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Distribution and effects of PACAP, VIP, nitric oxide and GABA in the gut of the African clawed frog *Xenopus laevis*

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Summary

The distribution and possible effects on gastrointestinal motility of pituitary adenylate cyclase-activating polypeptide (PACAP), vasoactive intestinal polypeptide (VIP), nitric oxide and γ-amino-butyric acid (GABA) were investigated in the African clawed frog (*Xenopus laevis*) using immunohistochemistry and *in vitro* strip preparations. PACAP- and VIP-immunoreactive nerve fibres were common in the myenteric plexus as well as in the longitudinal and circular muscle layers all along the gastrointestinal tract. Double labelling demonstrated a close correlation between PACAP and VIP immunoreactivities, indicating that the two neurotransmitters are colocalised within the enteric nervous system. Occasionally, PACAP- and VIP-positive nerve cell bodies were seen in the myenteric or submucous plexa. In addition, VIP immunoreactivity coexisted with helospectin immunoreactivity. Nitric oxide synthase (NOS)-immunoreactive nerve cells were found in the myenteric plexus at an average density for the whole gastrointestinal tract of 4584±540 cells cm^{-2}. The NOS-immunoreactive nerve cells were usually multipolar with an average size of 11.3±3.7 × 23.2±6.6 μm. Some NOS-immunoreactive nerve fibres were VIP-immunoreactive but not all VIP-positive fibres showed NOS immunoreactivity. GABA immunoreactivity was found in nerve fibres and nerve cells in the myenteric plexus of all regions of the gut. Few GABA-immunoreactive nerve fibres were VIP-immunoreactive. PACAP 27, VIP, sodium nitroprusside (a nitric oxide donor; NaNP) and GABA caused similar responses on spontaneously contracting circular preparations of the cardiac stomach of *X. laevis*. The mean force developed was decreased, mainly by a reduction in resting tension, while the amplitude of contractions was not necessarily affected. The NOS inhibitor N^{G}-nitro-L-arginine methyl ester (L-NAME) increased the mean force developed, indicating a nitrergic tone in the preparations. In contrast, PACAP 27, VIP, NaNP, GABA and L-NAME had no significant effect on longitudinal strip preparations from the duodenum. These results indicate that PACAP, VIP, nitric oxide and GABA, which are known to be important inhibitory neurotransmitters in other vertebrates, are widely spread in the enteric nervous system of *Xenopus laevis* and may be involved in the inhibitory control of gastric motility. Although no effect of PACAP, VIP, nitric oxide or GABA on the longitudinal strips of the duodenum was seen in this study, this does not rule out the possibility that they might play an important role in controlling intestinal motility as well.

Key words: amphibian, immunohistochemistry, gut motility, enteric nervous system, VIP, PACAP, nitric oxide, GABA, *Xenopus laevis*.

Introduction

Gastrointestinal motility depends on the actions of longitudinal and circular smooth muscle in the gut wall and the activity of the smooth muscle is controlled mainly by excitatory and inhibitory enteric neurons. So far, details of the control mechanisms have been investigated in greatest detail in mammals. However, as the number of known transmitter substances increases and the complexity of the control mechanisms begins to be revealed, it is of great interest to study different groups of non-mammalian species as well to try to understand the importance of each of the components involved. There are reports on the presence of different transmitters in the enteric nervous system of fish, amphibians and reptiles, for example, but apart from teleosts, knowledge of their actual effects is scarce.

The aim of this study was to investigate the distribution of members of the VIP/GRF-family, nitric oxide and GABA, and their possible roles as inhibitory neurotransmitters in the amphibian gut. In addition, the results obtained are compared with data from other groups of vertebrates. The African clawed frog, *Xenopus laevis*, is widely used as a model animal in studies of genetics and ontogeny, including the development of autonomic innervation. However, little is known about the distribution of neurotransmitters in the adult animal. Therefore, *Xenopus laevis* was chosen as a representative of the amphibians.

Vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) are two neuropeptides belonging to the same gene superfamily, the VIP/GRF-family. The sequences of both peptides have been determined in several different vertebrate species, including amphibians (Hoyle, 1998). PACAP usually exists in two C-terminally amidated isoforms, one form containing 27 amino acids (PACAP 27) and one C-terminally extended (PACAP 38). The sequence of PACAP has been well conserved during evolution; the amphibian (*Rana ridibunda*) sequence, for example, differs in only one position compared with the mammalian PACAP (Chartrel et al., 1991; Hoyle, 1998). Since this substitution occurs in the C-terminal region, PACAP 27 is identical in mammals and in this amphibian. The amphibian VIP shows 86 % identity to the mammalian VIP sequence and there is 75 % amino acid sequence identity between the amphibian VIP and PACAP (1–28) (Hoyle, 1998). Helospectin, another member of this gene family, has been isolated from the venom of the lizards *Heloderma suspectum* and *H. horridum*, and exists in two forms consisting of 37 and 38 amino acids, respectively (Parker et al., 1984). VIP has been found in the enteric nervous system in species from all vertebrate classes (Jensen and Holmgren, 1994) while the distribution of PACAP and helospectin is less investigated. In rainbow trout (*Oncorhynchus mykiss*) and Atlantic cod (*Gadus morhua*), as well as in some mammals, VIP, PACAP and helospectin are colocalised to a high degree in enteric nerve cells (Absood et al., 1992; Olsson and Holmgren, 1994; Sundler et al., 1992).

Nitric oxide can be synthesised by reduction of L-arginine to L-citrulline, a reaction carried out by the enzyme nitric oxide synthase (NOS). NOS exists in several isoforms including one nerve-specific isoform that can be demonstrated by using either immunohistochemistry or NADPH-diaphorase histochemistry (see Lincoln et al., 1997). Neuronal NOS is widely distributed in the enteric nervous system of most vertebrate species examined including one amphibian species, *Bufo marinus* (Costa et al., 1992; Ward et al., 1992; Li et al., 1992; Olsson and Karila, 1995; Timmermans et al., 1994). Frequently, a subpopulation of NOS-positive neurons coexpress VIP and/or PACAP (Costa et al., 1992; Li et al., 1993; Olsson and Karila, 1995).

In mammals, PACAP, VIP and nitric oxide usually inhibit gastrointestinal motility. Since these transmitters could be found within the same nerve cells, it has been suggested that they interact with each other in mediating this response. Several studies have shown that the release of VIP is facilitated by nitric oxide (Grider, 1993; Grider and Jin, 1993; Daniel et al., 1994; Grider et al., 1994). It has also been indicated that VIP stimulates nitric oxide production in some tissues (Li and Rand, 1990; Grider, 1993; Jin et al., 1993; Grider et al., 1994) although other groups could see no such effects (D'Amato et al., 1992; Grider and Jin, 1993; Ekblad and Sundler, 1997).

GABA (γ-amino-butyric acid) is an important inhibitory neurotransmitter in the central nervous system but has also been demonstrated in enteric neurons in several species, including frog (Hills et al., 1987; Furness et al., 1989; Gabriel and Eckert, 1989; Williamson et al., 1996; Wu et al., 1998). In the guinea-pig intestine, GABAergic neurons frequently coexpress NOS and/or VIP immunoreactivities, and a few fibres are substance P-immunoreactive (Nichols et al., 1995; Williamson et al., 1996). Most GABAergic enteric neurons act as interneurons and the effect on gastrointestinal motility is usually inhibitory (Grider and Makhlouf, 1992; Minocha and Galligan, 1993). In addition, GABA can stimulate the release of acetylcholine, causing contractions of the smooth muscles (Krantis et al., 1980; Grider and Makhlouf, 1992; Minocha and Galligan, 1993).

Materials and methods

African clawed frogs, *Xenopus laevis* L., of either sex (65–190 g) were obtained from Horst Kähler (Hamburg, Germany) and kept in aquaria at room temperature. The frogs were fed approximately 0.4 g of pellets twice a week and the water was changed every second day.

Before the experiments the animals were anaesthetised by immersion in carbonate-buffered tap water containing 0.1 % MS 222 (3-aminobenzoic acid ethyl ester; Sigma) and killed by decapitation.

Immunohistochemistry

Tissues were collected from seven regions of the gastrointestinal tract: the cardiac stomach, the pyloric stomach, the proximal small intestine (duodenum), the middle small intestine (upper ileum), the distal small intestine (lower ileum), the large intestine (colon and rectum) and the cloaca. The preparations were immersed in phosphate-buffered saline (PBS; 0.1 mol l^{-1} sodium phosphate, 0.9 % NaCl, pH 7.2) containing the smooth-muscle relaxant nifedipine (10^{-5} mol l^{-1}; Sigma) and then stretched and pinned onto dental wax. The tissue was fixed for 4 h in 4 % formaldehyde in 0.1 mol l^{-1} phosphate buffer (PB; pH 7.0) at 4 °C and subsequently washed in PBS. Whole-mount preparations were prepared by peeling off the mucosa, the submucosa and most of the circular muscle layer exposing the myenteric plexus. Preparations used for sectioning were kept overnight in PBS containing 30 % sucrose as cryprotectant before they were frozen in isopentane pre-chilled in liquid N_2 and cut into 10 μm sections on a cryostat.

The preparations were incubated with normal donkey serum (1:10; Jackson Immuno Research, USA) for 30 min before incubation for 2 days at room temperature with the primary antisera. Usually, double staining was performed, meaning that a mixture of two antisera was used. These were directed against different antigens and raised in different host species (see Table 1 for details). The preparations were washed in PBS (2 % NaCl), incubated for 1 h with the appropriate secondary antisera conjugated to Cy3 (indocarbocyanine), DTAF (dichlorotriazinyl amino fluorescein) or biotin (Jackson Immuno Research, USA) and washed in PBS. The biotinylated antibodies were visualised by incubation with streptavidin–Cy3

Table 1. *Primary antisera used*

Antigen	Host	Source	Code	Dilution
GABA	Rabbit	Sigma	A2052	1:400
Helospectin	Rabbit	Euro-Diagnostica	B63-1	1:400
NOS	Rabbit	Affinity	N31030	1:100
PACAP 38	Rabbit	Peninsula	IHC8920	1:800
VIP	Guinea-pig	Euro-Diagnostica	B-GP340-X	1:800
VIP	Rabbit	Euro-Diagnostica	B34	1:1000

complex (Jackson Immuno Research, USA) and finally washed in PBS. The preparations were mounted in carbonate-buffered glycerol and viewed with an Olympus fluorescence microscope. Normal serum and antisera were diluted with PBS (2 % NaCl) plus 0.1 % bovine serum albumin, 0.2 % NaN_3 and 0.2 % Triton X-100, pH 7.2.

The specificity of the two VIP antisera was tested by preincubation with excess amounts of VIP, PACAP 27, helospectin or secretin (10^{-5} mol l^{-1} diluted antiserum). No crossreactivity with PACAP 27, helospectin or secretin was seen with either antiserum, while all immunoreactivity was abolished after preincubation with VIP. Likewise, preincubation of the PACAP antiserum with PACAP 27 extinguished the immunoreactivity while no crossreactivity with VIP was detected. Furthermore, no immunoreactivity was found when the GABA antiserum was preabsorbed with GABA (10^{-4} mol l^{-1}; Sigma) (for details, see Furness et al., 1989).

The density (± S.D.) and size (± S.D.) of the NOS-immunoreactive nerve cells were determined using a ×40 objective. The number of cells in 0.021 cm^2 of each preparation was counted and a minimum of 25 nerve cells was measured in each preparation.

NADPH-diaphorase histochemistry

Whole mounts were prepared as above except that the fixation time was only 1 h. The preparations were incubated with a reaction medium containing 1 mg ml^{-1} β-NADPH (Sigma), 0.25 mg ml^{-1} nitroblue tetrazolium (Sigma) and 0.1 % Triton X-100 in 0.1 mol l^{-1} PB (pH 7.0). The reaction was carried out at 37 °C during 1 h and stopped by washing the preparations in PB.

For double staining, some preparations were incubated with NOS-antiserum prior to the NADPH/nitroblue tetrazolium mixture and finally visualised using Cy3-conjugated secondary antisera.

Pharmacological experiments

Circular strip preparations (approximately 2×10 mm), including the muscle layers and the mucosa, were prepared from the cardiac stomach, and similar longitudinal preparations were taken from the duodenum. All preparations were mounted in organ baths containing 5 ml McKenzie's solution (pH 7.9) (Lockwood, 1967) kept at room temperature and bubbled with 0.3 % CO_2 in air. The force developed by the smooth muscle preparations (which reflects the tension in the preparations) was recorded *via* an FT03 isometric force transducer and a Grass Model 7 polygraph, and simultaneously sampled on data-acquisition software (AD/DATA; P. Thorén, Karolinska Institute, Stockholm, Sweden). The sampling frequency was set to one sample s^{-1} and the mean values of 10 samples were calculated and stored. An initial force of 10 mN was applied to the preparations, which were left to recover for at least 1 h, until they had adopted a stable resting tension. During the recovery period, most preparations began to exhibit rhythmic contractions.

The following drugs were used: mammalian PACAP 27 (10^{-7} mol l^{-1}; Peninsula, UK), mammalian VIP (10^{-7}–10^{-6} mol l^{-1}; Auspep, Australia), GABA (10^{-7}–10^{-4} mol l^{-1}), the nitric oxide donor sodium nitroprusside (NaNP; 10^{-7}–10^{-6} mol l^{-1}) and the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 3×10^{-4} mol l^{-1}) (all Sigma).

The results are presented as the mean force developed (± S.E.M.). To normalise the sampled values, the resting tension (i.e. the tension level adopted by the preparations between spontaneous contractions) of the corresponding control period was subtracted from each data point in all experiments. Negative values indicate a reduction in resting tension, i.e. relaxation, caused by the treatment. The mean force developed during 5 min immediately before addition of the drug was compared with a 3 min (stomach) or 5 min (intestine) period after the full effect of the drug was reached or, when no apparent effect was achieved, after approximately 5 min. Wilcoxon matched-pairs, signed-ranks test was used for statistical evaluation of the results. Differences where $P<0.05$ were regarded as statistically significant.

Results

Histochemistry

PACAP-, VIP-, helospectin-, NOS- and GABA-immunoreactive nerve cell bodies or nerve fibres were found throughout the gastrointestinal tract of *Xenopus laevis*.

A high number of PACAP-immunoreactive nerve fibres occurred in all layers of the gut wall in all regions examined (Figs 1, 2). Numerous varicose fibres ran in nerve bundles in the myenteric plexus and parallel to the muscle fibres. Weakly PACAP-immunoreactive nerve cell bodies were seen in the myenteric plexus (Fig. 1C). In addition, PACAP immunoreactivity was found in endocrine cells in the mucosa (Fig. 1D).

Double labelling with PACAP and VIP (guinea pig) antisera showed a more or less complete colocalisation of the two neuropeptides in nerves (Fig. 2A–F) while no endocrine cells displayed any VIP immunoreactivity. In contrast, VIP-immunoreactive endocrine cells were demonstrated by an antiserum raised in rabbit. As mentioned in Materials and methods, preincubation of both VIP antisera with VIP quenched the immunoreactivity, including in the endocrine cells, while other members of the VIP family had no such effect. This suggests that these cells contain VIP or a very

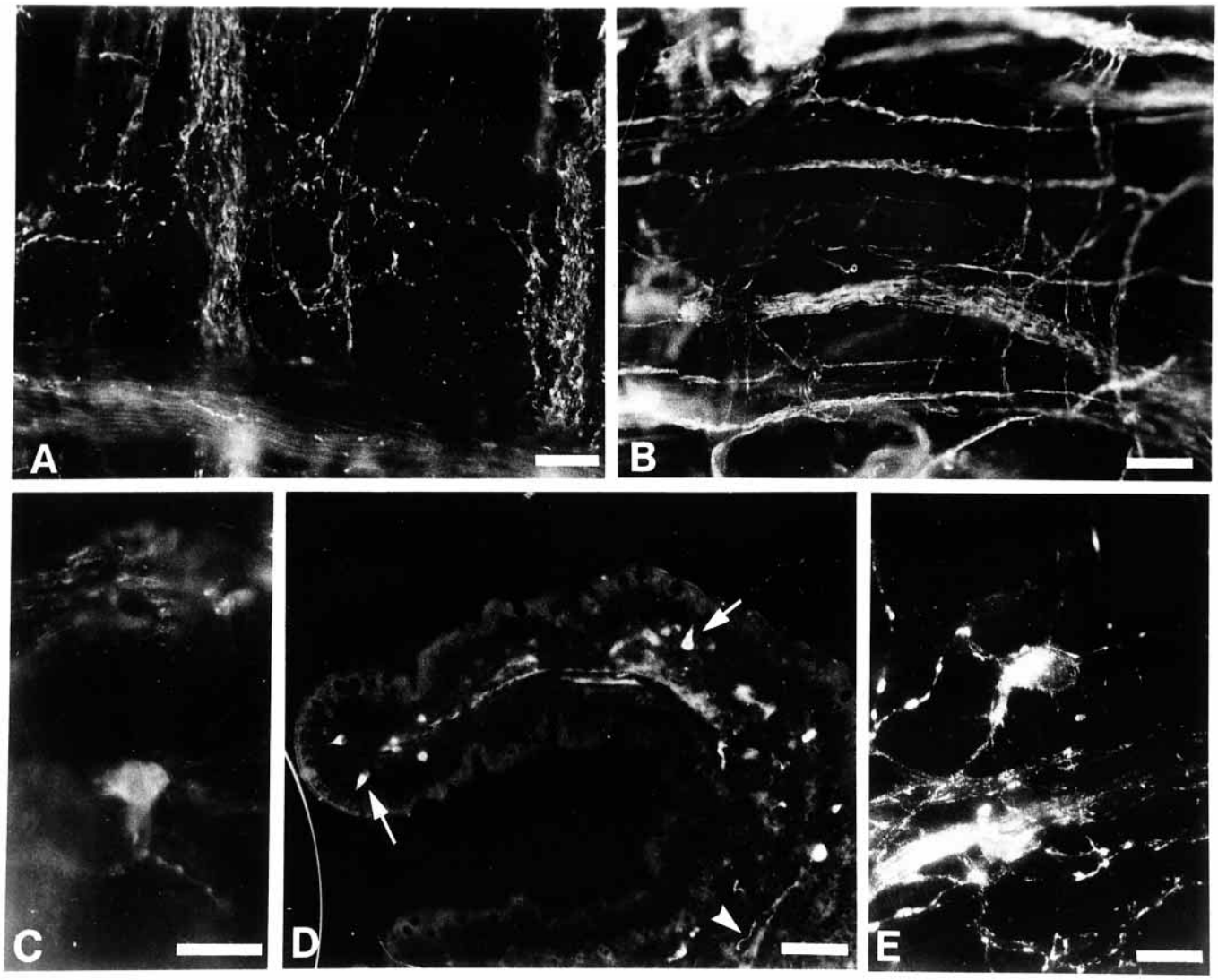

Fig. 1. PACAP and VIP immunoreactivities in the gastrointestinal tract of *Xenopus laevis*. (A,B) Extensive networks of PACAP-immunoreactive nerve fibres in the myenteric plexus of the distal intestine (A) and rectum (B). (C) PACAP-immunoreactive nerve cell body in the myenteric plexus of the pyloric stomach. (D) PACAP-immunoreactive endocrine cells (arrows) and nerve fibres (arrowhead) in the mucosa of the proximal intestine. (E) VIP-immunoreactive myenteric nerve cell in the distal intestine. Bars: 25 μm (C,E), 50 μm (A,D), 100 μm (B).

similar substance although this could not be detected using the guinea pig antiserum. Double labelling with VIP and helospectin antisera showed a high degree of colocalisation although the helospectin immunoreactivity in general was weaker (Fig. 2G–H).

Numerous NOS-immunoreactive or NADPH-diaphorase reactive nerve cell bodies were found in the myenteric plexus all along the gastrointestinal tract except in the cloaca (Fig. 3). Double staining demonstrated that the same nerve cells were stained with the two methods. Varicose nerve fibres were seen with both methods but were most intensely stained by the NADPH-diaphorase method (Fig. 3I). The NOS-reactive nerve cells were usually located in close association with nerve bundles (Fig. 3A,F,H,I) and most common in the middle intestine (Table 2). The average density for the whole gut was 4584±540 cells cm^{-2} (*N*=3, *n*=457 cells). A majority of the nerve

Table 2. *Average density and size of NOS-immunoreactive nerve cells in the gastrointestinal tract of* Xenopus laevis

Tissue	Density (cells cm^2)	Largest diameter (μm)	Smallest diameter (μm)
Cardiac stomach	3109±471	25.8±2.48	12.56±0.67
Pyloric stomach	4758±1143	21.63±1.03	10.47±0.36
Proximal small intestine (duodenum)	4900±535	24.54±2.29	11.55±1.84
Middle small intestine (upper ileum)	6172±2184	23.05±0.95	10.25±2.70
Distal small intestine (lower ileum)	4837±536	21.95±1.03	11.66±0.91
Rectum	2992±167	23.83±4.01	11.12±1.72

Values are means ± S.D. (*N*=3, except for rectum, *N*=2).

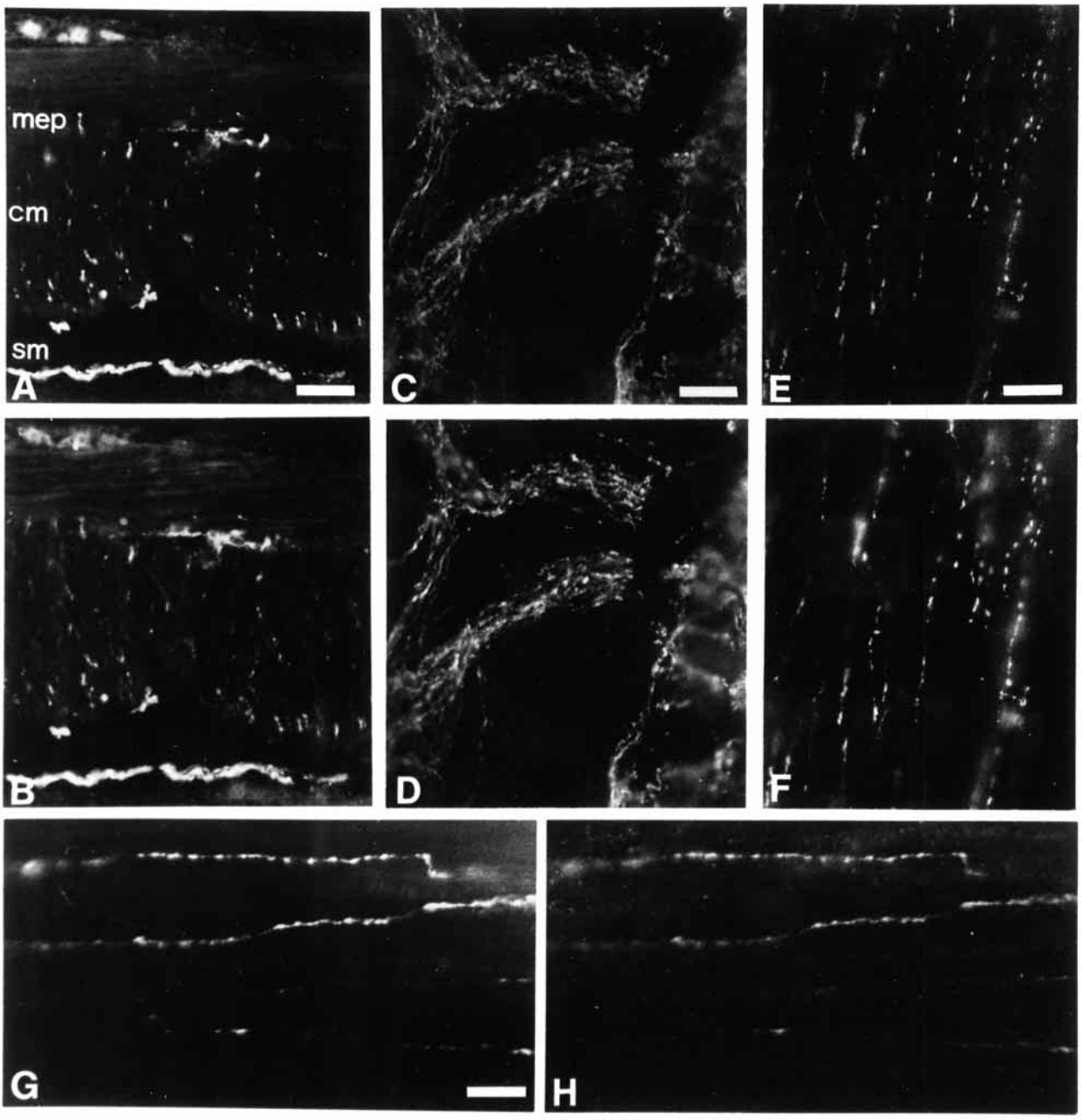

Fig. 2. PACAP, VIP and helospectin immunoreactivities in the gastrointestinal tract of *Xenopus laevis*. (A,B) Double-staining of a section through the cardiac stomach showing a high degree of colocalisation between PACAP (A) and VIP (B) immunoreactivities in the myenteric plexus (mep), the circular muscle layer (cm) and submucosa (sm). (C,D) A high degree of colocalisation between PACAP (C) and VIP (D) immunoreactivities in nerve fibres in the myenteric plexus of the pyloric stomach. (E,F) Colocalisation between PACAP (E) and VIP (F) immunoreactivities in varicose nerve fibres in the circular muscle layer of the proximal intestine. (G,H) Colocalisation between helospectin (G) and VIP (H) immunoreactivities in varicose nerve fibres along the circular muscle fibres in the cardiac stomach. Bars: 25 μm (E–H), 50 μm (A–D).

cells were multipolar with an average soma size of 11.3±3.7 × 23.2±6.6 μm (Table 2). Sections showed NOS-immunoreactive nerve fibres in both muscle layers, in the submucosa and the mucosa and in endocrine cells in the intestinal mucosa (Fig. 3C,D).

Double staining of the preparations showed partial overlap between NOS- and VIP-immunoreactive nerve fibres (Fig. 3). The degree of colocalisation was most easily observed in the muscle layers where a majority of NOS-positive nerve fibres were VIP-immunoreactive (Fig. 3D,E). The overall density of VIP-immunoreactive fibres was higher compared to the density of NOS-positive fibres, indicating a large number of NOS-negative VIP-immunoreactive fibres (Fig. 3A,B,F,G). There was also colocalisation in some nerve cell bodies, but due to

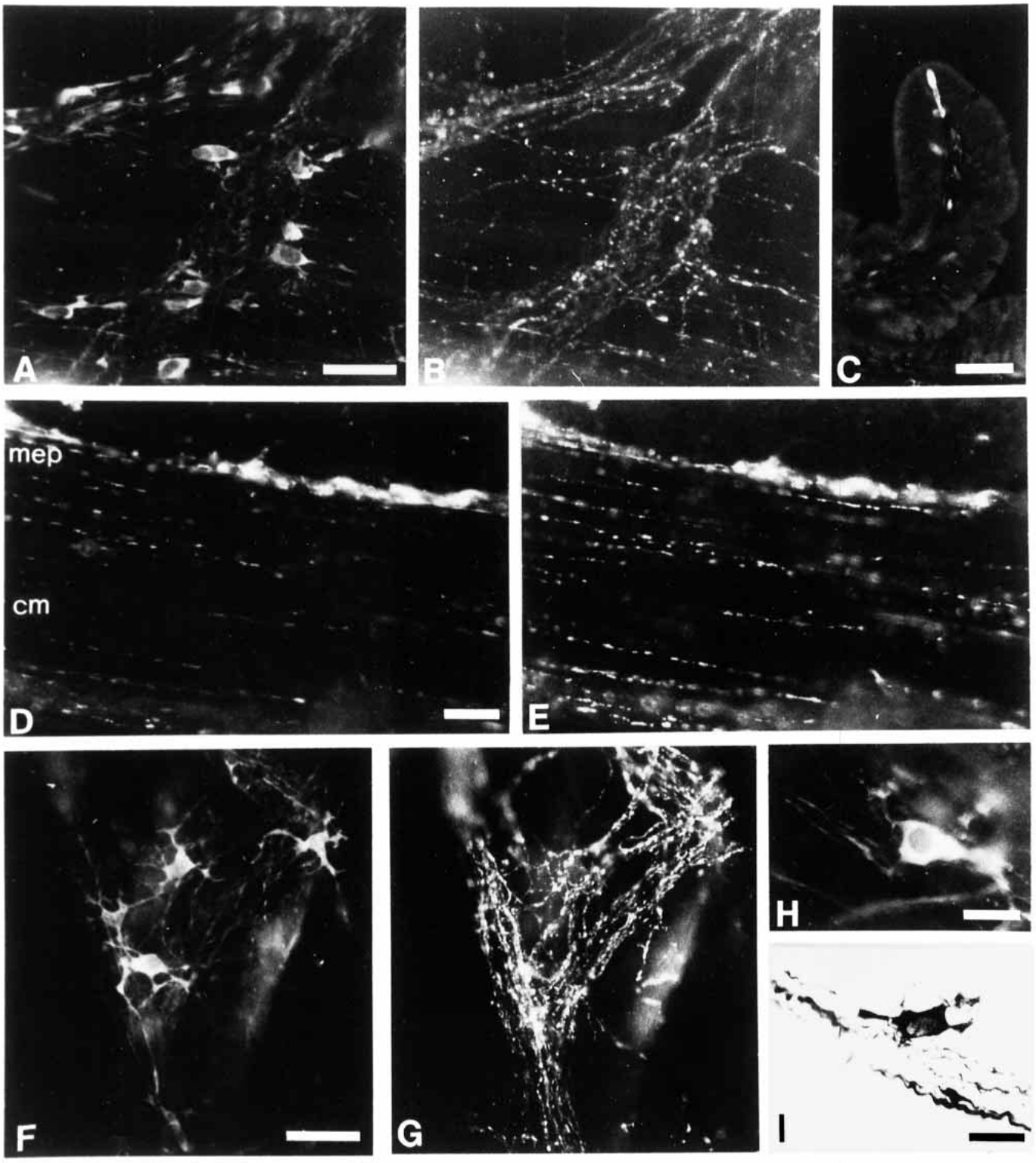

Fig. 3. NOS and VIP immunoreactivities and NADPH-diaphorase reactivity in the gastrointestinal tract of *Xenopus laevis*. (A,B) Double-staining of the myenteric plexus (mep) of the proximal intestine showing NOS- (A) and VIP-immunoreactive nerve fibres (B) but only NOS-immunoreactive nerve cell bodies. Most VIP-immunoreactive nerve fibres are not NOS-immunoreactive. (C) NOS-immunoreactive endocrine cell in the mucosa of the proximal intestine. (D,E) Section through the proximal intestine showing colocalisation between NOS- (D) and VIP-immunoreactive nerve fibres (E) in the circular muscle layer (cm). (F,G) Double-staining of the myenteric plexus of the distal intestine showing NOS- (F) and VIP-immunoreactive nerve fibres (G) but only NOS-immunoreactive nerve cells bodies. Most VIP-immunoreactive nerve fibres are not NOS-immunoreactive. (H) NOS-immunoreactive nerve cell body in the myenteric plexus of the rectum. (I) NADPH-diaphorase-reactive nerve cell body and nerve fibres in the myenteric plexus of the rectum. Bars: 25 μm (D,E,H), 50 μm (A–C,F,G,I).

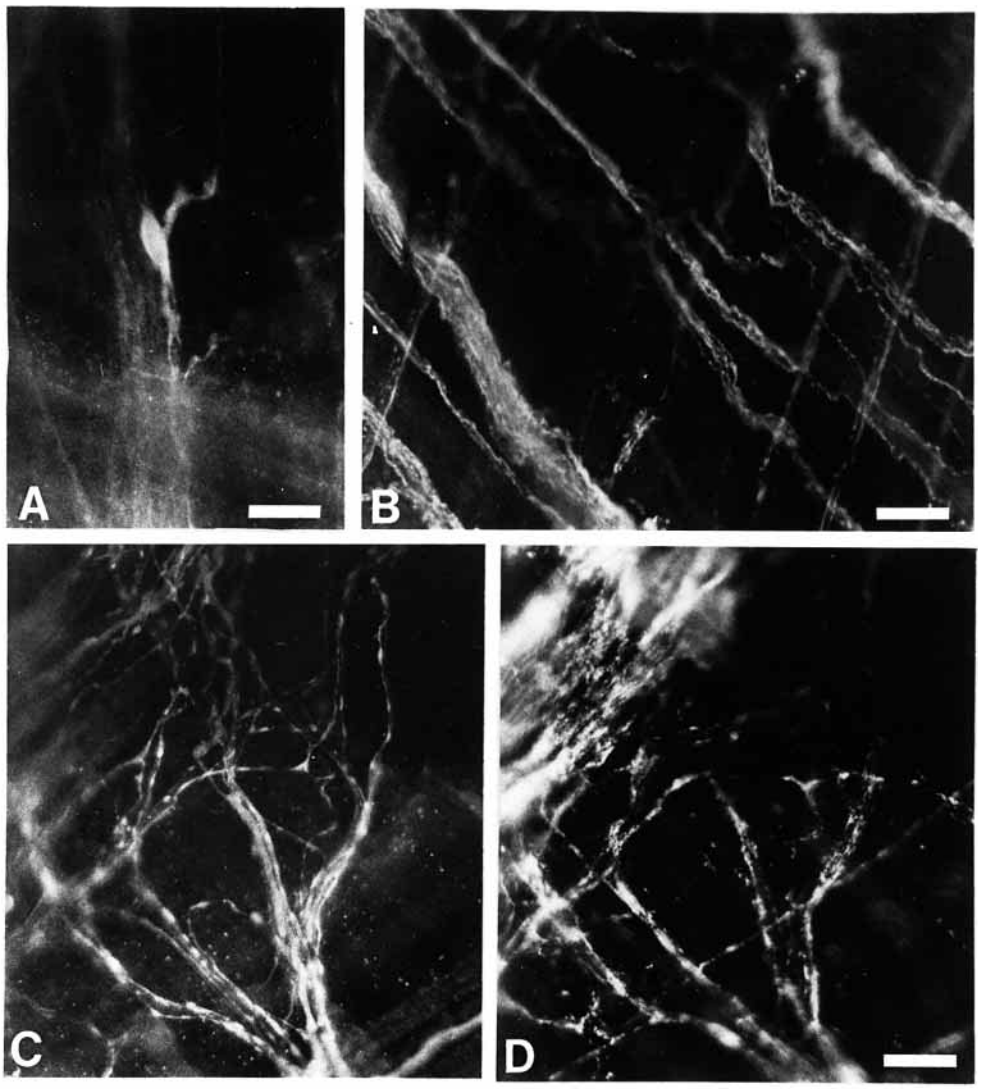

Fig. 4. GABA and VIP immunoreactivities in the gastrointestinal tract of *Xenopus laevis*. (A) GABA-immunoreactive bipolar nerve cell body in the myenteric plexus of the cardiac stomach. (B) Extensive network of GABA-immunoreactive nerve fibres in the myenteric plexus of the rectum. (C,D) Double-staining of nerve fibres in the myenteric plexus of the rectum. Most GABA-immunoreactive nerve fibres are not VIP-immunoreactive and *vice versa*. Bars: 25 μm (A,C,D) 50 μm (B).

weak staining of cell bodies with the VIP antiserum, no quantification was done.

The antiserum against GABA revealed nerve fibres and/or nerve cell bodies in the myenteric plexus in all regions examined (Fig. 4). GABA-immunoreactive nerve fibres were frequent in the submucosa and myenteric plexus and some were seen in the muscle layers. In the myenteric plexus, the vast majority of GABA-immunoreactive nerve fibres did not express VIP immunoreactivity (Fig. 4C,D), while in the submucosa, the number of fibres showing colocalisation of GABA and VIP was higher, although still less than 50 %. The GABA-immunoreactive nerve cell bodies were small, usually bipolar, or larger and multipolar. In the rectum, which showed a well developed network of GABA-immunoreactive nerve fibres (Fig. 4B,C), the number of immunoreactive nerve cell bodies was low, and no nerve cell bodies were seen in the cloaca.

Pharmacological experiments

The circular preparations of the cardiac stomach usually developed spontaneous contractions with varying amplitude and frequency (Fig. 5). PACAP 27, VIP, NaNP and GABA had similar effects on the gastric motility and reduced the mean force developed, mainly by lowering the resting tension, while not necessarily affecting the frequency or amplitude of the contractions (Fig. 5). PACAP 27 (10^{-7} mol l^{-1}) reduced the mean force developed from 0.65±0.17 mN to –0.42±0.26 mN (N=8) while VIP (10^{-7} or 10^{-6} mol l^{-1}) lowered the mean force developed from 0.39±0.07 mN to –0.34±0.16 mN and –0.73±0.18 mN (N=6), respectively (Fig. 6). The nitric oxide donor NaNP (10^{-7} or 10^{-6} mol l^{-1}) caused a reduction in mean force developed from 0.49±0.15 mN to –0.44±0.31 mN (N=6) and –1.06±0.25 mN (N=8) (Fig. 7). GABA had no effect at the lowest concentration tested (10^{-7} mol l^{-1}) but reduced the mean force developed from 0.63±0.17 mN to 0.05±0.21 mN at 10^{-6} mol l^{-1} and to –0.48±0.20 mN at 10^{-5} mol l^{-1} (N=6). No further reduction was seen at 10^{-4} mol l^{-1} (N=6) (Fig. 6). The L-arginine analogue L-NAME, increased the mean force developed from 0.46±0.09 mN to 1.15±0.37 mN (N=6), indicating a tonic release of inhibitory nitric oxide (Fig. 7). The increase in mean force was mainly due to an increase in amplitude of the spontaneous contractions.

The longitudinal preparations of the proximal small intestine

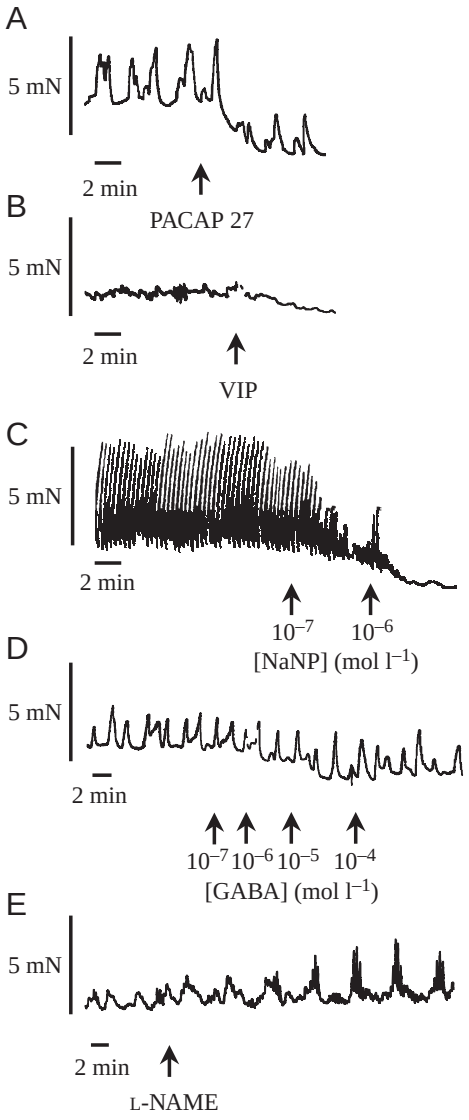

Fig. 5. Original tracings showing the effect of PACAP 27 (A; 10^{-7} mol l^{-1}), VIP (B; 10^{-6} mol l^{-1}), NaNP (C; 10^{-7} and 10^{-6} mol l^{-1}), GABA (D; 10^{-7}–10^{-4} mol l^{-1}) and L-NAME (E; 3×10^{-4} mol l^{-1}) on the cardiac stomach of *Xenopus laevis*. The appearance of spontaneous activity varied between individual preparations. PACAP 27, VIP, NaNP and GABA reduced the resting tension while the NOS inhibitor L-NAME enhanced the activity.

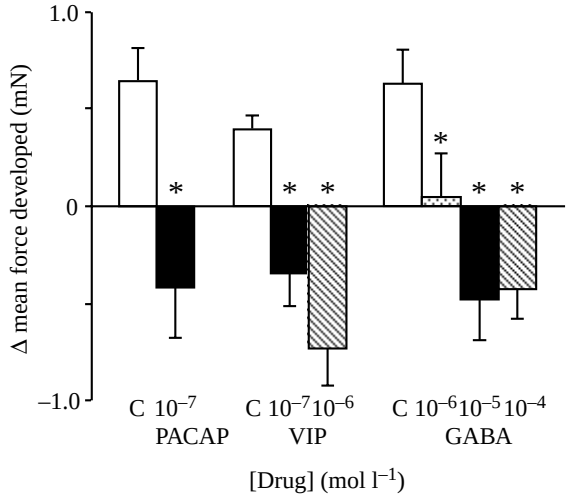

Fig. 6. The effect of PACAP 27 (10^{-7} mol l^{-1}, *N*=8), VIP (10^{-7} and 10^{-6} mol l^{-1}, *N*=6) and GABA (10^{-6}–10^{-4} mol l^{-1}, *N*=6) on the circular smooth muscles of the cardiac stomach of *Xenopus laevis*. PACAP 27, VIP and GABA reduced the mean force developed by the stomach. The results are presented as mean force developed (± S.E.M.) above resting level. Open bars represent values immediately before addition of drug. **P*≤0.05 compared to the control (C).

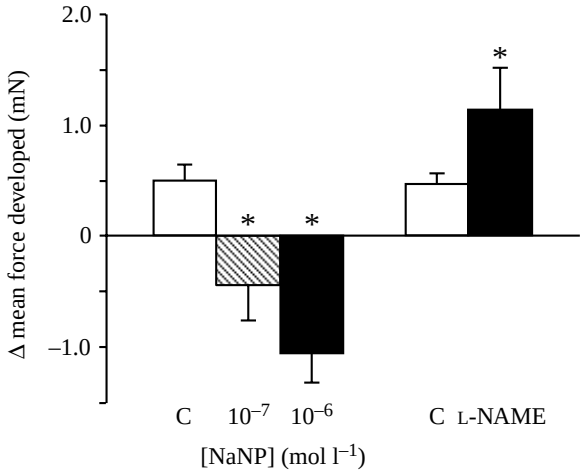

Fig. 7. The effect of NaNP (10^{-7} mol l^{-1}, *N*=6; 10^{-6} mol l^{-1}, *N*=8) and L-NAME (3×10^{-4} mol l^{-1}, *N*=6) on the circular smooth muscles of the cardiac stomach of *Xenopus laevis*. NaNP reduced the mean force developed while L-NAME enhanced the force developed. The results are presented as mean force developed (± S.E.M.) above resting level. Open bars represent values immediately before addition of drug. **P*≤0.05 compared to the control (C).

usually developed spontaneous contractions that were more regular, with larger amplitudes, compared to the stomach. PACAP 27, VIP, NaNP, GABA and L-NAME had no significant effect on the motility of these intestinal preparations (Figs 8, 9).

Discussion

This is the first study showing the distribution of PACAP immunoreactive material in the enteric nervous system of an amphibian. It demonstrates colocalisation of PACAP and VIP in enteric nerves and, in addition, the colocalisation of VIP with NOS and helospectin and, to a lesser extent, GABA immunoreactivities. It is also the first demonstration of the effects of PACAP, VIP, nitric oxide and GABA on gastrointestinal motility in anuran amphibians.

PACAP immunoreactivity has been demonstrated throughout the gastrointestinal tract in several mammals as well as in the Atlantic cod and the rainbow trout (Sundler et al., 1992; Olsson and Holmgren, 1994). The distribution in *X.*

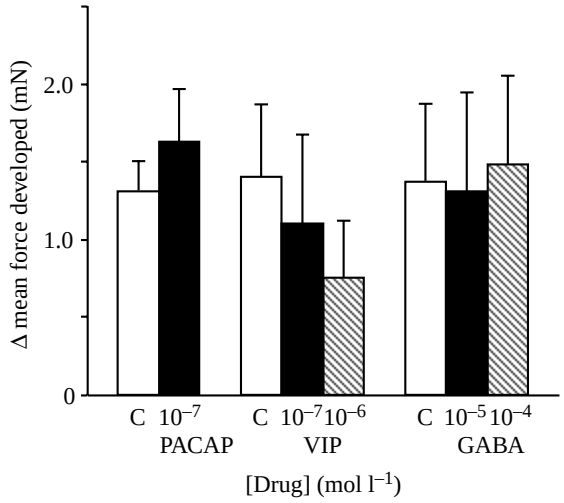

Fig. 8. The effect of PACAP 27 (10^{-7} mol l^{-1}, *N*=7), VIP (10^{-7} mol l^{-1}, *N*=6; 10^{-6} mol l^{-1}, *N*=5) and GABA (10^{-5} mol l^{-1}, *N*=5; 10^{-4} mol l^{-1}, *N*=6) on the longitudinal smooth muscles of the proximal intestine of *Xenopus laevis*. Neither PACAP 27 nor VIP nor GABA had any effect on the force developed by the intestine. The results are presented as mean force developed (± S.E.M.) above resting level. Open bars represent values immediately before addition of drug. **P*≤0.05 compared to the control (C).

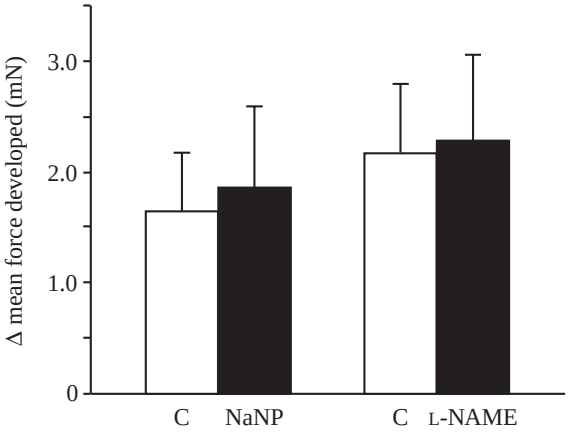

Fig. 9. The effect of NaNP (10^{-6} mol l^{-1}, *N*=6) and L-NAME (3×10^{-4} mol l^{-1}, *N*=5) on the longitudinal smooth muscles of the proximal intestine of *Xenopus laevis*. Neither NaNP nor L-NAME had any effect on the force developed by the intestine. The results are presented as mean force developed (± S.E.M.) above resting level. Open bars represent values immediately before addition of drug. **P*≤0.05 compared to the control (C).

laevis is in good agreement with these previous studies, with a dense PACAP innervation of the longitudinal and the circular muscle layers and the mucosa. The degree of colocalisation of PACAP and VIP varies somewhat between species. In *X. laevis*, as in cod, rainbow trout, chicken and human, the two immunoreactivities overlap to approximately 100 % (Sundler et al., 1992; Olsson and Holmgren, 1994). In contrast, in several other mammals the two peptides exist in separate nerve populations (Sundler et al., 1992). VIP and helospectin immunoreactivities are colocalised in enteric nerves in most species investigated (Absood et al., 1992; Olsson and Holmgren, 1994). Since the staining of VIP/PACAP/helospectin immunoreactive nerve cells bodies was weak in *X. laevis*, no quantification of the density of cells was done. The antiserum against VIP used in double-staining experiments did not reveal any endocrine cells, making it impossible to investigate any colocalisation of VIP and PACAP or VIP and helospectin in endocrine cells. In mammals, no PACAP-immunoreactive endocrine cells have been demonstrated, whereas they are common in chicken and fish (Sundler et al., 1992; Olsson and Holmgren, 1994). In the cod and the rainbow trout, these cells also contain VIP (Olsson and Holmgren, 1994). Helospectin-immunoreactive endocrine cells are found in mammals as well as in fish, but only the latter coexpress VIP. In summary, the distribution of VIP-, PACAP- and helospectin immunoreactivities in the enteric nervous system of *X. laevis* is similar to the patterns found in most other vertebrates (Buchan, 1986; Absood et al., 1992; Sundler et al., 1992; Olsson and Holmgren, 1994), indicating similar functions in different species.

The density of NOS-immunoreactive nerve cell bodies has been determined in several vertebrates (Li et al., 1992, 1994; Timmermans et al., 1994; Olsson and Karila, 1995). The present data show that the number of cells in the gastrointestinal tract of *X. laevis* is within the same range but the regional distribution may vary somewhat. A subpopulation of the NOS-immunoreactive nerve fibres were VIP-immunoreactive, but not all VIP-positive fibres were NOS-immunoreactive, which indicates the presence of three different neuronal subpopulations in *X. laevis*: one containing VIP/PACAP/helospectin/NOS, one containing VIP/PACAP/helospectin and one containing NOS. The same three populations are found in many species investigated although the relative number of each population may vary (Costa et al., 1992; Aimi et al., 1993; Li et al., 1993; Olsson and Karila, 1995; Olsson and Gibbins, 1999). While characterising several classes of descending and ascending myenteric neurons, Costa et al. (1996) demonstrated that VIP and NOS are found in both inter- and motorneurons. In the toad *Bufo marinus*, a majority of the VIP-immunoreactive nerve fibres were NOS-immunoreactive as well (Li et al., 1993) and VIP- and NOS-containing nerves project anally in the toad intestine, indicating an inhibitory function (Murphy et al., 1993).

GABA-immunoreactive nerve fibres, in addition to a low number of nerve cell bodies, have previously been described within the amphibian stomach (Gabriel and Eckert, 1989). In the present study, GABA immunoreactivity was demonstrated in nerves in all regions of the gut. In most regions, GABA-immunoreactive nerve cell bodies were common. Similar to porcine enteric neurons, both multipolar and bipolar cells are found in frog (this study; Gabriel and Eckert, 1989; Wu et al., 1998).

Few studies concerning the control of gastrointestinal motility have been performed on *X. laevis* or other amphibians. Since the investigated, presumed neurotransmitters were

common in the enteric nervous system of *X. laevis*, and had a distribution similar to other vertebrates, their effects on the stomach and intestine motility were examined. PACAP, VIP, nitric oxide and GABA all reduced the mean force developed by the circular muscles of the cardiac stomach, while giving inconsistent responses in the longitudinal preparations of the duodenum. The lack of an inhibitory effect on the intestine contrasts with findings in the Atlantic cod where PACAP and nitric oxide abolished the spontaneous contractions (Olsson and Holmgren, 2000). Previous studies have demonstrated that the rhythmic activity in the gastrointestinal tract of amphibians involves interstitial cells of Cajal located in the longitudinal muscle layer or in the myenteric plexus (Prosser, 1995). The rhythmic activity of the stomach and intestine in *X. laevis* is not affected by TTX (Å. Johansson and S. Holmgren, unpublished observations), indicating that the contractions are not dependent on enteric nerves. In cod, however, atropine abolished the contractions, demonstrating the presence of a cholinergic tone necessary for maintaining the spontaneous contractions (Olsson and Holmgren, 2000).

In mammals, the effect of PACAP and VIP on gastrointestinal motility is mediated mainly *via* $VPAC_1$ and $VPAC_2$ receptors, which stimulate adenylate cyclase and thereby raise the levels of cAMP (Harmar et al., 1998). Recently, a VIP/PACAP receptor has been cloned from the frog *Rana ridibunda* and it has also been demonstrated in the gastrointestinal tract. This receptor shows characteristics of both mammalian $VPAC_1$ and $VPAC_2$ receptors and it has been speculated that amphibians possess only one single receptor form (Alexandre et al., 1999). When the VPAC receptor was expressed in mammalian cells, VIP and PACAP were equally potent in stimulating cAMP production (Alexandre et al., 1999). Although no further characterisations of the receptor were carried out in the present study, it is likely that at least part of the effect of VIP and PACAP on gastric motility is mediated *via* a similar receptor. The lack of effect on the intestine could be due to low concentrations of the receptor or to a shift in affinity for the two peptides. Alexandre et al. (1999) showed that the amphibian VPAC receptor were common in the stomach while only low levels where detected in the intestine. Although so far only one type of VIP/PACAP receptor has been identified in the amphibian gut, other subtypes may occur, exhibiting slightly different affinities and effects on motor activity. For example, there is evidence for a PAC_1 (PACAP preferring) receptor in the frog, but its presence in the gastrointestinal tract has not yet been confirmed (Alexandre et al., 1999). Furthermore, the effect of PACAP and VIP (and similarly of nitric oxide and GABA) on the intestine could be masked by the high amplitude of the spontaneous contractions. The substances may also play a role in, for example, secretion and absorption.

The inhibitory effect of GABA on gastrointestinal motility is usually mediated *via* $GABA_A$ receptors (stimulatory) on inhibitory neurons and/or $GABA_B$ receptors (inhibitory) on cholinergic neurons (Grider and Makhlouf, 1992; Minocha and Galligan, 1993). In addition, $GABA_A$ receptors on the cholinergic neurons stimulate the release of acetylcholine, causing contractions of the smooth muscles (Krantis et al., 1980; Grider and Makhlouf, 1992; Minocha and Galligan, 1993). The nature and distribution of GABA receptors need to be further investigated before any conclusion can be drawn about how the effect of GABA is mediated in *Xenopus*.

Several studies have investigated the interactions between different inhibitory substances. For example, it has been shown that nitric oxide facilitates the release of VIP in several tissues (Grider, 1993; Grider and Jin, 1993; Daniel et al., 1994; Grider et al., 1994). Other studies have demonstrated that the production of nitric oxide is stimulated by VIP and/or GABA (Li and Rand, 1990; Boeckxstaens et al., 1991; Grider, 1993; Jin et al., 1993; Grider et al., 1994). Recently, Krantis and coworkers demonstrated that nitric oxide is responsible for the spontaneous relaxation in the gastric antrum and duodenum, and that VIP-, GABA- and ATP-induced relaxation are mediated by nitric oxide (Glasgow et al., 1998; Krantis et al., 1998). In addition, VIP can stimulate the release of GABA. However, in some studies no stimulatory effect of VIP/PACAP on the production of nitric oxide could be demonstrated (D'Amato et al., 1992; Ekblad and Sundler, 1997). It is probable that nitric oxide, VIP, PACAP and GABA cooperate in *Xenopus laevis* as well, but more research is needed.

To conclude, this study shows the presence of some putative neurotransmitters in enteric neurons in *Xenopus laevis*. The distribution of PACAP, VIP, NOS and GABA is similar to the situation observed in mammals as well as in some non-mammalian species including elasmobranchs, teleosts, other amphibians and reptiles. Furthermore, it is the first report of the inhibitory effect on gastrointestinal motility of these substances in an amphibian, as PACAP, VIP, nitric oxide and GABA cause relaxation of the cardiac stomach in *Xenopus*. The widespread distribution of PACAP, VIP, NOS and GABA in different groups of vertebrates, in addition to the similarities in effect on the gut smooth muscle, indicates that these substances play an important role in the inhibitory control of gut motility. These neurotransmitters, as well as some of their receptors, probably arose early in the evolution of vertebrates, or even before that as there are reports of NOS, for example, in invertebrate species (Johansson and Carlberg, 1995). The structures and the effects and intracellular pathways involved have been well preserved during this time. Although we did not see any effect on the duodenum in our experiments this does not necessarily rule out the possibility that PACAP, VIP, nitric oxide and/or GABA are involved in the control of intestinal as well as gastric motility.

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