Co-release of PHI and VIP in dog stomach by peripheral and central vagal stimulation

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1 The purpose of this investigation was to examine the outputs of peptide histidine isoleucine (PHI) and vasoactive intestinal peptide (VIP) from the gastric corpus in response to stimulation of the peripheral (PVS) and central (CVS) ends of the vagus nerve and to compare the effects of PHI on gastric motility and blood flow with those of VIP in atropine-treated dogs.

2 PVS and CVS caused parallel increases in gastric venous plasma concentrations of VIP and PHI. The molar ratios of the peptides (VIP/PHI) appearing in the gastric venous plasma were about 1.5 for PVS at 10 Hz, 0.65 for PVS at 40 Hz and about 0.7 for CVS at 10 Hz.

3 The molar ratio of peptide concentration extracted from the gastric corpus was about 1.5 in the muscle layer and 2.7 in the mucosal layer.

4 Intra-arterially-injected PHI was about 5 fold and 50 fold less effective in producing gastric relaxation and vasodilatation, respectively, than VIP.

5 These results indicate that PHI is co-released with VIP by peripheral vagal stimulation and by initiation of the vago-vagal reflex, and that PHI seems to have an important role in the regulation of gastric motility but not gastric blood flow in the dog.

Introduction

Peptide histidine isoleucine (PHI) has a marked sequence homology with vasoactive intestinal peptide (VIP) (Tatemoto & Mutt, 1981). Also, it has been demonstrated that a prepro VIP, the VIP precursor, contains a PHI-related peptide (peptide histidine methionine) in its sequence, suggesting that VIP and PHI are co-synthesized in the same precursor molecule (Itoh *et al.*, 1983). Recent immunohistochemical studies have also provided evidence that the two peptides coexist in neurones of various tissues (Bishop *et al.*, 1984; Lundberg *et al.*, 1984a; Ekblad *et al.*, 1985; Holst *et al.*, 1987).

PHI has been shown to be co-released with VIP in response to parasympathetic nerve stimulation (Lundberg *et al.*, 1984b; Holst *et al.*, 1987; Yasui *et al.*, 1987). In particular, vagal stimulation releases PHI with VIP into the portal plasma of dogs (Yasui *et al.*, 1987) and into the perfusate from the pig pancreas (Holst *et al.*, 1987). PHI and VIP share several biological activities, such as stimulation of pancreatic secretion (Dimoline & Dockray, 1980; Holst *et al.*, 1987), smooth muscle relaxation (Lundberg *et al.*, 1984a; Bardrum *et al.*, 1986) and vasodilatation (Lundberg & Tatemoto, 1982; Lundberg *et al.*, 1984a; Suzuki *et al.*, 1984; Bardrum *et al.*, 1986). However, their relative potencies vary in different tissues.

We have recently shown that peripheral or central (vago-vagal reflex) vagal stimulation releases VIP into the venous effluent from the dog gastric corpus. We suggested that VIP output evoked by peripheral vagal stimulation mainly originated from peptidergic (VIP) vasodilator nerves in the submucous plexus (Ito *et al.*, 1988a), whereas that released by reflex vagal stimulation originated from peptidergic (VIP) neurones in the myenteric plexus (Ito *et al.*, 1988b). The aim of the present study was to investigate whether or not PHI is released with VIP from the gastric corpus of the dog by peripheral and central vagal stimulation, and to compare the effects of PHI and VIP on the gastric motility and blood flow in atropine-treated dogs.

Methods

Experimental procedure

Twenty-three adult dogs of either sex weighing between 8 and 13 kg were used after overnight fasting. Dogs were anaes-

thetized with chloralose (50 mg kg⁻¹, i.v.) and urethane (100 mg kg⁻¹, i.v.) after induction with pentobarbitone (30 mg kg⁻¹, i.v.). Operative and experimental procedures were as previously described in detail (Ito *et al.*, 1988a). In brief, a tracheal cannula was inserted and polyethylene cannulae were inserted into the cephalic vein and the right femoral artery for systemic administration of drugs and for measurement of systemic blood pressure through a pressure transducer (Gold Stantham Inc., P23 IC), respectively. After the spleen and greater omentum had been removed, a thin polyethylene cannula was inserted retrogradely into a branch of the splenic artery for close arterial injection of drugs into the stomach. A 0.1 ml bolus of drugs dissolved in sodium phosphate buffered saline (PBS; sodium phosphate 10 mM, NaCl 150 mM, pH 7.4) was injected and then flushed in with 0.15 ml PBS. After heparinization (1,000 u kg⁻¹, i.v.), the left gastroepiploic-splenic vein was cannulated for measurement of gastric blood flow by a photo-electric drop counter (Sanei, 1321) and for collection of gastric venous blood. Venous flow was expressed as drops min⁻¹ (55 µl per drop). The venous effluent was continuously returned to the external jugular vein by a peristaltic pump after warming at 37°C. To measure the arterial peptide concentration, arterial blood was collected from a branch of the left femoral artery. Lateral thoracotomy was performed by removing the 8th and 11th ribs and the animals were maintained with artificial ventilation. The peripheral and central cut ends of a communicating branch of the left vagus nerve to the dorsal vagus trunk in the thorax were stimulated electrically with bipolar silver ring electrodes. To record gastric motility, a balloon was introduced into the stomach via the mouth and was connected to a water reservoir mounted on a force displacement transducer. The intraballoon pressure was set at 5–10 cmH₂O. Rectal temperature was maintained at 37°C by a heating table. Experiments were performed 30–60 min after the preparatory surgery and 20–30 min after the intra-venous injection of atropine (1 mg kg⁻¹) or hexamethonium (15 mg kg⁻¹).

Measurement of plasma VIP and PHI

The concentrations of VIP and PHI in venous and arterial plasma were determined by radioimmunoassay. When the blood was collected, a corresponding amount of PBS was infused from the jugular vein to compensate for blood loss. In each animal, two or three tests were performed at about

30 min intervals and the sequence of the tests was randomized. The blood samples were collected for 1 min in ice-cold tubes containing aprotinin (Sigma, 800 u) and EDTA (Dojin, 12.5 μ mol) and haematocrit was measured. Blood samples were centrifuged at 4°C and the plasma was extracted with a double amount of absolute ethanol. Following centrifugation (10,000 g) for 10 min, the extract was dried under vacuum and kept frozen at -20°C until assayed. Each sample was reconstituted in 40 mM sodium phosphate buffer (pH 7.4) at the original volume. Over 90% of the immunoreactive peptide in plasma was extracted, so that data were not corrected for recovery.

VIP (Protein Inc., Osaka, 0.6 nmol) was iodinated in Na¹²⁵I (Amersham, 1 mCi) by the cholramine T method and the iodinated product was purified by ion exchange chromatography as described previously (Ito *et al.*, 1988a). PHI (Sigma, 3 nmol) was also iodinated in the same manner as VIP. The reaction products were applied to a CM Sephadex C25 column (1 $\times$ 10 cm) equilibrated with ammonium acetate (50 mM, pH 6.6) and then eluted by a linear gradient of equal volume (30 ml) of 50 mM and 500 mM ammonium acetate at a flow rate of 0.3 ml min⁻¹. The radioactive peak fraction was collected in a tube containing 500 u aprotinin. The iodinated peptides were then aliquoted and lyophilized.

Radioimmunoassay procedures were conducted by the method described previously (Ito *et al.*, 1986). The VIP antiserum (VK208) used recognized the middle and carboxyl terminal regions of a VIP molecule (Ito *et al.*, 1988a). The PHI antiserum (AE104, Cambridge Research Biochemicals) reacted with the C-terminal but not with the N-terminal. The VIP antiserum did not cross-react with PHI and vice versa. Standards contained 1.56 fmol synthetic porcine VIP or PHI at the lowest level and the concentration was doubled to 100 fmol at the highest level. The detection limit was 0.8 fmol 0.8 ml⁻¹ for VIP and 2.0 fmol 0.8 ml⁻¹ for PHI with 95% confidence. The respective inter- and intra-assay ($n = 5$) coefficients of variation were between 5% and 14% for VIP radioimmunoassay and 8% and 15% for PHI radioimmunoassay.

Tissue extraction and gel filtration

Tissue extraction was performed as described previously (Ito *et al.*, 1988c). In brief, dogs not used for nerve stimulation were killed by a rapid injection of pentobarbitone and then the gastric corpus was isolated and separated into mucosal and muscle layers. The isolated tissues were frozen with liquid nitrogen and weighed. Following 5 min of boiling in 0.5 M acetic acid, the sample was homogenized for 20 s with Physcotron (Nition, NS310E) and centrifuged for 3 min (10,000 g). The supernatant was aliquoted, lyophilized and stored at -20°C until assayed. The concentrations of immunoreactive peptide in the extracts were determined at 3 or 4 different dilutions. Venous plasma extracts were fractionated by chromatography on a Sephadex G50 superfine column (1 $\times$ 90 cm) eluted with 0.5 N acetic acid at a flow rate of 2.7 ml h⁻¹. The effluent collected for 20 min was divided into two and each elution position of immunoreactive VIP or PHI was determined.

Statistical analysis

The results are expressed as means $\pm$ s.e.mean. Regression analysis was carried out by the method of least squares. Statistical significance was assessed by Student's *t* test and *P* values less than 0.05 were considered to be statistically significant.

Results

PHI and VIP outputs evoked by peripheral vagal stimulation

The resting concentrations of gastric venous and arterial peptides were 57.0 ± 6.7 and 40.8 ± 6.0 pM ($n = 12$) for PHI and

Figure 1 The effect of peripheral vagal stimulation on vasoactive intestinal peptide (VIP) (a) and peptide histidine isoleucine (PHI) (b) concentration in the plasma of the gastric vein (●) and femoral artery (○). (Δ) Gastric venous concentration of peptides after treatment with hexamethonium (15 mg kg⁻¹, i.v.). Peripheral vagal stimulation ($n = 6$, 10 Hz, 40 V, 0.5 ms) for 2 min (solid bar). Each point represents the mean and vertical lines show s.e.mean. **P* < 0.05 when compared with the resting level.

29.2 ± 2.6 and 23.6 ± 2.8 pM ($n = 12$) for VIP, respectively, in atropine-treated dogs (1 mg kg⁻¹, i.v.). The mean resting VIP and PHI levels in gastric venous plasma were slightly higher than those in arterial plasma, although these values were not statistically different.

The vagus nerve was stimulated for 2 min (10 Hz, 40 V, 0.5 ms) and gastric venous blood was collected continuously before and after the start of stimulation. Peripheral vagal stimulation elicited an increase in gastric blood flow and evoked gastric relaxation. As shown in Figure 1, it evoked a prominent increase in the venous plasma PHI as well as in VIP without any significant changes in the arterial concentration of either peptide. The concentrations of PHI and VIP in venous plasma increased within the first 1 min period of stimulation, attained a maximum in the next 1 min and returned to basal within 7 min after the end of stimulation. There was a statistically significant difference between the peak and resting concentrations of each peptide. These responses were inhibited by pretreatment with hexamethonium (15 mg kg⁻¹, i.v.). There was no statistically significant difference between the amounts of VIP and PHI released from the stomach in response to peripheral vagal stimulation at 10 Hz (Table 1). A positive correlation was obtained between the outputs of both peptides (Figure 3a), and the mean molar ratio of net VIP to PHI output was 1.5. Stimulation at 40 Hz evoked a gastric relaxation of similar magnitude to stimulation at 10 Hz, but it produced a less pronounced gastric vasodilatation and a smaller increase in the outputs of VIP and PHI than stimulation at 10 Hz. The mean molar ratio of VIP to PHI output decreased to 0.65 with stimulation at 40 Hz (Table 1).

PHI and VIP outputs evoked by central vagal stimulation

Central vagal stimulation for 2 min (10 Hz, 40 V, 0.5 ms) evoked slight vasodilatation and prominent gastric relaxation.

Table 1 The outputs of vasoactive intestinal peptide (VIP) and peptide histidine isoleucine (PHI) in response to peripheral (PVS) and central (CVS) vagal stimulation and the concentrations of peptides in the gastric corpus of the dog

			Peptide output (fmol for 7 min)		
		n	VIP	PHI	VIP/PHI
PVS	10 Hz	6	679.8 ± 133.1	536.0 ± 139.3	1.47 ± 0.20
	40 Hz	3	161.7 ± 27.2	247.0 ± 24.9	0.65 ± 0.05
CVS	10 Hz	6	146.7 ± 56.6	183.0 ± 43.0	0.71 ± 0.61

			Peptide concentration (pmol g ⁻¹ wet weight)		
		n	VIP	PHI	VIP/PHI
Muscle layer		5	300.9 ± 47.7	255.6 ± 60.4	1.51 ± 0.44
Mucosal layer		5	326.8 ± 94.7	122.9 ± 15.8	2.70 ± 0.61

The total peptide output from the gastric corpus was calculated as the veno-arterial difference in plasma peptide concentration multiplied by the gastric plasma flow, which was determined from a knowledge of blood flow and haematocrit. To estimate the amounts of vagal stimulation-induced release of the peptides into the venous effluent from the stomach, resting peptide outputs for 7 min were subtracted from the total peptide outputs for 2 min during stimulation and for 5 min after stimulation. We calculated the mean molar ratios (VIP/PHI) after determining the molar ratio of each individual dog. *n* = number of experiments.

PHI was released into the gastric venous plasma together with VIP in response to reflex vagal stimulation without any significant changes in arterial peptide levels. The time course of PHI output was similar to that of VIP (Figure 2). The concentrations of both peptides in the plasma in response to central vagal stimulation were significantly less than those evoked by peripheral vagal stimulation. These responses were inhibited by hexamethonium. There was a statistically significant difference between the resting and peak venous concentration for VIP but not PHI. Table 1 shows the output of both peptides from the stomach. A positive correlation was obtained between them (Figure 3b) and the mean molar ratio of VIP to PHI output was 0.71 (Table 1).

Figure 2 The effect of central vagal stimulation (vago-vagal reflex) on vasoactive intestinal peptide (VIP) (a) and peptide histidine isoleucine (PHI) (b) concentration in the plasma of the gastric vein (●) and femoral artery (○). (Δ) Gastric venous concentration of peptides after treatment with hexamethonium (15 mg kg⁻¹, i.v.). Central vagal stimulation (*n* = 6, 10 Hz, 40 V, 0.5 ms) for 2 min (solid bar). Each point represents the mean and vertical bars show s.e.mean. **P* < 0.05 when compared with the resting level.

Metabolism of exogenous PHI in the gastric circulation

To examine the metabolism of PHI in the gastric circulation, porcine PHI at doses of 1 and 2 pmol was injected intra-arterially into the stomach. The amount of PHI in the venous plasma was measured continuously for 5 min after injection and resting PHI output for 5 min was subtracted from this. The amount of PHI recovered from the venous plasma was

Figure 3 Correlation between peptide histidine isoleucine (PHI) and vasoactive intestinal peptide (VIP) released into the gastric venous plasma for a 7 min period in response to peripheral (a) and central (b) vagal stimulation at 10 Hz for 2 min. The regression line indicated in each figure was computer fitted (a, $Y = 0.75X + 28.0$, $r = 0.71$, $P < 0.05$; b, $Y = 0.67X + 85.8$, $r = 0.86$, $P < 0.05$)

Figure 4 The profile of gel chromatography of vasoactive intestinal peptide (VIP) (●) and peptide histidine isoleucine (PHI) (○) in plasma extract from gastric venous effluent collected during peripheral vagal stimulation. The elution positions for porcine VIP and PHI are indicated by arrows. V_0 ; void volume, V_t ; total mobile phase.

435.0 ± 68.4 fmol after an injection of 1 pmol ($n = 6$) and 977.5 ± 101.2 fmol after 2 pmol ($n = 4$), indicating that the mean recovery rate of exogenous PHI was $45.1 \pm 4.7\%$. This rate was significantly higher than that of porcine VIP ($28.5 \pm 0.02\%$; $P < 0.05$) as determined previously with the same techniques (Ito *et al.*, 1988a).

Gel filtration and tissue concentration

Figure 4 shows the gel filtration profile of venous plasma extract collected during peripheral vagal stimulation. PHI and VIP were eluted in the same position as the respective authentic porcine peptides and no extra peaks appeared. The plasma extract obtained during central vagal stimulation showed a similar result (not shown).

The concentrations of VIP and PHI in the muscle and mucosal layers of the gastric corpus were measured in dogs which were not used for nerve stimulation (Table 1). The concentration of VIP in the stomach was somewhat higher than that of PHI. The mean molar ratios of VIP to PHI concentration were 1.5 in the muscle layer and 2.7 in the mucosal layer. Dilution curves for plasma and gastric extracts were parallel to standard curves for porcine VIP and PHI.

The effects of VIP and PHI on gastric motility and blood flow

Representative gastric and vascular responses to PHI and VIP are shown in Figure 5. Close intra-arterial bolus injection of PHI or VIP caused a dose-dependent gastric relaxation and an increase in gastric blood flow without any significant effects on arterial blood pressure. Although the gastric relaxation induced by these peptides had a very slow onset and was long lasting, gastric vasodilatation developed quickly. The peak vasodilator responses to peptides increased with increasing doses, but a further increase in dose prolonged the duration of the responses without increasing the peak values. The dose-response curves for the effect of PHI and VIP on gastric relaxation and vasodilatation are shown in Figure 6. The gastric relaxation induced by both peptides attained a maximum, but PHI-induced vasodilatation did not reach a maximum at 1 nmol. The threshold doses required to elicit gastric relaxation and vasodilatation were 50 and 100 pmol

for PHI and 10 and 2 pmol for VIP, respectively. Simultaneous injection of PHI (20–500 pmol) and VIP (10–50 pmol) produced gastric relaxation and vasodilatation which equalled the sum of the responses to the two peptides injected separately.

Discussion

The present experiments indicate that PHI is released with VIP by peripheral and central vagal stimulation in the dog

Figure 5 Effects on mean arterial blood pressure (MABP), gastric blood flow and relaxant responses to intra-arterial injection (●) of vasoactive intestinal peptide (VIP) (a) and peptide histidine isoleucine (PHI) (b) at various doses. The data were obtained from the same animal. Numbers above the dots show the doses (pmol) of peptides.

Figure 6 Dose-response curves showing gastric vasodilator (●) and relaxant (○) responses to intra-arterial injection of vasoactive intestinal peptide (VIP) and peptide histidine isoleucine (PHI). The increment of the vasodilator response above the resting level (total change in blood flow) and the peak value of the relaxant response are expressed as a percentage of those responses to isoprenaline (Iso, 0.1 nmol) and noradrenaline (NA, 5 nmol), respectively ($n = 5$). Continuous and dashed lines respectively indicate the effects of VIP and PHI. Abscissa scale, doses of VIP and PHI (pmol) on a log scale. Each point represents the mean and vertical bars show s.e.mean.

stomach. The VIP concentration in the dog gastric corpus is higher than that of PHI as is the case in many tissues (Bishop *et al.*, 1984; Christofides *et al.*, 1984; Lundberg *et al.*, 1984a; Fahrenkrug *et al.*, 1985; Bardrum *et al.*, 1986). These differences may be attributable to differences in the processing scheme of the VIP precursor.

Dog VIP has the same amino acid sequence as porcine VIP (Eng *et al.*, 1986). Although the primary structure of dog PHI has not yet been elucidated, dog PHI is considered to have a similar amino acid composition to porcine PHI (Yiangou *et al.*, 1983). However, a large form of dog PHI has been found by using N-terminal region specific PHI antiserum (Yasui *et al.*, 1987). In our experiments, C-terminal specific antiserum was used so that gel chromatography of plasma extracts from the dog stomach showed a single peak of immunoreactive PHI in the same position as porcine PHI.

Although co-release of PHI and VIP from the stomach occurred with both methods of stimulation, a molar ratio of VIP to PHI output was different (VIP/PHI; peripheral vagal stimulation, 1.5; central vagal stimulation, 0.7). It is unlikely that the ratio of VIP/PHI evoked by peripheral vagal stimulation reflects a physiological abnormality of the stimulus because vagal stimulation elicits frequency-dependent gastric

relaxation and vasodilatation up to 10 Hz. Central vagal stimulation at 10 Hz elicits gastric relaxation of similar magnitude to peripheral vagal stimulation at 10 Hz, but is less effective in producing gastric vasodilatation. We previously suggested that VIP output evoked by peripheral vagal stimulation mainly originates from peptidergic (VIP) vasodilator neurones (Ito *et al.*, 1988a) and that evoked by reflex vagal stimulation from peptidergic (VIP) myenteric neurones (Ito *et al.*, 1988b). Therefore, the difference in the ratio of VIP/PHI could be due to the difference in their origins. The mucosal and submucosal layers of the guinea-pig are rich in peptidergic (VIP) neurones innervating the blood vessels (Costa & Furness, 1983) and circular smooth muscles are exclusively innervated by peptidergic (VIP) fibres coming from the myenteric plexus but not from the submucous plexus (Furness *et al.*, 1988). It is, therefore, suggested that the molar ratio of peptide concentration (VIP/PHI) is higher in the peptidergic (VIP) vasodilator neurones (2.7) in mucosal layers than peptidergic (VIP) myenteric neurones (1.5) in muscle layers. If this is the case, the molar ratio in the tissue is somewhat higher than that appearing in the plasma. However, the ratio decreases if both peptides are released into the plasma, because VIP is metabolized more easily than PHI in the gastric circulation.

Peripheral vagal stimulation at 40 Hz caused less pronounced vasodilatation and VIP output than stimulation at 10 Hz, although both frequencies elicited a similar magnitude of gastric relaxation, as in our previous experiments (Ito *et al.*, 1988a). We have proposed that the gastric peptidergic (VIP) vasodilator neurones are less effective during stimulation at such a high frequency, but peptidergic (VIP) myenteric neurones are not influenced by stimulation frequency (Ito *et al.*, 1988a,b). This contention is supported by the fact that the ratio of peptides appearing in the plasma in response to peripheral vagal stimulation at 40 Hz is almost the same as that in response to central vagal stimulation.

PHI was a much less potent gastric vasodilator than VIP. However, in regard to gastric relaxation, PHI was only slightly less effective than VIP. PHI has been shown to be a less potent vasodilator in submandibular gland (Lundberg & Tatemoto, 1982) and brain (Suzuki *et al.*, 1984). These results therefore suggest that PHI released by vagal nerve activation is able to affect gastric motility but not vascular tone. Simultaneous injections of both peptides cause only additive effects, suggesting that PHI does not modify the actions of VIP.

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