Br. J. Pharmacol. (1994), **113**, 541–549

Peptide histidine isoleucine-like immunoreactivity release from the rat gastric fundus

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1 Longitudinal muscle strips from the rat gastric fundus were subjected to *in vitro* electrical field stimulation (EFS) under non-adrenergic non-cholinergic (NANC) conditions to study the release of peptide histidine isoleucine-like immunoreactivity (PHI-LI) and the correlation between PHI-LI release and NANC relaxation.

2 Different radioimmunoassay (RIA) systems employing C-terminal- and N-terminal-specific anti-PHI sera were used to determine the relative contributions of PHI and its C-terminally extended forms, peptide histidine glycine (PHI-Gly) and peptide histidine valine [PHV(1–42)], to the PHI-LI released by the rat gastric fundus.

3 In the presence of atropine (1 μM) and guanethidine (5 μM), EFS (120 mA, 1 ms, 0.25–32.0 Hz, trains of 2 min) induced frequency-dependent relaxations of 5-hydroxytryptamine (3 μM) pre-contracted strips.

4 EFS at frequencies of 8–32 Hz evoked significant increases in PHI-LI outflow. The increases in PHI-LI outflow evoked by 16-Hz EFS were abolished by tetrodotoxin (3 μM) and by a calcium-free medium, indicating an active release process from intramural nerves.

5 The EFS-induced release of PHI-LI measured with the N-terminal-specific antiserum was significantly greater than that detected with the C-terminal-specific antisera.

6 Sephadex G-25 gel permeation chromatographic analysis was performed on the PHI-LI released in response to 32-Hz EFS. A C-terminal-specific antiserum revealed one peak co-eluting with the rat PHI standard. When PHI-LI was measured with the N-terminal-specific antiserum, two peaks were found that co-eluted with the rat PHV(1–42) and rat PHI-Gly/PHI standards, respectively.

7 The present data suggest that the extended forms of PHI are the primary components of the PHI-LI released by NANC inhibitory neurones in the rat gastric fundus and support a NANC inhibitory neurotransmitter role for PHI and its extended forms in this tissue.

Keywords: Peptide histidine isoleucine (PHI); peptide histidine glycine (PHI-Gly); peptide histidine valine [PHV(1–42)]; rat gastric fundus; non-adrenergic non-cholinergic (NANC); relaxation

Introduction

Peptide histidine isoleucine (PHI) is a 27-amino-acid peptide (P) with an N-terminal histidine (H) and a C-terminal isoleucine (I). It was originally isolated from porcine small intestine and shows remarkable sequence homology with vasoactive intestinal polypeptide (VIP), sharing 13 amino-acid residues in identical positions (Tatemoto & Mutt, 1981). PHI and VIP are co-synthesized from the same precursor molecule (Itoh *et al.*, 1983) and co-localize in the neurones of various tissues (Yanaihara *et al.*, 1983; Bishop *et al.*, 1984; Christofides *et al.*, 1984; Lundberg *et al.*, 1984a; Fahrenkrug *et al.*, 1985), including the rat gastric fundus (Ekblad *et al.*, 1985). The two peptides have also been shown to produce similar biological effects, such as stimulation of pancreatic (Dimaline & Dockray, 1980; Holst *et al.*, 1987), intestinal (Anagnostides *et al.*, 1983) and hormonal secretions (Samson *et al.*, 1983; Szecówka *et al.*, 1983; Grunditz *et al.*, 1986), vasodilatation (Lundberg & Tatemoto, 1982; Lundberg *et al.*, 1984a; Suzuki *et al.*, 1984; Bardrum *et al.*, 1986) and relaxation of genitourinary (Bardrum *et al.*, 1986; Blank *et al.*, 1986), bronchial (Lundberg *et al.*, 1984a; Ellis & Farmer, 1989) and gastrointestinal (Biancani *et al.*, 1989; Nurko *et al.*, 1989; D'Amato *et al.*, 1990) smooth muscles. They are also co-released in response to parasympathetic nerve stimulation (Lundberg *et al.*, 1984b; Holst *et al.*, 1987; Yasui *et al.*, 1987; Ohta *et al.*, 1990). These findings strongly support the hypothesis that VIP and PHI act as co-transmitters.

Non-adrenergic non-cholinergic (NANC) inhibitory innervation is responsible for receptor relaxation of the gastric fundus during food intake (Abrahamsson, 1986). Under *in vitro* NANC conditions, electrical field stimulation (EFS) of rat gastric fundus strips causes relaxation (Heazell, 1977), which can be reduced by peptidases (De Beurme & Lefebvre, 1987; Kamata *et al.*, 1988; D'Amato *et al.*, 1992a; Boeckxstaens *et al.*, 1992), anti-VIP and anti-PHI sera (Kamata *et al.*, 1988; De Beurme & Lefebvre, 1988; D'Amato *et al.*, 1990; Li & Rand, 1990) and nitric oxide (NO)-synthase inhibitors (Li & Rand, 1990; Boeckxstaens *et al.*, 1991; 1992; D'Amato *et al.*, 1992a). Nitric oxide (Boeckxstaens *et al.*, 1991), VIP and PHI (D'Amato *et al.*, 1990; Lefebvre *et al.*, 1991) all cause relaxation of the smooth muscle of the rat gastric fundus. Moreover, EFS of rat gastric fundus strips has been shown to elicit the release of VIP (D'Amato *et al.*, 1992b) and of a vasorelaxant substance(s) that is biologically similar to NO (Boeckxstaens *et al.*, 1991). Therefore, NO (or an NO-releasing substance), VIP and PHI are considered to be putative neurotransmitters released by NANC neurones in this tissue.

The aim of this study was to determine whether PHI release could be induced by EFS in the rat gastric fundus. If so, PHI would fulfil both the 'identity of action' and 'detectable release' criteria for a neurotransmitter. Two C-terminal-extended forms of PHI, peptide histidine glycine (PHI-Gly) and peptide histidine valine [PHV(1–42)], co-localize with PHI in a number of rat tissues, including the stomach, where they account for 65% of the detectable PHI-like immunoreactivity (PHI-LI) (Cauvin *et al.*, 1989b) and produce biological effects identical to those of PHI (Lefebvre *et al.*, 1991). We, therefore, used radioimmunoassay (RIA) systems employing C-terminal- and N-terminal-specific antisera to

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determine whether PHI or its extended forms are preferentially released by the rat gastric fundus.

Methods

General

Male and female Wistar rats weighing 150–250 g were fasted overnight with free access to water. The animals were killed by decapitation and exsanguinated. A mid-line abdominal incision was made, and the gastric fundus was removed and immediately placed in Krebs solution at room temperature. One or two longitudinal muscle strips (3 × 20 mm) were prepared from each fundus, according to the method of Vane (1957). The strips were immediately transferred to organ baths or glass tubes (see below) containing Krebs solution, which was maintained at a constant temperature of 37°C and bubbled with a 95% O_2:5% CO_2 mixture. Under these conditions, the pH of the solution was 7.4. Different strips were used for functional and release experiments (see below). In both cases, the composition of the Krebs solution was as follows (mM): NaCl 118.5, KCl 4.8, $CaCl_2$ 1.9, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 10.1

Functional experiments

The strips were suspended between two parallel platinum electrodes (20 mm long, 4 mm wide, 5 mm apart) inside 5 ml organ baths and connected to auxotonic transducers (Harvard smooth muscle) under a 1 g load. Smooth-muscle activity, magnified 5–10 times, was recorded with Rikadenki R-01 single-pen recorders. For these experiments, the bath solution also contained atropine 1 μM and guanethidine 5 μM. Strip tone was induced with 5-hydroxytryptamine (5-HT) 3 μM to enable recording of NANC relaxations. After a 60-min equilibration period, during which the bath solution was changed every 10 min, the strips were stimulated with rectangular, bipolar electrical pulses of constant duration (1 ms) and amplitude (120 mA) delivered with a Palmer Bioscience 6012 Stimulator linked in series with a Basile Biological Research Apparatus constant-current unit. The pulse train duration was 2 min; frequencies ranged from 0.25–32.0 Hz. After equilibration, the bath solution was changed every 5 min between trains of EFS.

Release experiments

Each strip was mounted under a 1 g load between two parallel platinum electrodes (20 mm long, 3.5 mm wide, 3 mm apart) inside a 4 ml silanized glass tube containing 2 ml of the Krebs solution plus atropine 1 μM, guanethidine 5 μM, 5-HT 3 μM, bovine serum albumin (BSA) 50 mg l^{-1}, bacitracin 30 mg l^{-1} and aprotinin 100,000 kallikrein inhibitor units (KIU) l^{-1}. Additional experiments were performed to determine whether 5-HT had any effect on the evoked release of PHI-LI (see Experimental protocols below). After a 60-min equilibration period (during which the strip was transferred, every 10 min, to a different tube containing fresh solution), the strip was subjected to 2 min incubations (in different tubes) before, during and after the EFS described above. After each incubation, the solution was collected on ice and stored at − 20°C. At the end of each experiment, the strip was blotted and weighed. The samples were extracted with ethanol 2:1 (v/v), centrifuged at 2,000 *g* for 30 min at 4°C, vacuum-dried in a Speedvac Evaporator linked with a Savant refrigerated condensation trap and kept at − 20°C until assayed. Recovery of known amounts of PHI, added to the solution and extracted and dried in the same manner as the samples, was almost 100%.

PHI measurement

PHI-LI was measured by RIA using three different anti-PHI sera. Serum R8403 (N-terminal-specific) and R8801 (C-terminal-specific), raised against synthetic porcine PHI (14–27) and PHI(1–15), respectively, were kindly supplied by Prof. Dr N. Yanaihara. The third antiserum, R01, was obtained from New Zealand white rabbits immunized with synthetic porcine PHI (pPHI) which had been coupled to human serum albumin (HSA) with carbodiimide. Lyophilized pPHI (0.5 mg) was added to HSA (1.15 mg in 0.575 ml) and 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (5 mg in 0.5 ml), both dissolved in 0.25 M phosphate buffer, pH 7.4. The reaction mixture was stirred overnight at 4°C, and the conjugate was then dialysed against distilled water for 24 h at 4°C. The final yield of the coupling reaction, assessed by adding a trace amount of [^{125}I]-pPHI to the reaction mixture, averaged 40%. The conjugate was emulsified with a double volume of complete Freund's adjuvant and injected subcutaneously at multiple sites on the backs of two 6-week-old New Zealand rabbits at 3-week intervals. Each rabbit received 30 nmol of pPHI in each of the three initial injections and half this amount in each booster immunization. The rabbits were bled 15 days after the third injection and after each booster injection. The cross-reactivity of the antiserum with several other peptides and pPHI analogues was tested by adding up to 1 μg/tube corticotrophin-releasing factor (CRF), gastric inhibitory peptide (GIP), glucagon, growth hormone-releasing factor (GRF), helodermin, peptide histidine methionine (PHM), pPHI(14–27), rat PHI (rPHI), rat PHI-Gly (rPHI-Gly), rat PHV(1–42) [rPHV (1–42)], sauvagine, secretin, urotensin I and VIP.

Production and characterization of anti-pPHI sera R8403 and R8801 have been described previously (Yanaihara *et al.*, 1984; Yasui *et al.*, 1987). We tested their cross-reactivities with rPHI-Gly and rPHV(1–42). R8403 showed full immunological recognition of both C-terminal-extended forms of PHI, while R8801 cross-reactivity with these peptides was lower than 0.01%.

Iodinated pPHI was prepared using the chloramine T method and purified on a Sep Pak C18 cartridge, according to a slightly modified version of the method described by Fahrenkrug (1984). The column was eluted with a step-wise gradient of methanol from 0 to 80%. For each step, three 1-ml fractions were collected. Ten microlitres of each fraction were counted in a γ-counter and the radioactive peak fractions were pooled and used as labelled antigen in the RIA systems.

The RIA was performed in a buffer containing 10 mM sodium phosphate, NaCl 154 mM, ethylene diamine tetraacetic acid (EDTA) 25 mM, thimerosal 0.01%, BSA 0.5% and Tween 20 0.03%, pH 7.2. The diluent for sera contained aprotinin 500 KIU ml^{-1}. The dried samples were reconstituted with buffer and concentrated five fold in the RIA systems employing R8403 and R8801 sera and 10 fold in the assay performed with our serum R01. Krebs solution, extracted and reconstituted in the same manner, was used to assess non-specific binding and for calculation of standard curves. The pH of the concentrated Krebs solution was 7.9, while the final pH of the incubation medium in the RIA system at 4°C was 7.2. The anti-pPHI sera were used at final dilutions of 1:320,000 (R8403), 1:800,000 (R8801) and 1:80,000 (R01) to obtain approximately 40% binding of the iodinated peptide. The RIAs were performed as follows: an initial incubation was carried out at 4°C in disposable plastic tubes containing 0.1 ml of anti-pPHI serum, 0.1 ml of sample or standard solution and 0.1 ml of buffer. After 24 h, 0.1 ml of [^{125}I]-pPHI (5,000 c.p.m./tube) was added and the incubation was continued at 4°C for 48 h. Separation of free from bound pPHI was achieved by adding 0.1 ml of anti-rabbit goat serum, diluted 1:20, and 0.5 ml of a 6.6% polyethylene glycol solution. After a 2 h incubation at 4°C, the tubes were centrifuged at 3,000 *g* for 1 h at 4°C, the supernatant was

discarded and the pellet was counted in a γ-counter. The standard curves ranged from 1.95 to 1,000 pg/tube of rPHI. Each sample was assayed in duplicate; PHI-LI measurements with sera R8403 and R8801 were performed in the same sample.

Since the PHI-LI levels were not influenced by the wet weight of the tissues within the range 112–167 mg (137.7 ± 4.6 mg), the results were expressed as pg per fraction. The total evoked PHI-LI release was calculated by subtracting the basal values from each value observed during and after the EFS and summing the differences. For each experiment, these sums are reported as absolute values (pg).

Experimental protocols

EFS frequency-response curves Strip relaxation and PHI-LI release induced by isolated EFS at increasing frequencies (0.25–32.0 Hz) were studied. In functional experiments, strips were allowed to recover basal tone prior to subsequent EFS. In the release experiments, the interval between stimulations was increased progressively from 10 to 60 min as higher frequencies were used. All relaxations were expressed as both amplitudes and areas under the curves (estimated using the trapezoidal rule for planar area). The results are shown as percentages of the maximal response (to 32 Hz) obtained in each strip.

Effect of 5-HT on PHI-LI release Two strips from each fundus were subjected to EFS at 1, 4 and 16 Hz, the first in the normal incubation medium, the second in the same solution without 5-HT, 3 μM. PHI-LI was measured in the incubation medium before, during and after each EFS with antisera R8403 and R8801.

Effects of tetrodotoxin and calcium-free Krebs solution Relaxations and PHI-LI release elicited by EFS, 16-Hz, were measured before and after 15 min incubation with tetrodotoxin (TTX) 3 μM. Sixty minutes elapsed between the pre- and post-TTX stimulations. In the functional experiments, the post-TTX relaxation was expressed as a percentage of the first.

The influence of calcium removal from the incubation medium was tested only on EFS-induced PHI-LI release, since this experimental condition abolished smooth muscle tone and made it impossible to evaluate the mechanical responses. PHI-LI release was measured in response to EFS (16 Hz) delivered before and after 30 min incubation in calcium-free Krebs solution containing an equimolar concentration of Mg^{2+}. Sixty minutes elapsed between the two determinations.

Effect of thiorphan on PHI-LI release Two strips from each fundus were subjected to EFS at 32 Hz, the first in the normal incubation medium, the second in the same medium with 1 μM thiorphan, a neutral endopeptidase inhibitor. PHI-LI was measured in the medium before, during and after EFS with antisera R8403 and R8801.

Gel permeation chromatography The chromatographic study of the PHI-LI released in response to EFS was performed on a 1 × 100 cm column containing Sephadex G-25 fine gel. The column was equilibrated and eluted with the buffer used for the RIA at a flow rate of 5 ml h^{-1}. The PHI-LI used in this analysis was obtained by stimulating four strips at 32 Hz and pooling the 2-ml fractions collected during the EFS and the 4-min period that followed it. The resulting sample (24 ml) was concentrated on two Sep Pak C18 cartridges, each equilibrated with 10 ml of a 10 mM HCl-10% CH_3CN solution and eluted with 4 ml of a 50% CH_3CN in 10 mM HCl solution. The eluates were vacuum-dried, reconstituted with buffer in a total volume of 1 ml and loaded onto the top of the column. A small amount of dextran blue was added to calculate the void volume (Vo). PHI-LI was measured in 0.5 ml collection fractions with antisera R8403 and R8801.

Drugs used

Atropine sulphate, BSA, chloramine T, guanethidine sulphate, human CRF, human GRF, HSA, 5-HT creatinine sulphate, polyethylene glycol, porcine GIP, porcine glucagon, pPHI, porcine secretin, sauvagine, tetrodotoxin, thimerosal, DL-thiorphan, urotensin I (Sigma, St Louis, MO, U.S.A.); aprotinin (Lepetit, Milan, Italy); bacitracin (Lusofarmaco, Milan, Italy); complete Freund's adjuvant (Difco Laboratories, Detroit, MI, U.S.A.); 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (Calbiochem, La Jolla, CA, U.S.A.); EDTA (Carlo Erba, Milano, Italy); helodermin, pPHI(14–27) (Novabiochem, Laufelfingen, Switzerland); Na ^{125}I (Amersham, Buckinghamshire, U.K.); PHM, rPHI, VIP (Bachem, Bubendorf, Switzerland); Sephadex G-25 fine gel (Pharmacia, Uppsala, Sweden). rPHI-Gly and rPHV(1–42) were kindly supplied by Prof. Dr P. Robberecht.

Statistical analysis

The results were evaluated by means of Dunnett's test for multiple comparisons with a control. All values are reported as mean ± s.e.mean. $P<0.05$ was considered statistically significant.

Results

Antiserum characterization and RIA system performance

The anti-pPHI serum produced in our laboratory (R01) proved highly specific and capable of recognizing the C-terminus of PHI. Its cross-reactivity was <0.01% with rPHV(1–42), CRF, GIP, glucagon, GRF, helodermin, sauvagine, urotensin

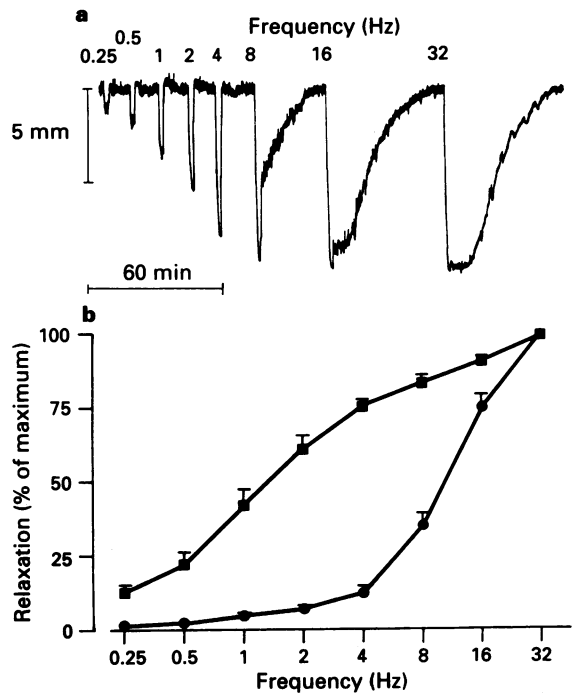

Figure 1 Relaxation of longitudinal muscle strips from the rat gastric fundus induced by electrical field stimulation (0.25–32.0 Hz, 120 mA, 1 ms, pulse trains of 2 min). (a) Representative tracing from one muscle strip. (b) Mean results ± s.e.mean based on responses of 12 strips. Relaxations were measured as peak amplitude (■) and as area under the curve (●) and are expressed as percentages of the maximal relaxation induced with 32-Hz EFS in each strip.

I and VIP; 0.2% with secretin; 0.94% with rPHI-Gly and PHM, and 100% with pPHI(14–27) and rPHI.

The IC_{50} of the standard curve from the RIA system employing serum R01 averaged 85.3 ± 1.9 pg/tube, and non-specific binding of the labelled ligand was less than 3%. The intra-assay ($n = 4$) and inter-assay ($n = 5$) coefficients of variation were ± 2.4% and ± 2.3% at the lowest level of standard (1.95 pg/tube) and ± 6.4% and ± 12.0% at the highest level of standard (1,000 pg/tube), respectively. The lowest concentration that could be measured with 95% confidence (i.e., 2 s.d. at zero) was 1.95 pg/tube. The detection limit was thus 1.95 pg ml^{-1} of the original sample, since each dried 1 ml sample was reconstituted in 0.1 ml in the RIA system.

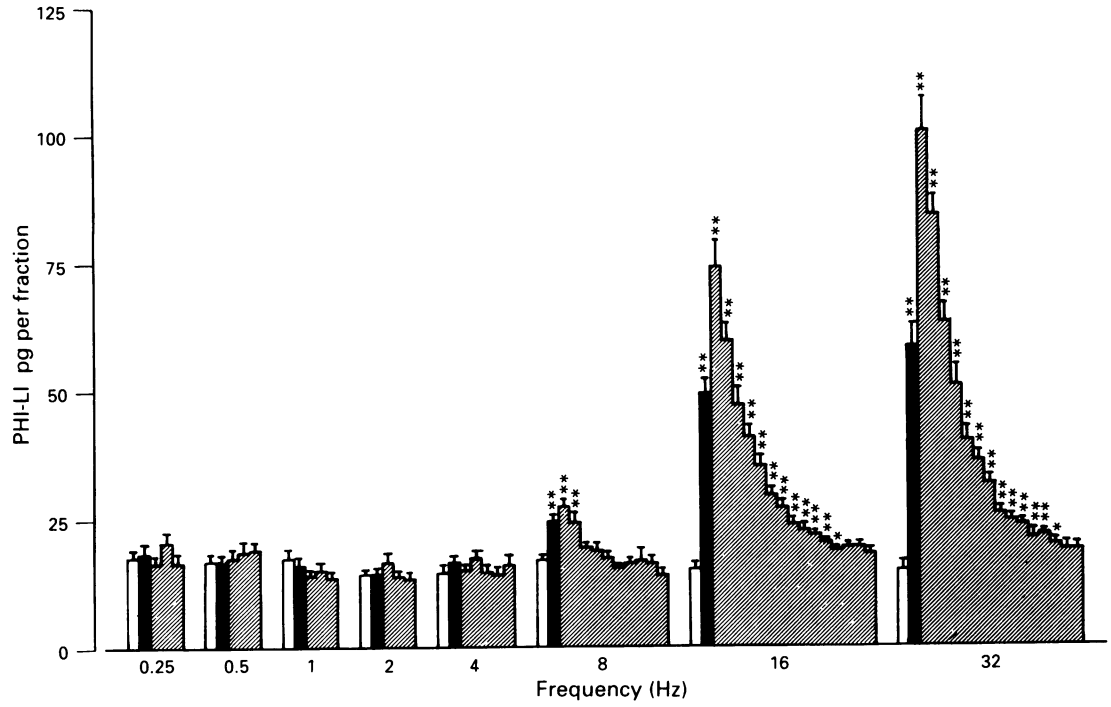

Figure 2 Peptide histidine isoleucine-like immunoreactivity (PHI-LI) concentrations measured with anti-pPHI serum R8403 in the incubation medium of longitudinal muscle strips of the rat gastric fundus before (open columns), during (solid columns) and after (hatched columns) electrical field stimulation (0.25–32.0 Hz, 120 mA, 1 ms, pulse trains of 2 min). Each column represents mean PHI-LI with s.e.mean found in 2-min collection fractions from 13 strips. Values significantly different from baseline levels: $*P<0.05$; $**P<0.01$.

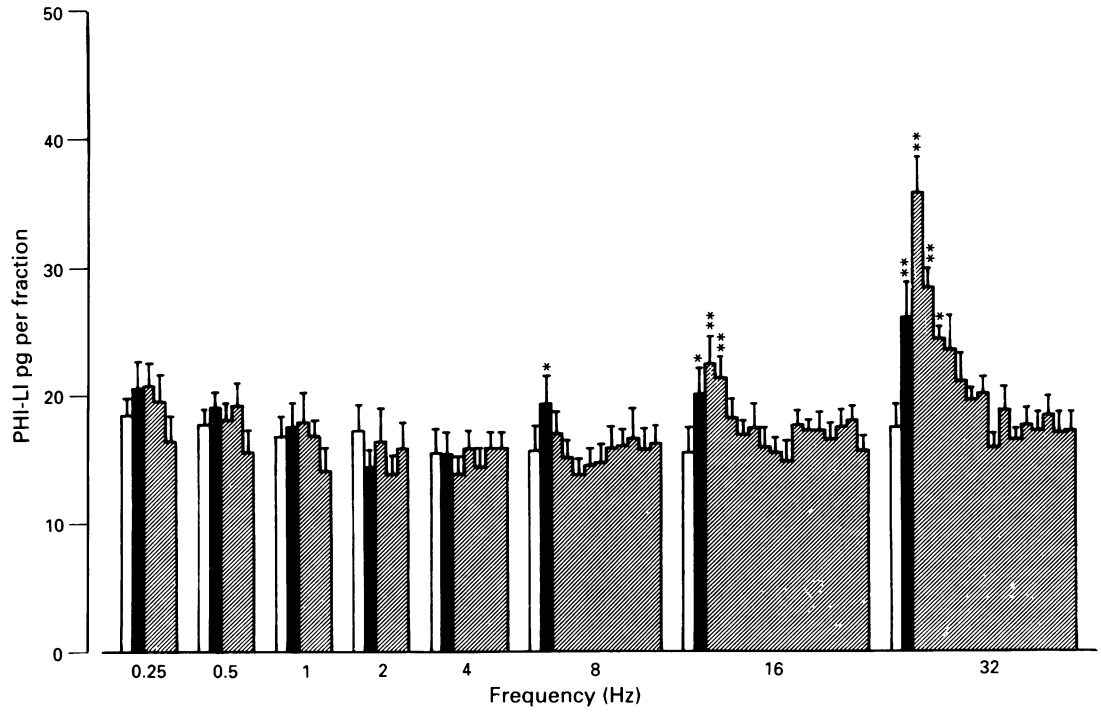

Figure 3 Peptide histidine isoleucine-like immunoreactivity (PHI-LI) concentrations measured with anti-pPHI serum R8801 in the incubation medium of longitudinal muscle strips of the rat gastric fundus before (open columns), during (solid columns) and after (hatched columns) electrical field stimulation (0.25–32.0 Hz, 120 mA, 1 ms, pulse trains of 2 min). Each column represents mean PHI-LI (s.e.mean shown by vertical lines) found in 2 min collection fractions from 13 strips. Values significantly different from baseline levels: $*P<0.05$; $**P<0.01$.

EFS frequency-response curves

Figure 1 shows an actual tracing, as well as the mean results, from the functional experiments. Within the range of 0.25–32.0 Hz, EFS elicited frequency-dependent NANC relaxations characterized by two distinct phases, which were well evident when higher frequencies were used. The initial relaxation, which began immediately after the initiation of EFS, developed rapidly to a near-maximal level. The second phase was characterized by a slight additional decrease in tone, which occurred much more gradually. At frequencies of 2 Hz or less, recovery of basal tone was almost immediate; the recovery period increased progressively thereafter to a maximum of about 1 h after 32-Hz EFS. Consequently, the frequency-response curves calculated on the basis of the amplitude are different from those based on the area under the curve (Figure 1).

EFS at frequencies of 8–32 Hz evoked statistically significant, frequency-dependent increases of PHI-LI outflow into the incubation medium (Figures 2, 3, 4). Although the basal (pre-EFS) levels of PHI-LI outflow measured with the three antisera were essentially the same, the increases detected with the N-terminal-specific serum R8403 were significantly greater than those measured with antiserum R8801 or R01, both of which are C-terminal-specific. In addition, in assays based on R8403, PHI-LI levels increased during the 2-min EFS, but peak release was consistently observed during the initial 2-min post-EFS period. This level then gradually decreased, reaching essentially basal values 6 min after EFS at 8 Hz, 26 min after 16-Hz EFS and 28 min after 32-Hz EFS (Figure 2). PHI-LI measured with either R8801 or R01 also peaked shortly after stimulation (except with 8-Hz EFS), but the recovery to basal levels was achieved much more rapidly after cessation of EFS (2, 6 and 8 min, respectively, after EFS at 8, 16 and 32 Hz) (Figures 3, 4).

Table 1 shows the mean total EFS-evoked PHI-LI release measured with the three antisera. The mean release observed with R8801 was not significantly different from that measured with serum R01, while both were significantly lower than that measured with R8403. The mean total release values observed in the R8801 assays following EFS at 8, 16 and 32 Hz were 15.5 ± 4.3%, 8.4 ± 1.2% and 12.3 ± 0.8% ($n = 13$), respectively, of those measured in the R8403 assays. An identical pattern was displayed by serum R01. The relationship between the total evoked PHI-LI release found with R8403 and strip relaxation is shown in Figure 5.

Effect of 5-HT on PHI-LI release

In the absence of 5-HT, the pattern of PHI-LI release produced by EFS was similar to that described above. There was no release in response to 1- or 4-Hz EFS, and the total

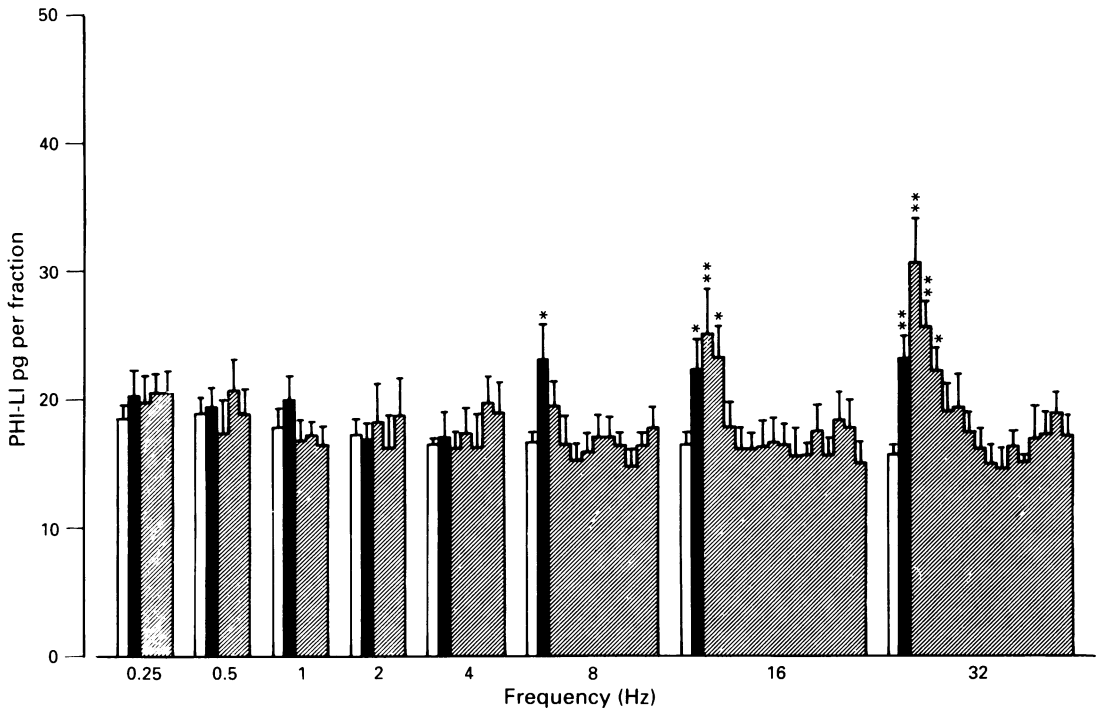

Figure 4 Peptide histidine isoleucine-like immunoreactivity (PHI-LI) concentrations measured with anti-pPHI serum R01 in the incubation medium of longitudinal muscle strips of the rat gastric fundus before (open columns), during (solid columns) and after (hatched columns) electrical field stimulation (0.25–32.0 Hz, 120 mA, 1 ms, pulse trains of 2 min). Each column represents mean PHI-LI with s.e.mean found in 2-min collection fractions from seven strips. Values significantly different from baseline levels: $*P<0.05$; $**P<0.01$.

Table 1 Total PHI-LI release from the rat gastric fundus detected by three different anti-pPHI sera in response to EFS

EFS frequency (Hz)	*R8403*	*R8801*	*R01*
		Total PHI-LI released (pg)	
8	34.8 ± 5.4	4.0 ± 0.8*	4.3 ± 0.9*
16	289.2 ± 19.5	24.3 ± 4.4*	25.0 ± 6.4*
32	430.3 ± 9.0	52.6 ± 3.1*	42.6 ± 6.2*

Numbers are means ± s.e.mean of 13 experiments for antisera R8403 and R8801 and seven experiments for antiserum R01; $*P<0.001$ vs R8403.

release evoked by 16-Hz EFS was no different from that induced in the control strips exposed to 5-HT: 289.6 ± 32.6 vs 291.4 ± 32.4 pg ($n = 4$) as measured by R8403 and 32.8 ± 8.9 vs 32.4 ± 8.1 pg ($n = 4$) with R8801, in the absence and presence of 5-HT, respectively.

Effects of TTX and calcium-free Krebs solution

In this series of experiments, responses were evaluated to a single frequency (16 Hz) EFS and PHI-LI release was measured only with antisera R8403 and R8801. The addition of 3 μM TTX had no effect on basal strip tone, but completely abolished the relaxant response to 16-Hz EFS ($n = 8$). Neither TTX nor the calcium-free Krebs solution influenced basal outflow of PHI-LI, but both abolished the response to EFS (Figure 6).

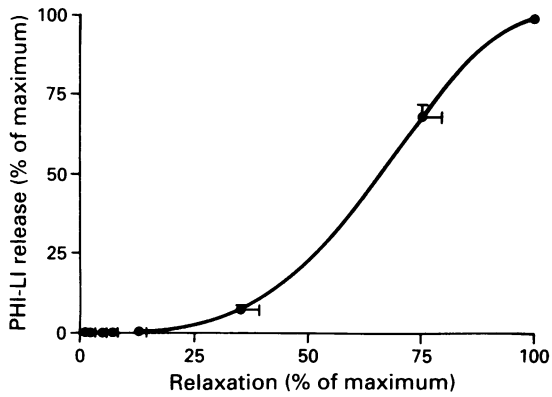

Figure 5 Relationship between relaxation (expressed as area under the curve) and total evoked peptide histidine isoleucine-like immunoreactivity (PHI-LI) release (calculated by subtracting the basal value from the value observed in each 2-min collection fraction during and after the stimulation and adding the differences) measured with anti-pPHI serum R8403. Data are expressed as percentage of maximal values and given as means ± s.e.mean from 12 strips for the relaxation and 13 strips for the release.

Effect of thiorphan on PHI-LI release

Thiorphan 1 μM had no effect on the EFS-induced PHI-LI release measured with antisera R8403 or R8801.

The total release evoked with 32-Hz EFS in control strips and in those incubated with thiorphan were 461.4 ± 22.5 and 454.9 ± 27.7 pg, respectively, when measured with R8403, and 67.8 ± 8.5 and 55.8 ± 12.2 pg, respectively, when measured with R8801 ($n = 4$).

Gel permeation chromatography

Figure 7 shows the gel filtration profile of the PHI-LI released in response to 32-Hz EFS and detected with R8403 or R8801. R8801 revealed a single immunoreactive peak that co-eluted with the synthetic rPHI standard, while R8403 detected two peaks, the first co-eluting with the synthetic rPHV(1–42) standard and the second in the same positions as the synthetic rPHI-Gly and rPHI standards. The first peak revealed by R8403 represented 50.1% of the total immunoreactivity measured with this antiserum, while the single peak detected with R8801 was only 38.8% of the total immunoreactivity measured with the N-terminal-specific antiserum. This value (38.8%) is quite similar to the ratio of the PHI-LI measured by R8801 and that revealed by R8403 (37.7 ± 3.1) in samples from the release experiments corresponding to the factions pooled for the chromatographic analysis (2 min during EFS plus the following 4 min). The second peak disclosed

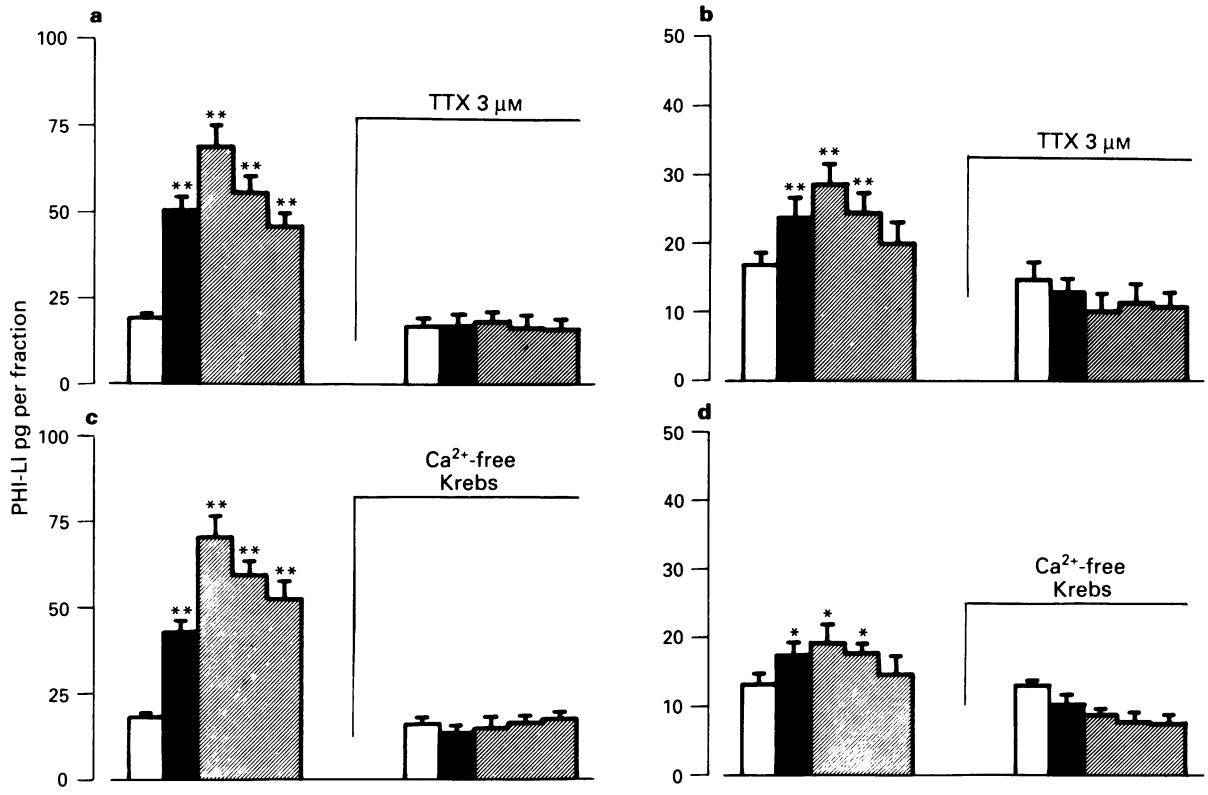

Figure 6 Release of peptide histidine isoleucine-like immunoreactivity (PHI-LI) from longitudinal muscle strips of rat gastric fundus induced by electrical field stimulation (16 Hz, 120 mA, 1 ms, pulse trains of 2 min) before and after addition of 3 μM tetrodotoxin (a, b) and before and after strip incubation with a calcium-free medium (c, d). Each column represents mean with s.e.mean PHI-LI measured with anti-pPHI serum R8403 (a, c) or anti-pPHI serum R8801 (b, d) in 2-min collection fractions from six strips. Open, solid and hatched columns, as in Figures 2, 3 and 4, refer to fractions collected before, during and after electrical stimulation. Values significantly different from baseline values: $*P < 0.05$; $**P < 0.01$.

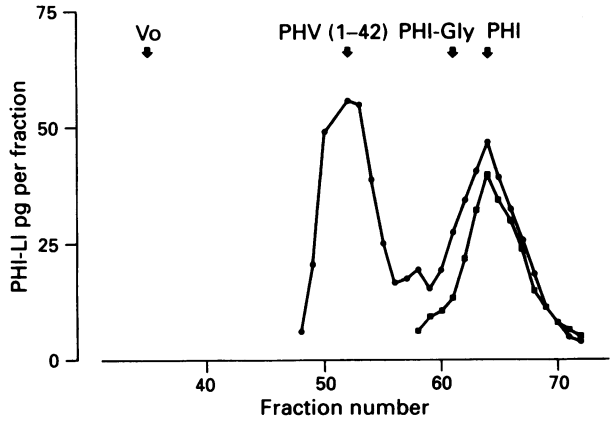

Figure 7 Sephadex G-25 gel filtration chromatographic profiles of the PHI-LI released by longitudinal muscle strips from the rat gastric fundus that were subjected to 32-Hz EFS (fractions of the incubation medium collected during and after EFS of four strips were pooled for this analysis). PHI-LI was detected with anti-pPHI serum R8403 (●) and with anti-pPHI serum R8801 (■). Arrows indicate the elution positions of dextran blue (Vo), synthetic rPHV (1–42), synthetic rPHI-Gly and synthetic rPHI.

by R8403, which appeared before and was larger than that detected by R8801, is probably due to the superimposition of an early, small peak representing PHI-Gly and a later, larger peak produced by PHI. In fact, when the R8801 peak is subtracted from the second peak detected by R8403, the result suggests a small peak co-eluting with the synthetic rPHI-Gly standard and representing the remaining 11.1% of the total PHI-LI.

Discussion

NANC inhibitory neurones are responsible for both the receptive and adaptive relaxations that occur in the proximal third of the stomach. There was initially a great deal of controversy about the identity of the neurotransmitters released by these neurones, but, until a few years ago, VIP and ATP were considered to be the most likely candidates. However, Bult *et al.* (1990) have recently demonstrated that NO acts as an inhibitory NANC neurotransmitter at the ileo-colonic junction, and Li & Rand (1990) have provided evidence that it plays an identical role in the rat gastric fundus. It currently appears that the NANC relaxation to EFS in the rat gastric fundus involves two components: a nitrergic component that is primarily responsible for the relaxation observed with low-frequency or short-duration EFS, and a peptidergic component that is mainly responsible for the relaxation induced by EFS at high frequencies or long duration (Li & Rand, 1990; D'Amato *et al.*, 1992a,b; Boeckxstaens *et al.*, 1992; Currò *et al.*, 1992). VIP is still considered as the most likely candidate for the peptidergic component, but there are also reasons to believe that PHI participates in this response, as follows: (a) both rat and porcine PHI (rPHI and pPHI) relax the smooth muscle of the rat gastric fundus, although they are about 2 and 30 times less potent than VIP; therefore, rPHI, which differs from pPHI for two amino-acid residues in the sequence, is approximately 15 times more potent than pPHI in relaxing the rat gastric fundus, although its potency in this respect is still half of that displayed by VIP (Lefebvre *et al.*, 1991); (b) electrically induced NANC relaxation in this tissue is reduced by a specific anti-pPHI serum (D'Amato *et al.*, 1990); (c) two C-terminally extended forms of rPHI, rPHI-Gly and rPHV(1–42), relax the rat gastric fundus, although their potencies are 4 and 3 fold less, respectively, than that of rPHI. These findings mean that PHI and its extended forms fulfil the 'identity of action' criterion for putative neurotransmitters in this NANC system. The results described here demonstrate that EFS of the rat gastric fundus elicits the release of PHI-LI through a TTX-sensitive, calcium-dependent mechanism, which means that PHI and its extended forms also satisfy the 'detectable release' criterion for inhibitory NANC neurotransmitters in this tissue.

The finding that significantly increased PHI-LI release is evoked only at EFS frequencies of 8 Hz or higher is consistent with the idea that PHI and VIP may act as co-transmitters in the rat gastric fundus, since the same pattern of release has been observed for VIP-LI in this tissue (D'Amato *et al.*, 1992b). These two peptides are also co-released in the dog gastric corpus in response to vagal stimulation (Ohta *et al.*, 1990). Our data support the hypothesis that VIP and PHI are primarily involved in the NANC relaxation elicited by high frequency EFS. With this type of stimulation, recovery to basal tone is more gradual than that observed after low frequency EFS. This gradual recovery is also observed when exogenously applied PHI and VIP are removed from the bath medium (results not shown). Therefore, there is a good correlation between the time course of the mechanical responses and the time course of the release of PHI-LI and VIP-LI elicited in these muscle strips by high frequency EFS. Moreover, peptidases and anti-VIP sera primarily inhibit the NANC relaxations provoked by high frequency EFS, while those induced by low frequencies of stimulation are mainly attenuated by NO-synthase inhibitors (Li & Rand, 1990; Boeckxstaens *et al.*, 1992).

PHI-LI release was measured under the same experimental conditions used in the functional study in order to correlate this release as closely as possible with the intensity of evoked relaxation. The question arose, however, as to whether the substances used to unravel the NANC relaxation, in particular 5-HT, might have exerted modulatory effects on PHI-LI release. This possibility was excluded in separate experiments conducted in the absence and presence of 5-HT.

The existence of C-terminally extended forms of PHI or PHM was first suggested for human neuroblastoma cells (Hoshino *et al.*, 1984) and later for porcine stomach (Yiangou *et al.*, 1985). In the latter study, an N-terminal-specific anti-pPHI serum revealed levels of PHI-LI that were 2–4 times higher than those measured by a C-terminal-specific antiserum. Subsequent gel-permeation chromatographic analysis of these extracts revealed a PHI-LI component that eluted before PHI itself and reacted exclusively with an N-terminal-specific antiserum. Similar chromatographic profiles were observed for stomach extracts from cats, rats and man. This larger molecular form of PHI was later found in the nasal mucosa and genitourinary tissue from various species (Yiangou *et al.*, 1986), in the gastrointestinal tracts of dogs (Yasui *et al.*, 1987), in human adrenal phaeochromocytoma (Yiangou *et al.*, 1987) and in rat small intestine (Cauvin *et al.*, 1989b) and characterized as PHV(1–42). A third extended form of PHI, isolated from rat small intestine, was characterized as PHI-Gly (Cauvin *et al.*, 1989a). In the present study, the total EFS-induced PHI-LI release revealed by the C-terminal-specific antisera was approximately 12% of that measured by the N-terminal-specific antiserum, regardless of the stimulation frequency used. Since the former sera (R8801 and R01) do not recognize rPHI-Gly or rPHV(1–42), it is reasonable to assume that PHI-LI detected by these sera represents PHI itself. In contrast, serum R8403 showed full cross-reactivity with both rPHI-Gly and rPHV(1–42), and the immunoreactivity revealed by this serum represents the total produced by PHI and its extended forms. These data suggest that PHI itself accounts for only a small portion (12%) of the PHI-LI observed with EFS. It is also possible, however, that at least part of the PHI released under these circumstances undergoes cleavage by endogenous peptidase(s) (such as neutral endopeptidase, which has been described in the rat gastric fundus; Bunnett *et al.*, 1993) that are not inhibited by aprotinin or bacitracin. This might give rise to

N-terminal fragments that are fully recognized by serum R8403 with short C-terminal fragments that fail to react with sera R8801 or R01. This may provide an alternative explanation for the low levels of PHI-LI detected by the C-terminal-specific antisera. However, the addition of thiorphan, which selectively inhibits neutral endopeptidase, had no effect on the amounts of PHI-LI recovered with either the C- or the N-terminal-specific antisera, which excludes PHI degradation by this peptidase and weakens the above mentioned peptidase-degradation hypothesis. The chromatographic data clearly identify both PHV(1–42) and PHI as components of the PHI-LI released in response to EFS and strongly suggest the presence of PHI-Gly as well. A distinct peak corresponding to the PHI-Gly standard could not be obtained, but the second peak detected by serum R8403 was somewhat earlier and larger than that revealed by serum R8801. It is possible that this R8403 peak contains a small peak that represents PHI-Gly, as well as a larger one produced by PHI itself. In fact, when the R8801 peak is subtracted from the second peak detected by R8403, the remainder is a small peak that co-elutes with the synthetic PHI-Gly standard. The samples used for this analysis were composed of fractions that were collected during EFS and the 4-min period that followed it and, for this reason, the relative proportions of PHI, PHI-Gly and PHV(1–42) in the total PHI-LI released in response to EFS (measured in fractions collected up to 30 min after stimulation) could not be determined. The higher PHI/total PHI-LI ratio in the pool used for chromatographic analysis, with respect to that found in the total (30 min) PHI-LI release measured in the release experiments, is a result of these differences in sampling time. Nevertheless, our data strongly suggest that PHV(1–42) is the predominant molecular form released by the rat gastric fundus in response to EFS.

Using chromatographic separation, Cauvin *et al.* (1989b) found that PHI-Gly, PHV(1–42) and PHI accounted for 32%, 33% and 36%, respectively, of the total PHI-LI detected in tissue extracts of rat stomach. The fact that the PHI/total PHI-LI ratio reported by these authors (1:3) is somewhat higher than that observed in the present study (1:8) is probably due to the fact that the tissue extracts used in the former study were obtained from the stomach as a whole. This ratio has, in fact, been shown to be lower in the gastric fundus (1:7) than it is in the antrum (1:3) (Yiangou *et al.*, 1986), and the former ratio is quite similar to the one that we have reported for our fundus strips.

The concentration of exogenous rPHI and its extended forms that elicited a strip relaxation comparable to that produced with 32-Hz EFS was approximately 30 nM (data not shown). The total PHI-LI detected with serum R8403 during and after EFS at this frequency was about 430 pg. Considering the volume of the incubation medium and the molecular weights of rPHI and its extended forms, this figure translates into a concentration of 0.07 nM, i.e. approximately 400 times lower than the concentration of exogenous rPHI required to produce the same biological effect. However, the PHI-LI concentration produced at the neuromuscular junction during nerve stimulation is much higher than that measured in the incubation medium, and it would be more appropriate to compare this concentration with that of exogenous rPHI. It is also important to remember that the EFS-induced relaxation is not mediated exclusively by PHI-related peptides, since VIP is also released in response to this stimulus.

In conclusion, the present data demonstrate that PHI-LI release occurs in response to activation of intramural neurones of rat gastric fundus and support the NANC inhibitory neurotransmitter role for PHI and its extended forms in this tissue.

Supported by National Research Council (CNR), targeted project 'Prevention and control of disease factors', subproject 2. The authors are indebted to Prof. Dr N. Yanaihara (Laboratory of Bioorganic Chemistry, Shizuoka College of Pharmacy, Shizuoka, Japan) for the generous gift of the anti-pPHI sera R8403 and R8801, to Prof. Dr P. Robberecht (Department of Biochemistry and Nutrition, University of Brussels, Belgium) for providing us with rPHI-Gly and rPHV (1–42), and to Mrs M. Kent for editorial help in preparing the manuscript.

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(Received January 4, 1994
Revised June 6, 1994
Accepted June 8, 1994)