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Vasoactive intestinal polypeptide-like immunoreactivity and effects of VIP in the swimbladder of the cod, *Gadus morhua*

Kersti Lundin and Susanne Holmgren
Department of Zoophysiology, University of Göteborg, Box 25059, S-40031 Göteborg, Sweden.

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Summary. 1. Immunohistochemistry has been used to localize the presence of vasoactive intestinal polypeptide (VIP)-like immunoreactivity (IR) in different parts of the swimbladder and in the coeliac and swimbladder arteries of the cod, *Gadus morhua*. The effects of exogenous porcine VIP on the swimbladder have been studied in a perfused gas gland preparation and on isolated strips from the secretory mucosa (including the muscularis mucosae), the oval edge and the coeliac and swimbladder arteries.

2. VIP-like IR was present in nerve fibres in the muscularis mucosae and submucosa of the swimbladder wall, the oval edge region, the swimbladder artery and the coeliac artery (Figs. 1–5). No VIP-like IR was encountered in the gas gland of the swimbladder. A few IR fibres were seen in the extrinsic nerves supplying the swimbladder (Fig. 6).

3. VIP (10^{-7} M) in the perfusion fluid increased the flow through the gas gland of the swimbladder perfused in situ (Fig. 8), and isolated strips of the coeliac and swimbladder arteries showed a small decrease in tension in response to prolonged exposure to VIP. VIP also produced a slowly developing, profound decrease in tension of isolated strips of the secretory mucosa and the oval edge of the swimbladder (Fig. 7).

4. It is concluded that a functional innervation by VIP-containing and -releasing nerves may exist in the swimbladder and swimbladder arteries of the cod.

Introduction

The swimbladder of the cod, *Gadus morhua*, is of the physoclist (closed) type, with a gas gland in the anterior ventral part. A thick, relatively gas-impermeable mucosa covers the main part of the inner wall (the secretory part) of the swimbladder, while the dorsal, resorbent, part is covered by a thin mucosa only, allowing resorption of gas to take place into the blood vessels of this area. The resorbent part is more or less covered by a fold of secretory mucosa, depending on the degree of opening of a sphincter, the oval. Circular and radial smooth muscle fibres in the oval edge, and the tonus of the muscularis mucosae of the secretory mucosa determine the degree of opening of the oval, thus regulating the area exposed directly to the swimbladder gas and the rate of resorption into the blood. The muscularis mucosae is densely innervated (Woodland 1910; Saupe 1940; Fänge 1953; Nilsson 1971).

Gas secretion from the physoclist gas gland is initiated by an 'inflatory reflex', triggered e.g. by the balance organ or by stretch receptors in the swimbladder wall when the volume of the swimbladder is reduced during diving. Efferent autonomic fibres innervating the gas gland run in the vagi, and cause gas production due to metabolic changes in the gas gland secretory cells. Indications accumulated throughout the years suggest that these fibres are cholinergic (Dreser 1892; Bohr 1894; Deineka 1905; Fänge 1953, 1976; Fänge et al. 1976; McLean and Nilsson 1981; Fänge and Holmgren 1982). Gas production is also affected by the flow of blood through the gas gland (Fänge 1953). Adrenergic constrictory nerve fibres innervate the gas gland blood vessels and regulate the blood flow through the gas gland via alpha-adrenoceptors (Fahlén et al. 1965; Nilsson 1972; Fänge et al. 1976; McLean and Nilsson 1981).

Gas resorption in the swimbladder of gadid fish is facilitated by opening of the oval. This is controlled by adrenergic fibres contracting the radial smooth muscles of the oval edge via alpha-adreno-

Abbreviations:IR immunoreactive, immunoreactivity; *VIP* vasoactive intestinal polypeptide.

ceptors and simultaneously relaxing the circular smooth muscles of the oval edge via beta-adrenoceptors (Fänge 1953; Nilsson 1971; Ross 1978).

In view of the increasing knowledge of the presence of non-adrenergic, non-cholinergic nerves in the autonomic innervation of visceral organs not only in mammals (see Furness and Costa 1980; Schultzberg et al. 1980) but also in fish (e.g. Langer et al. 1979; Holmgren et al. 1982; Holmgren and Nilsson 1983a, b), we found it of interest to study the distribution and function of neuropeptides in nerves of the swimbladder of a teleost fish. Recent work has shown a probable presence of functioning VIP-containing nerves in glands (Lundberg 1981), in vessel walls (Järhult et al. 1982) and in the gut smooth muscle layers (Fahrenkrug et al. 1978; Eklund et al. 1979) of mammals. Our aim has been to determine the possible presence and function of VIP in the swimbladder, a well vascularized organ with a gas producing gland and a smooth muscle coat derived from the gastrointestinal canal.

Materials and methods

Atlantic cod, *Gadus morhua,* weighing 400–1,000 g were captured outside the Swedish west-coast, and kept in circulating aerated sea-water at 10 °C until used. The fish were killed by a sharp blow on the head.

Immunohistochemistry

Whole mount preparations were made according to the method described by Costa et al. (1980). The tissue pieces were pinned flat on dental wax and fixed floating for 18 h at 4 °C in a mixture of formaldehyde (2%) and picric acid (15%) in 0.1 M phosphate buffer (pH 7.2). After repeated rinsing in 80% alcohol, the tissue pieces were dehydrated, treated for 30 min with xylene, and rehydrated. Incubations with diluted antiserum were made in a moist chamber at room temperature for 18 h. The antiserum was VIP antiserum L85 (Dockray), raised against synthetic 18–28 VIP and C-terminal specific (Dimaline et al. 1980), diluted 1:400. After incubation the preparations were repeatedly rinsed in phosphate buffered saline (PBS; pH 7.2) and finally incubated for 1 h with a second antibody (swine antirabbit IgG, diluted 1:20, Dako immunoglobulins) tagged with FITC (fluorescein iso-thiocyanate). After rinsing for 1 h in PBS the preparations were mounted on slides in carbonate-buffered glycerol (1:1, pH = 8.5).

Gas glands were fixed in parabenzoequinone (Bishop et al. 1978) in 0.1 M sodium cacodylate buffer (pH = 7.2) at 4 °C for 4 h and rinsed in buffer containing 20% sucrose for 24–48 h at 4 °C. The fixed glands were snap-frozen in liquid nitrogen and sections of 10 µm were cut on a cryostat. The sections were collected on slides covered with chrome alum gelatine and stored at −20 °C until used. Incubations were made with diluted antiserum according to the indirect fluorescence method by Coons (1956), which is essentially the same as that described above for whole mounts.

Controls were carried out by replacing the specific antiserum with specific antiserum preincubated for 48 h at 4 °C with natural porcine VIP (10 nmol/ml; Prof. V. Mutt). This preincubated serum failed to produce specific immunoreactivity in all types of tissues studied.

Ligation of nerves

Ligations of the splanchnic nerves and the vagus nerve were performed in anaesthetized fish according to Abrahamsson (1979). The fish were killed after 60 h, and the nerves examined for the presence of accumulated VIP-like IR in the fibres proximal to the ligature.

Perfusion experiments

Prior to killing, the fish were injected with heparin (ca. 3,000 IU/kg body weight) in the caudal vein.

The fish were opened laterally on the right side to expose the swimbladder and the swimbladder vessels. The coeliac artery – swimbladder artery and the portal vein were catheterized for inflow and outflow of perfusion fluid respectively (see Nilsson 1972). All other branches of the coeliac artery were ligated to prevent leakage, and the stomach and intestine were removed to avoid interference from gut movements with the perfusion flow through the swimbladder.

Perfusion was carried out with constant pressure as described by Nilsson and Grove (1974). Inflow pressure was kept at 3.5–4 kPa and outflow pressure at zero. The outflow was recorded with a photoelectric drop counter triggering a 7P4D tachograph in a Grass Polygraph mod 79.

A filtered cod Ringers solution (see Holmgren and Nilsson 1974), bubbled with a mixture of O_2/CO_2 (97/3%), was used for the perfusions. Porcine VIP was dissolved in cod Ringer containing bovine serum albumin (10^{-5} g/ml), and injected in 1 ml boluses into the lower funnel of the perfusion apparatus or continuously perfused through the preparation (see Nilsson and Grove 1974).

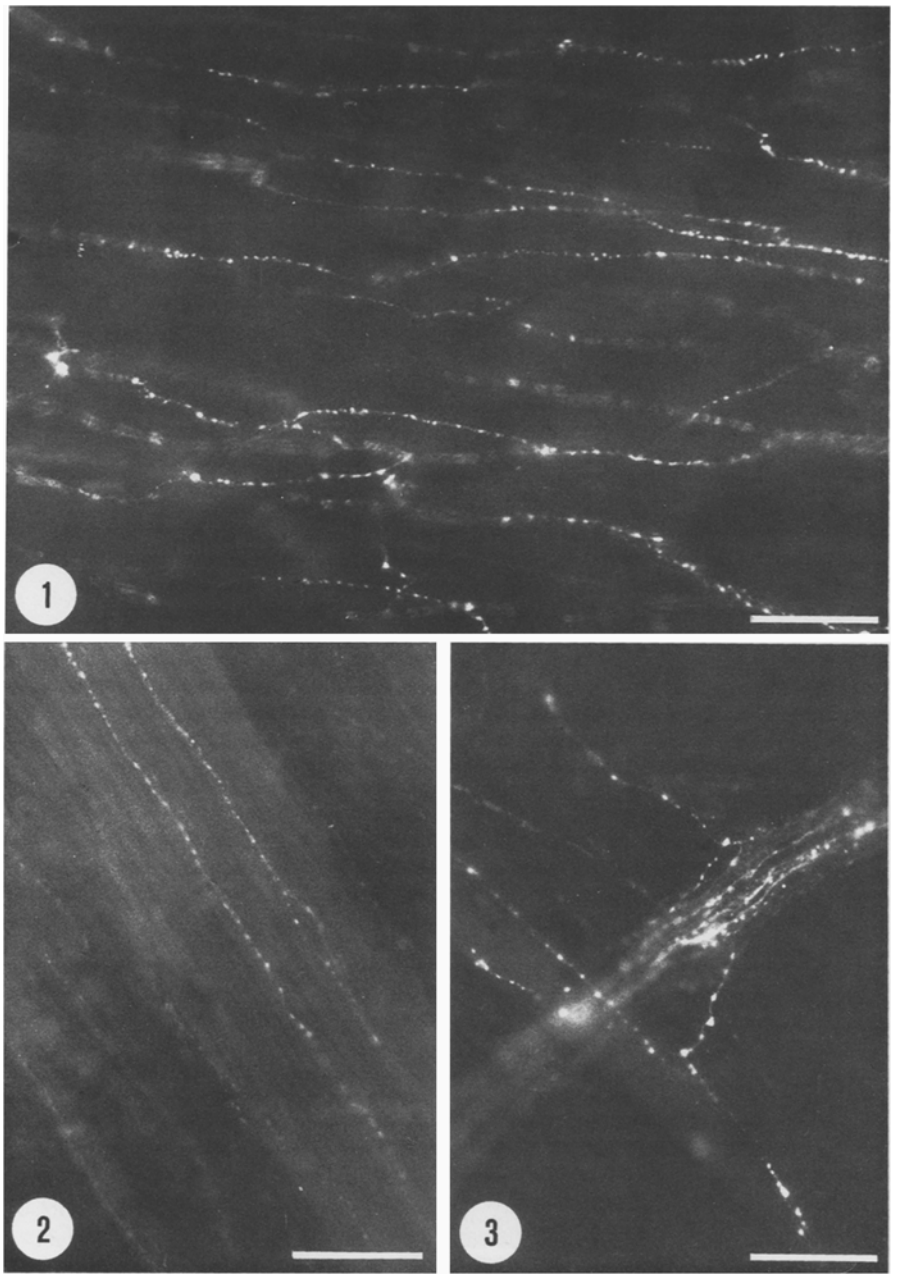
Isolated strip preparation experiments

Strip preparations were made from the muscularis mucosa of the secretory part of the swimbladder, from the oval edge (circular preparation) and from the coeliac and swimbladder arteries. The strips were mounted in 10 ml organ baths containing bubbled cod Ringer's solution at 10 °C. The tension of the strip was recorded on the Grass mod 79 Polygraph via Grass FT03 isometric transducers.

Strips from the swimbladder muscularis mucosae and oval edge were mounted with an initial tension of 0.0012 or 0.0020 N. (The two groups showed no difference in response relative to their initial tension, and the results have therefore been pooled). Carbachol (10^{-5} M), which is excitatory on these preparations (Nilsson 1971) was added to the bath and present throughout the experiments. Without this treatment the preparations would relax to base-line tension, and inhibitory effects of VIP could not be monitored.

Spiral strips were made of the coeliac artery and swimbladder artery. The preparations were mounted with an initial tension of 1.75–2.00 mN. Cod arteries are insensitive to cholinergic agonists (Holmgren and Nilsson 1974), and adrenaline (10^{-5} M) was therefore used in the bath in these experiments to increase the tension of the preparations.

The effect of VIP on the strips was calculated in per cent of the initial tension of the strips. The effect on the perfusion flow was calculated in per cent of the flow immediately prior to the addition of VIP.



Figs. 1–3. VIP-like immunoreactivity in nerves of the swimbladder of the cod, *Gadus morhua*

Fig. 1. Fibres running in the mucosa-submucosa of the secretory part of the swimbladder

Fig. 2. Fibres running along the radial smooth muscle of the oval edge

Fig. 3. Single fibres branching from fibre bundles in the secretory part. Calibration bars in all figures = 50 μm

Results

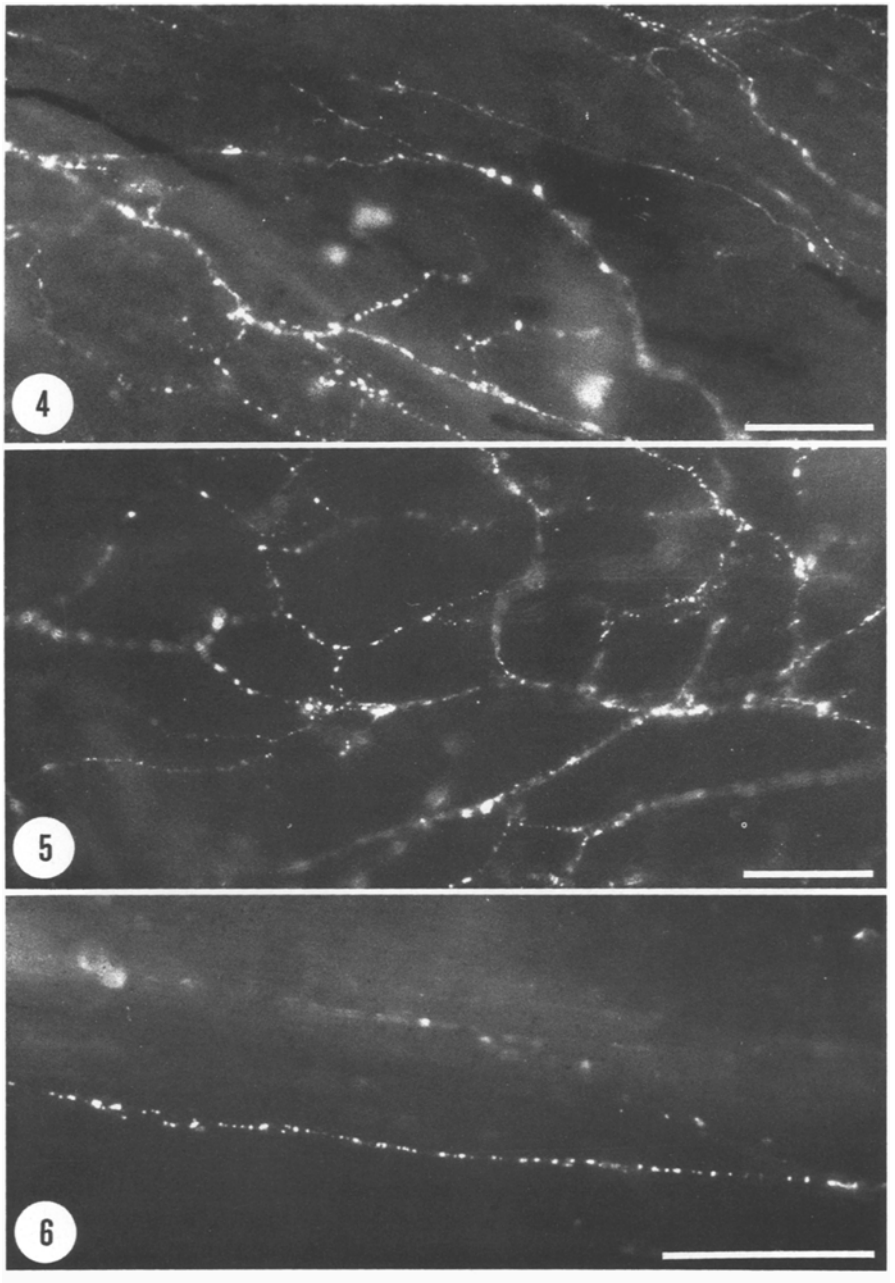
Immunohistochemistry

A network of single varicose fibres showing VIP-like immunoreactivity (IR) was present in the swimbladder muscularis mucosae-submucosa preparations (Fig. 1). The density of fibres was highest in the secretory part of the swimbladder and lower towards the oval region. Only few fibres were seen in the oval edge, all running along the radial muscle fibres (Fig. 2). Networks of fibres were also observed in the walls of some of the small vessels running in the submucosa.

Bundles of IR fibres, with no special orientation, were occasionally encountered in all preparations from the secretory part. Single varicose fibres could be seen leaving these bundles (Fig. 3).

No IR fibres were encountered in the gas gland, neither in thin pieces peeled from stretch preparations nor in sections. The rete mirabile was also apparently devoid of VIP-like IR.

A well developed plexus of single fibres and



Figs. 4–6. VIP-like immunoreactivity in arteries and nerves to the swimbladder of the cod, *Gadus morhua*

Fig. 4. IR nerve plexus in the wall of the swimbladder artery

Fig. 5. IR nerve plexus in the wall of the coeliac artery

Fig. 6. Occasional single nerve fibres were seen running along the splanchnic nerve to the swimbladder. Calibration bars in all figures = 50 μm

small fibre bundles showing VIP-like IR was observed in the walls of the coeliac artery (Fig. 5) and its branch to the swimbladder, the swimbladder artery (Fig. 4).

Occasional fibres were seen running in the intestinal branch of the vagus and in the splanchnic nerve (Fig. 6), including the nerve bundles entering the swimbladder in the hilus region of the gas gland. After 60 h of ligation the fluorescence intensity of the staining of these fibres was considerably increased proximal to the ligature in both nerves, while the number of visible immunoreactive fibres still was low.

Isolated strip preparations

Swimbladder. Porcine VIP (10^{-8}–10^{-6} M) produced a relaxation of the precontracted strips. The mode and magnitude of the effects was very similar between preparations from different parts of the muscularis mucosae (including the circular strips of the oval edge) from one fish, but varied consid-

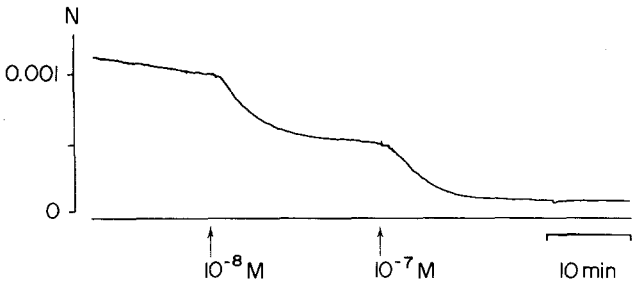


Fig. 7. The effect of porcine VIP on the tension of an isolated strip of muscularis mucosae from the secretory part of the swimbladder of the cod, *Gadus morhua*. The preparation is pretreated with carbachol to increase the initial tension.

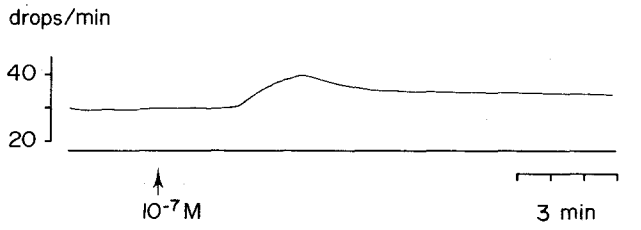


Fig. 8. The effect of porcine VIP on the perfusion flow through the swimbladder gas gland from cod, *Gadus morhua*. The preparation is pretreated with adrenaline to decrease the initial flow

erably between different specimens. The results from the different parts of the swimbladder are therefore pooled.

Of 38 preparations tested 7 were apparently insensitive to VIP (10^{-8}–10^{-6} M). The remaining strips responded to VIP 10^{-8} M with a decrease in tonus ranging from 0 to 50% ($\bar{x}=15\%$) of the initial tonus. An increase of the concentration of VIP to 10^{-7} M produced a further decrease in tonus of 0 to 49% ($\bar{x}=26\%$). 10^{-6} M, administered to 6 preparations only, produced a further relaxation of 0 to 41% ($\bar{x}=15\%$) (Fig. 7). Addition of tetrodotoxin (10^{-6} M, $n=3$) to the bath did not change the response to VIP.

It is notable that the decrease in tonus in response to addition of VIP is slow in onset and development. A full response is not obtained until 15–40 min after addition of the peptide. The effect is also longlasting

Arteries. Precontracted artery strips ($n=12$) were generally unaffected by addition of 10^{-8} M VIP, while higher concentrations (10^{-7}–10^{-6} M) in most cases produced a small relaxation.

Perfusions

During carefully controlled conditions (temperature, pH etc.) addition of 1 ml boluses of VIP (10^{-10}–10^{-7} M) to the perfused gas gland-swimbladder preparation produced no recordable effect on the flow through the preparation ($n=6$). However, prolonged exposure to VIP (10^{-7} M in the bulk of perfusion fluid) produced an increase in flow with a peak of $125\pm5\%$ (SE; $n=5$) (Fig. 8).

Discussion

The immunohistochemical results indicate that nerve fibres of the swimbladder mucosa-submucosa and in the swimbladder arteries of the cod, *Gadus morhua*, may contain VIP or a VIP-like peptide. Previous reports on the presence of VIP in fish mainly concerns the gastrointestinal canal. Thus Dockray (1974), Langer et al. (1979), Fouchereau-Peron et al. (1980), Van Noorden and Patent (1980), Holmgren et al. (1982) and Holmgren and Nilsson (1983a, b) all report the occurrence of VIP-like immunoreactive material in the gut of different species of teleost and other fish. The collected evidence strongly suggests that the presence of VIP or a VIP-like peptide (VIP-oid) in gastrointestinal neurons is a general feature in fish. The present study, a study of 'the gut in some teleosts' (Van Noorden et al. 1980) and own preliminary studies of *Salmo gairdneri, Ctenolabrus rupestris* and *Pollachius virens* demonstrate a well developed innervation by nerves showing VIP-like IR also of the swimbladder, originally a derivative of the alimentary canal.

VIP or a VIP-like peptide thus seems to be present in gut neurons in fish, but little is known about the physiological effects of release of this peptide. Holstein and Humphrey (1980) report that porcine VIP reduces gastric acid secretion in cod. In the rainbow trout, *Salmo gairdneri,* VIP has inconsistent effects on the spontaneous activity of stomach smooth muscle, sometimes inhibiting and other times exciting smooth muscle preparations (Holmgren 1983). The present study reports VIP-like IR in nerves of the cod swimbladder muscularis mucosae and submucosa and in the walls of the arteries supplying the swimbladder. This, together with the observations of the marked effects of exogenous VIP on swimbladder wall preparations and on the flow through the perfused gas gland is compatible with the view that a functional inhibitory innervation of smooth muscle by neurons releasing VIP or a VIP-like peptide exists in this species.

The effect of exogenous VIP was not blocked by tetrodotoxin, suggesting that the effector site is on the smooth muscle cells and not on intrinsic nerves. Only few fibres in the vagal and splanchnic

branches showed VIP-like IR, even after ligation, indicating that the cell bodies of the VIP-neurons innervating the swimbladder may be located closer to the swimbladder, e.g. in the swimbladder or gas gland ganglia (see McLean and Nilsson 1981).

A presence of inhibitory VIP-releasing neurons in the swimbladder muscularis mucosae does not necessarily contradict the report by Nilsson (1971), which concludes a solely adrenergic innervation of this tissue. Nilsson studied the effects of a comparatively short stimulation of the nerve supply. The response to VIP in the present study is, however, slow in onset and development and of a long duration, indicating that the response to VIP-release from nerves may not be readily seen after a short period of stimulation.

The slow onset of the inhibitory effect obtained in all preparations may of course be explained by a slow diffusion rate for the large VIP molecule from the external medium (Ringer bath) to its receptor, and may not mirror the time course of the response obtained after release of VIP from nerve endings. On the other hand, the mode of action with both a slow onset and a long duration of the effect may suggest that VIP acts to keep a certain tonus of the arteries and the muscularis mucosae, rather than to induce rapid changes. An inhibitory tonus by VIP-releasing fibres could thus act to balance an excitatory tonus maintained by adrenergic nerves or circulating catecholamines (Nilsson 1971; Wahlqvist 1980) on the arteries and secretory mucosa, while the effect on the circular muscle of the oval edge (if VIP neurons reach this muscle) rather would be in parallel with the effect of catecholamines.

The network of varicose fibres in the swimbladder wall is dense, and may not only be involved in control of the tonus of the muscularis mucosae. Other possible functions include control of secretion of mucous from the epithelium. Fibres were also seen surrounding vessels running in the submucosa, suggesting a vaso-active role of a VIP-like peptide in the swimbladder.

The gas gland was devoid of nerve fibres showing VIP-like IR, and a direct effect of VIP-releasing nerves on the gas gland cell activity thus seems unlikely. However, our experiments indicate that VIP may affect the blood flow through the gas gland and thus indirectly affects the gas production from the gland (cf. Fänge 1953; Nilsson 1972, 1983).

A high percentage of the preparations was apparently insensitive to VIP, while others showed a great variability in the response. It is possible that VIP released from nerve terminals acts together with some other substance (cotransmission) and that this substance needs to be present to produce the full effect of VIP.

In conclusion, the study indicates the presence of an innervation of the swimbladder and its vascular supply by fibres containing VIP or a VIP-like peptide. It is possible that these fibres provide a functional inhibitory control of the muscularis mucosae and a dilatory control of the swimbladder vascular supply, thus indirectly affecting the gas gland activity.

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