PACAP AND NITRIC OXIDE INHIBIT CONTRACTIONS IN THE PROXIMAL INTESTINE OF THE ATLANTIC COD, GADUS MORHUA

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Accepted 18 November 1999; published on WWW 17 January 2000

Summary

The possible inhibitory roles of pituitary adenylate cyclase-activating polypeptide (PACAP), vasoactive intestinal polypeptide (VIP) and nitric oxide in the control of intestinal motility were investigated in the Atlantic cod, Gadus morhua. Circular and longitudinal smooth muscle preparations developed spontaneous contractions that were inhibited by atropine (10⁻⁵ mol l⁻¹). PACAP 27 and PACAP 38 (10⁻⁷ mol l⁻¹) reduced the amplitude of the contractions but did not usually affect the resting tension. In the circular preparations, the mean active force developed (above resting level; ± S.E.M.) was reduced from 0.51±0.12 mN to 0.38±0.12 mN (N=8) and from 1.49±0.36 mN to 0.33±0.22 mN (N=7), respectively. However, preincubation with L-NAME before a second addition of PACAP 27 (10⁻⁷ mol l⁻¹) did not affect the response to PACAP, neither did preincubation with guanylate cyclase inhibitor 6-anilinoquinoline-5,8-quinone (10⁻⁷ mol l⁻¹), while the inhibitory response to NaNP (3×10⁻⁷ mol l⁻¹) was abolished by LY83583. The PACAP analogue PACAP 6–27 (3×10⁻⁷ mol l⁻¹) had no effect on the response to either NaNP (3×10⁻⁷ mol l⁻¹) or PACAP 27 (10⁻⁷ mol l⁻¹) in the circular preparations. These findings indicate the presence of both a cholinergic and a nitrergic tonus in the smooth muscle preparations of the cod. Although PACAP and NaNP both inhibit contractions, there is no evidence of any interactions between the two substances. In addition, NaNP, but not PACAP, probably acts via stimulating the production of cyclic GMP. In conclusion, both PACAP and nitric oxide may act as inhibitory transmitters, using distinct signalling pathways, in the control of intestinal motility in the Atlantic cod.

Key words: teleost, PACAP, VIP, sodium nitroprusside, L-NAME, motility, Atlantic cod, Gadus morhua.

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal polypeptide (VIP) genes evolved from a common ancestral gene more than 750 million years ago (Campbell and Scanes, 1992), and the amino acid sequences of the corresponding peptides have been determined in species from most groups of vertebrates (Hoyle, 1998). Amongst fish, cod VIP and salmon PACAP show 82% and 92% identity to their mammalian counterparts, respectively (Thwaites et al., 1989; Parker et al., 1993). In mammals, PACAP exists in two separate isoforms; the full-length peptide contains 38 amino acids (PACAP 38) while PACAP 27 is C-terminally truncated. Whether both isoforms exist in other vertebrates remains to be elucidated.

PACAP and VIP are found in enteric nerves of most vertebrate species investigated (Sundler et al., 1992; Jensen and Holmgren, 1994; Olsson and Holmgren, 1994). In the Atlantic cod, Gadus morhua, the two peptides coexist in nerve cells in the myenteric plexus as well as in nerve fibres innervating the circular and longitudinal muscle layers and the mucosa all along the gastrointestinal canal (Olsson and Holmgren, 1994). In addition, a majority of the myenteric neurons contain nitric oxide synthase (NOS), the enzyme responsible for nitric oxide production, and a sub-population of these neurons co-express NOS, PACAP and VIP (Olsson and Karila, 1995).

In mammals, the effect of PACAP, VIP and nitric oxide on...
gastrointestinal motility is mainly inhibitory. PACAP and VIP usually bind to G-protein-coupled receptors, causing stimulation of adenylyl cyclase and subsequently increased levels of cyclic AMP (cAMP; Jin et al., 1993; Harmar et al., 1998), while nitric oxide diffuses into the target cells and activates soluble guanylyl cyclase leading to cGMP production (see Shuttleworth and Sanders, 1996). Nitric oxide is produced and released at low stimulation frequencies, while VIP requires higher frequencies (Li and Rand, 1990; Maggi and Giuliani, 1996). It has also been demonstrated that VIP and nitric oxide can be co-released from enteric neurons and that nitric oxide initiates a relaxation, which is sustained by VIP (Boeckxstaens et al., 1991; D’Amato et al., 1992). In addition, several studies indicate that the release of VIP or PACAP is facilitated by nitric oxide (Grider, 1993; Grider and Jin, 1993; Daniel et al., 1994; Grider et al., 1994; Allescher et al., 1996). Whether this includes nitric oxide produced within the same nerve cell as releases VIP, nitric oxide from neighbouring nerve cells or nitric oxide produced in muscle cells or interstitial cells of Cajal (ICC) is under debate. In addition, there are conflicting reports about the stimulatory effect of VIP on nitric oxide production (Li and Rand, 1990; Boeckxstaens et al., 1991; D’Amato et al., 1992; Grider, 1993; Jin et al., 1993; Keef et al., 1994; Maggi and Giuliani, 1996; Ekblad and Sundler, 1997; Murthy et al., 1997).

Little is known about the mechanisms behind the inhibitory control of gastrointestinal motility in non-mammalian species. In the cod intestine, as well as in the stomach of the rainbow trout, Oncorhynchus mykiss, NOS inhibitors reduce the inhibitory responses to electrical stimulation (Green and Campbell, 1994; Karila and Holmgren, 1995). Mammalian VIP does not affect the rhythmic activity of the perfused intestine of the Atlantic cod (Jensen and Holmgren, 1985) while the effect of PACAP has not previously been investigated in any fish species. In an attempt to elucidate the inhibitory mechanisms involved in the control of fish gut motility, we wanted to examine further the effects of nitric oxide on the proximal intestine of the Atlantic cod, Gadus morhua, and to compare the effects of PACAP and mammalian and cod VIP.

Materials and methods

Atlantic cod (Gadus morhua L.), of either sex and with a body mass of 150–1100 g, were used. They were captured by local suppliers on the Swedish west coast and kept in aerated, recirculating sea water at 10 °C. The fish were not fed in captivity (maximum 4 weeks).

Prior to the experiments, the cod were killed by a sharp blow to the head. Longitudinal strips (approximately 2×10 mm) or ring preparations (approximately 5 mm wide) were prepared from the proximal intestine. The preparations were mounted in organ baths containing cod Ringer’s solution (in mmol l⁻¹: NaCl, 150.1; KCl, 5.2; MgSO₄, 1.8; CaCl₂, 1.9; Na₂HPO₄, 1.9; NaHCO₃, 7.0; glucose, 5.6, pH 7.8, Karila et al., 1993) kept at 10 °C and bubbled with 0.3 % CO₂ in air. The force developed by the preparations (reflecting the tension of the smooth muscles) was recorded using FT03 force transducers 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 and they were left to recover. Within 1–2 h, the preparations had usually developed rhythmic activity with a stable basal (resting) tension.

Data analysis

Data 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 contractions) of the corresponding control period was subtracted from each data point. Negative values indicate a reduction in resting tension (i.e. relaxation) caused by the treatment. The mean activity during 10 min immediately before addition of the agonist or antagonist was compared to a 3 min (agonist) or 5 min (antagonist) period after the full effect of the drug was reached. Wilcoxon matched-pairs, signed-ranks test was used for statistical evaluation of the results. In the case of repeated testing, a sequentially rejective Bonferroni test (Holm, 1979) was used to reduce the risk of discarding any true null hypothesis. Differences where P<0.05 were regarded as statistically significant.

Drugs

The following drugs were used: atropine sulphate, apamin, N⁵-nitro-L-arginine methyl ester (L-NAME), N⁵-nitro-L-arginine (L-NOARG) and sodium nitroprusside (NaNP) (all from Sigma), 6-anilinoquinoline-5, 8-quinone (LY83583, Calbiochem), pituitary adenylate cyclase-activating polypeptide (PACAP) 27, PACAP 38 and PACAP 6-27 (all from Peninsula), mammalian vasoactive intestinal polypeptide (VIP) (Auspep), cod VIP (Genosys).

The drugs were dissolved according to the recommendations of the suppliers and stock solutions were kept at −40 °C. They were diluted to the final working concentrations in cod Ringer’s solution on the day of the experiment.

Results

Preparations that did not develop spontaneous contractions were not used for further studies. The appearance, amplitude and frequency of the contractions varied substantially between individual preparations. In some preparations, the contractions occurred in bursts of peaks at a frequency of approximately one burst every 2–10 min (e.g. Fig. 1D) while other preparations had continuous contractions with single peaks (e.g. see Fig. 6B). The longitudinal preparations had higher resting levels than the circular preparations, the latter being close to zero. The spontaneous activity could be blocked by atropine (10⁻⁵ mol l⁻¹; Fig. 1A).
PACAP

PACAP 27 and PACAP 38 reduced the amplitude of the contractions on both circular and longitudinal muscle preparations while, usually, the resting tension was little affected (Figs 1B, 2, 3). On the circular preparations, PACAP 27 reduced the mean force developed from 0.62±0.18 mN to 0.21±0.07 mN at 10⁻⁸ mol l⁻¹ and to 0.03±0.03 mN at 10⁻⁷ mol l⁻¹ (N=10; Fig. 2). PACAP 38 had no effect at 10⁻⁸ mol l⁻¹ but reduced the mean force developed from 0.53±0.20 mN to 0.31±0.13 mN at 10⁻⁷ mol l⁻¹ (N=7; Fig. 2).

VIP

Neither mammalian (N=6) nor cod VIP (N=7) had any effect on the amplitude or frequency of contractions or on the resting tension in the circular muscle preparations within the concentration range tested (10⁻¹⁰–10⁻⁶ mol l⁻¹; Figs 1D, 2). Preliminary studies showed no effect of the highest concentrations of cod VIP on the longitudinal preparations either.

Nitric oxide

The nitric oxide donor sodium nitroprusside (NaNP) reduced the amplitude of the contractions on the circular and longitudinal preparations (Figs 1C, 2, 3). On the circular preparations, NaNP at 10⁻⁶ mol l⁻¹ and 10⁻⁵ mol l⁻¹ almost abolished the contractions, reducing the mean force developed from 0.47±0.05 mN to 0.02±0.06 mN (N=9) and ±0.07 mN (N=8), respectively (Fig. 2). On the longitudinal preparations, NaNP reduced the force developed from 2.03±0.36 mN to 0.33±0.22 mN (10⁻⁷ mol l⁻¹; N=8) and 0.19±0.30 mN (10⁻⁵ mol l⁻¹; N=8) (Fig. 3).

L-arginine analogues

The effect of repeated doses of PACAP was tested by adding...
a second dose of PACAP 27 (10^{-7} \text{mol l}^{-1}) when the preparations had regained stable activity after washout of the first dose (minimum 1 h). No statistically significant differences (P>0.05) between the first and the second response to PACAP 27 were recorded for either the circular (N=11) or the longitudinal preparations (N=7) (not shown).

To test whether PACAP stimulates release of nitric oxide, the preparations were preincubated (20–30 min) with a NOS inhibitor before the second addition of PACAP 27 (10^{-7} \text{mol l}^{-1}). The L-arginine analogue L-NAME (3\times10^{-4} \text{mol l}^{-1}) alone enhanced the spontaneous contractions on both the circular (Fig. 4) and the longitudinal preparations (Fig. 5). This caused an increase in the mean force developed from 0.51\pm0.12 \text{mN} to 0.94\pm0.21 \text{mN} on the circular preparations (N=8; Fig. 4B) and from 1.49\pm0.36 \text{mN} to 3.34\pm0.67 \text{mN} (N=7; Fig. 5B) on the longitudinal preparations, following L-NAME treatment. However, L-NAME did not affect the response to the second dose of PACAP 27 (Fig. 4B, 5B). Neither did L-NAME treatment affect the response to PACAP 38 on the longitudinal preparations (Fig. 5C). Similar results were obtained with L-NOARG, another L-arginine analogue, although no significant increase in mean force developed was recorded. However, in four of six preparations an increase in force was seen after L-NOARG treatment.

**PACAP 6-27**

No differences (P>0.05) in the response to NaNP were recorded when a second dose of NaNP (3\times10^{-7} \text{mol l}^{-1}; N=7) was added to the circular preparations after washout and recovery after the first dose (minimum 1 h; not shown).

Preincubation (10 min) with the PACAP analogue PACAP 6-27 (3\times10^{-7} \text{mol l}^{-1}), did not affect the response to either

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**Fig. 3.** The effect of PACAP 27 (10^{-8} and 10^{-7} \text{mol l}^{-1}, filled and hatched bars, respectively; N=8); PACAP 38 (10^{-7} \text{mol l}^{-1}; N=5) and NaNP (10^{-6}, 10^{-5} \text{mol l}^{-1}; N=8) on longitudinal muscle activity in the proximal intestine of the Atlantic cod, *Gadus morhua*. Values are mean force developed (\pm S.E.M.) above resting level. The open bars (control) represent the values immediately before addition of the drugs. *P*\leq0.05 compared to the control.

**Fig. 4.** The effect of L-NAME on the response to PACAP 27 on circular muscle activity in the proximal intestine of the Atlantic cod, *Gadus morhua*. (A) L-NAME (3\times10^{-4} \text{mol l}^{-1}) increased the amplitude of the spontaneous contractions, indicating a nitrergic tone in the preparation. However, the response to PACAP 27 (10^{-7} \text{mol l}^{-1}) was not affected by the presence of L-NAME. The recovery period between the two panels is approximately 90 min. (B) The effect of PACAP 27 (10^{-7} \text{mol l}^{-1}) and L-NAME (3\times10^{-4} \text{mol l}^{-1}) presented as mean force developed (\pm S.E.M.) above resting level (N=8). The open bars (C) represent the values immediately before addition of the drugs. *P*\leq0.05 compared to the control.
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NaNP (3×10^{-7} mol l^{-1}; N=7) or PACAP 27 (10^{-8} mol l^{-1}; N=7) on the circular preparations (not shown).

**Guanylate cyclase inhibitors**

Preincubation (approximately 25 min) with the guanylate cyclase inhibitor LY83583 (6-anilinoquinoline-5,8-quinone; 10^{-5} mol l^{-1}) did not affect the response to PACAP in the circular (N=9; Fig. 6A,C) or longitudinal (N=8; Fig. 7) preparations. However, the inhibitory response to NaNP (3×10^{-7} mol l^{-1}) was abolished by LY83583 (N=8; Fig. 6B,D).

**Apamin**

Preincubation (approximately 20 min) with the bee venom apamin (10^{-6} mol l^{-1}), which is supposed to block a specific type of PACAP receptors in mammals, did not affect the response to PACAP 27 in the circular preparations (N=8; Fig. 8).

**Discussion**

This study demonstrates inhibitory actions of PACAP and nitric oxide on intestinal motility in the Atlantic cod, *Gadus morhua*. Both longitudinal and circular strip preparations of the untreated proximal intestine develop rhythmic contractions that are inhibited by atropine, indicating a cholinergic tone. Since inhibition of nitric oxide synthase with L-NAME enhances the contraction, there is probably a nitrergic tone in the intestinal preparations as well. This supports earlier studies on the cod intestine (Karila and Holmgren, 1995). The origin of the rhythmicity, however, has not yet been determined. In mammals and amphibians, electrophysiological investigations have demonstrated that the occurrence of electrical slow waves is dependent on interstitial cells of Cajal (ICCs) in the submucous and/or myenteric plexa (Prosser, 1995; Cayabyab et al., 1997). The ICCs form networks, mainly via gap junctions, and they lie in close contact with smooth muscle cells (Sanders and Ward, 1996; Shuttleworth and Sanders, 1996). The ICCs are innervated, e.g. by VIP- and NOS-containing nerves, and they respond to nitric oxide by the release of more nitric oxide (Shuttleworth and Sanders, 1996). In the canine intestine, inhibition of NOS increased the amplitude of both electrical activity and contractions of smooth...
muscles (Cayabyab et al., 1997; Keef et al., 1997). It was suggested that nitric oxide, released from nerve cells, acts either directly on smooth muscle cells or on interstitial cells to modulate the pacemaker activity, but nitric oxide is not required for induction of the slow waves. It was also suggested that nitric oxide can be released from a non-neural source as well, presumably from ICCs (Cayabyab et al., 1997; Keef et al., 1997). The possible presence and function of ICCs has not yet been investigated in the cod.

In the cod, the cholinergic tone seems necessary to maintain the spontaneous contractions. Atropine abolished the contractions even after L-NAME treatment (results not shown), indicating that nitric oxide acts to depress the cholinergic, tonic activity. Whether this effect of nitric oxide is presynaptic, on the release of acetylcholine, or if nitric oxide acts postsynaptically, needs to be further investigated.

In the stomach of the rat and rabbit, L-arginine analogues potentiated the response to electrical (cholinergic) stimulation but had no effect on metacholine- or acetylcholine-evoked contractions (Lefebvre et al., 1992; Baccari et al., 1993). Subsequent studies on canine and guinea-pig intestine have shown that the levels of electrically evoked release of acetylcholine are affected by nitric oxide donors and NOS inhibitors (Hryhorenko et al., 1994; Hebeiss and Kilbinger, 1996). This suggests that tonically released nitric oxide inhibits the release of acetylcholine.

NaNP abolished the spontaneous contractions in the cod intestine, indicating that nitric oxide has the potential not only to depress the basal contractile activity, but also to effectively inhibit gastrointestinal motility. Similar to isolated mammalian smooth muscle cells where the guanylate cyclase inhibitor LY83583 blocked NaNP-induced relaxation (Jin et al., 1993),

**Fig. 6.** The effect of the guanylate cyclase inhibitor LY83583 on the response to PACAP 27 (A,C) and NaNP (B,D) on circular muscle activity in the proximal intestine of the Atlantic cod, *Gadus morhua*. (A) A slight increase in the amplitude of the contractions was seen after incubation with LY83583 (10^{-5} mol l^{-1}) while the response to PACAP 27 (10^{-7} mol l^{-1}) was not affected. (B) The response to NaNP (3\times10^{-7} mol l^{-1}) was blocked by preincubation with LY83583 (10^{-5} mol l^{-1}). (C,D) Values are mean force developed (±S.E.M.) above resting level (C, N=9; D, N=8). The open bars (C) represent the values immediately before addition of the drugs. *P*≤0.05 compared to the control.
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The response to NaNP in the cod intestine was abolished by LY83583. This suggests that the inhibitory effect of NaNP in cod, as well as in other vertebrates, is achieved by increased production of cGMP.

In mammals, both VIP and PACAP inhibit gastrointestinal motility (Grider et al., 1985; De Beurme and Lefebvre, 1987; Mungan et al., 1992; Katsoulis et al., 1993; Grider et al., 1994). Previous studies on cod have shown inconclusive effects of VIP on intestinal motility. In perfused intestine, mammalian VIP did not significantly alter the spontaneous contractions (Jensen and Holmgren, 1985). This is in agreement with the present study where neither mammalian nor synthetic cod VIP affected the spontaneous contractions. The sequence of cod PACAP has not yet been determined but PACAP from another teleost species, the sockeye salmon (Oncorhynchus nerka), differs from the mammalian sequence at only three positions (Parker et al., 1993). All the amino acid substitutions are in the C-terminal part of the full peptide, and consequently PACAP 27 should be identical in teleosts and mammals. This could be one explanation why mammalian PACAP 27 seems to be more potent than PACAP 38, at least on the circular preparations, in inhibiting the contractions in the cod intestine. However, PACAP 27 was more potent in relaxing rat ileum as well (Ekblad and Sundler, 1997). In mammals, there are three main types of PACAP/VIP receptors: PAC1, VPAC1 and VPAC2 (Harmar et al., 1998). The two latter have approximately equal affinity for VIP and PACAP while the former prefers PACAP 27 and PACAP 38 to VIP. PAC1 is located predominantly in the central nervous system while VPAC1 and VPAC2 are found both centrally and in the periphery. The VPAC receptors usually act via stimulation of adenylate cyclase (Harmar et al., 1998). Recently, VPAC receptors have been cloned from goldfish (Carassius auratus) and frog (Rana ridibunda) brain (Chow, 1997; Alexandre et al., 1999). In addition, these receptors have been demonstrated in the intestine of the goldfish and frog, respectively. Interestingly, VIP was about 100-fold more potent than PACAP in stimulating cAMP production when the goldfish receptor was expressed in mammalian cells while the two peptides were equally potent in stimulating cAMP production via the frog receptor (Chow, 1997; Alexandre et al., 1999). The frog receptor shares similarities with both human VPAC1 and VPAC2 and it was indicated that there is only one VPAC receptor form present in the frog (Alexandre et al., 1999). Although the goldfish receptor was thought to be most like the human VPAC1 (Chow 1997), it has been suggested that the gene duplication that yielded the two receptor forms occurred after the amphibians had evolved (Alexandre et al., 1999). If this is true, the relative affinity of the receptor for VIP and PACAP seems to vary between different species since, unlike the situation in frog and goldfish, only PACAP had any effect on the cod intestine. In contrast, preliminary studies show that both mammalian and cod VIP inhibit motility in the cardiac stomach of the cod (C. Olsson and S. Holmgren, unpublished data). This suggests the presence in the stomach of a distinct type of receptor (from that in the intestine) with equal affinity for VIP and PACAP, or a
second type of receptor, with high affinity for VIP only, present together with the PACAP-preferring receptor. In either case, two types of receptors are indicated in the cod. In mammals, there is physiological evidence for apamin-sensitive receptors that act via K⁺-channels in some tissues, in addition to the above-mentioned receptors (Jin et al., 1994; McConalogue et al., 1995; Ekblad and Sundler, 1997). Although there was no evidence of apamin-sensitive PACAP receptors in the cod intestine it is possible that they exist in the stomach.

Since VIP (and/or PACAP) and NOS coexist in a sub-population of myenteric neurons in most vertebrate species, many studies dealing with their possible interactions have been performed. Several studies have demonstrated that VIP and PACAP can stimulate the production of nitric oxide (Li and Rand, 1990; Grider, 1993; Jin et al., 1993; Murthy et al., 1997). Most of these studies have been performed on isolated smooth muscle cells indicating the presence of a constitutive, non-neuronal NOS (Grider, 1993; Jin et al., 1993; Murthy et al., 1997). VIP and PACAP increase the levels of nitric oxide as well as the levels of cAMP and cGMP in these cells (Jin et al., 1993; Murthy et al., 1997). In contrast, in other tissues no effect of L-arginine analogues on VIP- or PACAP-induced relaxation has been demonstrated (Boeckxstaens et al., 1991; D’Amato et al., 1992; Jin et al., 1993; Maggi and Giuliani, 1996; Ekblad and Sundler, 1997). In addition, Keef et al. (1994) saw no elevation in the levels of NOₓ measured after treatment with VIP. In the cod, neither NOS inhibitors nor guanylate cyclase inhibitors influenced the inhibitory response to PACAP, suggesting that PACAP does not stimulate the production of nitric oxide in nerve cells or in muscle cells.

There is also evidence that nitric oxide enhances release of VIP and PACAP (Grider, 1993; Grider and Jin, 1993; Daniel et al., 1994; Grider et al., 1994; Allescher et al., 1996). The source of nitric oxide is usually nerve cells, but it has been suggested that nitric oxide produced within muscle cells can act as a retrograde messenger on VIP-containing neurons (Grider, 1993). Most of these studies involve measurements of the peptides since there are only few antagonists of the VIP/PACAP receptors available. The peptide analogues PACAP 6-27, PACAP 6-38 and VIP 6-28 (which lacks the five most N-terminal amino acids of the respective peptide), or antibodies directed against PACAP or VIP, have been used to inhibit the electrically or stretch-induced relaxation as well as to block the effect of exogenous peptides (Grider et al., 1994; Murthy et al., 1997). Although our data indicate that preincubation with PACAP 6-27 does not alter the response to NaNP, suggesting that nitric oxide (NaNP) does not stimulate the release of PACAP in the cod intestine, it is not possible to draw any further conclusion from these experiments, since PACAP 6-27 was unable to block the response to PACAP 27 as well.

In conclusion, this study demonstrates inhibitory effects of PACAP and NaNP (presumably forming nitric oxide) on intestinal motility in the Atlantic cod, Gadus morhua. No evidence of interactions between nitric oxide and PACAP in producing these effects has so far been established. Whether PACAP and nitric oxide act directly on the smooth muscles or, for example, via other enteric neurons or interstitial cells of Cajal, remains to be further investigated.

This study was supported by the Swedish Natural Science Research Council, Adlerbertska Research Foundation, Helge Ax:son Johnson Foundation and Wilhelm and Martina Lundgren Research Foundation. We thank Mrs Ann Wikström for technical assistance.

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