP_{6-38}$ were examined. Relaxations are expressed as a percentage of those obtained before addition of $PACAP_{6-38}$ (control). Points are means±s.e.mean for 3–9 experiments. For further details, see Methods and text. *Significantly different from control, $P<0.05$.

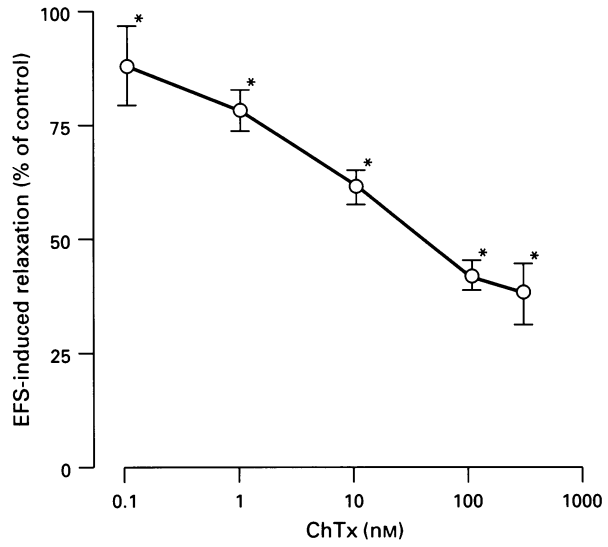

Figure 3 Effect of charybdotoxin on EFS-induced relaxation: relaxations induced by EFS (100 pulses) in the presence of various concentrations of charybdotoxin (ChTx) are expressed as a percentage of those obtained before addition of charybdotoxin (control). Points are means±s.e.mean for 3–7 experiments. For further details, see Methods and legend of Figure 2. *Significantly different from control, $P<0.05$.

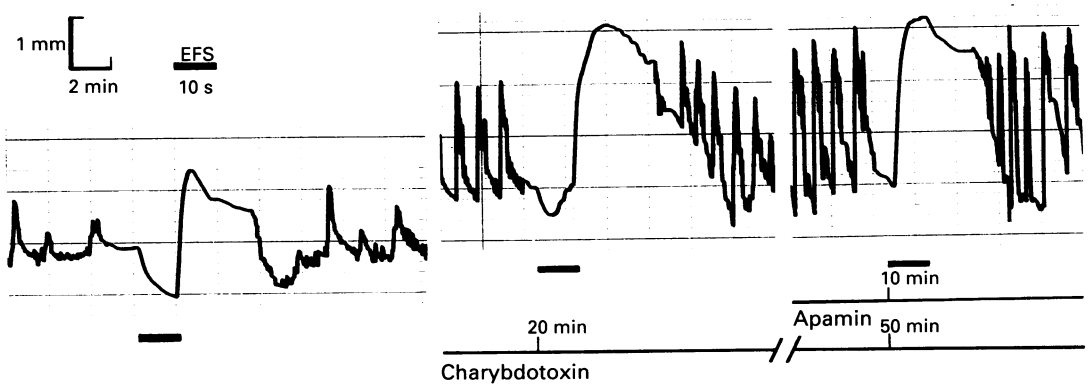

Figure 4 Effects of charybdotoxin and apamin on EFS-induced relaxation of longitudinal muscle of rat distal colon. Relaxation was induced by EFS (100 pulses at 10 Hz, 50 V) in the absence or presence of 100 nM charybdotoxin without or with 1 μM apamin. For further details, see legend of Figure 1.

Effects of combinations of VIP_{10-28} or $PAPCAP_{6-38}$ and K^+ channel antagonists on EFS-induced rapid relaxation

Next, we examined the effect of the combination of the receptor and channel antagonists on EFS-induced rapid relaxation to confirm the involvement of charybdotoxin-sensitive K^+ channels in the action of VIP, and apamin-sensitive K^+ channels in the action of PACAP. After the inhibitory effect of VIP_{10-28} (3 μM) on the EFS-induced relaxation reached the maximum, addition of apamin (1 μM) to the bathing fluid resulted in further inhibition, while charybdotoxin (100 nM) did not have such effect (Table 1b).

On the other hand, the inhibition induced by $PACAP_{6-38}$ (3 μM) in the presence of charybdotoxin, but not apamin, was more pronounced than with $PACAP_{6-38}$ alone (Figure 1b, Table 1b).

From these data it seems that VIP and PACAP activate charybdotoxin- and apamin-sensitive K^+ channels, respectively.

Effects of apamin, VIP_{10-28} and $PACAP_{6-38}$ on the changes in membrane potentials induced by EFS or agonists in the longitudinal smooth muscle cells of the rat distal colon

The resting membrane potential of longitudinal muscle cells of the rat distal colon was -63.1 ± 1.8 mV ($n=211$). In the presence of atropine (1 μM) and guanethidine (5 μM), EFS with a single pulse or two pulses at 10 Hz, induced inhibitory junction potentials (i.j.ps). I.j.ps induced by two pulses at 10 Hz often consisted of two phases, rapid and subsequent slow hyperpolarization. But the latter was scarcely recorded by a single pulse. The amplitude of the slow i.j.ps varied from one cell to another. Apamin did not have any significant effect on the resting membrane potentials at 20–100 nM. Apamin at 100 nM completely inhibited i.j.ps induced by a single pulse, whereas i.j.ps induced by two pulses at 10 Hz partially remained even after the treatment with 1 μM apamin (Figure 6a, c). $PACAP_{6-38}$ (5 μM) also significantly inhibited a single pulse-induced i.j.ps (58.0 ± 8.6% inhibition, $n=11$: Figure 6b). VIP_{10-28} (3 μM) did not have any appreciable effect on a single pulse-induced i.j.ps (not shown). But the antagonist significantly inhibited the delayed phase, slow hyperpolarization induced by two pulses at 10 Hz and abolished the i.j.ps in the presence of 500 nM apamin ($n=9$; Figure 6d).

Bath application of PACAP (1 μM) induced slow hyperpolarization in longitudinal muscle cells (10.3 ± 1.8 mV, $n=4$). Apamin at 100 nM completely inhibited the hyperpolarization as well as the i.j.ps (Figure 7a, b). Bath application of VIP (1 μM) also induced slow hyperpolarization of membrane potentials in longitudinal muscle cells (7.8 ± 1.6 mV, $n=5$). Although VIP_{10-28} at 2 μM completely inhibited the VIP-induced hyperpolarization, apamin at 100 nM did not have any significant effect on the hyperpolarization (Figure 7c, d).

Table 2 Effects of K^+ channel antagonists on VIP- or PACAP-induced relaxation

	Antagonist	*% inhibition*
VIP-induced relaxation	Apamin (1 μM)	3.6 ± 3.6 (4)
	Charybdotoxin (100 nM)	61.1 ± 7.5 (4)
	$PACAP_{6-38}$ (3 μM)*	10.2 ± 1.8 (3)
PACAP-induced relaxation	Apamin (1 μM)	71.7 ± 5.5 (3)
	Charybdotoxin (100 nM)	29.0 ± 11.5 (6)
	VIP_{10-28} (3 μM)	9.4 ± 2.9 (4)

Relaxations induced by 300 nM VIP or 100 nM PACAP in the presence of the indicated K^+ channel antagonists are expressed as a percentage of those obtained in the absence of the antagonists. *Effect of 3 μM $PACAP_{6-38}$ was examined for 3 μM VIP-induced relaxation. For further details, see legend of Table 1.

Discussion

We previously suggested that nitric oxide does not have any role in NANC inhibitory transmission in longitudinal muscle of the rat distal colon, and that VIP partly mediates the NANC inhibitory response in this tissue (Suthamnatpong *et al.*, 1993a). It seems that PACAP mediates the component of the relaxation other than the VIP-mediated one, from the

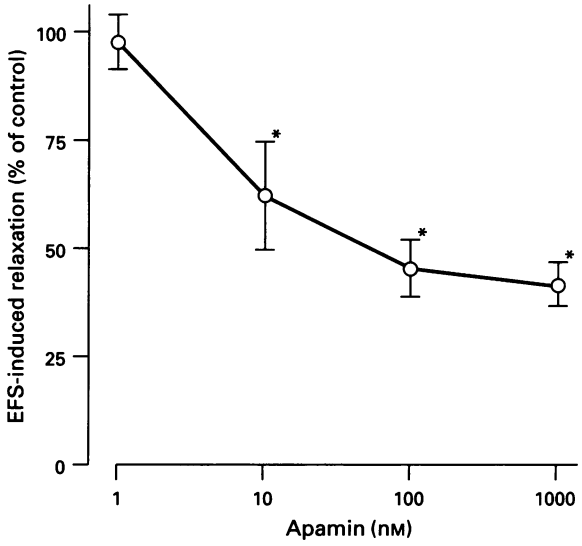

Figure 5 Effect of apamin on EFS-induced relaxation: relaxations induced by EFS (100 pulses) in the presence of various concentrations of apamin are expressed as a percentage of those before addition of apamin (control). Points are means ± s.e.mean for 4–14 experiments. For further details, see Methods and legend of Figure 2. *Significantly different from control, $P<0.05$.

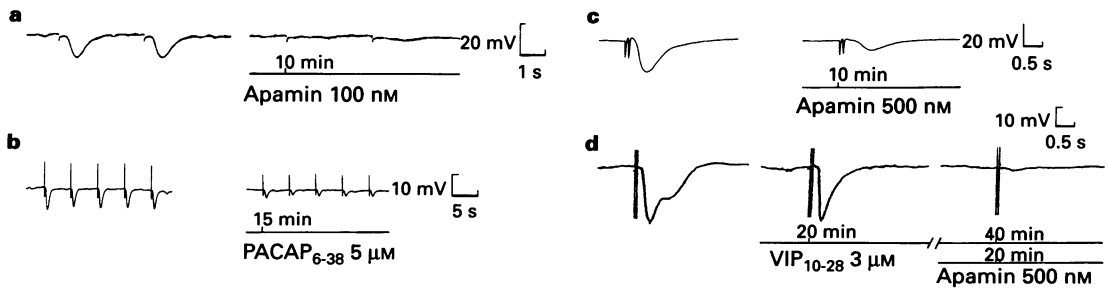

Figure 6 Effects of apamin, $PACAP_{6-38}$ and VIP_{10-28} on EFS-induced i.j.ps in longitudinal smooth muscle cell of the rat distal colon: i.j.ps were induced by a single pulse (a, b) or two pulses at 10 Hz (c, d) in the absence or presence of antagonists. Lines indicate the presence of the drugs. Times noted on the lines are those after addition of the drugs. All records in (a), (b), (c) or (d) were from the same longitudinal muscle cell.

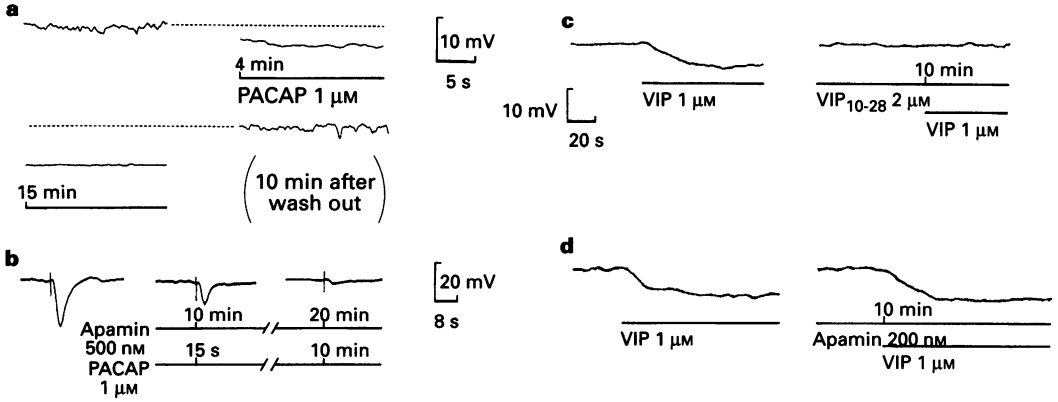

Figure 7 Effects of apamin on exogenous PACAP- and VIP-induced hyperpolarization in longitudinal smooth muscle cell of the rat distal colon: (a) Exogenously added PACAP (1 μM) induced slow hyperpolarization. (b) Inhibition of PACAP-induced hyperpolarization by apamin (500 nM). Exogenous VIP-induced slow hyperpolarization was inhibited by VIP_{10-28} (c) but not by apamin (d). VIP_{10-28} and apamin were added 10 min before addition of VIP. Lines indicate the presence of the drugs. Times noted on the lines are those after addition of the drugs. All records in (a), (b), (c) or (d) were from the same longitudinal muscle cell.

following findings in the present study. First, $PACAP_{6-38}$ concentration-dependently inhibited the EFS-induced NANC relaxation in longitudinal muscle of the rat distal colon. Second, exogenous PACAP induced slow gradual relaxation. Third, apamin at the concentration which partly inhibited the EFS-induced relaxation significantly inhibited exogenous PACAP-induced relaxation. Fourth, apamin and $PACAP_{6-38}$ inhibited a single pulse-induced i.j.ps and exogenous PACAP-induced hyperpolarization. Apamin also inhibited the rapid phase of the i.j.ps induced by 2 pulses. These findings also suggest that PACAP induces inhibitory responses by activating apamin-sensitive K^+ channels. The present finding of a stimulatory effect of PACAP on apamin-sensitive K^+ channels is consistent with findings in the guinea-pig tenia coli (Schworer *et al.*, 1992), stomach (Katsoulis *et al.*, 1994) and human colon (Schworer *et al.*, 1993). In contrast, PACAP-induced relaxation in guinea-pig tracheal smooth muscle was partly associated with activation of charybdotoxin-, but not apamin-sensitive K^+ channels (Hiramatsu *et al.*, 1995). Thus, activation of apamin-sensitive K^+ channels by PACAP is not general in different tissues.

Apamin completely inhibited the i.j.ps induced by a single pulse of EFS. $PACAP_{6-38}$ also significantly inhibited the i.j.ps. But the maximal inhibitory effect of apamin on relaxations induced by a single pulse and pulse trains was about 60%. This discrepancy between the effects of apamin on relaxation and i.j.ps cannot be explained at present. However, apamin and VIP_{10-28} inhibited the rapid and delayed phase of i.j.ps induced by two pulses at 10 Hz, respectively, and a combination of apamin and VIP_{10-28} completely inhibited the i.j.ps. Thus, it seems likely that PACAP and VIP mediate i.j.ps of longitudinal muscle cells induced by excitation of myenteric neurones. We could not confirm electrophysiologically the effect of charybdotoxin on the i.j.ps induced by EFS and on hyperpolarization induced by exogenous VIP, since we were unable to record continuously the membrane potentials of the smooth muscle cells due to the stimulatory effect of charybdotoxin on smooth muscle contractility. Nevertheless, activation of charybdotoxin-sensitive K^+ channels by VIP was strongly suggested by the pharmacological study on mechanical responses. That is, charybdotoxin partly inhibited the EFS-induced relaxation and significantly inhibited VIP-induced relaxation of the longitudinal muscle and charybdotoxin did not further inhibit the relaxation which persisted after VIP_{10-28} treatment. VIP has been suggested to mediate NANC inhibitory responses in different parts of the gastrointestinal tract, such as in the guinea-pig (Grider *et al.*, 1985), rat (Li & Rand, 1990; Lefebvre & Smits, 1992) and pig (Lefebvre *et al.*, 1995) gastric fundus, the rabbit (Biancani *et al.*, 1985) and opossum (Goyal *et al.*, 1980; Nurko & Rattan, 1988) lower oesophageal sphincter, and the chicken rectum (Komori & Ohashi, 1990). Charybdotoxin has also been reported to inhibit nitric oxide-independent relaxation in rabbit abdominal aorta and carotid artery (Cowan *et al.*, 1993), and to inhibit partly the nitric oxide-induced relaxation in rabbit aortic smooth muscle cells (Bolotina *et al.*, 1994). To our knowledge, the present study is the first to suggest activation of charybdotoxin-sensitive K^+ channels by VIP.

A sequential link between VIP and NO, VIP mediating NO release, was suggested in the gastric fundus of guinea-pigs (Grider *et al.*, 1992), the circular muscle of the middle or distal colon of rats (Grider, 1993) and the internal anal sphincter of opossums (Rattan & Chakder, 1992). However, L-NOARG did not inhibit NANC relaxation induced by EFS in longitudinal muscle of the distal colon of rats (Suthamnatpong *et al.*, 1993a) and this inhibitor also did not inhibit VIP- or PACAP-induced relaxation (data not shown). Therefore, this type of interrelation between VIP and NO seems unlikely in longitudinal muscle of the rat distal colon.

PACAP (PACAP 38 and PACAP 27) belongs to the VIP/secretin/glucagon family. PACAP 27 exhibits 68% homology with VIP (Miyata *et al.*, 1989). The PACAP receptor was classified into two types; type 1 receptors exhibit a high affinity for PACAP, but a low affinity for VIP and type 2 receptors exhibit similar affinity for them (Ishihara *et al.*, 1992; Spengler *et al.*, 1993). The amino acid sequence of rat PACAP receptors showed a high degree of similarity with that of rat VIP receptors (Hosoya *et al.*, 1993). The structural homologies between VIP and PACAP, and between VIP receptors and PACAP receptors suggest a related action of VIP and PACAP on the smooth muscle. Furthermore, it is generally accepted that VIP and PACAP have a similar affinity for VIP_1 and VIP_2 receptors. As in the present study, the VIP-induced relaxation was not inhibited by $PACAP_{6-38}$ and PACAP-induced relaxation was not inhibited by VIP_{10-28}, and the maximal inhibitory effects of VIP_{10-28} and $PACAP_{6-38}$ were additive, as were those of charybdotoxin and apamin, it seems that VIP and PACAP separately activate charybdotoxin- and apamin-sensitive K^+ channels, respectively, suggesting the involvement of type 1 PACAP receptors and a third type of VIP receptors, which are not activated by PACAP, in longitudinal smooth muscle cells of the rat distal colon. Indeed, such VIP receptors were also suggested in guinea-pig tania coli: VIP but not PACAP increased the cyclic AMP content and the VIP- but not PACAP-induced relaxation was inhibited by both inhibitors of cyclic AMP- and cyclic GMP-dependent protein kinases (Jin *et al.*, 1994). The findings in the present study indicate that VIP and PACAP are involved in NANC inhibitory responses in long-

itudinal muscle of the rat distal colon via interactions at separate receptors. However, some cross-action of both agonists cannot be excluded completely, since the inhibitory effects of the receptor antagonists and K^+ channel blockers on EFS-induced relaxation did not add up completely (Table 1), and charybdotoxin inhibited PACAP-induced relaxation to some extent (Table 2). A role for other mediator(s) than VIP and PACAP can thus not be excluded, since some small component of relaxation always persisted after blockade of both pathways, VIP-charybdotoxin-sensitive K^+ channels and PACAP-apamin-sensitive K^+ channels (Table 1).

Charybdotoxin- and apamin-sensitive K^+ channels are activated by Ca^{2+} ions. An increase in intracellular Ca^{2+} concentration above a certain level results in smooth muscle contraction. It seems, therefore, that the concentration of Ca^{2+} needed for activation of K^+ channels is lower than that for muscle contraction. An alternative possibility for activation of K^+ channels is an increase in Ca^{2+}-sensitivity of the K^+ channels. Several reports suggest that phosphorylation of the K^+ channels increases their sensitivity to Ca^{2+} and resulted in activation of the channels at a low concentration of Ca^{2+} ions (Ewald *et al.*, 1985; Sadoshima *et al.*, 1988; White *et al.*, 1993; Robertson *et al.*, 1993; Archer *et al.*, 1994; Esguerra *et al.*, 1994; Perez & Toro, 1994; Meera *et al.*, 1995).

Electrical field stimulation induced a rapid relaxation of the longitudinal muscle, but exogenous VIP or PACAP produced a slow relaxation. The slow spread of the peptides into the muscle tissue due to their high molecular weight may delay responses of the muscle to the peptides.

Our previous studies suggested that NANC relaxation in longitudinal muscle of the rat distal colon was not associated with the nitric oxide-cyclic GMP generating system (Suthamnatpong *et al.*, 1993a), although EFS or exogenous nitric oxide significantly increased cyclic GMP content of the colonic longitudinal muscle with myenteric plexus preparations (Suthamnatpong *et al.*, 1993b; Maehara *et al.*, 1994). Our present study strongly suggests that NANC-induced relaxation in the longitudinal muscle of the rat distal colon is mediated by VIP and PACAP which activate charybdotoxin- and apamin-sensitive K^+ channels, respectively. On the other hand, studies on circular muscle of the rat distal colon showed an important role of nitric oxide in NANC inhibition (Middleton *et al.*, 1993). It was also shown, in circular muscle of the rat middle or distal colon, that VIP and PACAP released from inhibitory neurones produced nitric oxide in smooth muscle cells which in turn resulted in relaxant responses (Grider, 1993; Grider *et al.*, 1994). It seems that NANC inhibitory mediators in the rat distal colon are different between longitudinal and circular muscle, in spite of a small restricted intestinal area.

Thus, the present findings suggest two essential sequences, VIP-charybdotoxin-sensitive K^+ channels and PACAP-apamin-sensitive K^+ channels, in the NANC-induced relaxation of longitudinal muscle of the rat distal colon.

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