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Localization of vasoactive intestinal polypeptide (VIP) to central and peripheral neurons

(gastrointestinal hormones/gastrointestinal nerves/hypothalamic peptides/peptidergic nerves/endocrine cells)

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ABSTRACT The localization of the vasoactive intestinal polypeptide (VIP) has been studied with immunohistochemistry and radioimmunoanalysis. VIP immunoreactivity is present in gastrointestinal nerves, which constitute a quantitatively important nerve population that may be intrinsic to the gut wall. VIP-immunoreactive neurons are also found within the ventromedial hypothalamus and give off processes that travel lateral to the third ventricle. Results of radioimmunoanalysis strongly indicate that the immunoreactive material represents true VIP. Thus VIP, at present a gastrointestinal hormone candidate, appears to represent a new neuronal peptide occurring in both the central and peripheral nervous system.

Vasoactive intestinal polypeptide (VIP), a potent hypotensive and vasodilatory agent, has been isolated from the porcine small intestine and shown to have an amino acid sequence related to that of secretin, glucagon, and gastric inhibitory polypeptide (1–3). The corresponding polypeptide has also been isolated from chicken intestine and its structure has been determined (4, 5). The almost complete inactivation of VIP reported to occur in the liver has led to the suspicion that its actions are confined to the gut (6). In contrast to a recent report on the exclusive localization of immunoreactive VIP in a population of endocrine-like cells of the gut (7), we wish to report that VIP immunoreactivity occurs mainly in neurons of both the central and peripheral nervous system.

MATERIALS AND METHODS

Antisera. Antisera were raised in rabbits to highly purified porcine VIP (generous gift from Prof. V. Mutt) covalently coupled to bovine serum albumin. Each rabbit received 30 nmol of the antigen emulsified in Freund's complete adjuvant subcutaneously at multiple sites with 8 week intervals. Sera collected after the third immunization were used for radioimmunoassay and immunohistochemistry.

Tissue Sources. Material was collected from adult human, pig, cat, rat, and mouse. Three rats underwent bilateral abdominal vagotomy 2 weeks prior to sacrifice and three mice received 6-hydroxydopamine (100 mg/kg intravenously) (chemical sympathectomy) 24 hr before sacrifice. Specimens of apparently normal human gut and pancreas were collected at surgery (for carcinoma or peptic ulcer). Material from pig was obtained immediately after death. Cats were killed by an overdose of mebumal (Nembutal) and rats and mice with diethyl ether.

Radioimmunoassay. Tissue material was immediately frozen on dry ice, crushed, and homogenized in acidified ethanol (i.e., 70% ethanol containing 0.74% hydrochloric acid). Solids were removed by centrifugation and the supernatant was decanted and dried *in vacuo*. The samples were reconstituted in 0.04 M sodium phosphate buffer at pH 7.4 containing 58 μM human serum albumin and 100 mM sodium chloride. Each sample was assayed in triplicate in three different dilutions. Antiserum no. 5601 was routinely used. ^{125}I-Labeled VIP was prepared by a chloramine-T method and highly purified porcine VIP was used as standard. Separation of free from antibody-bound VIP was carried out using charcoal coated with human plasma. The detection limit of the assay was 25 pmol/liter. At a level of 75 pmol/liter, the within-assay reproducibility expressed as coefficient of variation was 0.09 and the between-assay reproducibility was 0.15. Dilution curves of the extracts were superimposable on the standard curves. Concentrations were expressed as pmol/g (wet weight).

Immunohistochemistry. Specimens were taken from the pancreas and from various segments of the gut. Specimens from the heart were obtained from cats and hypothalamic material was from mice and cats. The specimens were frozen to the temperature of liquid nitrogen in a propane–propylene mixture, freeze-dried, and vapor-fixed with diethylpyrocarbonate, parabenzoquinone, or formaldehyde (8, 9). They were embedded in paraffin *in vacuo*, sectioned at 3 μm, and subjected to an indirect immunofluorescence method for the demonstration of VIP immunoreactivity. All VIP antisera (nos. 5597–5603) produced brilliant immunohistochemical staining of what evidently were the same structures. VIP antiserum no. 5603 was routinely used (dilution 1:80) and the site of antigen–antibody reaction was revealed with fluorescein isothiocyanate-labeled antiserum to rabbit IgG, prepared in sheep (SBL, Stockholm, Sweden) (diluted 1:20). Sections were examined in a Leitz Orthoplan fluorescence microscope equipped with an epi-illumination system (standard filter setting no. 3; peak excitation at 490 nm). Controls were run as recommended by Sternberger (10) and included the application of antigen-inactivated antiserum (30 nmol of VIP per ml of serum diluted 1:80).

Specificity of Antisera. In our radioimmunoassay none of the VIP antisera crossreacted with pure natural porcine secretin‖ highly purified porcine gastric inhibitory peptide**, monocomponent porcine pancreatic glucagon††, highly purified porcine enteroglucagon††, highly purified human pancreatic polypeptide‡‡, synthetic bovine substance P or synthetic ovine somatostatin. In order to exclude crossreactivity at the immunohistochemical level the VIP antisera were applied in different

Abbreviation: VIP, vasoactive intestinal polypeptide.

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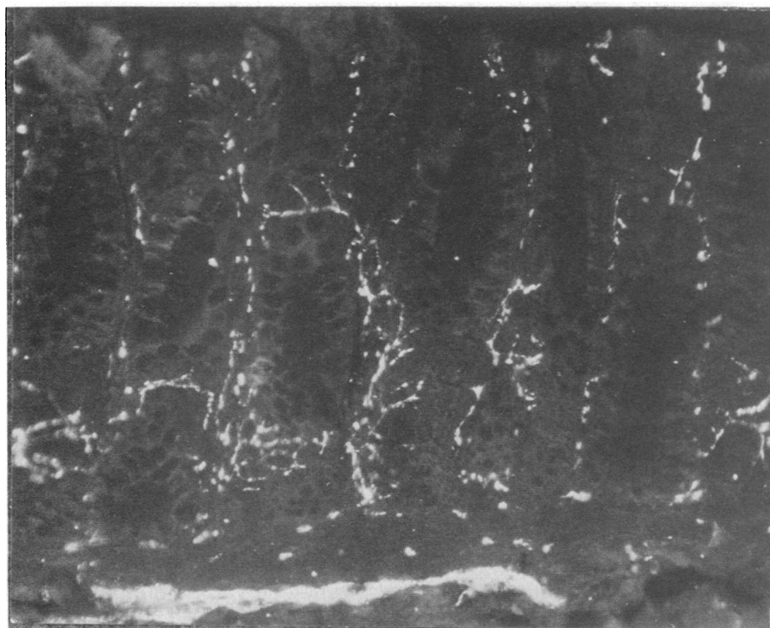
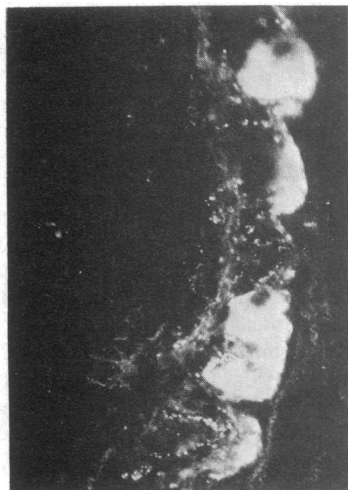


FIG. 1. Cat colon. VIP immunofluorescence occurs in numerous nerves in the mucosa and in nerves surrounding cells of the submucous plexus. ×180.

dilutions (from 1:10 to 1:80) onto tissue sections from locations known to contain large amounts of the peptides mentioned above, whose presence was verified by staining adjacent sections with potent antisera against them. No crossreactivity could be detected at the immunohistochemical level.

RESULTS

Diethylpyrocarbonate and parabenzoquinone fixation both gave optimal preservation of VIP immunoreactivity, whereas formaldehyde fixation yielded much weaker staining. All controls were negative. When applied to sections from the gastrointestinal tract the VIP antisera produced intense staining of numerous nerve fibers in all layers of the gut wall in all species studied (Fig. 1). Most of the nerve fibers had a beaded appearance but smooth fibers were also seen. The submucous and myenteric plexuses invariably contained a dense network of immunoreactive nerve fibers. Occasionally, the plexuses also contained strongly immunoreactive nerve cell bodies, being especially numerous in the human intestines (Fig. 2, left). In the stomach, only few fibers were detected in the mucosa, whereas the submucosa and the external muscle layer showed more extensive innervation. In the small and large intestines nerve fibers were abundant also in the mucosa; here they formed a network around the crypts and in the small intestines extended up in the cores of the villi. The mucosal nerve fibers often appeared to follow small blood vessels. Surgical vagotomy (rats) or chemical sympathectomy (mice) did not cause any change in the number or distribution of the VIP-immunoreactive nerve fibers. The effectiveness of the sympathectomy was tested fluorescence histochemically by the method of Falck and Hillarp, which induces strong fluorescence of adrenergic nerves due to their content of norepinephrine (6). Examination of such sections disclosed an almost complete disappearance of adrenergic nerves. From observations on control animals it was evident that the VIP-immunoreactive fibers by far outnumbered the adrenergic fibers.



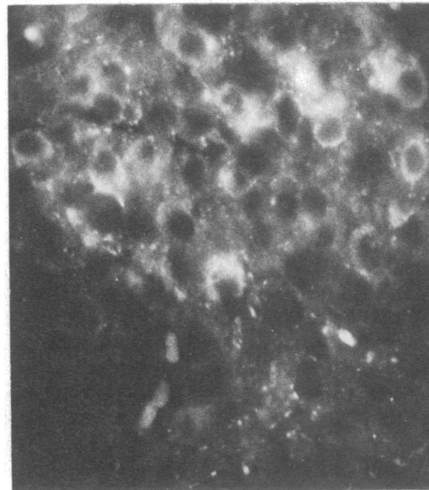


FIG. 2. (*Left*) Human colon, external muscle wall. VIP immunofluorescence occurs in nerve cell bodies and nerves of the myenteric plexus. ×240. (*Right*) Mouse hypothalamus. VIP immunofluorescence occurs in numerous nerve cell bodies and nerves situated in the ventromedial part. ×525.

Table 1. Distribution of VIP immunoreactivity in the gastrointestinal tract

Location	Species		
	Human	Porcine	Feline
Gastric fundus			
Mucosa + submucosa	—	146 (3)	—
Muscle layer	—	294 (3)	—
Gastric antrum			
Mucosa + submucosa	—	357 (3)	347 (1)
Muscle layer	—	401 (3)	497 (1)
Duodenum			
Mucosa + submucosa	110 (1)	625 (4)	1044 (1)
Muscle layer	797 (1)	1179 (4)	385 (1)
Jejunum			
Mucosa + submucosa	—	770 (2)	—
Muscle layer	—	475 (2)	—
Ileum			
Mucosa + submucosa	545 (1)	327 (3)	—
Muscle layer	342 (1)	345 (3)	—
Colon			
Mucosa + submucosa	730 (1)	497 (3)	1570 (1)
Muscle layer	330 (1)	359 (3)	378 (1)
Pancreas	—	121 (4)	—

— Denotes not tested. The amounts are expressed as pmol/g of wet weight. Numbers in parentheses indicate the number of animals tested.

Few VIP-immunoreactive nerve fibers were detected in the pancreas. They occurred in the connective tissue separating the pancreatic lobules and were only occasionally seen between acini or in islets.

Only in the antral mucosa of the cat were VIP-immunoreactive epithelial cells encountered. In no other location and in no other species were such cells detected, regardless of fixation. Addition of large amounts of aprotinin (Trasylol) to the VIP antiserum as suggested by Polak *et al.* (7) was without effect on the immunohistochemical results. In agreement with previous reports, however, a fair number of VIP cells are encountered in pancreatic tumors causing the pancreatic cholera syndrome (Larsson *et al.*, in preparation).

By radioimmunoassay, VIP-immunoreactive material was found in large amounts in the gastrointestinal tract of all species examined. The amounts in various locations along the gut and its distribution between the mucosal and muscular layers were in good agreement with the immunohistochemical observations (Table 1).

In the myocardium no VIP immunoreactivity could be detected. Examination of hypothalamic specimens from cats and mice revealed the presence of a large collection of nerve cell bodies in the ventromedial parts (Fig. 2, right), probably belonging to the arcuate nucleus. The cell bodies gave off processes that could be followed for a short distance lateral to the ventricle. Radioimmunoassay of extracts from mouse hypothalami confirmed the presence of significant quantities of VIP immunoreactivity (129 pmol/g of wet weight). By gel-permeation chromatography this immunoreactive material was found to have an apparent molecular size similar to that of material extracted from mouse colon and to highly purified porcine VIP (Fig. 3). The precise localization and distribution of the hypothalamic VIP immunoreactive neurons remain to be established. In specimens from cat hypothalamus were also included some pial vessels, the wall of which was found to contain VIP-immunoreactive nerve fibers. The fibers occurred mainly between the muscular and adventitial layers of the arteries. Their distribution and properties will be dealt with elsewhere (11).

DISCUSSION

VIP immunoreactivity thus occurs in both central and peripheral neurons. The VIP fibers apparently constitute a quantitatively important nerve population in the gut wall. Since VIP immunoreactivity was found in ganglionic cells of the gastrointestinal nervous plexuses and since neither vagotomy nor sympathectomy reduced their number, this extensive system of nerve fibers may be intrinsic to the gut wall. There is

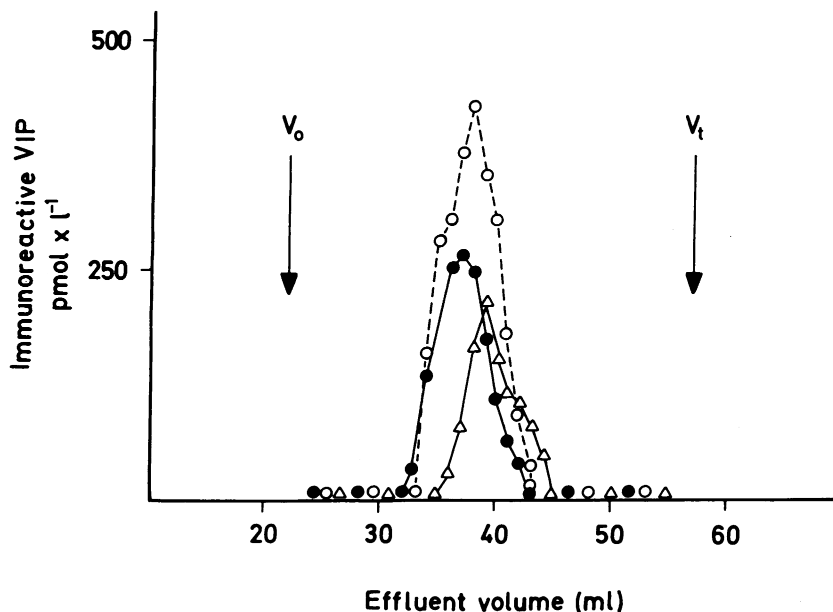


FIG. 3. Elution diagram of VIP immunoreactivity from an extract of mouse colon (○ - - - ○) and mouse hypothalamus (△——△). Gel-permeation chromatography was on a Sephadex G-50 superfine column (9 × 600 mm) eluted with 0.25 M ammonium acetate, pH 6.5, containing 72.5 μmol bovine serum albumin per liter at a flow rate of 12.0 ml per hour at 4°C. The column was calibrated with highly purified porcine VIP (●——●), ^{125}I-labeled albumin, and K^{125}I. V_o, Void volume; V_t, total mobile phase.

little doubt that the demonstrated VIP immunoreactivity is identical with the principle isolated by Said and Mutt. All antisera used displayed high specificity and high binding capacity, parallel dilution curves between highly purified VIP and all extracts were obtained, and the extracted gastrointestinal and hypothalamic VIP-immunoreactive material had an apparent molecular size similar to that of authentic VIP.

It seems pertinent here to recall the now classical observation made by Bayliss and Starling at the beginning of the century. While isolating secretin they noticed the occurrence of contaminating vasodilatory substances. They soon found, however, that secretin extracts prepared from desquamated intestinal epithelium contained no depressor activity (12). In retrospect it is likely that the vasodilatory material contained VIP (apart from histamine). These results are in excellent agreement with our observations indicating that VIP does not occur in the intestinal epithelium but is confined to the deeper strata of the gut wall.

The close interrelationship between hypothalamic and gastrointestinal and/or pancreatic peptides has recently been recognized. Thus, substance P immunoreactive material occurs in gastrointestinal and hypothalamic nerves as well as endocrine-like cells (13–15). Antisera against somatostatin react not only with a rich population of nerves in the hypothalamus but also with scattered gastrointestinal nerves and with pancreatic and gastrointestinal endocrine-like cells (D cells) (16–18). Antibodies to gastrin have been found to react with material in the vertebrate central nervous system (19). From its first recognized source, the gut, VIP now seems to have taken position also in the class of hypothalamic peptides.

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