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Mucosal peptidergic innervation of the opossum esophagus and anal canal: a comparison with snout skin

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Abstract

Nerves within and under the esophageal epithelium of the opossum esophagus were investigated morphologically with osmication and immunohistochemically for ten neuropeptides. The structurally similar but functionally diverse epithelia of the anal canal and snout skin, on which no immunohistochemical information exists, were similarly investigated for comparison. Total innervation was estimated from osmication, which revealed intraepithelial nerves in all three tissues in the following order of density: snout skin > anal canal > esophagus. Calcitonin gene-related peptide and substance P occurred in all three organs. The snout skin had intraepithelial galanin nerves but not vasoactive intestinal polypeptide, while conversely the esophagus and anal canal had vasoactive intestinal polypeptide but not galanin. All peptides found intraepithelially also occurred subepithelially. Calcitonin gene-related peptide, galanin, neuropeptide Y, substance P and vasoactive intestinal polypeptide subepithelial nerves occurred in all the tissues, while gastrin releasing peptide nerves occurred infrequently in the subepithelial regions of the esophagus and anal canal, but not the snout skin. As these epithelia neither secrete nor absorb, their nerves are presumably sensory. The peptides investigated could not account for all intraepithelial nerves demonstrated by osmium. Differences in the innervation of these epithelia may result from their differing sensory requirements.

Introduction

Free nerve endings that penetrate the basement membrane and lie between squamous epithelial cells are well described in the skin, but are not as widely recognized in the alimentary canal. Such endings are thought to mediate pain, particularly sharp pain as well as two-point discrimination [6,12,16] and stretch sensation [9] in the skin, and soft touch and chemical sensation in the cornea [21]. In the gastrointestinal tract the squamous epithelium of the esophagus [17,19] and anal canal [5,18] have been shown to possess intraepithelial nerves that are presumed to be sensory in nature. These studies employed classical metal impregnation methods which demonstrate the presence but not the immunocytochemical attributes of the nerve fibers in question. Additionally, immunocytochemical studies on the esophageal mucosa have demonstrated intraepithelial CGRP- (cat and monkey, ref. 20) and SP- (monkey, ref. 10; rabbit, ref. 11; opossum, ref. 2) containing nerve fibers.

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The immunocytochemical nature of the intraepidermal (i.e. intraepithelial) fibers of the skin has also been studied. These nerves have been shown to contain calcitonin gene-related peptide (CGRP), substance P (SP) and possibly neuropeptide Y (NPY) in various species such as the cat [22], mouse [25], guinea-pig [8] and human [26]. In sharp contrast, there is no immunohistochemical study on the innervation of the anal canal mucosa or of the opossum snout skin, though there is one metal-impregnation study [13] on the latter.

This study undertakes to provide an estimation of innervation density and peptidergic nature of intraepithelial (and subepithelial) innervation in the esophagus, and to compare it with that of the anal canal and epidermis of the snout skin. Since none of these epithelia secretes or absorbs, nerves within them are most likely sensory.

TABLE I

Details of antisera used

Antiserum to	Code	Dilution	Specificity (% cross-reactivities)
CCK	61007	1:300	100 with CCK-8 and CCK-33 *.
CGRP	RAS6009N	1:300	human CGRP, 100; rat calcitonin C-terminal adjacent peptide, < 0.001
GAL	RAS7153N	1:300	Gal, 100; secretin, 0; PHM-27, 0; VIP, 0
GRP	RAS7113N	1:300	GRP, 100; SP, 0; VIP 0; gastrin 1 (human), 0; *met*-enk, 0; CCK 26-33, 0; NPY, 0
Leu-Enk	61006	1:400	Some cross-reactivity with *Met*-Enk and N-terminal extension peptides; 0 with C-terminal extension peptides *
Met-Enk	61008	1:400	Low cross-reactivity with *Leu*-Enk, some with N-terminal extension peptides; 0 with C-terminal extension peptides *
NPY	RAS7172N	1:300	NPY, 100; human pancreatic polypeptide, 0.02; avian pancreatic polypeptide, 0.007; peptide YY, 0.003; VIP, < 0.001; rat corticotropin releasing factor, < 0.001; PHM-27 < 0.001; rat pancreatic polypeptide, 0; glucagon, 0; secretin, 0; PHI, 0
SOM	RAS8004	1:300	SOM-28, 100; SOM-14, 73
SP	RAS 7451N	1:300	SP, 100; SP 3-11, 87; SP 4-11, 75; SP 5-11, 75; SP 2-11, 68; SP 7-11, 55; SP 6-11, 38; SP 8-11, < 1; SP 9-11, < 1; Substance K, < 1; neurokinin B, < 1; neuromedin C, < 0.05; dynorphin 1-13, < 0.002; neurotensin, < 0.001; SOM, < 0.001; human β-endorphin, < 0.001; met^5-enk, 0; leu^5-enk, 0; ACTH 1--24, 0; Arg^8 vasopressin, 0; neuromedin B, 0; neuromedin N, 0; neuromedin U-8, 0
VIP	RAS7161N	1:300	VIP, 100; VIP 10-28, < 0.001; PHM-27, < 0.001

All antisera were from Peninsula Labs, Belmont, CA.
* Personal communication from Dr Y.N. Wang of Peninsula Labs

Material and Methods

The mid esophagi, anal canals and snout skin from seven pentobarbital-anaesthetized (40 mg/kg, i.p.) opossums (*Didelphis virginiana*) were

removed and immersed in either freshly-mixed Maillet's variation of Champy's fluid (2% OsO_4 + 3% ZnI_2, 1:4, v/v) [14] overnight for osmium staining of nerve fibers or in ice-cold Zamboni's fluid for 24 h [27] for immunohistochemistry. The first two organs were opened flat before fixation.

Maillet-Champy-fixed specimens were divided in two when fixation was complete. One received repeated changes of PBS for 24 h, was placed in 15% sucrose-enriched PBS overnight, and serially cryosectioned at 16 μm. The other was paraffin-embedded and serially sectioned at 16 μm. Both paraffin and cryosections were hydrated, and alternate sections were either (a) dehydrated, cleared and mounted in DPX, or (b) counterstained with 0.1% thionin before dehydration, clearing and mounting. This permitted visualization of nervous structures with and without histological counterstain.

Zamboni-fixed tissues received repeated changes of cold PBS (pH 7.4) for 24 h and were placed in PBS (pH 7.4) containing 15% sucrose for another 24 h. They were then serially cryosectioned at 16 μm. Sections were subjected to indirect immunofluorescence [4] for the demonstration of cholecystokinin (CCK), CGRP, GAL, gastrin-releasing peptide (GRP), leucine-enkephalin (*l*-Enk), methionine-enkephalin (*m*-Enk), NPY, somatostatin (SOM), SP and VIP. All antisera were obtained from Peninsula Laboratories, Belmont, CA; details and dilutions appear in Table I. Non-specific sites were blocked in 5% normal goat serum. Incubation in primary antiserum was for 20 h at 4°C, while the secondary antibody (FITC-conjugated affinity-purified goat anti-rabbit IgG, Calbiochem, La Jolla, CA, diluted 1:60) was applied for 1 h at room temperature. Observation and photography were performed under fluorescent epi-illumination on an Olympus BH-2 microscope. In many instances the coordinates of a given field of interest were marked, and the section re-stained in 0.1% thionin for re-photography to corroborate histological details. Three kinds of controls were performed: (a) omission of the primary antiserum; (b) substitution of the primary antiserum with normal rabbit serum at the same dilution, and (c) preabsorption of the antiserum with an excess of antigen (100 μg/ml, 24 h at 4°C) before incubation. An otherwise positive reaction was taken to be genuine only if it was eliminated in all three controls. Since the sharing of immunodeterminant sequences between various tissue components cannot theoretically be excluded, in the present account a description such as 'CGRP-immunoreactive' denotes CGRP-*like* immunoreactivity.

The nature of the nerve fibers, whether intraepithelial or subepithelial, was determined by careful examination. The histo-architecture of the tissues studied makes it very easy to misconstrue a fiber as intraepithelial even when it is not so. This is because the subepithelial region evaginates as a papilla into the epithelial region or rete peg, and carries along its contained nerve fibers. Frequently the point of evagination is not contained in a given section. Therefore the criteria used to decide whether a fiber was intraepithelial were both that (a) it lay above the basement membrane, and (b) it was free of structures characteristic of subepithelial regions. A 'true' intraepithelial fiber was one that passed cleanly between epithelial cells unaccompanied by collagen fibers.

Additionally a fiber was said to end freely, i.e. without any specialized ending, only if it was ascertained from serial sections that the whole length of the fiber had been examined.

Results

Osmication studies

In all epithelia studied, many orders of nerve fibers (based upon thickness) were accessible to metal impregnation by Maillet-Champy's method. They ranged from thick fascicles to single varicose terminal fibers. Terminal fibers were seen in both intraepithelial and subepithelial locations. The method produced a dark brown to black stain. However, as with many other metal-impregnation methods, the deposit also occurred in non-neural structures, at several planes where tissue density changed abruptly (see Fig. 3).

The opossum mid-esophagus had 5–15 layers of epithelial cells forming the rete peg, while the lamina propria between the rete pegs formed the

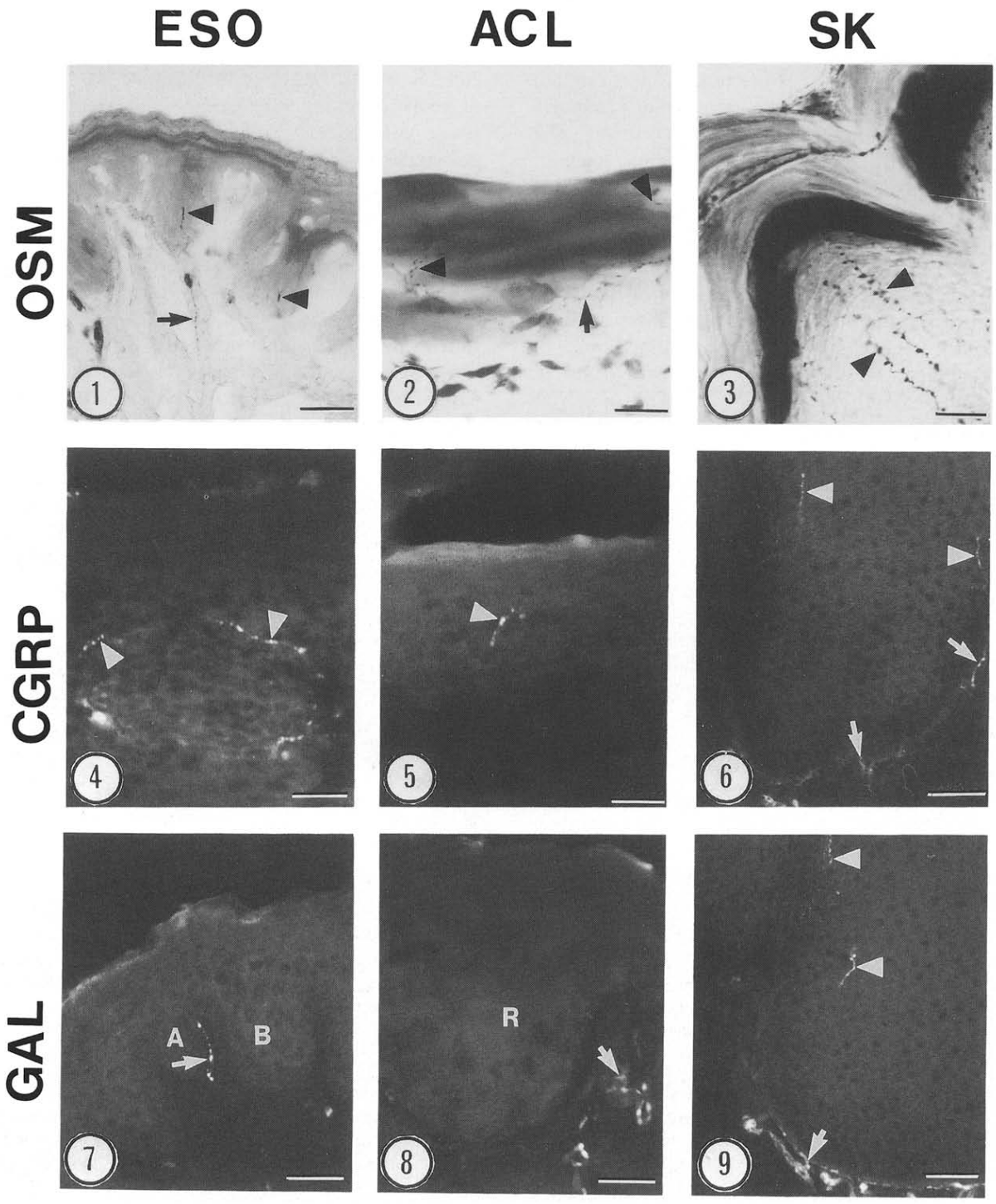

Fig. 1–9. The free epithelial surface and the lumen (esophagus and anal canal) are at the top in all figures. Abbreviations: ACL, anal canal; CGRP calcitonin gene-related peptide; ESO, esophagus; GAL, galanin; IIF, immunofluorescence; IR, immunoreactivity; NPY, neuropeptide Y; OSM, osmication with Maillet-Champy; SK, snout skin; SP, substance P; VIP, vasoactive intestinal polypeptide.

TABLE II

Opossum mucosal innervation

	Esophagus		Anal canal		Snout skin	
	Intepithelial	Subepithelial	Intepithelial	Subepithelial	Intepithelial	Subepithelial
CCK	−	−	−	−	−	−
CGRP	+ +	+ +	+ +	+ + +	+ + + +	+ + + +
GAL	−	+ +	−	+ +	+ + + +	+ + +
GRP	−	+ [1]	−	+ [1]	−	−
l-ENK	−	−	−	−	−	−
m-ENK	−	−	−	−	−	−
NPY	−	+	−	+ +	−	+ + +
SOM	−	−	−	−	−	−
SP	+	+	+ +	+ + +	+ + +	+ +
VIP	+ +	+	+ + +	+ + +	−	+ + + +

Intepithelial = intraepithelial.

[1] found on 2 out of 7 animals only.

−, absent; +, + +, + + +, and + + + +, increasing degrees of subjective positivity.

papilla. Successive rete pegs were separated by thinner epithelium, one to three cell layers thick. Fascicles, a plexus and individual fibers (Fig. 1) were noted in the subepithelial regions. Very few fibers penetrated the basement membrane. Some ended immediately after crossing it, while others traversed up to six cell layers (Fig. 1). Compared to anal canal and snout skin, innervation density was the lowest in the esophagus, 0–5/high power field (h.p.f., 40 ×), but variation of density was the greatest out of all tissues studied. Some stretches of section showed as many as five fibers/h.p.f., while others had none. Intraepithelial fibers were single, unbranched, and ended without specialization (Fig. 1), as determined from observation of serial sections.

The opossum anal canal is lined by stratified squamous epithelium distal to the 'pectinate line'. This epithelium was generally 4–8 cell layers thick at the maximum height of the rete pegs, though it sometimes reached 15 cell layers, and 2 to 4 cells thick in the intervening thin regions. Subjacent to it lay a well-developed plexus of osmiophilic nerve fibers (Fig. 2). Once more, osmicated fibers were

Fig. 1. Esophagus after osmication showing two varicose fibers in the intraepithelial region (arrowheads). Note also the fine varicose fiber in the subepithelial region (arrow). Maillet-Champy/thionin, paraffin section. Bar = 50 μm. Fig. 2. Anal canal after osmication. Intraepithelial fibers can be seen in two places (arrowheads), while a subepithelial fiber (arrow) continuous with the intraepithelial fiber at left traverses the field. Maillet-Champy/thionin, paraffin section. Bar = 100 μm. Fig. 3. Snout skin after osmication. Several fine varicose fibers can be seen between the epithelial cells (arrowheads). The massive black deposits occur at the interface of the keratinized and non-keratinized layers. Maillet-Champy, paraffin section. Bar = 50 μm. Fig. 4. CGRP in the esophagus: two bright intraepithelial CGRP-IR fibers (arrowheads) in the esophageal epithelium. The bright spot near the fiber at the left and the one at the luminal surface at the top are artifacts. IIF, cryosection. Bar = 100 μm. Fig. 5. CGRP in the anal canal. A single intraepithelial varicose fiber (arrow) is seen to run between the epithelial cells. IIF, cryosection. Bar = 100 μm. Fig. 6. CGRP in the snout skin: CGRP-like IR occurs both in the intraepithelial fibers (arrowheads) and the subepithelial plexus (arrows), which follows the basal curve of the rete peg closely. IIF, cryosection. Bar = 50 μm. Fig. 7. Galanin in the esophagus. A single bright subepithelial fiber (arrow) lies between two rete pegs (A,B) and pushes up between them within a papilla of the lamina propria, but does not cross the basement membrane. The luminous dots are from non-specific fluorescence. IIF, cryosection. Bar = 100 μm. Fig. 8. Galanin in the anal canal. GAL-IR fibers occur in a well-developed subepithelial plexus which closely follows the curve of the rete peg, but never penetrates the basement membrane. The bright spot and line at the luminal surface are artefactual. IIF, cryosection. Bar = 100 μm. Fig. 9. Galanin in the snout skin. In contrast to the distribution seen in Fig. 8, galanin-like IR is seen both intraepithelially (arrowheads) and subepithelially in the plexus (arrows). IIF, cryosection. Bar = 50 μm.

seen to traverse the basement membrane and to lie clearly between the epithelial cells (Fig. 2). They were varicose, and innervation density was lower than that of snout skin, ranging from 2–5 fibers/h.p.f. The fibers traversed 2–5 cell layers, ended freely, and were all single and varicose in the intraepithelial region.

In the snout skin the thickness of the rete pegs (between dermal papillae) was 15–25 cell layers between the stratum germinativum and the keratinized stratum corneum, while the thin portions intervening between adjacent rete pegs were about five cells thick. The snout was richest in terminal innervation revealed by osmication. A dense plexus of nerve fibers occurred subepithelially. Fibers were seen to penetrate the basement membrane of the germinal layer and to enter the cells of the rete pegs as long, wavy, slender strands (Fig. 3) traceable up to a height of 15–20 cell layers. Fiber density here was the highest, varying from 2 – 10 fibers/h.p.f. All were varicose and ended freely, i.e. there was no specialized end structure.

Immunohistochemical studies

Immunohistochemical observations on the intraepithelial and subepithelial fibers are summarized in Table II. Details of individual peptide distribution follow.

Calcitonin gene-related peptide (CGRP)

In the esophagus there was an abundant CGRP-positive subepithelial plexus supplying the esophageal glands, lamina propria and muscularis mucosae. Far fewer fibers were seen to penetrate the basement membrane and become truly intraepithelial. Many long, apparently intraepithelial fibers were actually carried up by evaginated lamina propria. Truly intraepithelial fibers were much shorter and traversed 3–5-cell thicknesses only (Fig. 4). Many fields were devoid of fibers, but on average there were about 0–2 CGRP-positive fibers/h.p.f. The maximum number seen was 5/h.p.f.

In the anal canal an extensive CGRP-positive subepithelial plexus was seen and because the epithelium is thin in places, parts of this subepithelial plexus appeared very superficial. However, the anal canal had, in addition, a true intraepithelial fiber supply (Fig. 5) whose density was roughly the same as that of the upper esophagus (0–2/h.p.f.). These CGRP fibers in the anal canal were short, ending within 3–4 cell heights of the basement membrane.

The snout skin was densely innervated by CGRP-positive fibers. Most fields showed them, and their numbers ranged from 1–5/h.p.f., though up to 10 fibers/h.p.f. were seen on occasion. CGRP fibers were fairly long in the snout skin, ascending up to 15 cell layers into the stratified epithelium of the rete pegs (Fig. 6).

Thus the distribution of CGRP-positive fibers was ubiquitous in the sub- and intra-epithelial regions of all the organs studied.

Galanin

In the opossum esophagus, galanin fibers occurred only subepithelially (Fig. 7), where they formed a rich, plexiform meshwork around esophageal glands (situated deeper and thus not visible in Fig. 7) and throughout the connective tissue of the lamina propria. However, no intraepithelial galanin fiber was seen in the esophagus.

No galanin-IR fiber was observed intraepithelially in the anal canal, though, as in the esophagus, an extensive subepithelial plexus was always seen (Fig. 8).

In the snout skin, terminal fibers immunoreactive for galanin were seen both sub- and intra-epithelially (Fig. 9). The latter showed the same density as the CGRP-positive fibers, i.e. 1–3/h.p.f., but their distribution was far more even. Nearly every field showed at least one fiber, and no field showed more than five.

Gastrin-releasing peptide (GRP)

GRP-like immunoreactivity was consistently absent intraepithelially in all organs studied. A few GRP-immunoreactive fibers were seen in the subepithelial regions of the esophagus and anal canal in two out of seven animals. The snout skin showed no GRP at all.

Neuropeptide Y (NPY)

There were no intraepithelial NPY fibers in the opossum esophagus despite their presence in the

subepithelial plexus (Fig. 10). No NPY fiber crossed the basement membrane in the anal canal, though there was a well-developed subepithelial meshwork (Fig. 11). There was no NPY in the snout skin intraepithelially, whereas a well-developed plexus and fascicles were seen subepithelially (Fig. 12).

Substance P (SP)

Varicose fibers showing substance P-like immunoreactivity were seen intraepithelially in the esophagus (Fig. 13), but they were much rarer than CGRP fibers. There was sparse epithelial innervation of the opossum esophagus with SP, the fiber density rarely exceeding 1/h.p.f. The fibers were relatively long, traversing 4-5 cell layers.

Substance P-immunoreactive fibers were sparse intraepithelially in the anal canal (0–2/h.p.f.; Fig. 14), though they were more frequent in the subepithelial region.

A rich supply of SP-immunoreactive fibers lay intraepithelially in the opossum snout skin. The fibers were long, traversing up to 20 cell layers, single, unbranched, and ending abruptly without specialization (Fig. 15). They were less frequent than CGRP fibers, i.e. about 0–2/h.p.f. Substance P-immunoreactive fibers also formed a rich network in the connective tissue of the dermal papillae, supplying blood vessels and glands.

Vasoactive intestinal polypeptide

Nerves immunoreactive for VIP occurred both intra- and sub-epithelially in the opossum esophagus (Fig. 16). There were 0–3 VIP-immunoreactive fibers/h.p.f. intraepithelially. They were variably long, ranging from just inside the basement membrane to six cell thicknesses. The subepithelial VIP supply was rich, and projected to the glands and blood vessels.

The anal canal had 0–3 VIP-positive fibers/h.p.f. intraepithelially. They were short, terminating within 1–3 cell layers (Fig. 17). Subepithelially, VIP supply was rich, particularly to the glands.

The snout skin had no VIP-immunoreactive nerves intraepithelially. However, a well-developed subepithelial plexus was seen (Fig. 18), and nerves with VIP-like immunoreactivity in the dermal papillae supplied glands and blood vessels.

Other neuropeptides

The presence of CCK, *l*- and *m*-Enk and somatostatin was also studied. All were absent in all tissues examined.

Discussion

This study shows that (1) there is a gradient of density of intraepithelial nerves in the order snout skin > anal canal > esophagus; (2) substantially more nerves are detected with osmication than with peptide immunohistochemistry; (3) the intraepithelial nerves are reactive for CGRP and SP in all three organs; (4) the epithelia of the esophagus and anal canal also have VIP-reactive, but not galanin-reactive nerve fibers, while (5) snout skin epithelium has galanin-reactive but not VIP-reactive fibers.

Intraepithelial CGRP and SP-immunoreactive nerves were present in all the epithelia examined. Although these have not been previously described in the anal canal or snout skin, CGRP-reactive fibers have been described in esophageal mucosa of monkeys [20], as have SP-reactive fibers in the esophageal mucosa of rat, cat and monkeys [20], opossums [2] and rabbits [11]. There are also several reports of CGRP- and SP-reactive nerves in the skin [8,22,26].

While there is no direct proof that CGRP and SP are sensory peptides, there is much circumstantial evidence from a voluminous body of varyingly conclusive literature to support this belief. For example, these peptides are found in ganglia generally regarded as sensory (SP, ref. 23; SP and CGRP, ref. 7; CGRP, ref. 24). Also CGRP and SP are found in organs that have no motor function such as the cornea (SP, ref. 15). The analgesic morphine has been shown to prevent the release of substance P in tooth pulp [1], and there is experimental evidence linking the decline of sensitivity or increase in threshold of pain directly to peptide depletion (SP, ref. 3). Both peptides are depleted by the sensory nerve poison capsaicin, and co-localization of CGRP and SP has been described [7].

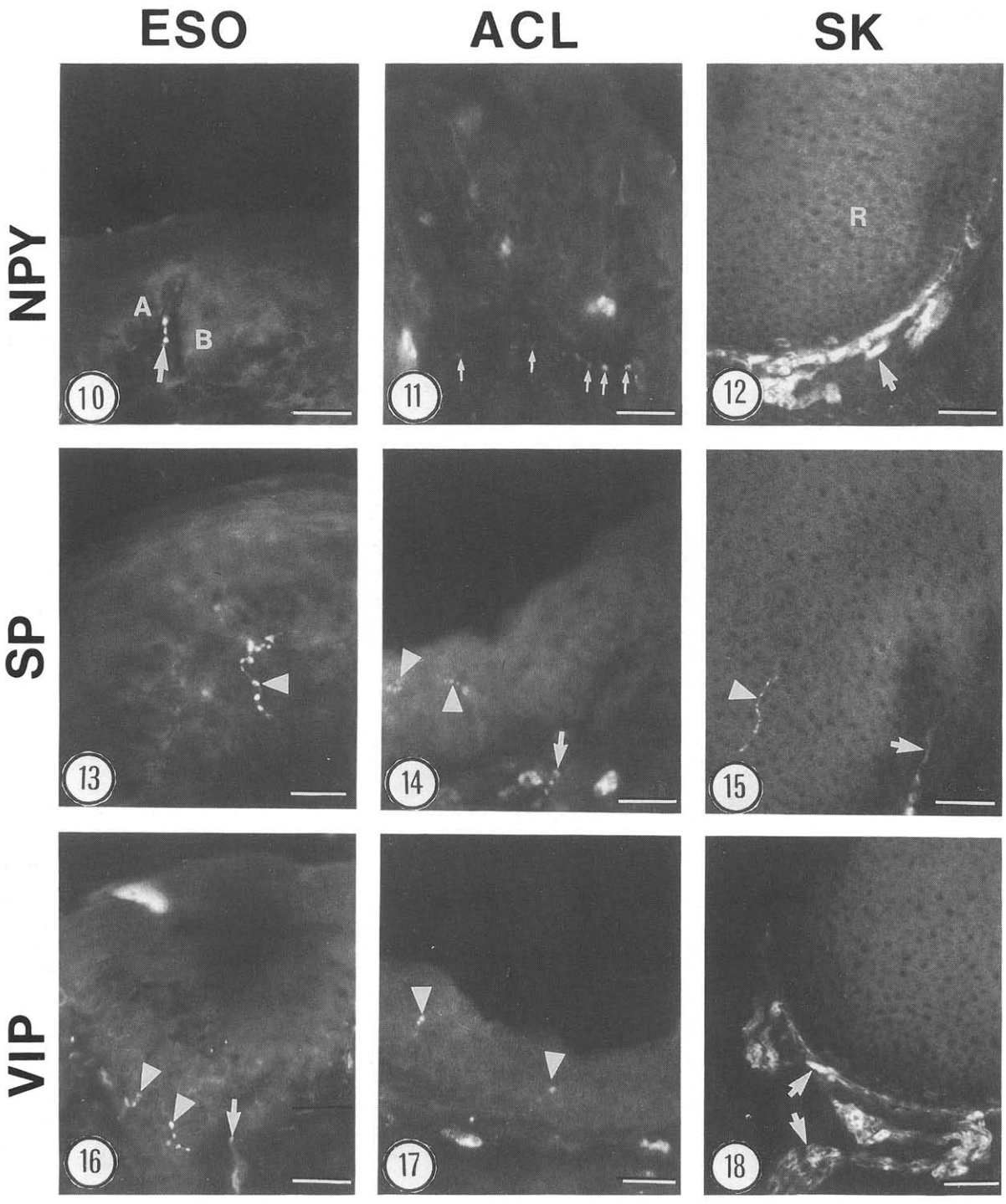

Fig. 10–18. For abbreviations see legend to Figs. 1–9

These two peptides are common to all three organs examined in this study. Their presence supports a sensory role for these nerve fibers, but the uniform occurrence of these peptides cannot account for the diversity of the sensory function served by these organs.

An explanation for that diversity may lie in differences in peptide content, in the density of innervation, or in an as yet unaccounted-for peptidergic nervous component. The snout skin is characterized by possession of galanin and the absence of VIP, while conversely the two gastrointestinal epithelia are characterized by the absence of galanin and the possession of VIP.

Osmication revealed far more fibers than did peptide immunohistochemistry. It is not clear what the 'missing component of innervation' may be. One possibility is that the antisera did not detect all fibers containing their respective antigens, but this is unlikely as varicosities contain high concentrations of neurotransmitters and are easily labelled. A second possibility is that there is a population of fibers containing peptides other than the ten examined. Thirdly, some of these fibers may not be peptidergic. Further studies are called for to resolve the nature of all the intraepithelial fibers and their functional roles.

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Fig. 10. NPY in the esophagus. A bright varicose fiber is seen subepithelially between two rete pegs (A,B), but it does not traverse the basement membrane. IIF, cryosection. Bar = 100 μm. Fig. 11. NPY in the anal canal. Immunoreactivity is confined to the subepithelial region, where a single long fiber can be identified as a string of varicosities (small arrows) running across the field just beneath the basement membrane. The three large bright spots are mast cells showing largely non-specific fluorescence which persists in controls. IIF, cryosection. Bar = 100 μm. Fig. 12. NPY in the snout skin. A well-developed subepithelial plexus (arrow) is positively immunoreactive, but no fiber crosses the basement membrane into the mass of the rete peg (R). IIF, cryosection. Bar = 50 μm. Fig. 13. SP in the esophagus. A single intraepithelial immunoreactive fiber (arrow) is seen between the epithelial cells. IIF, cryosection. Bar = 100 μm. Fig. 14. SP in the anal canal. Two immunoreactive fibers are seen intraepithelially (arrowheads) and one subepithelially (arrow). The two luminous bodies are mast cells showing largely non-specific fluorescence. IIF, cryosection. Bar = 100 μm. Fig. 15. SP in the snout skin. The lighter portion of the photograph is the rete peg, which has a single intraepithelial fiber (arrowhead), while the darker part is the dermal papilla, which has a subepithelial plexus (arrow). IIF, cryosection. Bar = 50 μm. Fig. 16. VIP in the esophagus. Two varicose intraepithelial fibers (arrowheads) are seen close to the basement membrane. A single strand of subepithelial plexus is also seen (arrow). IIF, cryosection. Bar = 100 μm. Fig. 17. VIP in the anal canal. Intraepithelially, two fibers are identifiable by their varicosities (arrowheads). The two bright mast cells in the subepithelial region show largely non-specific fluorescence. IIF, cryosection. Bar = 100 μm. Fig. 18. VIP in the snout skin. IR occurs only subepithelially in the well-developed plexus (arrows). No fiber crosses into the rete peg (R). IIF, cryosection. Bar = 50 μm.

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