Shimane J. Med. Sci. Vol. 5, pp. 123–129, 1981

Ultrastructural Profiles of Non-Adrenergic, Non-Cholinergic Enteric Nerve and Localization of VIP-like Immunoreactivity

(VIP/axons/intestine)

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(Received October 17, 1981)

In the nerve endings of the canine intestine, three types of the large granular vesicles found to coexist with small agranular vesicles were identified by conventional electron microscopy, on the basis of shape, size and electron density of their contents. They are characterized by the following profiles : (1) so-called large dense-cored vesicles (80–120 nm in diameter) whose contents were highly osmiophilic. Most of such vesicles had a clear-electron-transparent halo between the vesicle membrane and the granular core. These vesicles (LGV) usually coexist with a varying number of small agranular vesicles (50–70 nm in diameter) whose contents are assumed to be acetylcholine. (2) Large, electron-opaque vesicles (LOV) with a diameter of 140 to 200 nm. (3) electron-faint vesicles (LFV ; 100–140 nm in diameter) containing osmiophilic granular materials, with or without halos. Immuno-electron microscopy showed that vasoactive intestinal polypeptide (VIP)-like immunoprecipitates were localized to the large granular vesicles (Type 1 vesicles) which coexisted with small agranular vesicles (SAV), and VIP-immunoreactivity was not evident within the small granular vesicles (SGV).

Four types of axonal profiles in the myenteric plexus of the mammalian gut have been classified by electron microscopy. These include adrenergic, cholinergic, purinergic or P-type and sensory nerves (1–4). Of these, the purinergic (1, 2) and the P-type (3) nerves are similar with respect to shape, size, and electron-density of the contents of the axonal vesicles ; these axons mainly contain large, membrane-bound granular vesicles (80 – 200 nm in diameter, mostly 90–160 nm) and a varying amount of agranular vesicles (40–60 nm in diameter) (1–4). Baumgarten *et al.* (3, 4) suggested that P-type axons might contain peptides, whereas Burnstock (1, 2) postulated that purinergic axons contained adenosine 5'-triphosphate or its related compound. It has also been proposed that the large granular vesicles contain substance P (5) or 5-hydroxytryptamine (6,7). Furthermore, recent immunohistochemical and immunoassay studies have shown that several peptides are present in the nerves of the gastrointestinal tract ; these include substance P (8–11), vaso-active intestinal polypeptide (VIP) (11–13), somatostatin (11, 14, 15), enkeph-alin (11, 16–18), and so forth. However, crucial immunohistochemical evi-dence at the ultrastructural level is lacking for the localization of these peptides

in the nerves of the gastrointestinal tract. Therefore, the type of the peptides present in the large granular vesicles of the enteric nerve terminals should be determined. In previous studies at the light microscopic level by immunohistochemistry (19), we reported the localization of both substance P and VIP in the enteric nerves ; and, each of the peptides was detectcd in the separate axons.

In this report we describe the ultrastructural analysis of the P-type nerves in the canine intestine, and, based on the immunohistochemical findings, possible features of the VIP-containing vesicles in the enteric nerve are discussed.

MATERIALS AND METHODS

Sixteen dogs of either sex, weighing 8 to 10 kg were used after fasting for 20—24 h. In all experiments, the dogs were anesthetized with sodium pentobarbital (Pitman Moore Inc. 30 mg/kg i. v.). The intestine was perfused through the left ventricular cannula, first with 1.5 to 2 liters of Tyrode's solution, then with equal volumes of the fixatives containing 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). The tissues dissected from the intestine were immersed in the same fixatives for 60—90 min at 4° C, followed by immersion for 2—3 h in 4% paraformaldehyde solution containing 0.1 M sucrose. These tissues were sectioned on a vibratome in a thickness of 30—100 μm and processed for immunohistochemistry.

Immunohistochemical Procedures

The sections were incubated in 100—200 μl of anti-VIP-rabbit antiserum (R-502, Yanaihara) (21) diluted 1 : 500 to 1 : 1000 for 90 min. After washing in a solution of phosphate buffer (PBS ; pH 7.1) for 1—2 h with several changes, the sections were incubated with unlabeled goat anti-rabbit immunoglobulin antiserum (Research Institute for Microbial Diseases, Osaka), diluted 1 : 30, for 40—60 min at room temperature. After further washing in PBS, the sections were incubated with horseradish peroxidase-antiperoxidase (PAP) complex diluted 1 : 30, for 30—40 min. The PAP was prepared according to the methods by Sternberger (22). Following these procedures, the sections were treated with a solution of 0.003% of 3.3′-diaminobenzidine tetrahydrochloride (DAB : Sigma Chemical Company) and 0.001% of hydrogen peroxide in Tris-HCl buffer (pH 7.6) and finally immersed in 2% OsO_4. As controls for immunocytochemistry, some sections were incubated in normal rabbit serum (omitting anti-VIP antiserum) and treated with DAB and hydrogen peroxide. Additional controls include the application of anti-VIP-antisera preabsorbed with an excess amounts of synthesized porcine VIP. Specific, VIP-like immunoreactive deposits were not detected in the control sections.

Conventional Electron Microscopy

Tissues from the same dogs were also prepared for routine morphological

investigation. After perfusion with the fixatives as described before, the tissues were dissected out and immersed in a cold solution of 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2—7.4) for 2 h at 4 °C. The tissues were then washed with PBS, postfixed in 1% OsO_4 in 0.1 M phosphate buffer for 1 h at 4 °C, washed with PBS, dehydrated and embedded in Epon 812. Ultra-thin sections, uncontrasted or contrasted with uranyl acetate and lead citrate, were examined with a Philips 301 electron microscope operated at 60 kV.

RESULTS

In normal profiles of axons fixed with both glutaraldehyde and Osmic acid, at least three morphologically different types of large granular vesicles coexisting with small agranular vesicles could be distinguished. Identification was made on the basis of the size, shape and electron-density of the contents of the axon vesicles. Fig. 1 shows that these vesicles varied from 80 to 120 nm in diameter with a highly osmiophilic dense core and most appeared to have a clear electron-transparent halo between the vesicle membrane and the granular core. These large granular vesicles (LGV) were usually intermingled with varying amounts of small agranular vesicles (50—70 nm in diameter) and have been assumed to contain acetylcholine. The large granular vesicles of the second type shown in Fig. 2 had diameters ranging between 140 to 200 nm and contained electron-opaque fine granular materials, most of which filled the entire vesicles. These vesicles (LOV) are also intermingled with agranular vesicles and are often found in both ganglion plexus and interganglionic nerve bundles. The third type is characterized by those of a medium-size (100—140 nm in diameter), electron-faint vesicles (LFV) containing osmiophilic, granular materials (Fig. 3) ; some of these vesicles lacked a prominent electron-transparent 'halo' and often intermingled with the vesicles of the type 1 (LGV). After incubation of the sections with the anti-VIP-antiserum, a number of axons, situated in the muscle layer and in the ganglion plexus, were found to contain the electron-dense, VIP-like immunoreactive product ; and, these reaction products were mainly restricted to the type 1 vesicles (Fig. 4). Control sections incubated with antigen-inactivated antiserum or with normal rabbit serum were devoid of the reaction product.

DISCUSSION

The present study confirms that at least three types of large granular vesicles are present in the nerves of the canine intestine. Such types of the vesicles have been demonstrated mainly in the axons of the gastrointestinal tract (2, 3, 23) ; In agreement with these findings, occasional axons contained mainly LGV, LFV, and sometimes LOV. In addition to the above mentioned types of axonal profiles, axon varicosities containing many LGV as well as a variable number of small agranular vesicles (SAV) were widely distributed in

both Auerbach's and Meissner's plexus and in the muscle layers. Baumgarten *et al.* (3, 4) suggested that these axons might be peptidergic (polypeptide-containing) nerve endings. However, cholinergic and adrenergic axon varicosities contain a few similar LGV or LFV, and there are nearly always some SAV in the varicosities. As described in a previous study (20), a heterogeneous distribution of these vesicles is often observed when longitudinal sections are made of axon varicosities in the intestine. Thus, orientation of the sectioning will reveal various distribution or ratios of these vesicles in the axons ; and, profiles identified as belonging to peptidergic nerve endings may be a sampling artifact.

Using immunohistochemical techniques, we found that theVIP-immunoprecipitates were restricted to the Type 1 vesicles which coexisted with SAV ; and, VIP-immunoreactivity was not evident within the SGV. Therefore, the SGV may not contain VIP. These findings are supported by the data of Johansson and Lundberg (25). However, Larsson (24) reported that VIP was restricted to the granular vesicles in the terminals of the so-called P-type nerve (2,3) ; and, whether only one type of P-type nerves contain VIP remains to be elucidated.

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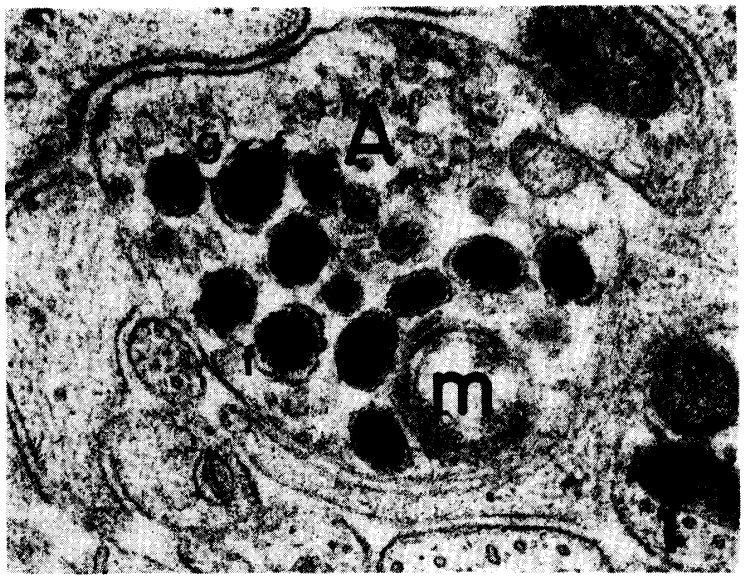
EXPLANATION OF FIGURES

Fig. 1. Presynaptic axon containing so-called large dense-cored vesicles (g ; 80–120 nm in diameter) and electron-faint vesicles (LFV ; (f) ; 100–140 nm in diameter) together with small agranular vesicles. (A ; 50–70 nm in diameter) × 63,000

Fig. 2. So-called large dense-cored vesicles (g) and large electron-opaque vesicles (LOV ; arrowhead ; 140–200 nm in diameter) ; the small agranular vesicles (SAV) are also seen in the axon. longitudinal section. n, neurotubule. × 62,000

Fig. 3. The large electron-faint vesicles (LFV ; arrowhead) together with the SAV (A). The LFV appear to contain granular materials. longitudinal section. n, neurotubule. × 96,000

Fig. 4. VIP-like immuno-reactive products (arrow), which were mainly localized to the large dense-cored vesicles in the axon. The SAV (arrowheads), which have been considered to contain acetylcholine, are also present in the same axon. Circular muscle layer in the ileum. SM, smooth musle, non-stained specimens. × 40,000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