A HISTOCHEMICAL APPROACH TO THE CHOLINERGIC INNERVATION OF ENDOCRINE-LIKE CELLS IN DOG ANTRO-PYLORIC MUCOSA

Isamu YAMAGUCHI, Jo MORI, Fumio HONDA, Hiroyuki NISHIZAKI* and Shigenobu KUMADA
Research Laboratories, Fujisawa Pharmaceutical Co. Ltd., Yodogawa-ku, Osaka and *Department of Surgery, Tokyo Medical and Dental University, Tokyo, Japan
Accepted June 10, 1974

Abstract—The cholinergic innervation of endocrine-like cells (probably identical with gastrin containing cells, G-cells) in dog antro-pyloric mucosa was studied histochemically, and was compared with that of secretory cells in fundic mucosa. Endocrine-like cells and cholinergic nerve fibers were detected by formaldehyde-HCl treatment and acetylcholine esterase (AChE) staining, respectively. In the fundic mucosa, cholinergic nerve fibers were widely observed along the oxyntic glands, particularly in their middle area. Cholinergic nerve fibers were seen in close proximity to the bases of secretory cells, and the distribution of cholinergic nerve fibers well coincided with that of parietal cells and chief cells. Cholinergic nerve fibers were detected also in the antropyloric mucosa, but they were rather restricted to the basal half of the mucosa. Demonstrating cholinergic nerve fibers and endocrine-like cells simultaneously in one section, we found that the former came in close proximity to the bases of endocrine-like cells. These results suggest that cholinergic nerve fibers control not only the production of gastric juice from the fundic mucosa but also the release of gastrin from the antropyloric mucosa.

There have been few histochemical studies of the cholinergic innervation in the gastric mucosa, though the important role of the cholinergic innervation in gastric secretion has been repeatedly emphasized on the basis of pharmacological studies (1-3). Histochemical studies of nerve fibers have shed light on the innervation between the submucous and longitudinal muscle layers, and elucidated the relationship between nerve supply to the smooth muscles and gastrointestinal motility (4, 5).

The authors found in a previous study that a small dose of atropine blocks food- and morphine-induced gastrin release in dogs (6). This finding suggests that G-cells, which are believed to store and release gastrin (7), receive cholinergic innervation. The G-cells have been identified in the antro-pyloric mucosa by means of immunocytochemistry (8). On the other hand, a series of endocrine-like cells, which are postulated to be identical with the G-cells, has recently been detected by a simpler method, i.e. formaldehyde-HCl treatment (9).

In the present study the authors investigated cholinergic innervation of endocrine-like cells in such a way as to demonstrate the cells and AChE activity by the formaldehyde-HCl treatment and the acetylthiocholine method, respectively, and compared the cho-
linergic innervation of endocrine-like cells in the antro-pyloric area with that of exocrine cells in the fundic gland.

MATERIALS AND METHODS

Mongrel dogs of both sexes were anesthetized with sodium pentobarbital (25 mg/kg i.v.). The stomach was rapidly removed, and small pieces of tissues were dissected out of the fundic and antro-pyloric area along the greater curvature.

Light microscopy

AChE was stained by Karnovsky's thiocholine method (10). Tissues were removed during anesthesia and frozen with solid CO₂. Sections 8-10 μm in thickness were cut with a cryostat at −25—−30°C and placed on slides. The slides were incubated for 1 hr at 37°C. Butyrocholinesterase was selectively inhibited by pre-incubation with 10⁻⁴ M isoOMPA (tetra-isopropyl pyrophosphamide). The sections were mounted by Permount and examined under a light microscope.

For staining of secretory cells, i.e. parietal cells, chief cells and mucous cells, the specimens were embedded in paraffin, sectioned at 4-6 μm thickness, and deparaffinized in xylene. The sections were successively stained with periodic acid-Schiff reagent, hematoxylin and aurantia according to the method described by Marks and Drysdale (11). After being mounted in Permount, sections were examined under a light microscope.

Fluorescence microscopy and cytospectrofluorometry

Specimens were frozen in solid CO₂-isopentane, freeze-dried, and exposed to formaldehyde gas for 1 hr at 80°C according to the standard Falck-Hillarp technique (12). The preparations were embedded in paraffin, sectioned at 6 μm thickness, mounted in xylene, and examined under a fluorescence microscope. These preparations were subsequently exposed to HCl gas by Hakanson's method (9), and re-examined for fluorescent cells. The spectral characteristics of the cytoplasmic fluorophore were analysed under a modified Leitz microscopic spectrofluorometer (MPV) with a high pressure mercury light source (HBO 200) and vertical illuminator. The blank correction for background fluorescence and light scatter was made by subtracting the reading from the nonfluorescence area in the same preparation.

Simultaneous demonstration of AChE and endocrine-like cells

Tissue blocks of 1-2 mm³ were incubated for 60-70 min in the incubation medium used in the above AChE staining, washed with 0.1 M sodium hydroxide-maleate buffer (pH 6.0), and frozen in solid CO₂-isopentane. The specimens were freeze-dried and exposed to formaldehyde gas as mentioned above. Paraffin sections were cut at 6 μm thickness, and examined under light and fluorescence microscopes.

RESULTS

Fundic mucosa

An intense AChE activity was observed in the fundic mucosa (Fig. 1). Although the cholinergic nerve fibers in which AChE activity was detected were found to have a
wide distribution, most were in the middle area of the fundic mucosa. These fibers were found to be distributed along the oxyntic gland and in some cases constituted a network connecting each gland. As shown in Fig. 2, cholinergic nerve fibers are seen in close proximity to the base of secretory cells. A few single cholinergic nerve fibers were observed in the superficial one-fifth of the mucosa, and extended to almost the surface epithelium. In the basal portion of mucosa, comparatively large bundles of cholinergic nerve fibers emanated from the submucosa (Fig. 3).

Secretory cells were stained in order to study the relationship between their distribution and those of cholinergic nerve fibers. Following the method of Marks and Drysdale (11), parietal cells, chief cells and mucous cells were stained yellow, blue and purple, respectively, and could be well differentiated. Chief cells were found to be arranged in a single layer, to surround the gastric lumen, and to be distributed in the lower two-thirds of the mucosa. The cells increased in number as they descended deeper to the base of oxyntic gland where no secretory cells other than chief cells existed.

Parietal cells were arranged in the same manner as were the chief cells. Localization of parietal cells differed from that of the chief cells, and most were confined to the middle one-third of the mucosa. The upper one-third of the mucosa was almost completely devoid of parietal cells. In this area were mucous epithelial cells alone.

Mucous neck cells were few and located between the parietal cells in the neck of the gland.
Antro-pyloric mucosa

Most cholinergic nerve fibers were distributed in the basal half or one-third of the antro-pyloric mucosa (Fig. 4). These nerve fibers in the antro-pyloric mucosa were stained for the AChE activity more lightly than those in the fundic mucosa. Only a few fine cholinergic nerve fibers approached the surface epithelium (Fig. 5).

In the antro-pyloric region the gland extended straight down to the middle of the mucosa, and thereafter meandered in the basal portion of the mucosa. Even when the sections were cut perpendicularly, a transverse face of the gland was seen in the basal half of the mucosa. Cholinergic nerve fibers were found around these transversely cut glands (Fig. 6). In some sections the cholinergic nerve fibers around the gland just above the muscularis mucosa were found to be linked with long cholinergic nerve fibers in the submucosal layer.

Utilizing the standard Falck-Hillarp technique enterochromaffin cells can readily be detected by their intense fluorophore of serotonin. Between these enterochromaffin cells an extensive system of endocrine-like cells which had a faint fluorescence was observed in the basal half of the mucosa (Fig. 7-A). These cells were clearly visible following subsequent exposure to HCl gas (Fig. 7-B). The fluorescence of the endocrine-like cells varied...
Fig. 5. Antro-pyloric mucosa. AChE staining. A single cholinergic nerve fiber (Ch) is seen extending almost to the surface epithelium. ×200

Fig. 6. Antro-pyloric mucosa. AChE staining. Many transverse sections of pyloric glands are surrounded with cholinergic nerve fibers (Ch). ×300

Fig. 7. Basal part of antro-pyloric mucosa. A : standard formaldehyde treatment. Endocrine-like cells with cytoplasmic granules are seen arranged in a single layer around the gastric lumen. Two enterochromaffin cells (EC) and an adrenergic nerve fiber (Ad) are also seen. B : same section as A after subsequent exposure to HCl. Endocrine-like cells are made to be strongly fluorescent. Fluorescence of the enterochromaffin cells and adrenergic nerve fiber is reduced or abolished. ×400
in color from faint green to clearly yellow, and were localized in the cytoplasm. On the other hand, this procedure abolished the green fluorescence of norepinephrine and reduced the yellow fluorescence of serotonin. The endocrine-like cells had a round-shaped nucleus and cytoplasmic granules. Most were oval, with their bases in the lamina propria and their apices extending to the luminal surface. These cells could not be found in the fundic area.

Fluorescence excitation and emission spectra were measured from the cytoplasm of endocrine-like cells with yellow fluorescence after prolonged HCl exposure (Fig. 8). The excitation spectrum was biphasic and had two peaks at 400-410 m\(\mu\) and 430-440 m\(\mu\). The latter was the excitation maximum. The emission maximum was at 500-510 m\(\mu\).

Simultaneous staining of the AChE and endocrine-like cells in one section showed that cholinergic nerve fibers surrounded the transversely cut gland which contained enterochromaffin and endocrine-like cells and that a cholinergic nerve fiber is located close to the bases of the endocrine-like cells (Fig. 9).

**DISCUSSION**

The present histochemical studies showed the dense cholinergic innervation of the fundic mucosa. Cholinergic nerve fibers were seen to come in close proximity to the bases of secretory cells, the distribution of which coincided well with those of parietal cells and...
chief cells. These findings suggest the direct involvement of cholinergic innervation in the secretion of pepsin and hydrochloric acid. This interpretation is supported by pharmacological evidence (1-3). Cholinergic nerve fibers were rarely seen in the superficial one-fifth of the mucosa. Mucous secretion from the surface epithelium, thus, is unlikely controlled by cholinergic nerves. On the contrary, the mucous neck cells appeared to be innervated by cholinergic nerves, and indeed this may be the case as the mucous neck cells are located at the neck of the fundic gland where dense cholinergic innervation was detected. The mucigogue effect of pilocarpine observed by Andre et al. (13) is presumably due to the cholinergic innervation of mucous neck cells.

Cholinergic nerve fibers were also seen in the antro-pyloric area, but the distribution differed somewhat from that in the fundic area. Cholinergic nerve fibers in the antro-pyloric area were rather restricted to the basal half of the mucosa. This portion innervated by cholinergic nerve fibers coincided well with the zone which has been reported to contain gastrin (14). G-cells have also been shown by means of immunocytochemistry to be localized in the basal half of the antro-pyloric mucosa (8). Moreover, there is abundant pharmacological evidence indicating that gastrin is released by vagal activation (1, 6, 14, 15).

Hakanson et al. (9) reported that an extensive system of endocrine-like cells was detected by formaldehyde-HCl treatment in cat antro-pyloric mucosa, and the excitation maximum and emission maximum were at 440-450 m/z and 500-520 m/z. These cells were considered to be G-cells on the basis of their stainability and distribution. In our study, endocrine-like cells were detected in the basal half of the antro-pyloric mucosa of dogs by formaldehyde-HCl treatment. These cells were barely visible and had cytoplasmic granules with fluorophore after formaldehyde-treatment, and were made clearly fluorescent after subsequent exposure to HCl gas. The excitation maximum and emission maximum of their fluorophore were at 430-440 m/z and 500-510 m/z, respectively. Since these properties of fluorophore in dogs were identical with those in cats, these cells were considered to be G-cells. Furthermore, the dogs' cells had another smaller excitation peak at 400-410 m/z. This is probably due to the co-existence of dopamine, as the excitation maximum of dopamine fluorophore has been reported to be at 410 m/z (12). Pearse et al. (16) have reported that human G-cells can take up dopa and decarboxylate it into dopamine.

Demonstrating the AChE activity and endocrine-like cells simultaneously in one section, the authors showed that the pyloric gland, in which the endocrine-like cells existed, was surrounded by cholinergic nerve fibers. Furthermore, a cholinergic nerve fiber was found to run in close proximity to the bases of these endocrine-like cells which were possibly identical with G-cells that store and release gastrin (7). Such are compatible with recent evidence that a small dose of atropine reduced the serum levels of gastrin which had been elevated by food or morphine (6, 15). Thus, it can be concluded that gastrin release is controlled by the cholinergic nerve fibers innervating G-cells.
Acknowledgement: The authors are grateful to Mr. S. Mukumoto and Miss. Y. Nakahira of the Fujisawa Research Laboratories for skillful technical assistance.

REFERENCES

1) Grossman, M.I.: Fedn. Proc. 27, 1312 (1968)
2) Hirschowitz, B.I. and Sachs, G.: Gastroenterology 56, 693 (1969)
3) Yamaguchi, I.: Japan. J. Pharmacol. (in press)
4) Jacobowitz, D.: J. Pharmacol. exp. Ther. 149, 358 (1965)
5) Schofield, G.C.: Handbook of Physiology, Section 6: Alimentary Canal, Edited by Code, C.F., Vol. IV, p. 1579, American Physiological Society, Washington, D.C. (1968)
6) Yamaguchi, I., Fuke, H., Tsujita, M., Honda, F. and Nishizaki, H.: Japan. J. Pharmacol. Suppl. 24, 124 (1974)
7) Vasallo, G., Solcia, E. and Capella, C.: Z. Zellforsch. 98, 333 (1969)
8) McGuigan, J.E. and Greider, M.H.: Gastroenterology 60, 223 (1971)
9) Håkanson, R., Larsson, I.-I., Nishizaki, H., Owman, Ch. and Sundler, F.: Histochemie 34, 1 (1973)
10) Karnovsky, M.J.: J. Histochem. Cytochem. 12, 219 (1964)
11) Marks, I.N. and Drysdale, K.M.: Stain Tech. 32, 48 (1957)
12) Björklund, A., Falck, B. and Owman, Ch.: Methods in investigative and diagnostic endocrinology. Edited by Berson, S.A., Vol. I, p. 318, North Holland Publishing Company (1972)
13) Andre, R., Nezami, J.E. and Philips, J.P.: Am. J. Physiol. 209, 877 (1965)
14) Elvin, C.-E. and Uvnas, B.: Gastrin, Edited by Grossman, M.I., p. 69, University of California Press, Los Angeles (1966)
15) Nilsson, G., Simon, J., Yalow, R.S. and Berson, S.A.: Gastroenterology 63, 51 (1972)
16) Pearse, A.G.E., Coulling, I., Weaver, B. and Friese, S.: Gut 11, 649 (1970)