



# Involvement of cyclic AMP–PKA pathway in VIP-induced, charybdotoxin-sensitive relaxation of longitudinal muscle of the distal colon of Wistar-ST rats

<sup>1</sup>Masami Kishi, <sup>1</sup>Tadayoshi Takeuchi, <sup>1</sup>Hideo Katayama, <sup>1</sup>Yasuko Yamazaki, <sup>1</sup>Hideaki Nishio, <sup>\*,1,2</sup>Fumiaki Hata & <sup>3</sup>Tadashi Takewaki

<sup>1</sup>Department of Veterinary Pharmacology, College of Agriculture, Osaka Prefecture University, Sakai 599-8531, Japan;

<sup>2</sup>Department of Molecular Physiology and Biochemistry, Research Institute for Advanced Science and Technology, Osaka Prefecture University, Sakai 599-8531, Japan and <sup>3</sup>United Graduate School of Veterinary Sciences, Gifu University, Gifu 501-1193, Japan

**1** The intracellular mechanism of vasoactive intestinal peptide (VIP)-induced, charybdotoxin (ChTx)-sensitive relaxation of longitudinal muscle of the distal colon of Wistar-ST rats was studied.

**2** A single pulse or 100 pulses at 10 Hz of electrical field stimulation (EFS) induced rapid transient relaxation or that with a subsequent contraction of the longitudinal muscle in the presence of atropine and guanethidine, respectively.

**3** Rp-8 bromo cAMPS, an inhibitor of cyclic AMP dependent protein kinase (PKA), at 30  $\mu$ M inhibited the relaxations induced by EFS with a single or 100 pulses maximally by about 80 or 60%, respectively. It also inhibited VIP (300 nM)-induced relaxation by 82%.

**4** VIP (100 nM–1  $\mu$ M) increased the cyclic AMP content of longitudinal muscle myenteric plexus preparations obtained from the distal colon.

**5** ChTx at 100 nM almost completely inhibited 8 bromo cyclic AMP-induced relaxation of the distal segments.

**6** EFS with two or three pulses at 10 Hz induced inhibitory junction potentials consisting of two phases, rapid and subsequent slow hyperpolarization in the membrane potential of longitudinal smooth muscle cells. Rp-cAMPS, another inhibitor of PKA, inhibited the delayed slow hyperpolarization. It also inhibited the exogenously added VIP-induced hyperpolarization of the cell membrane.

**7** Thus, the present study suggests that activation of PKA *via* activation of VIP receptors is associated with activation of ChTx-sensitive K<sup>+</sup> channels in relaxation of longitudinal muscle of the distal colon of Wistar-ST rats.

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**Keywords:** VIP; cyclic AMP; ChTx-sensitive K<sup>+</sup> channel; nonadrenergic noncholinergic relaxation; rat distal colon

**Abbreviations:** 8-bromo cAMP, 8-bromo adenosine-3',5'-cyclic monophosphate; ChTx, charybdotoxin; EFS, electrical field stimulation; H-89, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide dihydrochloride; i.j.ps, inhibitory junction potentials; LMMP, longitudinal muscle-myenteric plexus; PACAP, pituitary adenylate cyclase activating peptide; PKA, cyclic AMP dependent protein kinase; Rp-cAMPS, Rp adenosine-3',5'-cyclic monophosphorothioate; Rp-8-bromo cAMPS, Rp-8 bromoadenosine-3',5'-cyclic monophosphorothioate; TCA, trichloroacetic acid; VIP, vasoactive intestinal peptide

## Introduction

We have suggested that the vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating peptide (PACAP) are involved in nonadrenergic noncholinergic inhibitory responses of longitudinal muscle of the distal colon of Wistar-ST rats *via* activation of charybdotoxin (ChTx)- and apamin-sensitive K<sup>+</sup> channels, respectively (Kishi *et al.*, 1996). Recently we have also suggested that the activation of tyrosine kinase is involved in PACAP-induced relaxation of the muscle, 'upstream of' the activation of apamin-sensitive K<sup>+</sup> channels (Takeuchi *et al.*, 1999). However, the intracellular mechanism of activation of ChTx-sensitive K<sup>+</sup> channels after activation of VIP receptors is unknown.

It has been widely accepted that there is a correlation between an elevated cyclic AMP content and smooth muscle

relaxation mediated by  $\beta$ -adrenoceptor stimulation (for review, see Kamm & Stull, 1985). In the gastrointestinal tract, the calcitonin gene related peptide (CGRP) was suggested to mediate relaxation of smooth muscle *via* elevation of the cyclic AMP content in isolated ileal cells (Rekik *et al.*, 1997) and longitudinal muscle myenteric plexus preparations (Sun & Benishin, 1995) of guinea-pigs, and the internal anal sphincter of opossums (Rattan *et al.*, 1991). VIP was also suggested to induce relaxation with a concomitant increase in cyclic AMP content in rat stomach (Ito *et al.*, 1990) and dispersed stomach muscle cells (Bitar & Makhoulf, 1982; Murthy *et al.*, 1993; Jin *et al.*, 1993; Murthy & Makhoulf, 1995), in the lower oesophageal sphincter (Torphy *et al.*, 1986) and in the internal anal sphincter of the opossum (Chakder & Rattan, 1993). Although the sequence of VIP-cyclic AMP-relaxant response was also suggested in dispersed ileal smooth muscle cells of guinea-pigs (Rekik *et al.*, 1996), there is no direct evidence for this in the rat intestine.

\*Author for correspondence at: Department of Veterinary Pharmacology, College of Agriculture, Osaka Prefecture University, Sakai 599-8531, Japan

Cyclic AMP dependent protein kinase (PKA) was suggested to activate  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels in myocytes from the circular layer of canine colon (Carl *et al.*, 1991) and in *Xenopus* oocytes which expressed *Drosophila* Slo Kca channels (Esguerra *et al.*, 1994) and in the myometrial cell line PHM1-41 (Meera *et al.*, 1995). PKA was also suggested to activate ATP-sensitive  $\text{K}^+$  channels in coronary arterial smooth muscle cells (Wellman *et al.*, 1998) and to activate the (4-aminopyridine-sensitive voltage-gated) delayed rectifier current in rabbit portal vein smooth muscle cells (Aiello *et al.*, 1995). The aim of the present study was to determine whether cyclic AMP-PKA system is involved in VIP-mediated relaxation in the distal colon of Wistar-ST rats. Here we report the sequential coupling of activation of the VIP receptor, PKA and ChTx-sensitive  $\text{K}^+$  channels in relaxation of longitudinal muscle of the distal colon.

## Methods

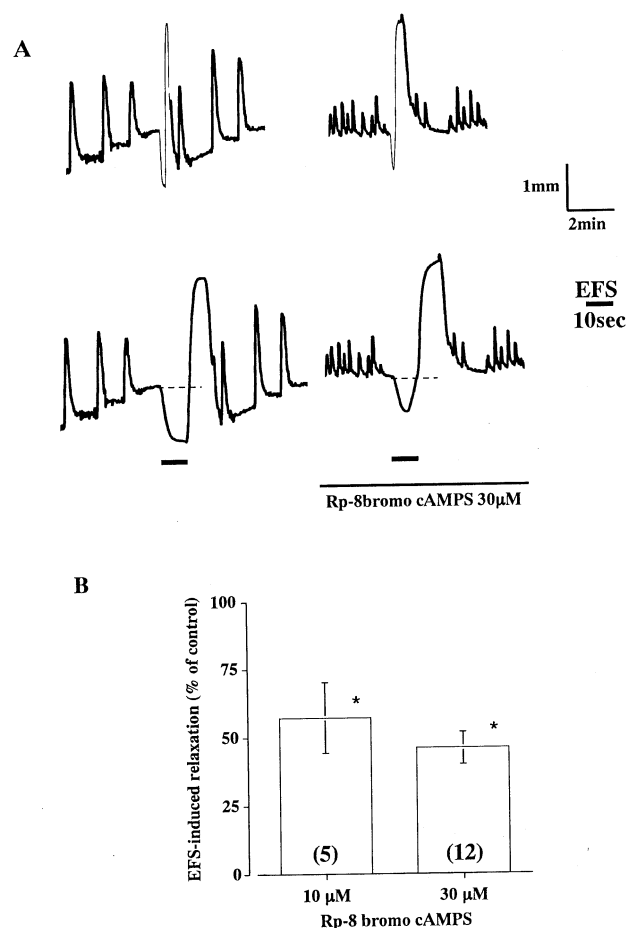
Male Wistar-ST strain rats (8–9 weeks-old) were purchased from Nippon SLC (Shizuoka, Japan). We indicated the strain of rats used simply as Wistar in previous papers (Suthamnatpong *et al.*, 1993; 1994; Kishi *et al.*, 1996), Wistar-ST rats belonging to a subclass of the Wistar strain, were used in those studies. The rats 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,  $\text{CaCl}_2$  1.8,  $\text{MgCl}_2$  1.1,  $\text{NaH}_2\text{PO}_4$  0.42,  $\text{NaHCO}_3$  11.9 and glucose 5.6. The contents of the excised segments 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 electrical field stimulation (EFS)

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  $37^\circ\text{C}$  and bubbled with 95%  $\text{O}_2$ :5%  $\text{CO}_2$ . After an equilibration period of 30 min, responses of the longitudinal muscle to EFS with a single or trains of 100 pulses of 0.5-ms width at 30 V, 10-Hz frequency in the presence of atropine (1  $\mu\text{M}$ ) and guanethidine (5  $\mu\text{M}$ ), were recorded isotonicly with a 10-min interval between tests. These concentrations of atropine and guanethidine did not have any significant effect on the tone or on the spontaneous contractile activity of the segments. The longitudinal muscle was subjected to a resting load of 0.75 g. The responses of the segments to EFS were nearly constant throughout the experiments of 120 min. EFS and drug-treatment were always started at the most relaxed state of resting spontaneous contractile activity: i.e. the bottom of the trace of resting activity.

In the experiments to examine the effect of Rp-8-bromo cAMPS on EFS-induced relaxation, the drug was added into an organ bath 30 min before EFS, since the magnitude of inhibition induced by 1 h-incubation was similar to that induced by 30 min period.

The extent of relaxation was expressed as the area under the line of resting tone that was drawn on the bottom of resting activity (Figure 1 dotted lines). The extent of relaxation induced by exogenous VIP was expressed as the depth of the relaxation.



**Figure 1** Effect of Rp-8-bromo cAMPS on EFS-induced relaxation of longitudinal muscle in the rat distal colon. (A) Relaxation was induced by EFS (100 pulses at 10 Hz) before and after treatment of the segments with 30  $\mu\text{M}$  Rp-8-bromo cAMPS for 10 min. The continuous line indicates the presence of the drug (upper traces). After recording normal spontaneous movements, the chart was run fast immediately before the stimulation to make the relaxant response clear, (lower traces). Bold black lines indicate the duration of EFS for 10 s. (B) Relaxations induced by EFS (100 pulses) in the presence of Rp-8-bromo cAMPS are expressed as percentages of the control response before addition of the drug. Columns and bars represent means with standard errors in 5 (10  $\mu\text{M}$ ) or 9 (30  $\mu\text{M}$ ) experiments. \*Significantly different from control,  $P < 0.05$  (Student's *t*-test).

### Measurement of cyclic AMP content of longitudinal muscle myenteric plexus preparations

Longitudinal muscle preparations with adhering myenteric plexus were prepared from the distal colon as described by Paton & Zar (1968). The preparations (10–20 mg wet weight) were equilibrated for 20 min with Tyrode solution at  $37^\circ\text{C}$  and aerated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . The preparations were then incubated with various concentrations of VIP (100 nM–1  $\mu\text{M}$ ) for 5 min. In another series of experiments to determine the effect of EFS on the cyclic AMP content, either end of the preparation was tied with threads allowing it to be hand held. This allowed easy mounting of the preparations between two platinum plate electrodes in a specially designed laboratory dish-type organ bath and quick removal from the electrodes at the end of EFS. The parameters for electrical field stimulation were the same as those used for measurement of the mechanical response. After the incubation or stimulation period, preparations were quickly frozen within a few seconds by putting them between two frozen metal plates on dry ice. Frozen preparations were then homogenized in 2 ml of 6.0 N

trichloroacetic acid (TCA) solution. After removal of TCA with ether, the cyclic AMP contents were determined with a cyclic AMP assay kit (Amersham Japan, Tokyo). All determinations of cyclic AMP were carried out in the absence of phosphodiesterase inhibitor.

#### *Recording of inhibitory junction potentials (i.j.ps) in longitudinal muscle cells 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<sup>-1</sup>. Atropine (1 µM) and guanethidine (5 µM) were added to the bathing solution throughout the experiment to block cholinergic and adrenergic responses, respectively. Membrane potentials were recorded with a conventional glass microelectrode filled with 3 M KCl with a resistance of 50–80 MΩ. The electrode was impaled into the longitudinal muscle cells of the superficial layer from the serosal side (Takewaki & Ohashi, 1977). Intramural nerves within the segment were stimulated with a pair of Ag wire electrodes, one on the serosal surface 1–2 mm from the impaled glass microelectrode and the other in the solution. The distance between the two electrodes was approximately 20 mm.

#### *Drugs*

Charybdotoxin, VIP, VIP<sub>10–28</sub>, pituitary adenylate cyclase activating peptide (PACAP) and PACAP<sub>6–38</sub> were from the Peptide Institute, Osaka, Japan. Atropine sulphate was from Wako Pure Chemical, Osaka, Japan. 8-Bromo adenosine-3',5'-cyclic monophosphate (8 bromo cAMP) and its Rp isomer, Rp-8 bromo adenosine-3',5'-cyclic monophosphorothioate (Rp-8 bromo cAMPS) and Rp adenosine-3',5'-cyclic monophosphorothioate (Rp-cAMPS) were from BIOLOG Life Sci, Inst., Bremen, Germany. Drugs were added to the organ bath in a volume of less than 1.0% of the bathing solution. These volumes of the vehicle of the drugs in redistilled water did not affect the spontaneous contractile activity or muscle tone.

#### *Statistical analysis*

Data are expressed as means ± s.e.means. Statistical analysis of the data for two groups was carried out with a Student's *t*-test and for more than three groups with a one-way analysis of variance and Bonferroni's test. *P* values of less than 0.05 were regarded as significant.

## **Results**

#### *Effects of an inhibitor of PKA on EFS- or VIP-induced relaxation of longitudinal muscle in the distal colon of Wistar-ST rats*

The distal colonic segments exhibited moderate spontaneous contractile activity and very small relaxation on EFS in the presence of 1 µM atropine and 5 µM guanethidine. However, 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 in this way, the segments began to exhibit clear relaxation on a single pulse of EFS and rapid transient relaxation with a subsequent contraction on pulse trains at 1–50 Hz for 10 s. Rp-8 bromo cAMPS, an inhibitor of PKA, inhibited relaxation induced by pulse trains at 10 Hz and inhibited it maximally by about 60% at 30 µM (Figure 1). Amplitude and duration of the relaxation were

significantly inhibited, but the basal tone of the segments and induced contraction was not affected (Figure 1A). The rapid relaxation induced by a single pulse was also inhibited by the antagonist (78.2 ± 1.8% inhibition at 30 µM Rp-8 bromo cAMPS, *n* = 5). However, the contraction subsequent to the relaxation was not affected.

VIP (300 nM) induced gradual slow relaxation of longitudinal muscle of the segments and inhibited the spontaneous contractile activity. Rp-8 bromo cAMPS at 30 µM significantly inhibited 300 nM VIP-induced relaxation by 82.4 ± 5.3% (*n* = 5; Figure 2). Since these results suggest that VIP-mediated relaxation in the distal colon is associated with activation of PKA, we next examined whether the cyclic AMP content of the longitudinal muscle was elevated by EFS or VIP application.

#### *Effects of VIP, PACAP and EFS on the cyclic AMP content of longitudinal muscle of the distal colon of Wistar-ST rats*

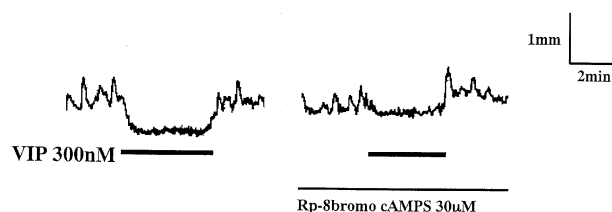
Although whole segments of the distal colon were used to record mechanical activity, only the longitudinal muscle with the myenteric plexus was used for measurement of cyclic AMP; this approach was used to avoid contamination by circular smooth muscle. VIP increased the cyclic AMP content of preparations in a concentration dependent manner (Figure 3). EFS at 10 Hz for 10 s also increased the cyclic AMP content from 165.0 ± 1.6 to 239.2 ± 11.3 fmol mg<sup>-1</sup> wet tissue and the increase was abolished in the presence of 3 µM VIP<sub>10–28</sub> (Figure 3).

PACAP was suggested to mediate the relaxation of longitudinal muscle of the distal colon partially *via* activation of apamin-sensitive K<sup>+</sup> channels (Kishi *et al.*, 1996). So, we next examined the correlation of the cyclic AMP content with PACAP-induced relaxation of the LMMP preparations. Although VIP and EFS increased the cyclic AMP content of LMMP preparations, PACAP at 100 nM which induced a maximal relaxant response in the distal segments did not affect the content (Figure 3).

EFS-induced relaxation was partially inhibited by a PKA inhibitor (Figure 1) and the relaxation which persisted after the PKA inhibitor-treatment was further inhibited by apamin but not ChTx (Figure 4). 8-Bromo cyclic AMP-induced relaxation was not affected by apamin (Table 1). Thus, it seems that cyclic AMP is involved only in the VIP-mediated component of the NANC relaxation.

#### *Effect of ChTx on 8 bromo cyclic AMP-induced relaxation of longitudinal muscle of the distal colon*

8-Bromo cyclic AMP, a membrane permeable analogue of cyclic AMP, induced slow relaxation of longitudinal muscle of the segments. Since ChTx-sensitive K<sup>+</sup> channels were suggested to



**Figure 2** Effect of Rp-8 bromo cAMPS on VIP-induced relaxation. Relaxation was induced by 300 nM VIP before and after treatment of the segments with 30 µM Rp-8-bromo cAMPS for 10 min. The continuous line indicates the presence of the drug. Bold black lines indicate the duration of the presence of VIP.

be involved in the VIP-mediated relaxation of the preparation in our previous study (Kishi *et al.*, 1996), the effect of ChTx on the 8-bromo cyclic AMP-induced relaxation was examined. ChTx at 100 nM, which exhibited a maximal inhibitory effect on the EFS-induced relaxation of the segments (Kishi *et al.*, 1996) almost completely inhibited the 8-bromo cyclic AMP-induced relaxation (Table 1).

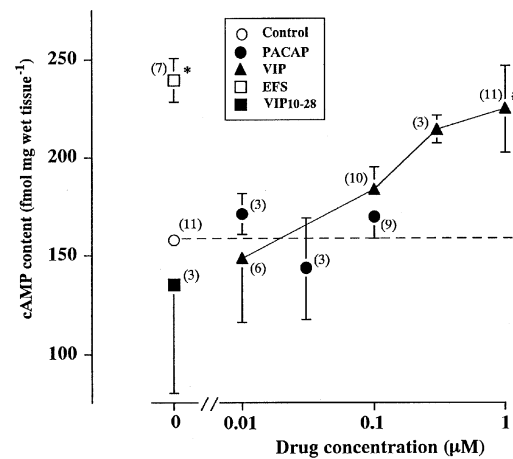
#### Effects of Rp-8 bromo cyclic AMPS on the changes in membrane potentials induced by EFS or VIP in the longitudinal muscle cells of the rat distal colon

The resting membrane potential of longitudinal muscle cells of the rat distal colon was around  $-60$  mV as reported previously (Kishi *et al.*, 1996). In the presence of  $1 \mu\text{M}$  atropine and  $5 \mu\text{M}$  guanethidine, EFS with a single pulse, or two or three pulses at 10 Hz induced inhibitory junction potentials (i.j.ps). The i.j.ps induced by two or three pulses at 10 Hz often consisted of two phases, rapid and subsequent slow hyperpolarization. But the latter was rarely recorded on a single pulse. The rapid and slow hyperpolarizations were suggested to be mediated by PACAP and VIP, respectively, in a previous study (Kishi *et al.*, 1996). Rp-cyclic AMPS at  $50 \mu\text{M}$ , another inhibitor of PKA, inhibited only the delayed phase, slow hyperpolarization, suggesting that VIP induced the delayed phase of i.j.ps via a cyclic AMP-PKA pathway (Figure 5).

Bath application of VIP induced slow hyperpolarization of membrane potentials in longitudinal muscle cells. Rp-8 bromo cyclic AMPS at  $30 \mu\text{M}$  completely inhibited 100 nM VIP-induced hyperpolarization,  $2.2 \pm 0.8$  mV ( $n=6$ ; Figure 6A) and significantly inhibited 300 nM VIP-induced hyperpolarization from  $6.5 \pm 1.1$  mV to  $1.7 \pm 0.9$  mV ( $n=5$ ; Figure 6B).

## Discussion

All layers of every region of the gut contain vasoactive intestinal peptide (VIP)- immunoreactive nerve fibres (for reviews, see Gillespie, 1982; Fahrenkrug, 1991). However, the role of VIP as a mediator of the inhibitory response has been

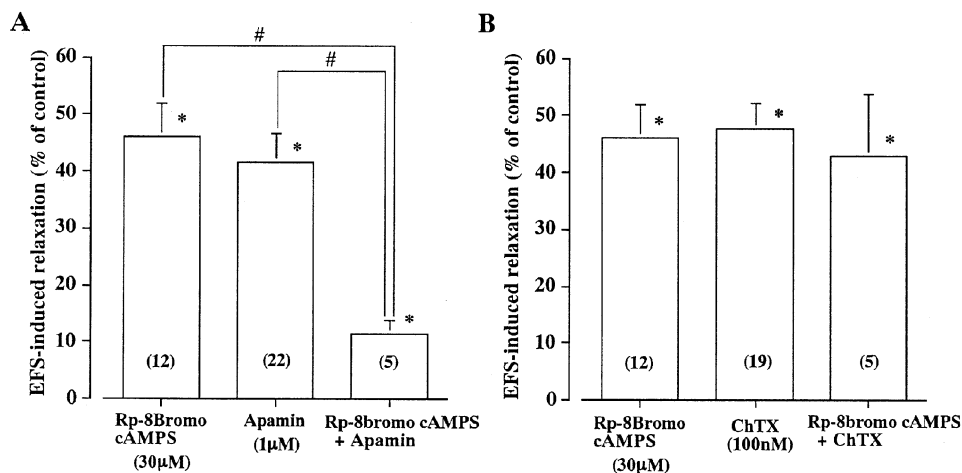


**Figure 3** Effects of VIP, PACAP and EFS on the cyclic AMP content of longitudinal muscle myenteric plexus (LMMP) preparations of rat distal colon. LMMP preparations were incubated without or with the indicated concentrations of VIP or PACAP for 5 min, or stimulated with electrical pulses at 10 Hz for 10 s in the absence or presence of  $3 \mu\text{M}$  VIP<sub>10-28</sub>. Values are means with standard errors for the numbers of experiments shown in parentheses. \*, # Significance of difference from the value in the absence of agonist,  $P < 0.05$  (\*Student's *t*-test, #Bonferroni's test). For further details, see Methods.

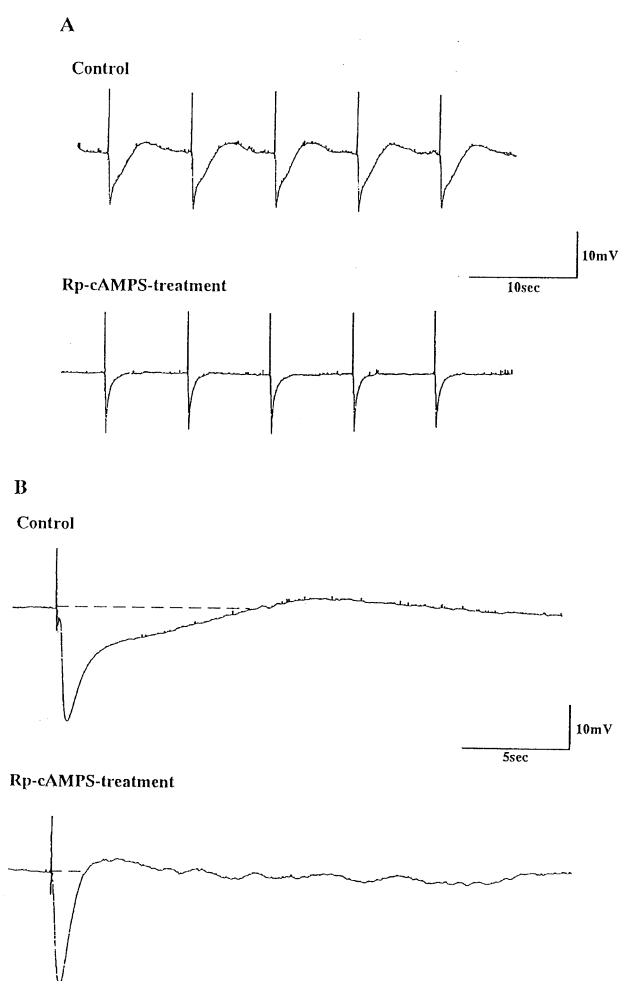
**Table 1** Effects of ChTx and apamin on 8-bromo cyclic AMP-induced relaxation

8-bromo cAMP ( $\mu\text{M}$ )	Treatment		
	Control	ChTx	Apamin
30	13.1 $\pm$ 1.2 (10)	0 (5)*	11.1 $\pm$ 2.8 (5)
100	31.8 $\pm$ 4.3 (10)	3.2 $\pm$ 2.2 (5)*	31.3 $\pm$ 2.9 (5)

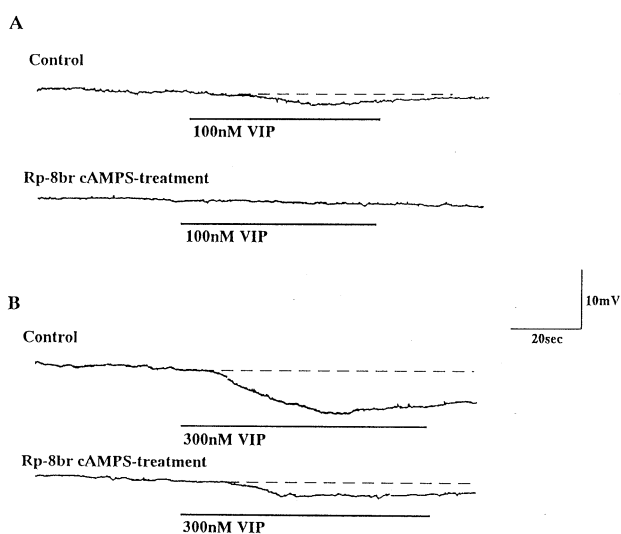
Relaxations were induced by 8-bromo cyclic AMP in the absence (control) or presence of ChTx (100 nM) or apamin ( $1 \mu\text{M}$ ) and expressed as percentages of  $30 \mu\text{M}$  papaverine-induced maximal relaxation. Values are means with standard errors of the number of experiments noted in parenthesis. \*Significantly different from corresponding control value at  $P < 0.05$  (Student's *t*-test).



**Figure 4** Effects of Rp-8 bromo cAMPS, ChTx and apamin on EFS-induced relaxation of longitudinal muscle in distal colon of Wistar-ST rats. (A) Relaxations induced by EFS (100 Pulses) in the presence of  $30 \mu\text{M}$  Rp-8 bromo cyclic AMPS or  $1 \mu\text{M}$  apamin, or both drugs are expressed as percentages of that obtained before addition of drugs (control). (B) Relaxations were induced by EFS in the presence of Rp-8 bromo cyclic AMPS or 100 nM ChTx, or both drugs. Columns and bars represent means with standard errors of 5–22 experiments shown in parentheses. \*Significantly different from the control value,  $P < 0.05$  and #Significant difference between the values at  $P < 0.05$  (Student's *t*-test).



**Figure 5** Effect of Rp-cAMPS on i.j.ps induced by EFS in longitudinal muscle cells of the distal colon of Wistar-ST rats. (A) I.j.ps induced by EFS with 2 pulses at 10 Hz were recorded before and 30 min after application of 50  $\mu$ M Rp-cAMPS. (B) The chart was run fast to make the i.j.ps clear. Note that i.j.ps consist of rapid and slow hyperpolarization. Atropine (1  $\mu$ M) and guanethidine (5  $\mu$ M) were added to the bathing fluid throughout the experiment.



**Figure 6** Effect of Rp-8 bromo cAMPS on VIP-induced inhibitory junction potentials of the longitudinal muscle cell membrane. Hyperpolarizations were induced by exogenously added 100 (A) or 300 (B) nM VIP before and 30 min after treatment of 30  $\mu$ M Rp-8 bromo cAMPS. Note that VIP-induced slow hyperpolarization was inhibited by Rp-8 bromo cyclic AMPS-treatment.

shown in only rather restricted regions of the gut: the lower oesophageal sphincter of the opossum (Goyal *et al.*, 1980) and rabbit (Biancani *et al.*, 1984), the stomach of the dog (Angel *et al.*, 1983), guinea-pig (Grider *et al.*, 1985a) and rat (Kamata *et al.*, 1988; De Beurme & Lefebvre, 1988), and the taenia coli of the guinea-pig (Grider *et al.*, 1985b). The role of VIP has not been shown in many regions of the intestine: the colon of mice (Fontaine *et al.*, 1986), the ileocolonic junction of dogs (Boeckxstaens *et al.*, 1991), the jejunum (Niioka *et al.*, 1997), proximal colon (Suthamnatpong *et al.*, 1993) and rectum (Takeuchi *et al.*, 1998) of Wistar-ST rats. However, the rat colon is exceptional. Namely, in this region VIP was also suggested to mediate descending relaxation (Grider & Makhoulf, 1986; Grider & Rivier, 1990). Our previous study also suggested that VIP and PACAP mediates about half NANC relaxation of longitudinal muscle of the distal colon of Wistar-ST rats *via* activating ChTx- and apamin-sensitive  $K^+$  channels, respectively (Kishi *et al.*, 1996). The present results suggest that a cyclic AMP-PKA-ChTx-sensitive  $K^+$  channel pathway is involved in the VIP-induced relaxation and that this pathway is independent of PACAP-mediated relaxation *via* activation of apamin sensitive  $K^+$  channels. First we found that exogenous VIP and EFS but not exogenous PACAP increased the cyclic AMP content of the longitudinal muscle; second, that a PKA inhibitor inhibited exogenous VIP- but not PACAP-induced relaxation; third, that ChTx but not apamin inhibited 8 bromo cyclic AMP-induced relaxation; fourth, that a PKA inhibitor inhibited EFS-induced relaxation additively with apamin but not ChTx; fifth, that a PKA inhibitor inhibited EFS-induced slow hyperpolarization of longitudinal muscle cell membranes which were inhibited by a VIP antagonist and also inhibited VIP-induced hyperpolarization of the membranes. This suggests a sequence of VIP-cyclic AMP-PKA-ChTx-sensitive  $K^+$  channels-relaxant response in the gut.

Although exogenously added VIP (100 nM–1  $\mu$ M) increased the cyclic AMP content of LMMP preparations, the extent of the increase was less than that in EFS (Figure 3). This inconsistency may be due to a different mode of action of VIP. Namely, VIP released toward the postsynaptic area by EFS induced fast elevation of the cyclic AMP content as a physiological response, while VIP applied exogenously diffuses into the LMMP preparation. Indeed, exogenous VIP induced a slow relaxation which took 5 min to reach a maximum in most cases.

PACAP (10–100 nM) did not increase cyclic AMP content of the preparations. We have suggested that PACAP induced relaxation of longitudinal muscle of the colonic segments *via* activation of tyrosine kinase-apamin sensitive  $K^+$  channel pathway (Takeuchi *et al.*, 1999). These results indicate that relaxant action of 100 nM PACAP which induced the maximal relaxation of the segments is not associated with an increase in cyclic AMP content.

EFS for 10 s induced relaxation with concomitant increase in the cyclic AMP content. For measurement of the cyclic AMP content, LMMP preparations were quickly frozen within a few second (see Methods). However, colonic segments contracted immediately after the stimulation, suggesting an increased cyclic AMP content in spite of muscle contraction. The contraction subsequent to NANC relaxation was not affected by treatments with Rp-8 bromo cAMPS (Figure 1), VIP<sub>10–28</sub> or ChTx (Kishi *et al.*, 1996). Therefore, the contraction seems to be induced by turn off-stimulation, independent of the cyclic AMP content.

In guinea-pig trachea smooth muscle, activation of ChTx-sensitive  $K^+$  channels was suggested to be involved in PACAP-

induced bronchodilation via a cyclic AMP–PKA pathway (Hiramatsu *et al.*, 1994; 1995). In intestinal smooth muscle, ChTx-sensitive  $K^+$  channel was also suggested to be constitutively activated for modulation of the activity of ileal smooth muscle cells of guinea-pig: ChTx increased spontaneous activity and inhibited the nerve stimulation-evoked hyperpolarization of the cell membrane (Hong *et al.*, 1997). Moreover, activation of ChTx-sensitive  $K^+$  channels is thought to be involved in cyclic AMP-induced hyperpolarization of cell membranes of canine colonic circular muscle (Du *et al.*, 1994), and its activation in setting the membrane potential, and in determining the excitability in longitudinal layer, and in limiting the responses to excitatory agonists in the circular layer of canine colon (Carl *et al.*, 1995). As mentioned in the Introduction, several reports suggest activation of  $Ca^{2+}$ -activated  $K^+$  channels by PKA (Carl *et al.*, 1991; Esguerra *et al.*, 1994; Meera *et al.*, 1995). The present results also suggest

that PKA activates ChTx-sensitive  $K^+$  channels in longitudinal muscle of the rat distal colon.

In summary, an inhibitor of PKA inhibited EFS-induced NANC relaxation and exogenous VIP-induced relaxation. The inhibitor also inhibited the delayed slow hyperpolarization of i.j.ps induced by EFS and exogenous VIP-induced hyperpolarization. Thus, the present results suggest that PKA is associated with activation of ChTx-sensitive  $K^+$  channels in longitudinal muscle of the distal colon, although the precise mechanism by which it activates them is unknown.

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