

THE IMMUNOHISTOCHEMICAL DETECTION OF SUBSTANCE P (SP) IN THE PANCREAS AND INTESTINE OF THREE SPECIES OF LOWER VERTEBRATES; LIGHT-MICROSCOPIC OBSERVATIONS

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ABSTRACT. The pancreas and gut of two amphibians and a reptile species were investigated immunohistochemically for the occurrence and topographic distribution of substance P (SP) by using peroxidase anti-peroxidase (PAP) procedure. A relative rich SP innervation consisting of nerve fibers with a peri-acinar distribution was seen both in frogs and turtle pancreas. In turtles, thick bundles of nerve fibers, localized in the pancreatic connective tissue, have been also observed. In addition to the neural structures, the pancreas of frogs and turtles displayed often singular immunostained cells of endocrine nature. Finally, both varieties of "closed" and "open" enteroendocrine cells have been immunodetected in the villi epithelium of these animals. The above findings are discussed in connection with those previously reported in other taxa of vertebrates.

Keywords: substance P (SP), amphibians, turtle, pancreas, intestine, immunohistochemistry

INTRODUCTION

The substance P was the first regulatory peptide with a dual localization (intestine and brain) proved since 1931 (von Euler and Gaddum, 1931). Isolated, chemically characterized and much later synthesized (Chang et al., 1971; Tregear et al., 1971), this neuropeptide, with a sequence of 11 amino acid residues, represents the prototype of a peptides family entitled tachikins (Eleodoisin, Physalemin, Uperolein a.o) involved in the prompt stimulation of the smooth musculature. Bertaccini (1976) was the first who relieved that the biological activity of SP is induced by the terminal pentapeptide fragment from the extremity -COOH of the molecule, exactly by the amino acid phenylalanine placed in 5th position in relation to the terminal -COOH extremity. This discovery was sustained by more studies on SP synthetic analogues like Physalemin and Eleodoisin (Yanaihara et al., 1977; De Castiglione, 1978), which were tested for their action on guinea pig ileum and rabbit large intestine. Still, the greatest amount of SP was identified in various brain formations such as substance nigra, hypothalamus, limbic system, dorsal rachidian bulb and dorsal pons of Varolli, where was detected immunohistochemically both in perikarya as well as in their neuronal extensions.

Extraneuroaxial SP appeared co-localized with somatostatin (SOM) in the perikaryons of paravertebral and perivertebral ganglia, including adrenal medulla, but also in the nonmyelinated nerve fibers (Bertaccini, 1980; Konturek et al., 1981; Trümper et al., 1982).

At the level of alimentary tract substance P was immunohistochemically identified alone or co-localized with serotonin (5HT) or with SOM in some endocrine cells disseminated from the esophagus to the

colon, as well as in the intrinsic nervous plexuses (Bertaccini, 1976; 1980). Finally, the occurrence of neuropeptide was revealed not only in the mucous cells of the gallbladder, in the respiratory and genito-urinary tracts, in the nervous fibers of the salivary glands and of the exocrine pancreas, but also in the hypophysis and pancreatic islets (Powell and Skrabanek, 1970; 1981). Taking into account the above results regarding the various SP localizations in the mammalian and human organs, the purpose of this study was to detect immunohistochemically in light-microscopy the occurrence and distribution of this neuropeptide in the pancreas and intestine of several lower vertebrates, so much the more its multiple functional involvements are still debated (Creagh et al., 1980). Another aim was to provide additional evidence for the phylogenetic perennality of this neuropeptide.

MATERIALS AND METHODS

Adult specimens of frogs and turtles, purchased in springtime (April-May) from commercial sources, were unfed in fresh water aquaria for 2-3 days. The species and the number of specimens (in brackets) employed have been the following: *Rana esculenta* L. (5), *Xenopus laevis* D. (4), *Emys orbicularis* L. (3).

All the animals were killed under chloroform anesthesia and the pancreas fragments and equal segments from proximal, middle and end regions of the intestine (depending of species) were then dehydrated through a graded ethanol series, cleared with toluene and paraffin-embedded (Möller, 1976). Further on, serial sections of 6 µm-thickness, prepared a rotary microtome, were mounted on poly-L-lysine (Sigma, USA) coated slides. The anti-substance P polyclonal serum has been purchased from Biotrend (Köln, Germany), goat anti-rabbit IgG from Sigma (USA) and

the peroxidase anti-peroxidase (PAP) complex from Dakko (Copenhagen, Denmark). The optimal immunostaining was achieved by increasing the dilutions of the primary antibody prepared in saline phosphate buffer (0.1M PBS; pH 7.4) supplemented with 0.3% Triton X-100. The 1:400 concentration which maximally stains the immunoreactive structures without any other unspecific reaction was chosen. The goat anti-rabbit IgG (dilution 1:40) and the PAP complex (dilution 1:80) were used in excess. Procedure-depending non-specificities were excluded by replacing the primary antibody, goat anti-rabbit IgG or PAP complex with phosphate saline (PS) or with Tris-saline (TS) buffers.

In addition, the anti-SP serum was tested by pre-adsorption (24h at 4°C) with various quantities of specific antigens or with structural related antigens.

RESULTS AND DISCUSSIONS

The optimal immunostaining was obtained by using the dilution 1:400 of the primary antibody, which didn't allow an unspecific reaction (background) in the pancreas and gut of all species of poikilotherm vertebrates under study. Thus, with this dilution variable amounts of immunoreactive endocrine cells and nerve fibers appeared disseminated in both organs. Their occurrence and density varied widely along the intestine and among the species investigated and only slightly among the individuals of the same species (Table 1). Mention should also be made that in the gut of animals studied no immunolabelled ganglions and intrinsic nerve plexuses were recorded.

Table 1

Occurrence and relative density of SP-immunoreactive structures in the pancreas and along the intestine of investigated lower vertebrates

SPECIES	IMMUNOSTAINED STRUCTURES			
	PANCREAS SECTIONS	PROXIMAL REGION	SECTIONS OF INTESTINE MIDDLE REGION	END REGION
<i>Rana esculenta</i>	EC/NF ++/+++	EEC/NF ++/+++	EEC/NF ++/+++	EEC/NF ++/+++
<i>Xenopus laevis</i>	EC/NF ++/+++	EEC/NF +/++++	EEC/NF ++/+++	EEC/NF ++/+++
<i>Emys orbicularis</i>	EC/NF +++/>++	EEC/NF ++/+++	EEC/NF +++/>++++	EEC/NF ++++/>++++

Abbreviations: EC=endocrine cells; EEC=enteroendocrine cells; NF=nerve fibers.

Symbols (amount of each category of immunostained structures/pancreas and intestine sections): +++, >12; ++, 8-12; +, 4-7; +, 1-3; ± occasional presence; -, none.

Frogs. Besides the relative abundant nerve fibers with a peri-acinar distribution in the pancreas of both amphibian species, a small number of neuroendocrine elements, most of them showing the morphological features of the "closed" cell variety, appeared scattered in this gland (Figure 1). As regard the gut, the most immunodetected cells were of "open" variety and localized in the lower half of the villi epithelium (Figures 2-5).

Turtles. The turtle pancreas displayed a lot of singular endocrine cells and also small clusters of 2-3 immunomarked cells disseminated randomly in exocrine tissue (Fig. 6). As compared to amphibian species the pancreas of these animals displayed frequently bundles of immunostained nerve fibers spread in the connective tissue septa (Fig. 7), whereas the intestine showed by far the most numerous labelled cells and nerve terminals (Figures 8-11). Apart from nerve fibers, immunostained cells, of both "open" and "closed" varieties, were encountered throughout the intestinal epithelium (Figures 8-11). The amount of these enteroendocrine cells slowly increased from proximal to end gut region (Figures 10, 11). A similar increasing trend was recorded also for SP-containing nerve fibers (Table 1).

Our results referring to the immunoreactive localizations for SP in the pancreas and intestine of the

two anuran amphibians (*Rana esculenta*, *Xenopus laevis*) and of the turtles *Emys orbicularis* seem to correspond in the main to those previously reported for this neuropeptide in the same organs of frogs (Gabriel, 1990; Li et al., 1993) or of other poikilotherm vertebrates (Gabriel et al., 1992; Liu et al., 2002).

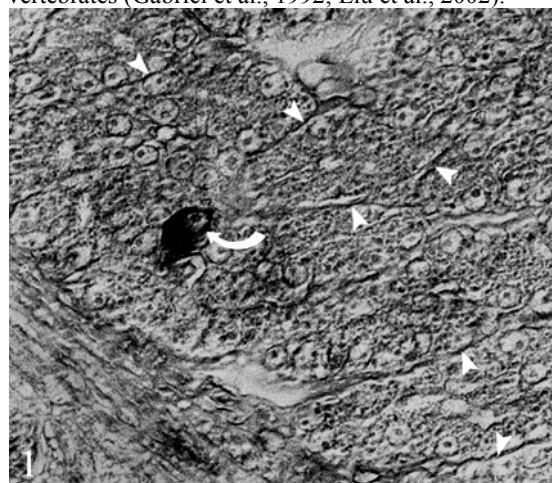


Fig. 1 Section through the pancreas of the frog *Rana esculenta*, showing a solitary immunostained cell (curved arrow) and a lot of peri-acinar nerve terminals (arrowheads). PAP- procedure, x160

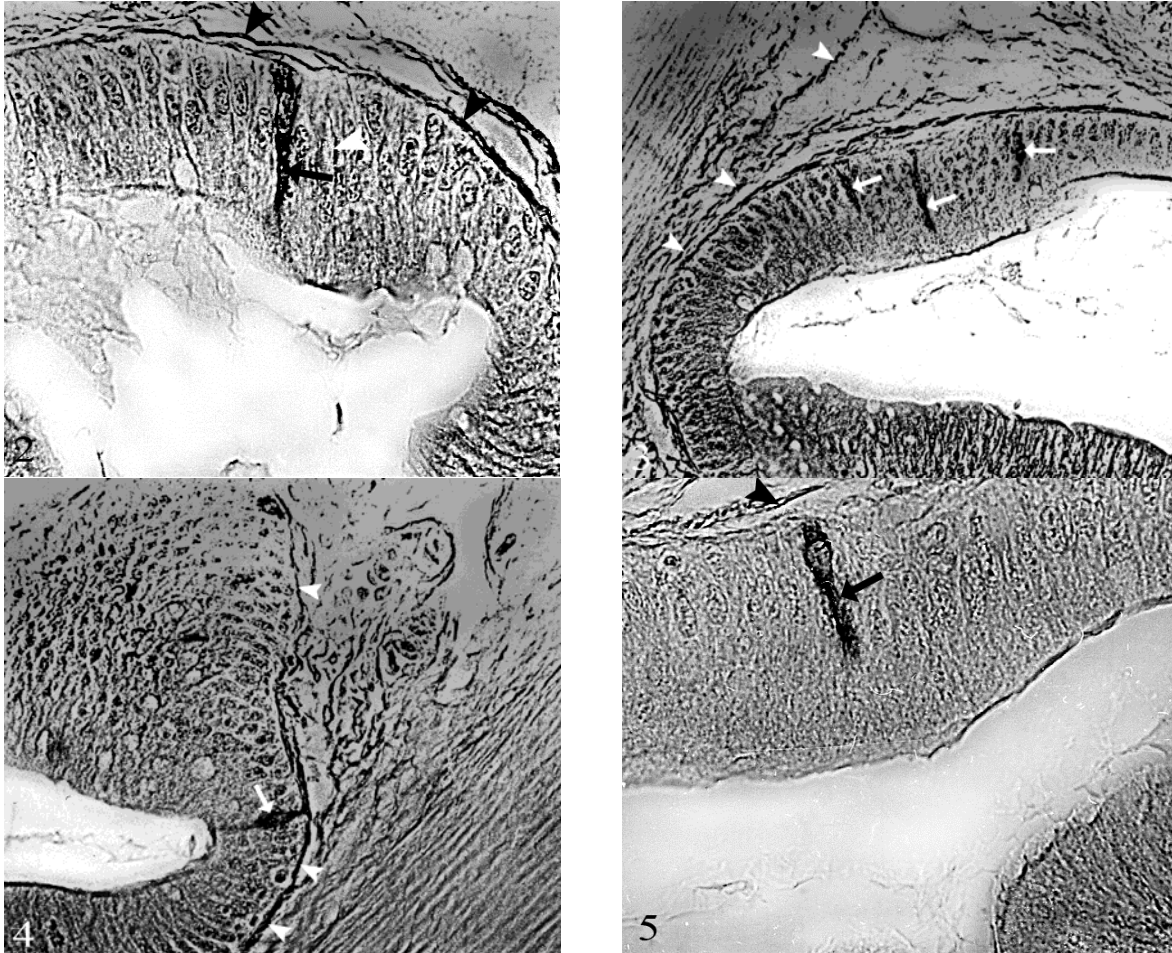


Fig. 2-5 Images displaying labeled nerve fibers (arrowheads) distributed among epithelial cells (Fig. 2) and at their base (Figs 2-5) in the proximal (Fig. 2), middle (Figs 3, 4) and distal (Fig. 5) regions of the frogs' intestine *Rana esculenta* (Fig. 2, 3) and *Xenopus laevis* (Figs 4, 5). To note also that all immunodetected enteroendocrine cells (arrows) are all of "open" variety. PAP-procedure. Fig. 2: x160; Fig. 3: x82; Fig.4: x 97; Fig.5:x160.

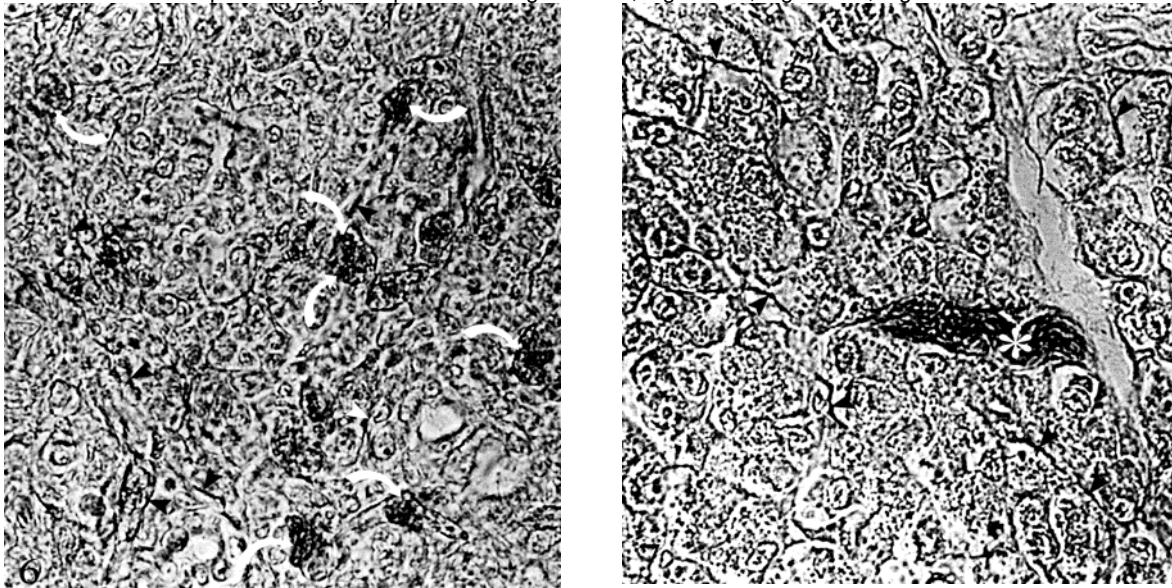


Fig. 6, 7 Portions of the turtle pancreas (*Emys orbicularis*) showing labeled nerve fibers (arrowheads) around acini, a thick bundle of nerve fibers (Fig. 7) in the connective tissue (asterisk), and small clusters or sole endocrine cells (Fig. 6) immunostained for SP (curved arrows). PAP- procedure. Figs. 6,7: x70

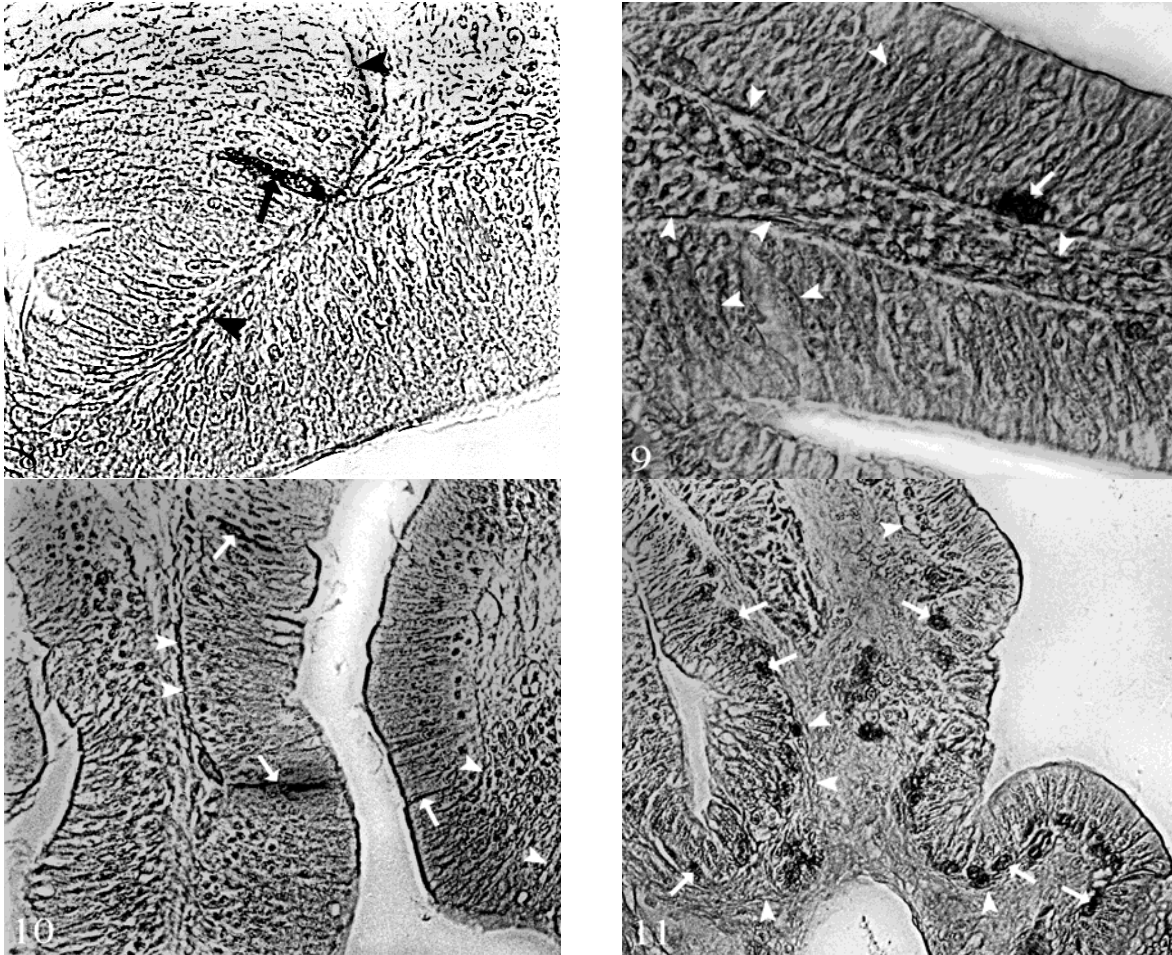


Fig. 8-11 Intestinal villi in the turtle *Emys orbicularis* exhibiting a lot of immunostained thin nerve fibers (arrowheads) spread among the epithelial cells (Figs. 8, 9) and in the villi axes (Figs 9, 10), as well as "open" (Figs. 8, 10, 11) and "closed" (Fig 9) varieties of labelled cells (arrows). Fig. 8=proximal intestine; Figs 9, 10=middle intestine; Fig. 11=distal intestine. PAP- procedure. Fig. 8: x160; Figs. 9, 11: x64; Fig.10: x82

Our findings illustrate also that, besides a number of similarities, species differences exist between frogs with regard the organization of the enteric nervous system (Junquera et al., 1987; Gabriel et al., 1992) and of other systems (Kusakabe et al., 1995), even along their metamorphosis (Maake et al., 1999). With other words, taking into account that amphibians' parasympathetic and sympathetic innervations are limited, it could be considered that in lower vertebrates the intestinal peptidergic innervation, like SP-innervation, is phylogenetically earlier and hence better developed than in mammals. On the other hand, a series of biochemical and experimental investigations on mammalian organs and tissues (Fernández et al., 2002; Johansson et al., 2002), inclusive on the pancreas and gut (Bailey et al., 1986; Fernández et al., 2002; Arciszewski and Zacharko-Siembida, 2007) pledge for a unitary distribution pattern and for similar functional significances of the substance P and related tachykinins neuropeptides. Considering the above, a special attention should be paid to the functional involvements

of SP in the motility of gastrointestinal tract through its stimulatory and inhibitory actions on circular and longitudinal musculature as claimed in mammals by numerous authors (Bertaccini, 1976; 1980; Holzer and Lembeck, 1980; Mukhopadhyay et al., 1980). Among the involments of this neuropeptide were listed those on the insulin- glucagon- and somatostatin- releases from pancreas (Lunquist et al., 1979; Hermansen, 1980), on the release of pancreatic amilase and inhibition of sodium bicarbonate secretion (Sjödin et al., 1980) and the last but not the least the qualities of this neuropeptide as neurotransmitter and neuromodulator of motor and sensitive nervous inputs (Chan-Palay, 1979).

CONCLUSIONS

This paper is one of the few immunohistochemical demonstrations dedicated to the occurrence and topographic distribution of SP in the pancreas and intestine of poikilotherm vertebrates. Its purpose is to enrich our knowledge on the presumed phylogenetic

perenity of this multifunctional neuropeptide in the above glandular organs, considering that its multiple functional involvements are still debated and even contradictory.

The main expression of SP-innervation in both organs could be considered phylogenetically earlier and better developed than in mammals even when the presence of immunolabelled ganglion cells and of intramural nervous plexuses (Meissner and Auerbach) could not be observed in our preparates. Regarding the pancreatic endocrine cells and the enteroendocrine ones immunomarked for SP, their presence and distribution pledge undoubtedly for similar functional involvements as those reported already in mammals.

Considering the aforesaid, detailed investigations on the occurrence, distribution and functional involvements of this neuropeptide in the pancreas and gut of other species and taxa of poikilotherm vertebrates are requested.

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