Immunohistochemical study on the distribution of galanin-containing nerves in the chicken pancreas

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Summary. The distribution of galanin-containing nervous elements in the chicken pancreas was investigated by use of immunohistochemical method. Galanin-immunoreactive nerve fibres formed a perivascular plexus and a dense network in the subepithelial layer of secretory ducts. The muscle layers of secretory ducts were also supplied with galanin-immunoreactive nerve fibres. The exocrine pancreas received a supply of varicose nerve fibres showing galanin immunoreactivity. In the endocrine part, B-islets were innervated by galanin-immunoreactive nerve fibres, whereas A-islets received fewer nervous elements. Double staining combined with the immuno-fluorescence method for galanin and acetyl-cholinesterase histochemistry showed at least two types of ganglion cells in the interlobular connective tissue; one showing both acetylcholinesterase activity and galanin immunoreactivity, and the other showing acetylcholinesterase activity only. The present results demonstrate that the chicken pancreas is innervated by galanin-containing nerves of intrinsic origin and suggest that galanin coexists with acetylcholine in the chicken pancreas.

Key words: Pancreas, Innervation, Galanin, Acetyl-cholinesterase, White leghorn chicken

Introduction

Galanin is known to have an inhibitory effect on hormone secretion from pancreatic islet cells in mammals (McDonald et al., 1985; Dunning et al., 1986; Lindskog and Ahrén, 1989). This peptide, which was initially isolated in a porcine intestinal extract by Tatemoto et al. (1983), has a wide distribution in the nervous system, particularly in the enteric nervous system (Rökaeus et al., 1984; Melander et al., 1985; Rattan, 1991). Immunohistochemical investigations have revealed that the pancreas is innervated by galanin-containing nerves in several mammalian species (Su et al., 1987; Ahrén et al., 1990, 1991; Messell et al., 1990; Lindskog et al., 1991). In the dog pancreas, galanin colocates with sympathetic neurotransmitters, nor-adrenaline and neuropeptide Y, in the extrinsic nerves derived from the coeliac ganglion (Ahrén et al., 1990). However, in the rat and mouse pancreas, galanin is contained in intrinsic and non-adrenergic nerves (Su et al., 1987; Lindskog et al., 1991). It is plausible that differences exist among species regarding the nature of galanin nerves in the pancreas (Ahrén and Lindskog, 1992). Little is known about the distribution and the nature of galanin-containing nerves in the avian pancreas.

The present study was aimed at the elucidation of the distribution of galanin-containing nerves in the chicken pancreas by means of the immunoperoxidase method and additionally at clarifying the probability of a colocalization of galanin with the classical neuro-transmitter, acetylcholine, in combination with the indirect the immunofluorescence method and acetyl-cholin-esterase (AChE) histochemistry.

Materials and methods

Animals

Ten adult white leghorn chickens of both sexes (weighing 1.8-2.2 kg) were used in this study. Chickens, anaesthetized with sodium pentobarbitone, were perfuxed with saline followed by a mixture of 4% para-formaldehyde, 0.1% glutaraldehyde and 0.2% picric acid in 0.1M phosphate buffer (pH 7.6). Pancreata were immediately removed, dissected into small blocks and immersed in the same perfusate at 4 °C for 6 h and then in 20% buffered sucrose (0.1M phosphate buffer, pH 7.6) at 4 °C overnight. Tissue blocks were frozen with dry ice-acetone. Frozen sections were serially cut at 16 μm with a cryostat.

Immunohistochemistry

The streptavidin-biotin method (Guesdon et al., 1979) was applied on sections which were pre-treated by

immersion in a solution of 0.03% hydrogen peroxide in 75% methanol for 20 min and then incubated with 10% normal goat serum (Vector, USA, S-1000) for 20 min at room temperature. The rabbit serum against porcine galanin (Chemicon, USA, AB 1985) was used at a dilution of 1:1000, and incubation was for 24 h at room temperature. Thereafter, sections were treated with biotinated goat anti-rabbit IgG diluted to 1:100 (E-Y Laboratories, USA, BA-2307) for 20 min and then with peroxidase-conjugated streptavidin diluted to 1:100 (Zymed, USA, 43-4323) for 20 min at room temperature. Sections were washed with PBS three times for 5 min after each step of incubation. Visualization of the immunocomplex was achieved by a 3,3'-diamino-benzidine-hydrogen peroxide solution. Sections counterstained with methyl green were dehydrated, cleared in xylene, mounted in Entellan and observed under a light microscope.

To identify A- and B-islets, a serial section adjacent to the section made with the immunohistochemistry procedure for galanin was stained immunohisto-chemically with anti-glucagon or -insulin sera (Milab, Sweden, B39-100 or B31-100), respectively.

For controls, some sections were incubated with the galanin antiserum preabsorbed with 10 µg/ml porcine galanin or normal rabbit serum instead of the primary antibody. In these cases, no immunoreactivity was observed.

Double staining for galanin and AChE

To examine the colocalization of galanin and acetylchone, the indirect immunofluorescence method and AChE histochemistry were carried out on the same section. Frozen sections treated with 10% normal goat serum were incubated with antiserum against porcine galanin (diluted to 1:250) at room temperature overnight. After several washes with PBS, the sections were incubated with FITC (fluorescein isothiocyanate)-conjugated goat anti-rabbit IgG (diluted to 1:100; E-Y Laboratories, USA, FA-2307-2) for 1 hr at room temperature. They were then rinsed with PBS and coverslipped with a mixture of glycerol and PBS (3:1). After photography under a fluorescence microscope, coverslips were subsequently removed and AChE histochemistry was carried out on the same section. Details of AChE histochemistry have been described

previously (Hiramatsu et al., 1988). Sections were observed and photographed under a light microscope.

Results

Ganglia containing some perikarya showing positive immunoreactivity for galanin were detected in the interlobular connective tissue (Fig. 1a). A combination of galanin immunohistochemistry and AChE histo-chemistry revealed the colocalization of galanin and AChE. Most of the galanin-immunoreactive ganglion cells also showed AChE activity (Fig. 1b). A few AChE-positive ganglion cells, however, were immunonegative for galanin (Fig. 1, small arrow). The percentage of ganglion cells showing both galanin immunoreactivity and AChE activity was 72.7% (497/684). There was no ganglion cell which showed only galanin immuno-reactivity.

Galanin-immunoreactive nerve fibres were never observed within nerve bundles running along the pancreatico-duodenal artery (Fig. 2) or within nerve bundles in the interlobular connective tissue.

Galanin-immunoreactive nerve fibres were distributed throughout the entire gland. Dense networks of galanin-immunoreactive nerve fibres were found around intrapancreatic arterioles (Fig. 3). Galanin-immunoreactive nerve fibres were also found in the adventitia of the pancreatico-duodenal artery (Fig. 2). Both extra- and intrapancreatic secretory ducts received varicose nerve fibres showing galanin immunoreactivity. They were distributed within the lamina propria of secretory ducts and were often found immediately beneath the epithelium (Fig. 4). The muscular layer of the secretory ducts was poorly innervated. In the exocrine parenchyma, varicose nerve fibres were found showing galanin immunoreactivity (Fig. 5) and appeared to have a close association with acinar cells. There was no difference in the distribution of galanin-immuno-reactive nerves between the three (ventral, dorsal and splenic) lobes.

A- and B-islets differenced in the distribution pattern of galanin-immunoreactive nerve fibres. Galanin-immunoreactive nerve fibres showing a beaded appearance were densely distributed in B-islets (Fig. 6a,b). They were found both around and within B-islets and sometimes formed a peri-insular plexus. Galanin-immunoreactive nerve fibres were only rarely found in

Fig. 1. Paired micrographs revealing colocalization of galanin **(a)** and AChE **(b)** in intrapancreatic ganglion cells. Many ganglion cells show both galanin immunoreactivity and AChE activity (large arrows). One ganglion cell shows AChE only (small arrow). x 540
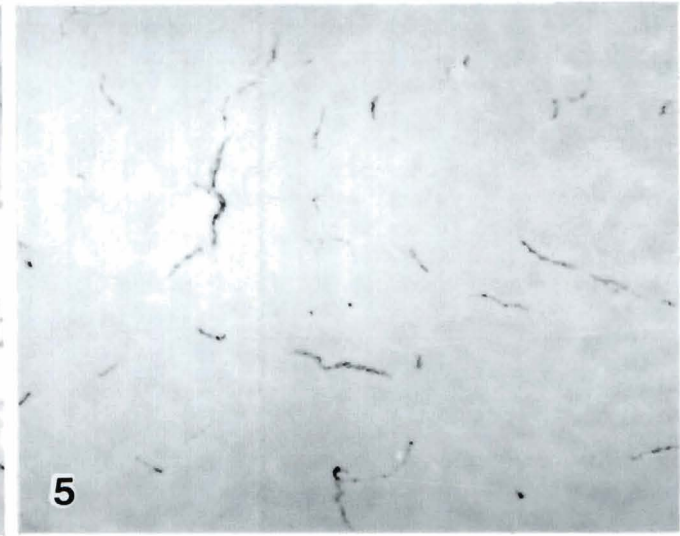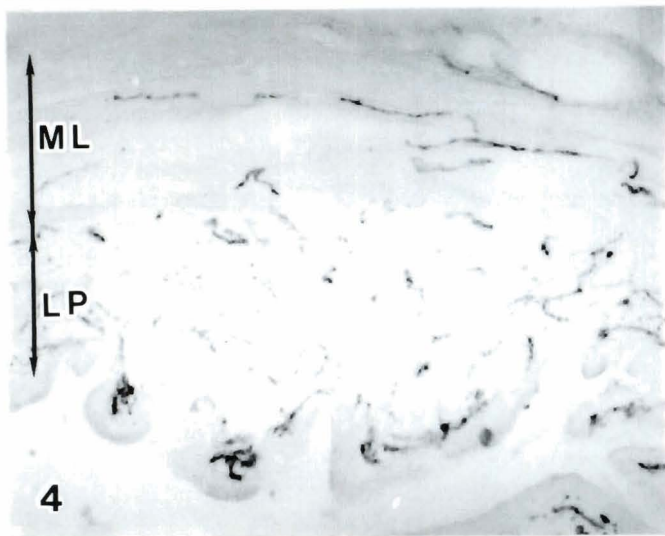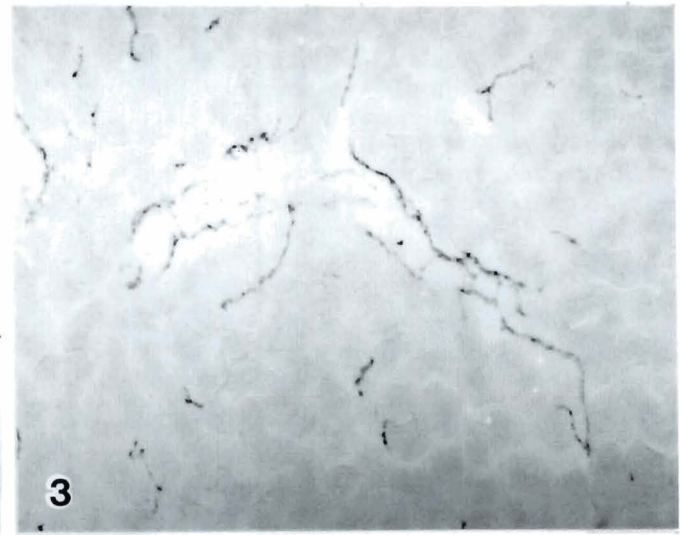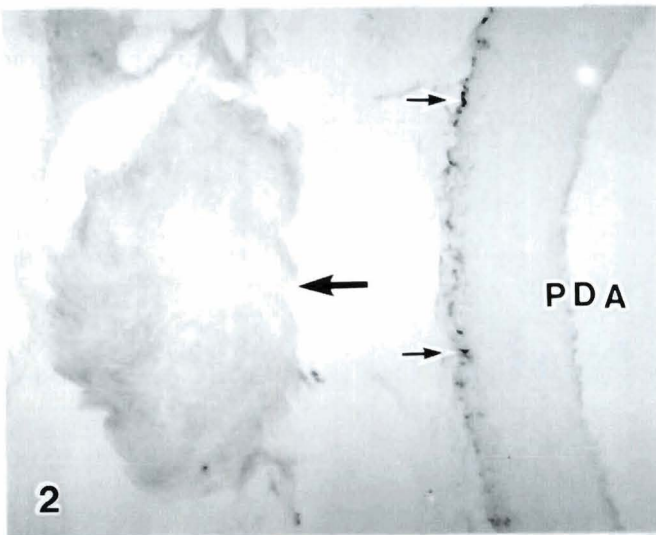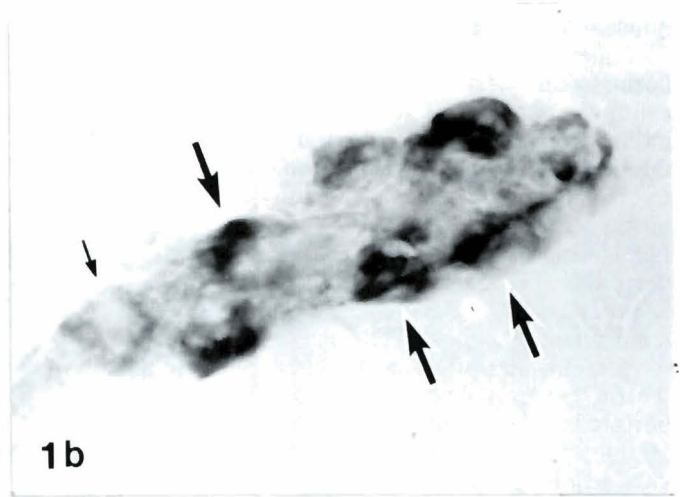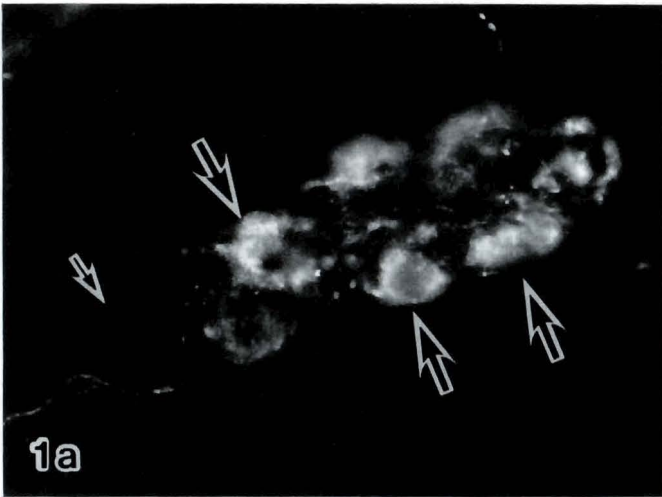
Fig. 2. Galanin-immunoreactive nerve fibres (small arrows) are observed in the adventitia of the pancreatico-duodenal artery (PDA). No nerve fibres showing the immunoreactivity for galanin were found in the extrapancreatic nerve bundle accompanying PDA (large arrow). x 215

Fig. 3. Galanin-immunoreactive nerve fibres form a dense network around blood vessels. x 215

Fig. 4. Cross section of the main pancreatic duct. Galanin-immunoreactive nerve fibres are densely distributed in the lamina propria (LP), but are fewer in the muscular layer (ML). Beneath the epithelium, varicose nerve fibres show galanin immunoreactivity. x 215

Fig. 5. A moderate number of nerve fibres showing galanin immunoreactivity are scattered in the exocrine parenchyma. x 215

Galanin in the chicken pancreatic neuron

A-islets (Fig. 6c,d).

Discussion

The present study demonstrates that the pancreas of the chicken is innervated by galanin-immunoreactive nerves. There appears to be species differences in the nature of galanin-containing nerves in the pancreata of various species. An immunohistochemical study in the dog (Ahrén et al., 1990) revealed that galanin-containing nerves in the pancreas showed immunoreactivity for tyrosine hydroxylase (TH), a marker of adrenergic
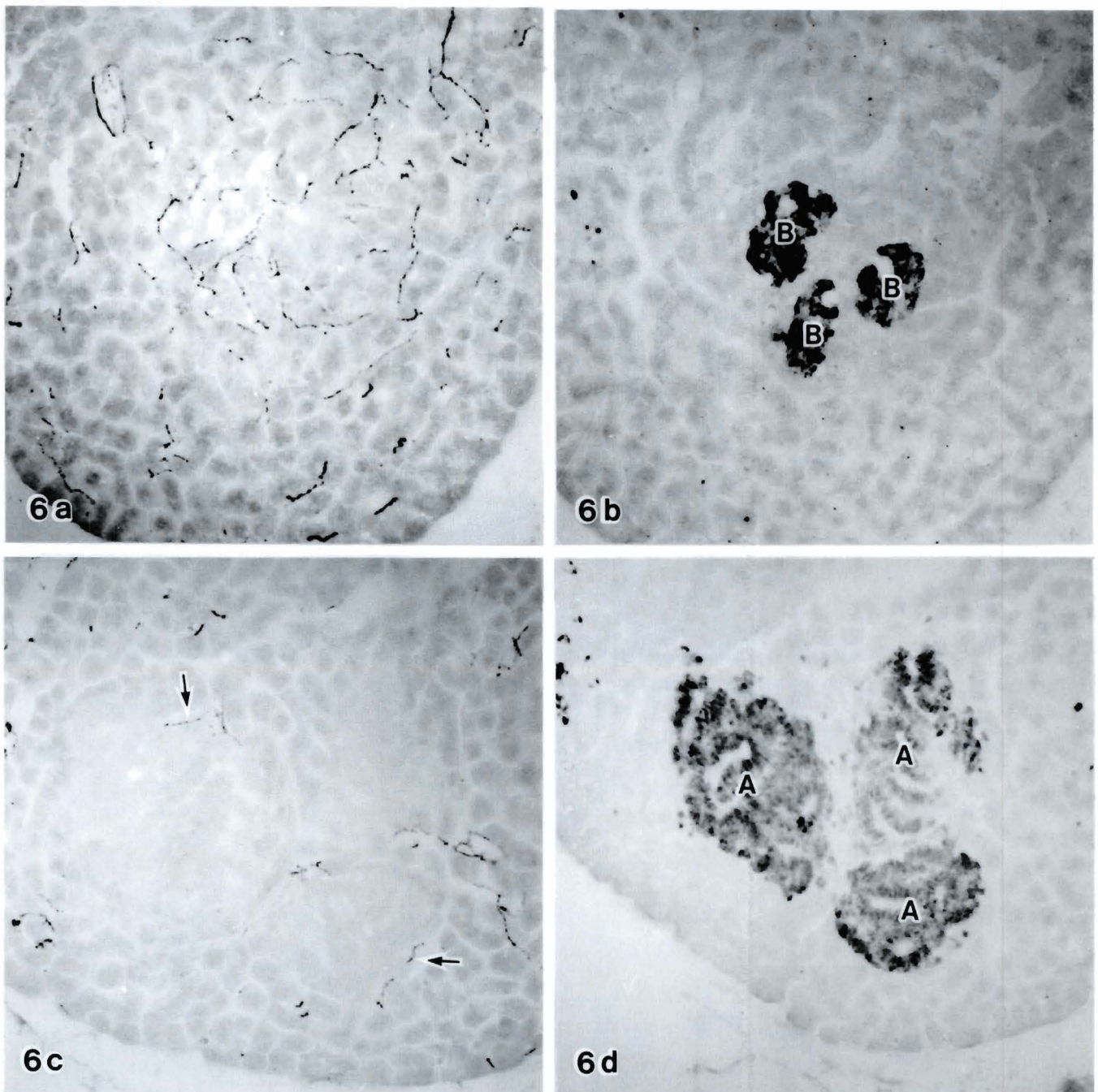

Fig. 6. Two pairs of serial sections showing distribution patterns of galanin-immunoreactive nerve fibres in B-islet **(a,b)** and A-islet **(c,d)**. a, c: Sections stained with antiserum against galanin. Many galanin-immunoreactive nerve fibres are distributed within and around B-islets (a), but only a few galanin-immunoreactive nerve fibres are found around A-islets (c, arrows). b: Serial section adjacent to a stained with antiserum against insulin. d: A section adjacent to c stained with antiserum against glucagon. A: A-islet, B: B-islet. a-d x 140

nerves, and the coeliac ganglion contained galanin-immunoreactive nerve cells. Thus, galanin-containing nerves are postganglionic, extrinsic and adrenergic in the dog pancreas. In the porcine pancreas, galanin-containing nerves might be extrinsic in origin, because no ganglion cells showing galanin immunoreactivity were detected (Messell et al., 1990). In rat and mouse pancreas, galanin-containing nerves were observed even after chemical sympathectomy by means of 6-hydroxy-dopamine (Su et al., 1987; Lindskog et al., 1991). Nerve cells showing galanin immunoreactivity also exist in the human pancreas (Ahrén et al., 1991). It appears that galanin-containing nerves in the pancreata of these species have an intrinsic origin and a non-adrenergic nature.

In the present study, many ganglion cells showing immunoreactivity for galanin were detected in the chicken pancreas. Moreover, nerve bundles accompanying the pancreatico-duodenal artery did not contain galanin-immunoreactive nerve fibres. These nerve bundles contain nerves derived from the coeliac ganglion (Watanabe and Paik, 1973) and the abdominal vagus (Hiramatsu and Watanabe, 1993). These data clarify that galanin-containing nerves in the chicken pancreas are intrinsic in origin.

A combination of immunohistochemistry and AChE histochemistry demonstrated that all galanin-immunoreactive ganglion cells showed AChE activity simultaneously. It is probable that galanin colocates with acetylcholine in the chicken intrapancreatic neurons. Moreover, at least two kinds of ganglion cells could be detected: one showing both galanin immunoreactivity and AChE activity; the other only AChE activity. It is not clear whether a difference exists in the target organ between these two kinds of ganglion cells. Considering the cellular composition of A- and B-islets (Iwanaga et al., 1983) and the distribution pattern of AChE-positive nerve fibres (Hiramatsu et al., 1988), it is plausible that each of the two neurons has its specific target cell.

Melander et al. (1985) revealed the co-existence of galanin and vasoactive intestinal polypeptide (VIP) in cell bodies of the myenteric plexus in the pig duodenum and the guinea-pig colon. In the human, rat and pig gastrointestinal tract, Bishop et al. (1986) demonstrated a similarity in the distribution pattern of galanin immunoreactive nerve fibres to those containing VIP. The distribution pattern of galanin-immunoreactive nerve fibres in the chicken pancreas was very similar to that of VIP-immunoreactive elements (Hiramatsu and Watanabe, 1989). In the human pancreas, these two peptides colocated in a large proportion of nerve cells (Shimosegawa et al., 1992). Recently, Salakij et al. (1992) reported the coexistence of galanin with VIP in the intrapancreatic ganglion cells of the chicken. Galanin and VIP may be colocated in neurons of the chicken pancreas and it is suggested that these two neuropeptides have a functional relationship.

Galanin-immunoreactive nerve fibres were distributed throughout the entire gland. Galanin-immunoreactive nerve fibres were distributed within the lamina propria and in the smooth muscle of secretory ducts. Galanin has a direct constrictive effect on intestinal smooth muscle (Tatemoto et al., 1983). It is likely that galanin plays a role in the physiological regulation of muscle tone and the motility of secretory ducts. The appearance of varicose nerve fibres showing galanin immunoreactivity just beneath the epithelium suggests that galanin-containing nerves may be functionally coupled with the activity of ductal epithelial cells.

Pharmacological studies in the rabbit have shown that blood vessels did not respond to galanin (Ekblad et al., 1985). A relation of galanin-immunoreactive nerves to blood vessels has been consistently reported in the mammalian pancreas (Su et al., 1987; Ahrén et al., 1990, 1991; Messell et al., 1990; Lindskog et al., 1991). In this study, a dense network of fine nerve fibres showing galanin immunoreactivity was found around blood vessels. In view of the present immunohistochemical data, it is plausible that galanin-containing nerves present the major vasomotoric elements in the pancreas.

Acini of the mammalian pancreas appear to be innervated by galanin-containing nerves. However, there is a difference in the density of innervation of the exocrine tissue among animal species. Galanin-immunoreactive nerve fibres were observed only occasionally in the exocrine parenchyma of the dog pancreas (Ahrén et al., 1990). In the human pancreas galanin-immunoreactive fibres were often seen to run close to acini (Ahrén et al., 1991). The present study reveals that the exocrine parenchyma of the chicken pancreas receives a rich supply of galanin-immunoreactive nerve fibres. Thus, our immunohistochemical data suggest that galanin-immunoreactive nerve fibres have a close relation to exocrine secretion as well as vasomotor and ductal functions.

In the dog (Ahrén et al., 1990), human (Ahrén et al., 1991) and mouse and rat pancreas (Lindskog et al., 1991), pancreatic islets were innervated by galanin-immunoreactive nerves. Physiological studies on the mammalian pancreas have revealed an inhibitory effect of galanin on the secretion of islet hormones (McDonald et al., 1985; Dunning et al., 1986; Lindskog and Ahrén, 1989). It is well established in the mammalian pancreas that galanin acts as a neurotransmitter on the islets.

Chicken pancreatic islets are divided into two types: A-islets consist of many A cells and some D cells, B-islets are made up of many B cells and a few D cells (Iwanaga et al., 1983). We revealed a difference in the distribution pattern of galanin-immunoreactive nerve fibres between the two types of pancreatic islets. A small number of galanin-immunoreactive nerve fibres was found in the A-islets. In contrast, many beaded nerve fibres showing galanin immunoreactivity were detected in B-islets. These findings suggest that galanin may play an important role in the control of insulin secretion from the chicken pancreatic islets.

In conclusion, galanin-immunoreactive nerve fibres,

innervate both endocrine and exocrine tissue in the chicken pancreas and are of intrinsic origin. Co-localization of galanin with acetylcholine is suggested.

References

Ahrén B. and Lindskog S. (1992). Galanin and the regulation of islet hormone secretion. Int. J. Pancreatol. 11, 147-160.

Ahrén B., Böttcher G., Kowalyk S., Dunning B.E., Sundler F. and Taborsky G.J.Jr. (1990). Galanin is co-localized with noradrenaline and neuropeptide Y in dog pancreas and celiac ganglion. Cell Tissue Res. 261, 49-58.

Ahrén B., Ar'Rajab A., Böttcher G., Sundler F. and Dunning B.E. (1991). Presence of galanin in human pancreatic nerves and inhibiton of insulin secretion from isolated human islets. Cell Tissue Res. 264, 263-267.

Bishop A.E., Polak J.M., Bauer F.E., Christofides N.D., Carlei F. and Bloom S.R. (1986). Occurrence and distribution of a newly discovered peptide, galanin, in the mammalian enteric nervous system. Gut 27, 849-857.

Dunning B.E., Ahrén B., Veith R.C., Böttcher G., Sundler F. and Taborsky G.J.Jr. (1986). Galanin: a novel pancreatic neuropeptide. Am. J. Physiol. 251, E127-E133.

Ekblad E., Håkanson R., Sundler F. and Wahlestedt C. (1985). Galanin: neuromodulatory and direct contractile effects on smooth muscle preparations. Br. J. Pharmacol. 86, 241-246.

Guesdon J.-L., Ternyck T. and Avrameas S. (1979). The use of avidin-biotin interaction in immunoenzymatic techniques. J. Histochem. Cytochem. 27, 1131-1139.

Hiramatsu K. and Watanabe T. (1989). Immunohistochemical study on the distribution of vasoactive intestinal polypeptide (VIP) containing nerve fibres in the chicken pancreas. Z. Mikrosk. Anat. Forsch. 103, 689-699.

Hiramatsu K. and Watanabe T. (1993). Ultrastructural and morphometric studies in the peripheral course of the vagus in the domestic fowl, with particular reference to the cholinergic innervation of the pancreas. Ann. Anat. 175, 335-341.

Hiramatsu K., Watanabe T. and Fujioka T. (1988). The distribution of acetylcholinesterase (AChE)-positive nerves in chicken pancreas demonstrated by light and electron microscopy. Arch. Histol. Cytol. 51, 159-168.

Iwanaga T., Yui R. and Fujita T. (1983). The pancreatic islets of the chicken. In: Avian endocrinology; environmental and ecological perspectives. Mikami S., Homma K. and Wada M. (eds). Japan Sci. Soc. Press. Tokyo. pp 81-94.

Lindskog S. and Ahrén B. (1989). Effects of galanin on insulin and glucagon secretion in the rat. Int. J. Pancreatol. 4, 335-344.

Lindskog S., Ahrén B., Dunning B.E. and Sundler F. (1991). Galanin-immunoreactive nerves in the mouse and rat pancreas. Cell Tissue Res. 264, 363-368.

McDonald T.J., Dupre J., Tatemoto K., Greenberg G.R., Radziuk J. and Mutt V. (1985). Galanin inhibits insulinn secretion and induces hyperglycemia in dogs. Diabetes 34, 192-196.

Melander T., Hökfelt T., Rökaeus Å., Fahrenkrug J., Tatemoto K. and Mutt V. (1985). Distribution of galanin-like immunoreactivity in the gastro-intestinal tract of several mammalian species. Cell Tissue Res. 239, 253-270.

Messel T., Harling H., Böttcher G., Johnsen A.H. and Holst J.J. (1990). Galanin in the porcine pancreas. Regul. Peptides 28, 161-176.

Rattan S. (1991). Role of galanin in the gut. Gastroenterology 100, 1762-1768.

Rökaeus Å., Melander T., Hökfelt T., Lundberg J.M., Tatemoto K., Carlquist M. and Mutt V. (1984). A galanin-like peptide in the central nervous system and intestine of the rat. Neurosci. Lett. 47, 161-166.

Salakij C., Watanabe T., Takahashi S., Ohmori Y. and Nagatsu I. (1992). Immunohistochemical studies on the intrinsic pancreatic nerves in the chicken. J. Auton. Nerv. Syst. 40, 131-140.

Shimosegawa T., Moriizumi S., Koizumi M., Kashimura J., Yanaihara N. and Toyota T. (1992). Immunohistochemical demonstration of galanin-like immunoreactive nerve in the human pancreas. Gastroenterology 102, 263-271.

Su H.C., Bishop A.E., Power R.F., Hamada Y. and Polak J.M. (1987). Dual intrinsic and extrinsic origins of CGRP- and NPY-immunoreactive nerves of rat gut and pancreas. J. Neurosci. 7, 2674-2687.

Tatemoto K., Rökaeus Å., Jörnvall H., McDonald T.J. and Mutt V. (1983). Galanin - a novel biologically active peptide from porcine intestine. FEBS Lett. 164, 124-128.

Watanabe T. and Paik Y.K. (1973). Comparative and topographical anatomy of the fowl. LXIV. Sympathetic nervous system of the fowl. Pat 3. Plexus celiacus and plexus mesentericus cranialis. Jpn. J. Vet. Sci. 35, 389-401 (in Japanese with English summary).