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Original investigations

Distribution and co-localization of immunoreactive helospectin with vasoactive intestinal polypeptide and peptide histidine methionine in human nasal mucosa, soft palate and larynx*

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Summary. Regulatory peptide immunoreactivities reported in the upper respiratory system of man include vasoactive intestinal polypeptide (VIP) and peptide histidine methionine (PHM), which are co-localized in a network of fine varicose nerve fibers. The present study was undertaken to examine the possible occurrence and distribution of the recently described VIP-like peptide helospectin. Double immunofluorescence labelling showed that helospectin is co-localized with VIP and PHM. In addition to nerve fibers containing all three peptides, scattered nerve fibers were detected that were only immunoreactive to helospectin but not to VIP and/or PHM. The distribution of helospectin/VIP/PHM immunoreactive nerve fibers around blood vessels and in close relationship to seromucous glands indicates their possible involvement in the regulation of blood flow and secretion.

Key words: Upper respiratory system – Helospectin – Vasoactive intestinal polypeptide – Immunocytochemistry – Neuropeptides

Introduction

Various peptide immunoreactivities and the presence of all components of the diffuse neuroendocrine system in the upper respiratory tract have been reported, indicating complex physiological mechanisms [2, 4, 9, 11, 15, 16, 20]. Nerve fibers immunoreactive to vasoactive intestinal polypeptide (VIP) are widely distributed in the peripheral nervous system [5–7, 13, 19] and also occur in the upper respiratory system, where VIP is co-localized with peptide histidine isoleucine (PHI) in various mammals [14, 16] or peptide histidine methionine (PHM) in man [12, 15].

Helospectin I and II are peptides isolated from the poisonous saliva of Gila monster (*Heloderma horridum* or *H. suspectum)* [17]. Helospectin I has the sequence

His-Ser-Asp-Ala-Thr-Phe-Thr-Ala-Glu-Tyr-Ser-Lys-Leu-Leu-Ala-Lys-Leu-Ala-Leu-Gln-Lys-Tyr-Leu-Glu-Ser-Ile-Leu-Gly-Ser-Ser-Thr-Ser-Pro-Arg-Pro-Ser-Ser

Helospectin II is identical to helospectin I except for the lack of Ser-38. Recently, it was found that helospectin immunoreactivity is present in VIP-immunoreactive nerve fibers of the mammalian gastrointestinal tract [1]. The present study was undertaken to investigate helospectin immunoreactivity in nerve fibers of the upper airways of man.

Materials and methods

Surgical specimens consisted of tumor-free parts from the larynx (5 vocal cords, 5 ventricular folds, 3 epiglottis and 1 subglottis) from patients undergoing laryngectomies for oncological reasons, parts of the inferior turbinates taken from 8 patients during septorhinoplasties, and portions of soft palates with uvulae from 3 patients following uvulopharyngoplasties. The tissue samples were fixed by immersion in Zamboni/Stefanini's solution [18, 22] for 4–6 h at 4°C and thoroughly rinsed in 0.01 *M* phosphate-buffered saline (PBS) containing 15% sucrose (at least overnight at 4°C).

Cryostat sections 14 µm thick were adhered on glass slides coated with poly-L-lysine [10] and then stained with the Coons' [3] indirect immunofluorescence method using monoclonal antibodies to VIP in combination with rhodamine- or fluorescein-isothiocyanate-conjugated second layer antibodies (Table 1). Double immunostaining was also carried out in combination with rabbit polyclonal antisera to PHM and to helospectin I in order to examine the possible co-existence of the three immunoreactants.

After cutting, tissue sections were air dried at room temperature for at least 1 h and then incubated for 30 min with PBS at pH 7.2 containing 0.1% Triton X-100. Following this, primary antibodies diluted in PBS containing 0.01% sodium azide and 0.1%

* Dedicated to the memory of Prof. Heinrich Spoendlin (Innsbruck)

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Table 1. Immunoreagents used to study regulatory peptide immunoreactivities. (The dilutions shown gave maximum detection of antigen with lowest levels of background)

Immunogen	Abbreviation	Code	Donor	Type	Dilution	Source
Vasoactive intestinal polypeptide	VIP	MaVIP	Mouse	Monoclonal	1/800	East Acres Biologicals, Southbridge, MA, USA
Peptide histidine methionine 27	PHM	Ras7176N	Rabbit	Polyclonal	1/400	Peninsula, Belmont, CA, USA
Helospectin I	HS	8843	Rabbit	Polyclonal	1/800	Milab, Malmö, Sweden
Mouse immunoglobulin G, RITC-coated		AP131R	Rabbit	Polyclonal	1/25	Chemicon, Temecula, CA, USA
Rabbit immunoglobulin G, FITC-coated		4171-1	Goat	Polyclonal	1/25	BioMakor, Rehovot, Israel

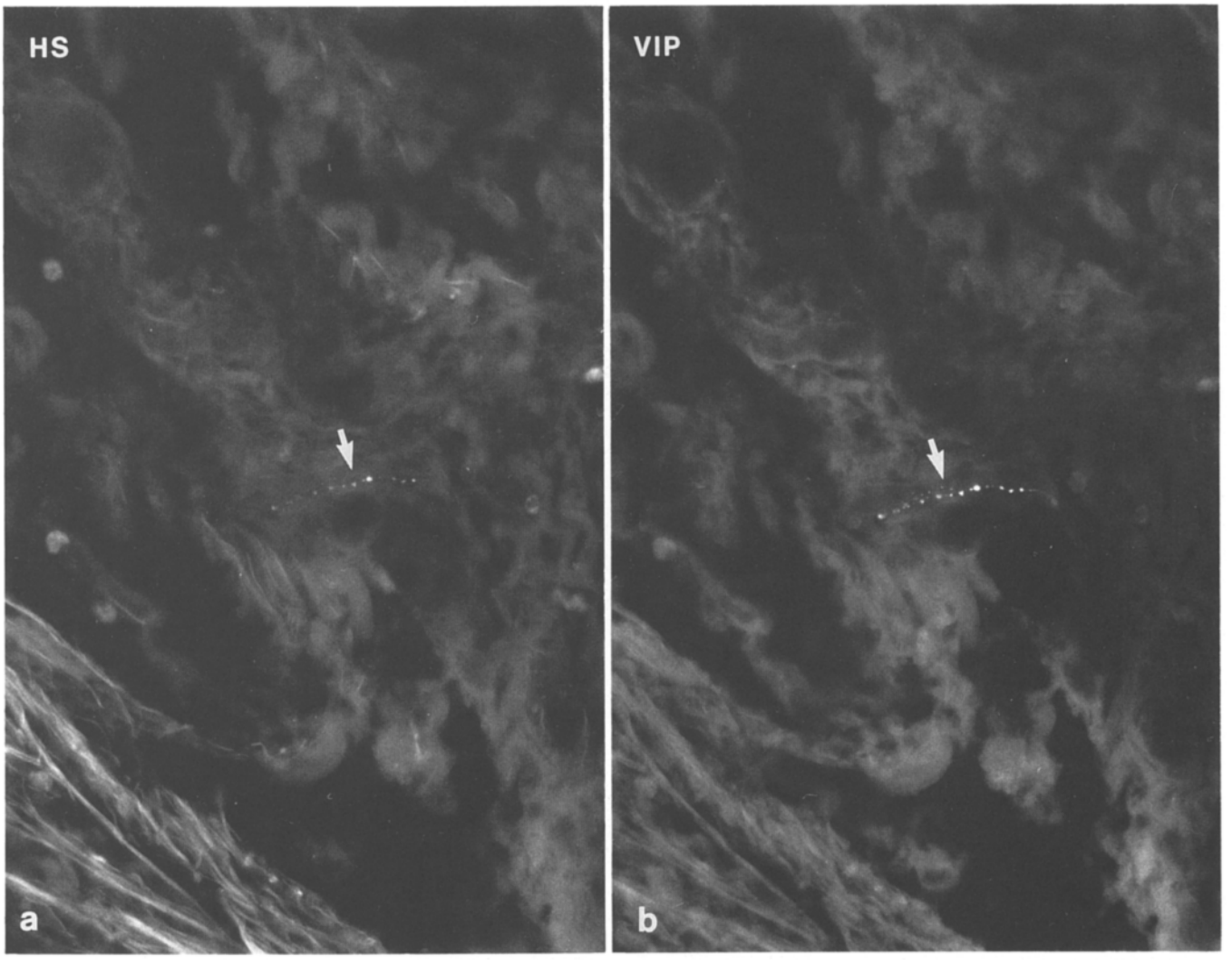

Fig. 1a, b. Double immunostaining using rabbit polyclonal antibodies to helospectin (HS, FITC) and monoclonal mouse antibodies to vasoactive intestinal polypeptide (*VIP*, RITC) in a 14-μm-thick frozen section of Stefanini/Zamboni's-fixed human epiglottis (lingual portion). A fine varicose nerve fiber *(arrow)* is labelled by both antibodies, showing a co-expression of helospectin (**a**) and VIP (**b**) immunoreactive material. Original magnification, × 470

bovine serum albumin were incubated in parallel for 48 h at 4°C in moist chambers, followed by parallel incubation of the appropriate second layer antibodies for 1 h at room temperature. Coverslips were mounted using Glycergel (Dakopatts, Glostrup, Denmark and Santa Barbara, CA, USA). Slides were examined using a Polyvar microscope (Reichert-Jung-Leica, Vienna, Austria) equipped for double fluorescence microscopy, using B1 filters for green fluorescein isothiocyanate (FITC) fluorescence and G2 filters for red rhodamine isothiocyanate (RITC) fluorescence.

Negative controls omitted primary antibodies, while positive-staining controls used tissues known to contain the antigen in question (e.g., stomach and duodenum). Absorption controls were performed on material from known positive tissue (gastrointestinal tract). The helospectin I antiserum cross-reacted with helospectin II and helodermin, but not with VIP, PHI, PACAP-27 and -38, salmon calcitonin and growth hormone releasing factor by adding 10 μg of each of the peptides per milliliter of diluted antiserum [1].

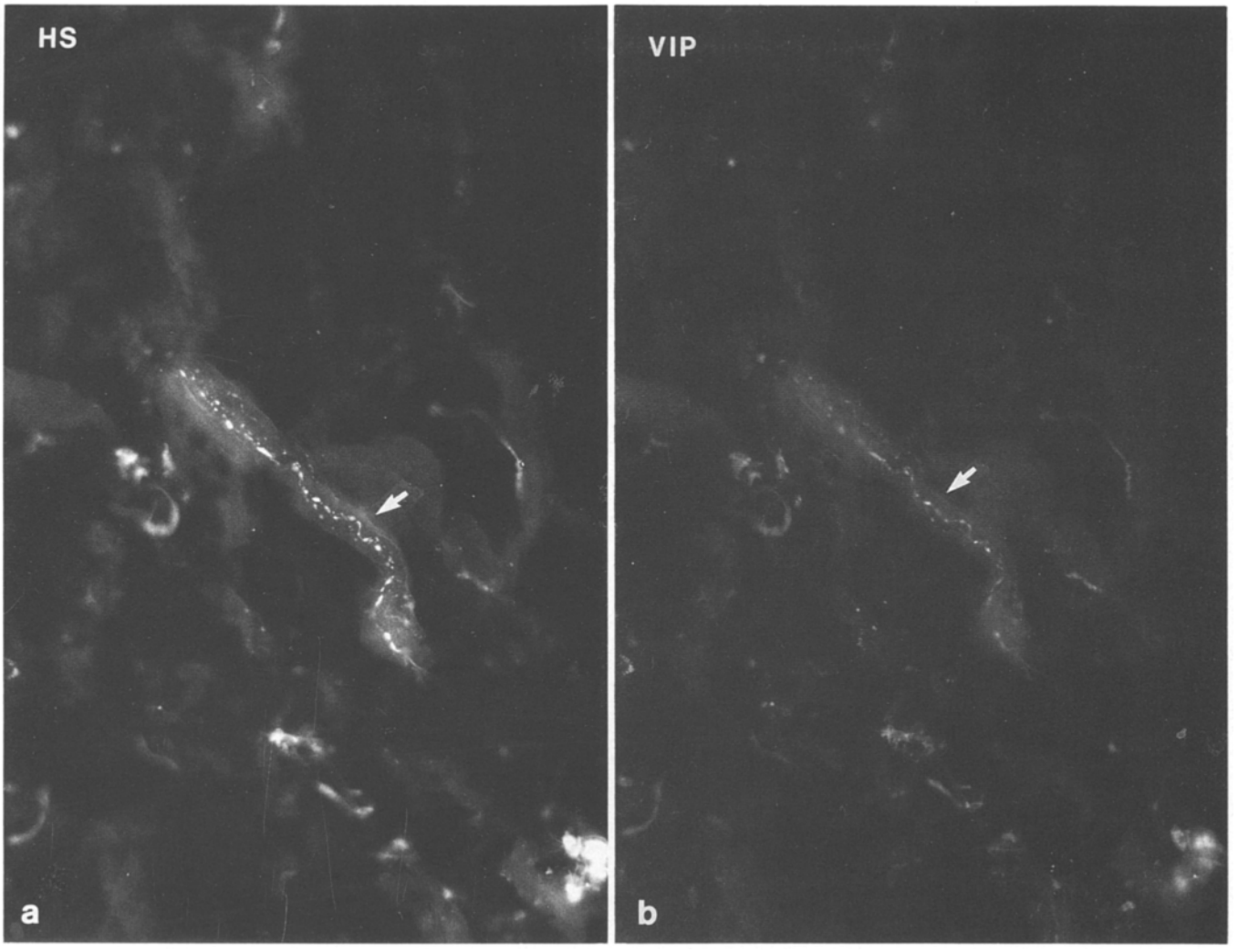

Fig. 2. Double immunofluorescence of a 14-μm-thick frozen section of Stefanini/Zamboni's fixed human vocal cord, showing co-expression of helospectin (**a**) and VIP (**b**) immunoreactive material in thin varicose nerve fibers *(arrow)* within a nerve bundle in the lamina propria. Original magnification, × 470

Results

Double immunofluorescence revealed nerve fibers immunoreactive to VIP, PHM and to helospectin antibodies in all tissues examined. Virtually all nerve fibers labelled by the antiserum to VIP were also immunoreactive to PHM and/or helospectin. However, nerve fibers exclusively immunoreactive with helospectin antibodies were occasionally detected.

Dense networks as well as numerous scattered varicose VIP/PHM immunoreactive nerve fibers showing co-expression of helospectin immunoreactivity were mainly found in close relationship to serous/mucous glands (Fig. 1a, b) and blood vessels (Fig. 2a, b) in the lamina propria of all tissues investigated.

All nasal mucosal samples investigated demonstrated the most dense innervation of VIP/PHM/helospectin-immunoreactive nerve fibers. The soft palate showed nearly the same distribution and number of immunoreactive structures. In contrast, the mucosa of the epiglottis and subglottis was less densely innervated with VIP/PHM/helospectin-immunoreactive nerve fibers. Single nerve fibers were located underneath the epithelium and were more numerous around exocrine glands. Generally, nearly all immunoreactive nerve fibers of the epiglottis were located at its lingual part. Finally, the laryngeal ventricular fold showed a more dense innervation of VIP/PHM/helospectin-immunoreactive nerve fibers than did the vocal cord.

Discussion

The findings of the present study indicate the existence of helospectin-like peptide(s) in the nasal mucosa, soft palate and larynx of man. Absorption controls also showed that the antibody to helospectin I reacts with helospectin II and therefore cannot distinguish between helospectin I and II. Most nerve fibers demonstrated with this antibody were also immunoreactive to VIP and PHM, while scattered fibers were only positive for helospectin. The

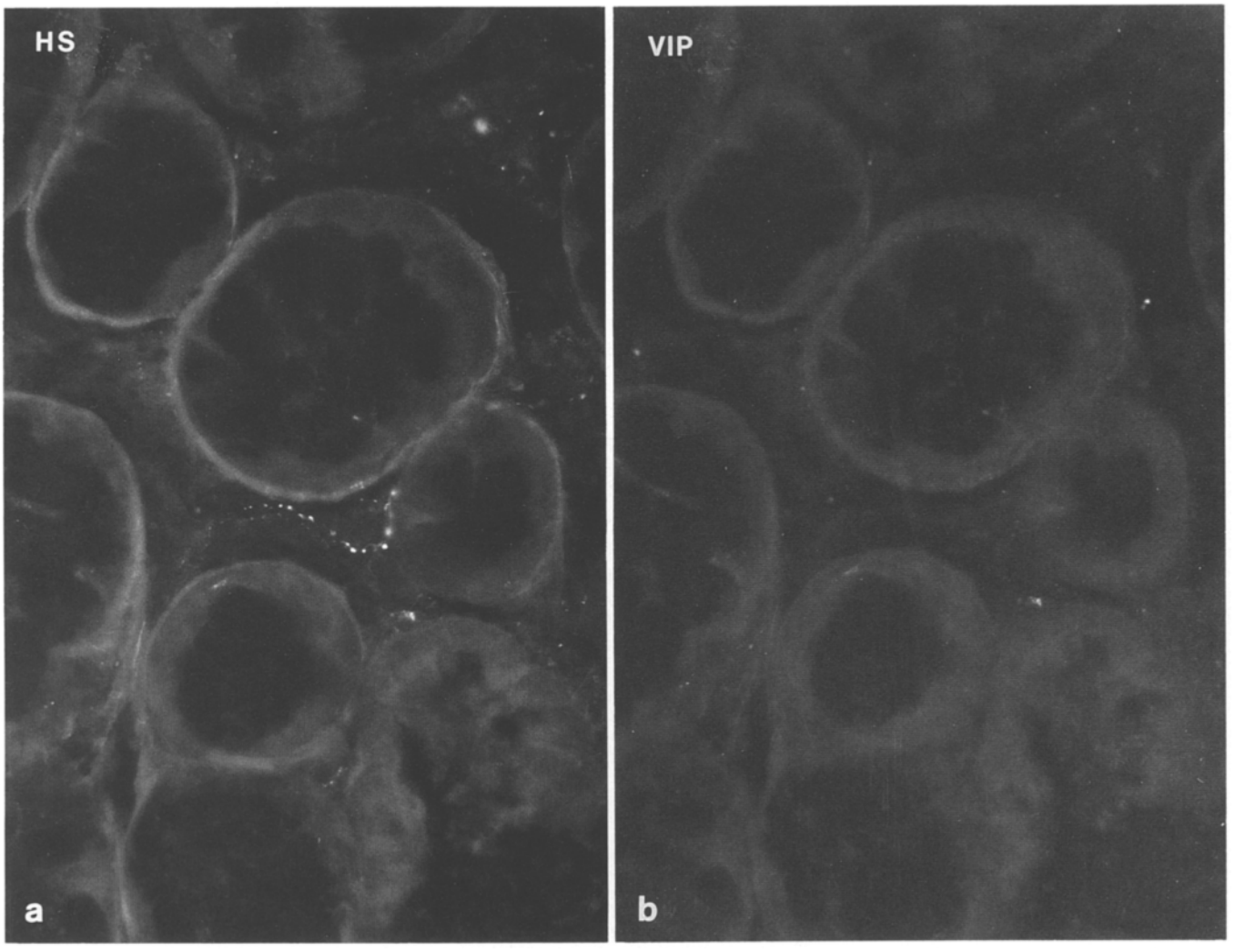

Fig. 3. Double immunofluorescence using polyclonal rabbit antibodies to helospectin (**a**) and monoclonal mouse antibodies to VIP (**b**) in a 14-μm-thick frozen section of Stefanini/Zamboni's fixed human epiglottis. A single varicose nerve fiber in between mucous glands is labelled by the anti-helospectin antibody, whereas the anti-VIP antibody shows no reaction in the same nerve fiber. Original magnification, × 470

distribution of immunoreactive nerve fibers demonstrated around blood vessels and in close relation to serous/mucous glands indicates a possible involvement of all three peptides in the regulation of blood flow and secretion.

The structural homology of VIP, PHM and helospectin I and II suggests comparable biological activities [1, 21]. In bioassay, the three peptides cause a stimulation of amylase release in the pancreatic acini of the guinea pig with relative potencies: i.e., VIP greater than helospectin greater than PHM. They also cause an increase in adenosine 3,5-cyclic monophosphate [23]. Helospectin I and II relax phenylepinephrine-contracted vessels to the same extent as VIP, but with a lower potency [8].

Since VIP and helospectin I and II have a similar profile of action, they may act on a common receptor [8]. The functional significance of the co-localization demonstrated by three structurally related but distinct peptide immunoreactivities is the subject of further examination. The finding of scattered nerve fibers only immunoreactive with the helospectin antiserum but not for VIP suggests alternative subpopulations of helospectin-immunoreactive nerve fibers and therefore also possible exclusive physiological mechanisms of helospectin without the presence of VIP.

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