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Release of vasoactive intestinal polypeptide from the rat gastric fundus

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1 Auxotonic responses and release of vasoactive intestinal polypeptide-like immunoreactivity (VIP-LI) induced by electrical field stimulation (EFS) were studied in longitudinal muscle strips from the gastric fundus of reserpinized rats suspended between parallel platinum electrodes in Krebs solution containing atropine (1 μM), 5-hydroxytryptamine (3 μM) and bovine serum albumin (50 mg l^{-1}).

2 EFS (supramaximal voltage, 1 ms, 0.25–32.0 Hz, trains of 2 min) induced frequency-dependent relaxations.

3 EFS at frequencies $\geqslant$8 Hz also produced significant increases in VIP-LI release.

4 VIP-LI release induced by EFS at 16 Hz no longer occurred in the presence of tetrodotoxin (1 μM) or a Ca^{2+}-free medium.

5 Detection of VIP-LI upon activation of inhibitory non-adrenergic, non-cholinergic neurones indicates that VIP meets the 'detectable release' criterion for an inhibitory neurotransmitter in the rat gastric fundus.

Keywords: Stomach (rat); non-adrenergic non-cholinergic (NANC) neurones; relaxation; vasoactive intestinal polypeptide (VIP); peptide histidine isoleucinamide (PHI)

Introduction

According to Orrego (1979), the following basic criteria must be met by any substance that is to be considered a neurotransmitter: 'detectable release' via a Ca^{2+}-dependent mechanism in response to neural stimulation and 'identity of action' between the effects of exogenous application of the putative neurotransmitter and those of neuronal stimulation. In many species, electrically induced relaxation of the smooth muscles of the gastric fundus has a non-adrenergic non-cholinergic (NANC) component but the identity of the neurotransmitter(s) that mediates this effect has not been definitively established. Vasoactive intestinal polypeptide (VIP) seems responsible for at least part of this relaxant response in the rat. Exogenously applied VIP has already been shown to mimic the effects of NANC neurone activation in this species (Lefebvre, 1986). In addition, both trypsin (De Beurme & Lefebvre, 1978) and specific anti-VIP antisera (De Beurme & Lefebvre, 1988; D'Amato *et al.*, 1990; Li & Rand, 1990), currently the most effective methods of antagonizing VIP-induced relaxation, partially reduce the inhibitory responses to NANC stimulation at concentrations that selectively prevent responses to VIP itself. Very recently, similarities have also been found between the mechanical, electrophysiological and cyclic nucleotide responses to VIP and those elicited by inhibitory NANC neurone stimulation (Ito *et al.*, 1990).

The aim of the present study was to determine whether VIP also meets the 'detectable release' criterion as an inhibitory NANC neurotransmitter in the gastric fundus of the rat.

Methods

Following intraperitoneal injection of reserpine (5 mg kg^{-1}) to deplete noradrenergic stocks (Lefebvre, 1986), male and female rats weighing 150–300 g were fasted, with free access to water, for 24 h. The animals were then killed and their stomachs were removed. Longitudinal muscle strips (3 mm × 20 mm) from the gastric fundi were prepared as described by Vane (1957). One strip was prepared from each fundus. Some of the strips were used for functional studies and others were used for release experiments (see below). The basic bath solution used for both types of studies was a Krebs solution containing (mM): NaCl 118.5, KCl 4.8, $CaCl_2$ 1.9, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 10.1. The bath solution was maintained at 37°C and bubbled with a 95:5 O_2/CO_2 mixture.

Functional experiments

The strips were mounted under a 1 g load between two parallel platinum electrodes (20 mm long, 4 mm wide, 5 mm apart) placed in 5 ml organ baths. The bath solution for these experiments also contained atropine 1 μM, 5-hydroxytryptamine (5-HT) 3 μM and bovine serum albumin (BSA) 50 mg l^{-1}. The tissues were allowed to equilibrate for 1 h during which the bath solution was changed every 10 min. Changes in strip length, magnified 5–10 times, were recorded auxotonically (Harvard smooth muscle transducers) with Rikadenky R-01 single-pen recorders. After the equilibration period the bath solution was renewed every 5 min between drug applications and/or periods of electrical field stimulation (EFS).

Rectangular, bipolar pulses or constant current output (120 mA) were delivered with a Palmer Bioscience 6012 Stimulator linked in series with a Basile Biological Research Apparatus constant-current unit. Throughout the period of EFS, output was controlled on a Hewlett Packard 141B oscilloscope. Stimulation voltage (supramaximal), duration (1 ms) and pulse train duration (2 min) were kept constant; frequency was varied as indicated below. At the end of all experiments, a supramaximal concentration (35 μM) of sodium nitroprusside (SNP) was added to the bath and left in contact with the strip until maximal relaxation had occurred.

Release experiments

Strips were fitted with a pair of platinum electrodes (20 mm long, 3.5 mm wide, 3 mm apart) and mounted under a load of 1 g inside 4 ml plastic tubes (Sarstedt No 55478, Numbrecht, Germany). The tubes contained 2 ml of the basic Krebs solution with atropine (1 μM), BSA (50 mg l^{-1}), bacitracin (30 mg l^{-1}) and aprotinin (100,000 iu l^{-1}). The bath solution was bubbled with a 95:5 O_2/CO_2 mixture and maintained at 37°C. The strips were placed in different tubes every 10 min

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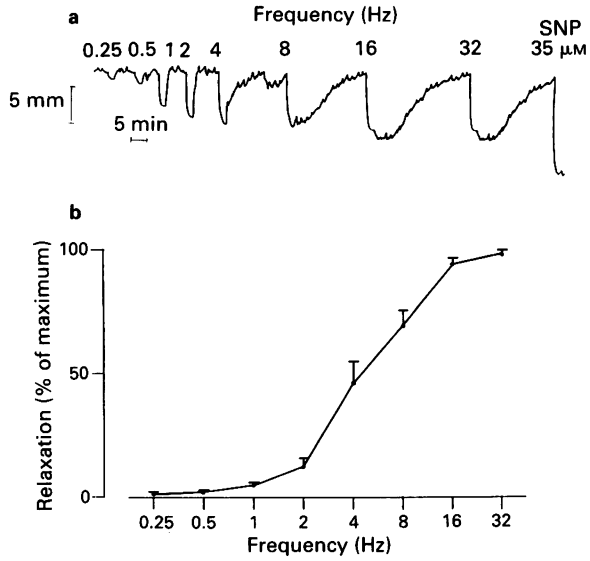

Figure 1 Relaxation of longitudinal muscle strips from the rat gastric fundus induced by electrical field stimulation (0.25–32.0 Hz, supramaximal voltage, 1 ms, pulse trains of 2 min). (a) Representative tracing from one muscle strip SNP = sodium nitroprusside. (b) Mean results ± s.e.mean (vertical bars) based on responses of 6 strips. Relaxations were measured as area under the curve and expressed as percentages of the maximal relaxation induced in each strip (32 Hz in 5 out of 6 strips).

during the 60 min equilibration period. Thereafter each strip was incubated for 2 min in different tubes before, during and after EFS delivered as described above.

At the end of each 2 min incubation period, the tube solution was collected on ice, divided into 1 ml aliquots and stored at −20°C until assayed. The wet weight of the strips was recorded at the end of all experiments.

A specific radioimmunoassay (RIA) was used to measure VIP-like immunoreactivity (VIP-LI). On the day of assay, the frozen aliquots were thawed, extracted in absolute ethanol (3/4 v/v), centrifuged at 3000 r.p.m. for 20 min at 4°C and evaporated in a Speedvac Evaporator linked with a Savant refrigerated condensation trap and vacuum pump. They were then reconstituted in assay buffer (NaCl 154 mM, phosphate 10 mM buffer, ethylene diaminetetraacetic acid (EDTA) 25 mM, thimerosal 0.01%, BSA 0.5%, pH 7.2) to a 4 fold concentration of the original.

Duplicates of each sample were assayed with an antiserum produced in white New Zealand rabbits as described elsewhere (D'Amato *et al.*, 1990). The serum showed cross-reactivity of less than 0.01% with synthetic porcine or rat peptide histidine isoleucinamide (PHI), extracted C-terminal extended forms of rat PHI, peptide histidine valine and peptide histidine glycine, secretin, corticotrophin releasing factor, human growth hormone releasing factor, sauvagine, helodermin, helospectin, gastric inhibitory polypeptide and glucagon in concentrations up to 1 μM.

For each assay, 0.1 ml of antiserum diluted 1:25,000 was mixed with 0.1 ml of the sample being tested or of VIP standard solution in disposable plastic tubes and incubated for 72 h at 4°C (with the exception of those used to assay non-specific binding). After incubation, 0.05 ml of an [^{125}I]-VIP solution (2000 Ci mmol^{-1}) containing approximately 5000 c.p.m. was added to each sample. After a second incubation period (48 h) under the same conditions, free VIP was separated from that bound to antibody by adding 0.5 ml of a 1% charcoal suspension in 0.2% BSA. The tubes were placed on a vortex mixer briefly and then centrifuged for 10 min at 3000 r.p.m. at 4°C. The supernatants were decanted into plastic tubes, and free and antibody-bound fractions of [^{125}I]-VIP were measured in a gamma counter.

Data were processed with the aid of a computer programmed to correct for non-specific binding. The IC_{50} of VIP standard curves averaged 12.9 (range 7.9–17.4) fmol per tube. The range of concentrations encountered in our samples was 9.3–94.6 fmol per tube.

Since the VIP-LI levels were influenced by the wet weight of the tissue (range: 120–210 mg), the results were expressed as fmol per fraction g^{-1} wet weight. The overall evoked VIP-LI release was calculated by subtracting the basal value from each value observed during and after the stimulus and adding them.

Experimental protocols

Frequency-response curves to electrical field stimulation In a first group of experiments the motor effect and the VIP-LI release induced by isolated EFS at increasing frequencies (0.25–32 Hz) were studied. In the functional experiments the strips were allowed to recover basal tone after each individual frequency of stimulation. All relaxations were expressed as areas under the curve, estimated by means of the trapezoidal rule for planar area. The results are expressed as percentages of the maximal response obtained in each strip. In the release experiments stimuli were delivered 30 min apart.

Effects of tetrodotoxin and of removal of extracellular Ca^{2+} The effect of tetrodotoxin (TTX) was studied in both functional and release experiments. The response to 16 Hz EFS was measured before and after 15 min incubation with TTX 1 μM. Sixty minutes elapsed between the two measurements. In the functional experiments, results are expressed as percentage of the relaxation induced before addition of the neurotoxin.

The effect of a Ca^{2+}-free medium was studied in release experiments only, because removal of Ca^{2+} reduced strip tone, making accurate quantification of the relaxant response impossible. Measurement of VIP-LI release induced by 16 Hz EFS was made before and 30 min after $CaCl_2$ had been removed from the Krebs solution and replaced with equimolar $MgCl_2$. Sixty minutes elapsed between the two determinations.

Drugs used

Aprotinin (Lepetit, Milan, Italy), atropine sulphate (Sigma, St Louis, U.S.A.), bacitracin (Lusofarmaco, Milan), bovine serum albumin (BSA, Sigma), ethylene diaminetetraacetic acid (EDTA, BDH, Poole, U.K.), 5-hydroxytryptamine creatinine sulphate (Sigma), isopropylnoradrenaline sulphate (Boehringer Ingelheim, Florence, Italy), nicotine (Merck, Munchen, Germany), reserpine (Sigma), sodium nitroprusside (Sigma), tetrodotoxin (Sigma), thimerosal (Sigma), highly purified synthetic vasoactive intestinal polypeptide (Bachem), [^{125}I]-VIP (Amersham, U.K.).

Drugs were dissolved in or diluted with distilled water. A stock solution of reserpine was prepared from powder (5 mg ml^{-1} dissolved in 10% ascorbic acid).

Drugs were kept on ice during each experiment and were added to the bath in a maximum volume of 0.1 ml. Atropine, 5-hydroxytryptamine and BSA (and bacitracin and aprotinin for the release experiments) were added directly to the reservoir containing Krebs solution.

Statistical analysis

Data are given as means ± s.e.mean. Responses were compared by means of the paired Student's *t* test.

Results

Frequency-response curves to electrical field stimulation

An actual tracing and the mean results from the functional experiments are shown in Figure 1. EFS (0.25–32.0 Hz)

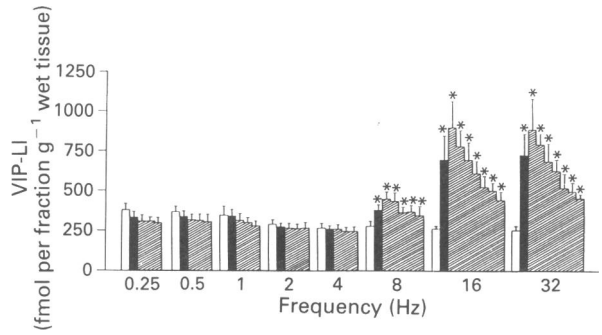

Figure 2 Release of vasoactive intestinal polypeptide-like immunoreactivity (VIP-LI) from longitudinal muscle strips of rat gastric fundus induced by electrical field stimulation (0.25–32.0 Hz, supramaximal voltage, 1 ms, pulse trains of 2 min). Each column represents mean VIP-LI (s.e.mean shown by vertical lines) found in 2 min collection fractions from 6 strips: open columns show baseline release just before electrical stimulation; closed columns show release during stimulation; hatched columns refer to post-stimulation fractions. *Values significantly different ($P < 0.05$) from baseline values.

induced frequency-dependent NANC relaxation that started almost immediately after stimulation was begun and quickly increased. Relaxation amplitude remained constant or increased slightly during the stimulation period. This pattern was more evident with higher frequencies. Recovery of basal tone after the end of stimulation was also slower when higher frequencies had been used.

Significant increases over basal levels were consistently seen in VIP-LI released in response to EFS at frequencies of 8 Hz or more (Figure 2). VIP-LI release at 16 Hz was significantly higher than that seen at 8 Hz, but no further increase was seen at 32 Hz. The VIP-LI released per unit mass of tissue during the 2 min EFS at 16 and 32 Hz was 693.7 ± 155.0 and 709.0 ± 132.0 fmol per fraction g^{-1} wet weight, respectively. VIP-LI release from the tissue continued to increase in the first fraction after the stimulation was stopped. Values returned to baseline levels within 14 min after stimulation at 8 Hz as opposed to 20 min or more after stimulation at 16 or 32 Hz (data not shown). The overall evoked VIP-LI release in response to EFS at 16 and 32 Hz was 3062.0 ± 672.8 and 3148.5 ± 545.6 fmol per fraction g^{-1} wet weight, respectively.

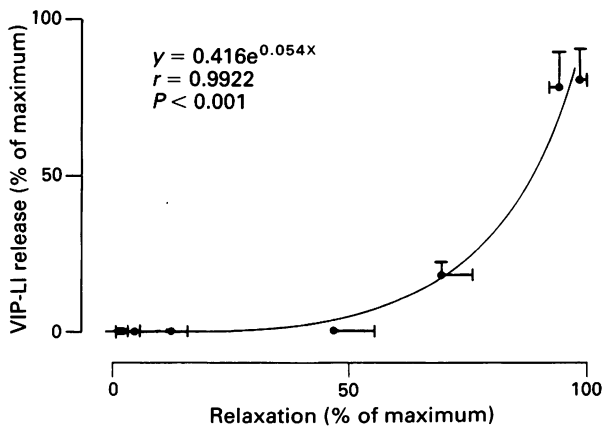

Figure 3 Relationship between relaxation (expressed as area under the curve) and overall evoked vasoactive intestinal polypeptide-like immunoreactivity (VIP-LI) release (calculated by subtracting the basal value from the value observed during and after the stimulus and adding them). Data are expressed as percentage of maximal value and given as means from 6 strips; s.e.mean shown by bars. Electrical field stimulation at 32 Hz produced maximal relaxation in 5 strips and maximal VIP-LI release in 3 strips; in the remaining strips, maximal values for both relaxation and VIP-LI release were observed at 16 Hz.

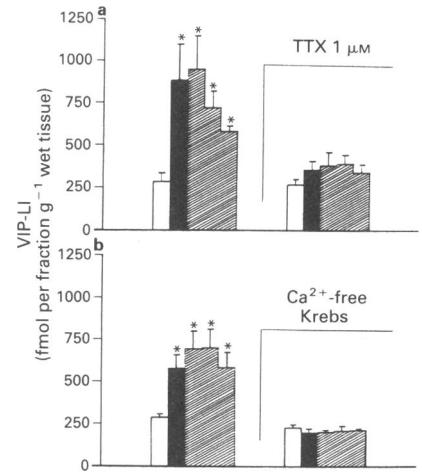

Figure 4 Release of vasoactive intestinal polypeptide-like immunoreactivity (VIP-LI) from longitudinal muscle strips of rat gastric fundus induced by electrical field stimulation (16 Hz, supramaximal voltage, 1 ms, pulse trains of 2 min) before and after addition of 1 μM tetrodotoxin (a) and before and after removal of extracellular Ca^{2+} (b). Each column represents mean VIP-LI (s.e.mean shown by vertical lines) found in 2 min collection fractions from 6 strips. Open, closed and hatched columns, as in Figure 2, refer to fractions collected before, during and after electrical stimulation. *Values significantly different ($P < 0.05$) from baseline values.

The relationship between the overall evoked VIP-LI release and muscle relaxation is illustrated in Figure 3.

Effects of tetrodotoxin and of removal of extracellular Ca^{2+}

Preliminary experiments had shown that 1 μM TTX was capable of selectively preventing relaxation induced by up to 1 μM of nicotine without affecting that induced by submaximal concentrations of VIP (3 nM), isopropylnoradrenaline (10 nM) or sodium nitroprusside (SNP, 300 nM) (data not shown). At this concentration, TTX did not affect the tone of the strips at all during the 15 min contact period. However, NANC relaxation induced by 16 Hz EFS in the presence of TTX was only 1.53 ± 0.68% ($n = 6$) of that observed in its absence. VIP-LI released in response to control periods of EFS at a single frequency of 16 Hz in this series of experiments (Figure 4) was comparable to that seen at 16 Hz in the experiments performed with increasing frequencies. Neither TTX nor the Ca^{2+}-free medium influenced baseline release of VIP, but each completely abolished the electrically stimulated release of this peptide.

Discussion

The major criteria that must be met by a neurotransmitter are 'identity of action' and 'detectable release' (Orrego, 1979). NANC inhibitory neurones of the rat gastric fundus mediate the gastric receptive relaxation during food intake (Abrahamsson, 1986) but the neurotransmitter(s) released from these neurones has (have) not been definitively established. In this study, we provide further evidence that VIP contributes to the NANC relaxation in the rat gastric fundus.

The responses to EFS that we observed were clearly NANC in nature: noradrenergic and cholinergic effects had been eliminated by reserpinization of the rats and addition of atropine to the organ baths. Details of the noradrenergic depletion that is induced with reserpine pretreatment have been described in a previous paper (Lefebvre, 1986).

In the functional experiments, muscle strip tone was increased with 5-HT. The objective here was to render the

relaxant responses more evident. The frequency-dependent relaxation that we observed was similar to that seen in previous studies (Lefebvre, 1986; D'Amato et al., 1990). Also when the tone of the rat gastric fundus strips is raised with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) NANC relaxant responses can be obtained (D'Amato & Currò, 1990; Belai et al., 1991). Preliminary experiments conducted prior to the present study showed that neither 5-HT nor $PGF_{2\alpha}$ influenced the release (basal or EFS-stimulated) of VIP-like immunoreactivity. Therefore, 5-HT was not used in our release experiments.

There is already a good deal of evidence to suggest that VIP contributes to the inhibitory NANC neurotransmission in the gastric fundus of the rat. It causes relaxation that closely mimics that induced by activation of inhibitory NANC neurones (Lefebvre, 1986). In addition, NANC relaxation is partially antagonized by VIP antiserum (De Beurme & Lefebvre, 1988; D'Amato et al., 1990; Li & Rand, 1990). Ito et al. (1990) have shown that both VIP and NANC neurone stimulation elicit increases in adenosine 3':5'-cyclic monophosphate (cyclic AMP) and guanosine 3':5'-cyclic monophosphate (cyclic GMP) and induce similar electrophysiological changes in the gastric fundas muscles of the rat.

In the present study, we have demonstrated that electrical stimulation of the inhibitory NANC neurones in longitudinal muscle strips from the gastric fundus of the rat elicits release of a VIP-like immunoreactive substance through a tetrodotoxin-sensitive, Ca^{2+}-dependent mechanism. These findings corroborate other recent findings of TTX-sensitive release of VIP-LI from a sheet of rat gastric fundus induced by EFS at 16 Hz (Belai et al., 1991) and from circular muscle strips of the rat gastric antrum with 20 Hz stimulation (Ito et al., 1990).

The maximal release of VIP-LI per unit mass of tissue during EFS occurred at 16 and 32 Hz and was approximately 700 fmol per fraction g^{-1} wet weight. Considering the size of the strips and the volume of the bath medium, this translates into a molar concentration of approximately 0.05 nM per tube. We have found that approximately 10 nM of exogenously applied VIP is necessary to produce the degree of relaxation induced by 16 Hz EFS (D'Amato et al., 1990). The amount we measured in the RIA described here is thus 200 times less than the amount of exogenous VIP required to produce the same biological effect. However, as Furness & Costa (1982) have pointed out, experiments with exogenously applied agents do not necessarily reflect the manner in which the same agent might act if it were released from axons within an organ. Neurally released transmitters may act at sites that are sequestered within the tissue and therefore less available to exogenous agents. In addition, although there is no way to calculate the concentration of VIP achieved at the synaptic level with neural stimulation, we can reasonably assume it to be higher than that detected into the bath medium, since the neuromuscular junction represents only a small fraction of the extracellular space, which is estimated in about 10% of wet tissue weight (Grider & Makhlouf, 1987).

The release of VIP-LI was observed only with stimulation frequencies of 8 Hz or more although a mechanical response could be detected with frequencies as low as 0.25 Hz. This finding conflicts with Grider & Makhlouf's (1987) observation of correlation between VIP-LI release and mechanical responses of taenia coli and gastric fundus strips from guinea-pigs at all stimulation frequencies tested. However, it has also been shown that peptide release requires higher stimulation frequencies than those needed for release of classical neurotransmitters (Bartfai et al., 1988; Agoston & Lisziewicz, 1989).

Our data suggest that the contribution of VIP to NANC relaxation is confined to that induced by higher frequency stimulation. Figure 1a clearly shows that recovery of tone after stimulation at frequencies of 8 or more Hz was slower than that observed at lower stimulation frequencies. Even when exogenously applied VIP was removed from the bath solution, recovery of baseline tone was still slow (results not shown). Thus, the mechanical response correlates with the VIP release measured, at least at higher frequencies. At lower stimulation frequencies, relaxation may be mediated by nitric oxide (NO). The L-arginine analogues, which inhibit the formation of NO from L-arginine, have been found to antagonize electrically-induced NANC relaxation of the gastric fundus of the rat, especially at lower frequencies (Li & Rand, 1990). EFS of the rat gastric fundus elicits release of a vasorelaxation agent with biological properties similar to those of NO; the substance has a short half-life and relaxes rabbit aortic rings with the epithelium removed (Boeckxstaens et al., 1991).

However, Li & Rand (1990) have recently shown that relaxation induced by EFS at 5 Hz is not completely blocked by a combination of VIP-antiserum and the L-arginine analogue L-N^G-monomethyl arginine. Other substances might, thus, be involved and PHI and ATP are two of the most likely candidates. It has been found that NANC relaxation of the rat gastric fundus is to some extent reduced by a specific PHI-antiserum (D'Amato et al., 1990). When rat gastric fundus strips were desensitized to ATP by α,β-methylene ATP, NANC relaxation decreased and rat gastric fundus sheets have also been found to release ATP upon EFS (Belai et al., 1991).

In conclusion, our finding of VIP-LI release elicited during electrical field stimulation of longitudinal muscle strips of the rat gastric fundus through a TTX-sensitive and Ca^{2+}-dependent mechanism indicates that VIP meets the 'detectable release' criterion for an inhibitory neurotransmitter in the rat gastric fundus.

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