VIP-Immunoreactive nerves in the pancreas of the snake, *Elaphe quadrivirgata* (Boie): Another model for insular neurosecretion

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ABSTRACT

1) VIP-immunoreactive nerve fibers were demonstrated around the blood capillaries in the pancreatic islet of the Japanese rat racer snake (shimahebi), *Elaphe quadrivirgata* (Boie), by the peroxidase-antiperoxidase (PAP) technique. 2) Electron microscope observation confirmed occurrence of numerous nerve terminals in the pericapillary spaces in the snake islet. They possessed both large endocrine-type granules and small synaptic-type vesicles. The Schwann cell sheath was often absent on the vascular side of the nerve profiles. 3) These findings suggest that VIP-immunoreactive substance released from the nerve terminals into the pericapillary space may flow into the blood stream, be transferred by the insulo-acinar portal vessels, and possibly stimulate the exocrine function of the pancreas, although the secretin-like activity of VIP remains to be examined in the snake.

KEY WORDS snake pancreas / insular neurosecretion / vasoactive intestinal peptide (VIP) / immunohistochemistry / *Elaphe quadrivirgata*

In various mammals it has been reported that the islet of the pancreas is supplied more or less amply with nerves (for review, see 17). The nerves in the islets are believed to control the secretory activity of the endocrine cells (3, 8, 13, 16, 17). A few authors suggested an additional role, that of controlling the function of the vascular walls (1, 2, 10, 13). In the dog pancreas, however, Fujita and Kobayashi (4) pointed out that rather few nerves seem to terminate on the endocrine cells on one hand, and on the other hand, the nerves reaching the pericapillary space apparently are much too abundant to be interpreted as controlling the permeability or contractility of the vascular wall. Fujita and Kobayashi obtained electron micro-

scope images suggesting that the secretions of the pericapillary nerve terminals enter the blood stream of the islet and proposed that the neurosecretions are conveyed to and act upon the exocrine tissue in high concentrations via the insulo-acinar portal system (5, 6). Among the candidates for the insular neurosecretions they gave attention to VIP because of its secretin-like activity, evidenced at least in the dog (9, 14). On the other hand, many of the neurons in the canine pancreas contain VIP-like immunoreactivity (11, 12).

In the course of our phylogenetic investigations on the neuro-endocrine system of the pancreas, we found numerous VIP immunoreactive nerves concentrated in the islets of a Japanese rat racer snake. This paper will report on the distribution and structure of these nerves and discuss the possible significance of this finding as another model of the insular

neurosecretory system.

The pancreas was removed from four adult snakes, *Elaphe quadrivirgata* (Boie) under ether anesthesia and fixed in Bouin's or Zamboni's solution for 8–24 hr. After embedding in paraffin, 4 μ m sections were cut. A part of the sections was stained with aldehyde-fuchsin-Masson-Goldner, while others were treated by immunocytochemistry.

Preparation of anti-VIP serum R-502 was described previously (18). Briefly, the antiserum was produced by injections of synthetic porcine VIP mixed with polyvinyl pyrrolidone (Mann Research Laboratory, N. Y.) and complete Freund's adjuvant (Calbiochem, Los Angeles, Calif.) into female rabbits. The synthetic preparation of VIP used as antigen had been identified with natural porcine VIP (Karolinska) in a radioimmunoassay. No cross reaction with secretin, pancreatic glucagon, gastrin, motilin, substance P, or calcitonin was observed at doses up to 100 ng/tube in a radioimmunoassay using antiserum R-502 and 125 I-synthetic porcine VIP as tracer. Before use for immunocytochemistry, the antiserum was incubated for 30 min at 56°C in order to inactivate the complements possibly contained.

After the blockage of endogenous peroxidase the sections were exposed to the anti-VIP serum (diluted 1 : 3000) for 24 hr at 4°C. The immunoreaction was demonstrated by the peroxidase-antiperoxidase (PAP) method of Sternberger (15; unlabeled swine anti-rabbit Ig G, diluted 1 : 10, PAP complex, diluted 1 : 50, DAKO, Copenhagen, Denmark). After the reaction, some sections were counterstained by Mayer's hematoxylin.

The specificity of the antisera was histologically checked by the following methods: 1) A series of control tests as described by Sternberger (15). 2) Absorption tests. The complement inactivated antiserum (0.9 ml) was incubated for 24 hr at 4°C with the following peptides (10 μ g): synthetic porcine VIP, porcine crystallized pancreatic glucagon and synthetic porcine secretin. 3) Staining patterns were compared, using variously diluted (1 : 50–6,000) antiserum.

All control studies, including absorption tests, supported the specificity of the antiserum. The staining patterns in every case using variously diluted sera were the same, though the reaction differed in intensity. Furthermore, the findings were not essentially different between

the specimens fixed in Bouin's and Zamboni's solutions.

The aldehyde-fuchsin-Masson-Goldner staining revealed large islets gathered in the juxta-splenic portion. The islet was continuous to the acini and ducts, without being covered by a connective tissue sheath. The endocrine A cells, stained red, and B cells, stained violet, were of tall-columnar shape and were arranged at random in irregularly branching cell cords. D cells, stained pale green, were rather round in shape.

The PAP technique revealed the occurrence of nerve fibers containing a VIP-like immunoreactive substance. In the islet, a number of beaded profiles of VIP-immunoreactive fibers were seen in the pericapillary space, i. e., between the endocrine cell cord and the endothelium of the capillary vessel (Fig. 1, 2).

Fig. 1 An islet of Langerhans in the Japanese rat racer snake treated with the PAP method for demonstration of VIP. Surrounding the islet, the exocrine pancreas is identified, being stained slightly darker by hematoxylin used as a counter-stain. The brown depositions surrounding the islet cell cords represent the VIP-like immunoreactivity. Bouin's fixiaton.

Fig. 2 Closer view of a part of the preceding micrograph. The VIP-like immunoreactivity is seen in nerve fibers which are located between the endocrine cell cords and blood capillaries which look pale and empty in this picture.

Fig. 3 Electron micrograph showing a nerve bundle in the intercellular space of the pancreatic islet in the rat racer snake. Endocrine cells are seen on the right and left sides. A blood capillary with a characteristically vesiculated endothelium (E) is shown at the bottom. Axons containing large, endocrine-like granules (g) and small, synaptic-like vesicles (v) are invested by the cytoplasm of Schwann cells (S). The axons devoid of the Schwann cell coverage and facing towards the blood capillary are indicated by arrows. $\times 26,000$

The exocrine pancreas contained much fewer VIP-immunoreactive nerves. The beaded portions of these nerves were mainly associated with arterioles, venules, and capillaries; some of them gave us the impression that they terminated in the pericapillary space. VIP immunoreactive nerves were also seen in the lamina propria of the bile duct penetrating the pancreatic parenchyme. Neither VIP immunoreactive endocrine cells nor nerve cell perikarya were detected in more than 60 sections of the pancreas.

Electron microscope study was performed to elucidate the fine structure of the nerves in the snake islet. The pancreas was perfusion fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The splenic portion of the pancreas was trimmed into small pieces, immersed in the same fixative for 2 hr, post-fixed in 1% osmium tetroxide in phosphate buffer and embedded in Araldite. Thin sections were cut on a Porter-Blum microtome and doubly stained with uranyl acetate and Reynolds' lead. Observation was made with an HU-125DS electron microscope.

Under the electron microscope, numerous swollen nerve profiles were found in the pericapillary spaces of the islet, where collagen fibers pervaded with dispersed fibroblasts. These apparently terminal and preterminal portions of axons contained simultaneously endocrine-type secretory granules and synaptic-type small vesicles. The former were round granules of about 120 nm diameter composed of a membrane sac, a large electron dense core and a narrow, less dense halo between them. The latter were small clear vesicles of oval and elongate profiles without any visible contents. The population ratio of the granules and vesicles varied in each nerve section. Some axon profiles were heavily laden with the granules, mingled with only a few vesicular elements, whereas others contained large areas packed with the vesicles and only dispersed granules (Fig. 3).

Schwann cells invested the nerve axons with their cytoplasm. In the swollen portions storing endocrine-type granules and synaptic-type vesicles, the Schwann cell coverage was often lacking on the vascular side and the axon was naked on the side directly facing towards the capillary. Furthermore, both endocrine-type granules and synaptic-type vesicles tended to be gathered on this side. The nerve profiles were located at a distance of no less than 100 nm from the outer surface of the capillary en-

dothelium. The endothelium showed only a few typically attenuated portions provided with fenestrae of 70 nm diameter. Otherwise it comprised rather thick cytoplasm apparently penetrated by a number of twisted channels, 70 nm in caliber (7).

Only occasionally could a direct juxtaposition of nerves against the endocrine cells be encountered.

This study revealed that in the snake pancreas VIP immunoreactive nerves of beaded structure are concentrated around the capillaries in the islet. As no VIP-immunoreactive nerve cell perikarya could be demonstrated in the pancreas, the origin of the VIP-immunoreactive nerve fibers seems to be extrinsic. By electron microscopy, numerous nerve profiles were demonstrated between the endocrine cells and the capillary wall, swollen by accumulations of granules and vesicles. On the basis of these light and electron microscope findings, we may reasonably call the pericapillary axon profiles in question nerve terminals and preterminals. The large 'peptidergic' type granules revealed by electron microscopy resemble those reported to be contained in the presumably VIP-ergic nerves of the cat in shape, size (120–150 nm), and electron density (10). Co-occurrence of small vesicles in large granule-containing neurons (neurosecretory cells and peptidergic-type neurons) is being recognized more and more widely, although it is not known whether the vesicles store additional secretory substances or are of some other nature.

It is noteworthy that on the vascular side of the axons the Schwann cell sheath investing them is very often lacking, so that the neurosecretions seem to be easily transferred to the opposed endothelium. This, in its turn, looked highly permeable to the substances like polypeptide messengers with its channeled structure (7). Thus, it seems most reasonable to suggest that VIP-like substance may be released from the nerve endings into the pericapillary space and traverse the endothelium to enter the blood stream, besides acting upon the endocrine cells presumably *per diffusionem*.

Our preliminary studies in this species of snake indicate that vasa efferentia emerge from the capillary bed in the islets into the capillary bed in the exocrine tissue, thus forming an insulo-acinar portal system corresponding to that in mammals. The VIP-like substance, thus, is believed to act upon the exocrine pancreas in high concentrations. VIP is closely related to

secretin in chemical structure and its secretin-like effect upon the pancreatic secretion has been confirmed in some mammals (9, 14). The secretagogous effect of VIP on the pancreas needs to be examined in the snake.

Our preliminary study indicates that VIP immunoreactive nerves occur in the pericapillary space of the pancreatic islet in the mamushi, *Agkistrodon halys* (Pallas) (= *A. blomhoffi* Boie). It is thus likely that the structure suggesting insular neurosecretion may be common among different species of Ophidia.

This report may strengthen the view of insular neurosecretion reached by Fujita and Kobayashi (4) in the dog by proposing a principally identical mechanism in the snake. It is likely that islets of Langerhans in the snake, as in the dog, represent a 'neuro-paraneuronal control center' of the pancreas.

The possible significance of VIP-immunoreactive nerves in the submucosal layer of the bile duct will be discussed elsewhere.

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REFERENCES

1. ALM P., CEGRELL L., EHINGER B. and FALCK B. (1967) Remarkable adrenergic nerves in the exocrine pancreas. *Z. Zellforsch.* **83**, 178–186
2. CEGRELL L. (1968) The occurrence of biogenic monoamines in the mammalian endocrine pancreas. *Acta physiol. scand.*, Suppl. **314**, 1–60
3. COUPLAND R. E. (1958) The innervation of pancreas of the rat, cat and rabbit as revealed by the cholinesterase technique. *J. Anat.* **92**, 143–149
4. FUJITA T. and KOBAYASHI S. (1979) Proposal of a neurosecretory system in the pancreas. An electron microscope study in the dog. *Arch. histol. jap.* **42**, 277–295
5. FUJITA T. and MURAKAMI T. (1973) Microcirculation of monkey pancreas with special reference to the insulo-acinar portal system. A scanning electron microscope study of vascular casts. *Arch. histol. jap.* **35**, 255–263
6. HENDERSON J. R. (1969) Why are the islets of Langerhans? *Lancet* **7618**, 469–470
7. KOBAYASHI S. (1970) Occurrence of unique colloidal particles in snake blood and their transport across the capillary wall. A proposal of a new hypothesis on the permeability of the blood capillaries. *Arch. histol. jap.* **31**, 511–528
8. KOBAYASHI S. and FUJITA T. (1969) Fine structure of mammalian and avian pancreatic islets with special reference to D cells and nervous elements. *Z. Zellforsch.* **100**, 340–363
9. KONTUREK S. J., PUCHER A. and RADECKI T. (1976) Comparison of vasoactive intestinal peptide and secretin in stimulation of pancreatic secretion. *J. Physiol.* **255**, 497–509
10. LARSSON L.-I. (1977) Ultrastructural localization of a new neuronal peptide (VIP). *Histochemistry* **54**, 173–176
11. LARSSON L.-I. (1979) Innervation of the pancreas by substance P, enkephalin, vasoactive intestinal polypeptide and gastrin/CCK immunoreactive nerves. *J. Histochem. Cytochem.* **27**, 1283–1284
12. LARSSON L.-I., FAHRENKRUG J., HOLST J. J. and SCHAFFALITZKY DE MUCKADELL O. B. (1978) Innervation of the pancreas by vasoactive intestinal polypeptide (VIP) immunoreactive nerves. *Life Sci.* **22**, 773–780
13. LEGG P. G. (1968) Fluorescence studies on neural structures and endocrine cells in the pancreas of the cat. *Z. Zellforsch.* **88**, 487–495
14. SINGH M. and WEBSTER P. D. (1978) Neurohormonal control of pancreatic secretion. *Gastroenterology* **74**, 294–309
15. STERNBERGER L. A. (1974) *Immunocytochemistry*. Prentice Hall Inc, Englewood Cliffs, New Jersey
16. WATARI N. (1968) Fine structure of nervous elements in the pancreas of some vertebrates. *Z. Zellforsch.* **85**, 291–314
17. WOODS S. C. and PORTE D. (1974) Neural control of the endocrine pancreas. *Physiol. Rev.* **54**, 596–619
18. YANAIHARA N., SAKAGAMI M., SATO H., YAMAMOTO K., HASHIMOTO T., YANAIHARA C., ITO Z., YAMAGUCHI K. and ABE K. (1977) Immunological aspects of secretin, substance P, and VIP. *Gastroenterology* **72**, 803–810