THE ULTRASTRUCTURE OF TWO TYPES OF ENTEROENDOCRINE PARANEURONS IN THE MOUSE DUODENUM

by

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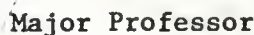

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INTRODUCTION

A system of basally-granulated endocrine-like cells has been known to be situated in the mucosa of the mammalian gastrointestinal tract since Heidenhain (1870) first observed them in the stomach of the dog. Certain cells of this system were found to react strongly to chromium salts (Schmidt, 1905) and have been named "enterochromaffin" (EC) cells since Ciaccio coined the term in 1907. There are several other cells of this system which react with silver salts after the addition of an extraneous reducing agent; these are called the argyrophil cells of the gastrointestinal mucosa (Dawson, 1970). Electron microscopic studies have shown that each of several types of gastrointestinal endocrine cells can be identified by the cytoplasmic granules which are of a characteristic size and shape (Forssmann et al., 1969; Solcia et al., 1975), although the exact number of distinct types of endocrine cells in the mammalian gut has yet to be determined (Forssmann, 1970; Ferreira, 1971; Kubes and Jirasek, 1974).

Based upon their morphological and biochemical properties, the enteroendocrine cells have been grouped with the Amine Precursor Uptake and Decarboxylation (APUD) cell series of Pearse (1969) and more recently with a larger system of "paraneurons" described by Fujita (1976). Cells occurring outside the digestive system, but classified either as belonging to the APUD series or the paraneuron system have been demonstrated to be closely associated with nerve fibers (Lauweryns et al, 1973; Eaton and Fedde, 1977). Within the digestive system, the endocrine cells of the pancreatic islets, including the pancreatic A cell, have been demonstrated at the electron microscopic level to be directly innervated (Stach, 1974). Although enteroendocrine cells of the small intestine

have been demonstrated to be intimately associated with enteric nerve fibers in the human fetus (Osaka and Kobayashi, 1976), the question of whether or not the same cells in the adult intestine are directly innervated has not been answered.

The purpose of this study is to determine the presence or absence of structural evidence of synaptic contact between nerve fibers of the subepithelial plexus and enteroendocrine cells in the intestine of the adult mouse using the techniques of serial thin sectioning and transmission electron microscopy. Fujita (1976) has shown enteroendocrine cells to be either "open" to the intestinal lumen, or "closed", that is not having a luminal border. The EC cell typifies the open-type cell (Forssmann et al, 1969) and the intestinal A cell is at present thought to be a closed-type cell in the adult mouse gastrointestinal mucosa (Solcia et al., 1975). The cell types chosen for this particular study are the EC cell and the intestinal A cell in the adult mouse duodenum. A second portion of the study is aimed at more precisely describing the relationship of these two types of cells with underlying tissue components in order to more fully understand the potential fate of secretory products released from these cells and thus gain a better understanding of the possible functional roles these cells might have, based upon ultrastructural analysis.

LITERATURE REVIEW

Enterochromaffin Cell

The term enterochromaffin (EC) sets this particular cell apart from chromaffin cells found in other organs such as the adrenal medulla, sympathetic paraganglia, lung and carotid body. All of these cells show similar histochemical properties (Hillarp and Hokfelt, 1955), and all of them appear histologically as lightly staining cells filled with cytoplasmic granules (Thompson, 1966). Early studies led to the characterization of the EC cell granular substance as "enteramine" (Vialli and Erspamer, 1937) which was later identified as 5-hydroxytryptamine (Erspamer and Asero, 1952).[1] Since then the correlation between chromaffin cells and high 5-hydroxytryptamine (5-HT, serotonin) has been studied in many parts of the mammalian body including connective tissue (blood platelets), neural tissue (brain and certain autonomic nerve fibers), and epithelium (gut and lung) (Twarog and Page, 1953; Hillarp and Hokfelt, 1955; Falck and Owman, 1968; Eaton and Fedde, 1977). Until recently the term enterochromaffin cell had been used interchangeably with enteroserotonin cell to denote the 5-HT containing cell of the gut.

Electron microscopic histochemistry and autoradiography studies have confirmed that 5-HT is stored within the granules of the EC cell (Rubin et al., 1971; Vassallo et al., 1971) and have recently shown the presence of another substance within the same granules. This additional

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1. Identification of enteramine as 5-HT occurred independently and after serotonin, a substance with vasoconstrictor properties, had been isolated and characterized as 5-HT (Page, 1968).

component of the EC cell granules has been identified as Substance P (Pearse and Polak, 1975; Heitz et al., 1976), a compound first isolated from rabbit gut (von Euler and Gaddum, 1931) and later found to be synonymous with the neurokinin found in brain and peripheral nerves (Bass and Bennett, 1968).

Even though the presence of characteristic biconcave or polymorphic granules has made the EC cell readily identifiable in the electron microscope, and the content of the granules has been determined biochemically, very little is known about the function of the EC cell. It has been suggested that this lack of understanding is due to the relatively small numbers and dispersed distribution of EC cells (Sonstegard et al., 1976). The physiological actions of serotonin and Substance P have been studied separately for many years and have been reviewed (Bass and Bennett, 1968; Page, 1968); both are known neurotransmitters and both have been shown to stimulate smooth muscle contraction. Exactly what function the two compounds serve as secretory products of the EC cell remains a mystery. It has been shown, however, that the EC cell might be derived from the neuroectoderm (Pearse, 1969) and that Substance P is primarily a sensory neurotransmitter (Heitz et al., 1976). The suggestion then, that this cell serves some receptive function seems quite plausible (Fujita, 1976), and is further substantiated by the recent localization of adenylate cyclase in the EC cell microvilli (Yamamoto and Ozawa, 1977).

Intestinal A Cell

A review of the literature on the intestinal A cell is complicated for a number of reasons. First of all, the A cell is not uniquely argentaffin like the EC cell; rather it is one of a group of endocrine cells

in the gut which are silver absorbing and therefore termed argyrophil (Polak et al, 1975). Secondly, this group of argyrophil cells are morphologically similar in that they all contain secretory granules which are spherically shaped, not uniquely polymorphic as in the case of the EC cell. Furthermore, the intestinal A cell has been given a variety of names (A-like, EG, Type II, L, S, and D cell) at one point or another in the literature. This cell has been variously described as containing spherical granules with diameters of anywhere from 200 nm (Vassallo et al., 1969) to 700 nm (Forssmann et al., 1969).

There is not a long history of information on the intestinal A cell as there is for the EC cell where the cell had been studied for some time and its content only recently revealed (see review of the EC cell above). The presence of a glycogenolytic substance in the gastrointestinal mucosa (Sutherland and de Duve, 1948) which was later shown to be immunoreactive to antiglucagon antisera (Unger et al., 1966) led to the ultrastructural evidence for "glucagon-producing cells" in the intestinal mucosa (Orci et al., 1967, 1968). Because of the striking similarity between this new enteroendocrine cell and the α_2 cell of the pancreatic islet, the cell was first described as the "intestinal A cell" (Forssmann et al., 1969). Since the initial description it has been confirmed by immunofluorescent localization and electron microscopy that the intestinal A cell is indeed the site of enteroglucagon production in the gut (Polak et al., 1971). Histochemistry at the electron microscopic level has allowed the distinction between the highly argyrophilic granules of the intestinal A cell and similar spherical but less- or non-reactive granules of other enteroendocrine cells (Vassallo et al., 1971), a distinction which cannot be made with routine electron microscopy stains.

Like the EC cell, much remains unknown about the actual functions of the intestinal A cell. The physiological function of the polypeptide it contains (enteroglucagon) has not been determined (Polak et al., 1971), nor has the amine portion of the granule content been identified although it is known that there is a possibility that dopamine or a mixture of dopamine and serotonin is contained in the intestinal A cell (Pearse, 1976). It has been shown that the pancreatic A cell is directly innervated (Stach, 1974); a similar finding with respect to the intestinal A cell has not been reported.

Subepithelial Nerve Plexus

The initial studies on intrinsic innervation of the gut were those detailing nerve structures found in the submucosa of the intestine in various mammals (Meissner, 1857). The submucous plexus was shown to be linked to the myenteric plexus (which lies between the longitudinal and circular smooth muscle layers of the gut wall) by bundles of nerve fibers. It was also shown that both of these plexuses are connected, either directly or indirectly, with a plexus lying just beneath the epithelial lining of the gut. This subepithelial plexus is comprised of a complex network of fibers, some of which were said to penetrate the epithelial surface of the intestinal villi and terminate around and between the epithelial cells of both the villi and the crypts of Lieberkühn (Hill, 1927). More recent work at the histological level has not added significantly to the information concerning the subepithelial plexus (Gunn, 1968; Schofield, 1968).

Sensory-like neurons situated within enteric ganglia, with dendrites terminating somewhere in the mucosa, were described very early in the literature (Dogiel, 1899). These findings were confirmed and it was further proposed that the subepithelial plexus is composed primarily of these sensory fibers which provide afferent innervation of the glandular crypts (Hill, 1927). Further histochemical studies, mainly cholinesterase and silver impregnation methods, have proven that the subepithelial plexus is the only one of the enteric plexuses to contain ganglion cells which distinctly resemble those found in dorsal root ganglia (i.e., they are bipolar, unipolar or pseudounipolar). This is interpreted as an indication that the subepithelial plexus is an afferent plexus that receives sensory impressions from the mucous membrane (Gunn, 1968).

Bülbring and co-workers have shown firm physiological evidence that the mucous membrane of the gut is in fact afferently innervated (Bülbring and Crema , 1958). Recordings from nerve fibers with chemosensitive and/or mechanosensitive endings have been reported (Paintal, 1957), but surprisingly little work has been done on afferent innervation of the gut using advanced microelectrode techniques (Davison, 1972). The fact that nerve elements can be observed "in close vicinity of the intestinal epithelium" has led to the circumstantial conclusion that some of these fibers are of "real" sensory nature (Feher, 1976). Although light microscopic studies have shown "innervation" of the gut epithelium (Schofield, 1968), no synaptic relationship between any element of the intestinal epithelium and fibers of the submucous plexus has been confirmed at the ultrastructural level.

MATERIALS AND METHODS

Perfusion Fixation

Seven adult white mice (35-50 g), all males, were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg body weight). The anesthetized animal was secured to a dissection board and a mid-ventral incision made from the upper thorax to lower abdomen. The thoracic cavity was opened along the sternum and spread with small retractors. A 21 gauge needle, primed with 0.1 ml Heparin solution was inserted into the left ventricle with a micromanipulator. This needle was connected to a three-way stopcock to which bottles containing flushing and fixative fluids were connected using solution administration sets (Travenol Labs, Inc.).

After inserting the needle into the heart, the stopcock was opened to begin the perfusion with Tyrode's balanced salt solution (Lillie, 1965) pH 7.4, equilibrated by aerating with a gas mixture of 95% O_2/ 5% CO_2. The right atrium was then slit open and the circulatory system flushed until the perfusate coming from the right atrium was cleared (this fluid was aspirated from the abdominal cavity). The stopcock was then turned to allow cold fixative (2% glutaraldehyde in 0.15 M sodium cacodylate buffer, pH 7.4 at 4° C, 274 mOsm) to perfuse through the animal for 5 minutes. Cold fixative was poured into the abdominal cavity and injected into the lumen of the upper small intestine while the perfusion fixation was proceeding.

Upon completion of the perfusion, the proximal 3 cm of small intestine was resected, divided into 1 cm lengths and rinsed with fresh, cold fixative. Total fixation time was 2 hours at 4° C.

Specimen Preparation

After fixation, tissues were rinsed in cold buffer, post-fixed in a 1:1 solution of 2% OsO_4/0.15 M sodium cacodylate buffer at pH 7.4 (at 4° C), rinsed in distilled water, dehydrated in graded ethanols (30, 50, 70, 95, and 100%), cleared with acetone and infiltrated with an embedding mixture of Epon 812 and Araldite resins (Ladd Research Industries, Inc.). The large (1 cm long) tissue specimens were trimmed, while infiltrating, to yield pieces small enough for ultramicrotomy (0.25 mm^2 x 0.5 mm). These pieces were transferred to fresh embedment in flat rubber molds and then polymerized and hardened in warming ovens (30° C for 12 hours, 45° C for 12 hours, and 60° C for 2-3 days).

Specimens processed for the silver impregnation studies were handled according to the method described by Vassallo (Vassallo et al., 1971).

Sectioning and Staining

Orientation of the embedded tissue was obtained through the study of 1 µm thick sections cut with a glass knife on a Sorvall MT-2 ultra-microtome. Thick sections were stained with either basic fuchsin-alkaline methylene blue (Huber et al., 1968) or with alkaline methylene blue alone. After staining, the sections were viewed under phase contrast illumination on a Zeiss RA Photomicroscope.

After locating the lighter staining, basally-granulated endocrine cells in the thick sections under the light microscope, the glass knife was replaced with a diamond knife and serial thin sections were cut at 60 - 90 nm thickness (based on interference colors, silver and gold respectively). Thin sections were stained 2 minutes in 75% ethanolic uranyl acetate (Stempak and Ward, 1964) and 45 seconds in the lead stain developed by Sato (1968).

Electron Microscopy

All thin sections were examined in either an RCA EMU-3G or Philips EM 301 transmission electron microscope at accelerating voltages of 40 kV for unstained, silver impregnated sections or 100 kV for stained sections.

OBSERVATIONS

Light Microscopy

Stained 1 μm thick sections, observed with the light microscope, showed lightly stained, basally-granulated enteroendocrine cells in the duodenal epithelium (Fig. 1). The cells were seen most often in the intestinal crypts where their distribution was either at the base of the crypt or at the crypt/villus junction and very infrequently along the lateral sides of the crypts. At the light microscopic level the enteroendocrine cells appeared to be either very elongated, reaching from the base of the epithelium to the lumen of the crypt, or more rounded and not reaching the lumen. Enteroendocrine cells were easily distinguished from the absorptive columnar epithelial cells, the mucus-secreting goblet cells and the zymogen-secreting Paneth cells. The differential morphology of enteroendocrine cell granules could not be determined at the light microscope level; therefore, observations at this level were primarily limited to localization of enteroendocrine cells. Specific identification of endocrine cell types and the study of tissues surrounding the cells was reserved for electron microscopic investigation.

Electron Microscopy

Enterochromaffin Cell Ultrastructure

Enterochromaffin (EC) cells are identified by the electron dense polymorphous granules in their cytoplasm. These granules average 375 nm by 145 nm in size and are variously biconcave, oblong, crescent or spherical in shape. Although they occurred throughout the cytoplasm, granules are concentrated in the infranuclear region (Fig. 2). The cytoplasm of

EC cells is noticeably more electron transparent than the cytoplasm of neighboring columnar epithelial cells. Several EC cells have been semi-serially sectioned and a model of one EC cell has been constructed from electron micrographs of complete serial thin sections through the entire cell. From this model the EC cell is viewed as an elongated, pyramidal or cone-shaped cell, extending from the basal lamina to the lumen of the intestinal crypt.

The very narrow (1.2 μm) apical pole of the EC cell consists of a tuft of microvilli protruding into the lumen of the intestinal crypt. From these microvilli, microtubules (oriented along the long axis of the cell) extend into the apical cytoplasm (Fig. 3). The apical portion of the EC cell is firmly attached to neighboring columnar epithelial cells by a distinct junctional complex consisting of three components: an outermost (closest to the crypt lumen) zonula occludens (fused plasma membranes), an intermediate zonula adherens (closely apposed plasma membranes with microfilaments extending into each cell) and below this, a macula adherens (closely apposed plasma membranes with cytoplasmic plaques of filaments). The entire junctional complex in one EC cell is approximately 0.9 μm long from zonula occludens to macula adherens.

All EC cells observed contain a single, very large (1.6 μm by 0.7 μm) phagosome centrally located in the apical cytoplasm and quite conspicuous by its extreme electron density and irregular shape (Fig. 4). Single or paired centrioles are also present in the apical cytoplasm and are not closely associated with either the Golgi complexes or the nucleus (Fig. 4). The apical and perinuclear regions of the EC cell contain a highly developed system of rough endoplasmic reticulum whereas the Golgi complexes were seen only in the supranuclear region of the cell (Figs. 2 and 5).

The nucleus of the EC cell is quite large (7.3 μm at its greatest diameter) compared to the nucleus of columnar epithelial cells and is located in or near the center of the cell. Mitochondria and the characteristic electron dense polymorphous granules, found throughout the EC cell, are concentrated primarily in the infranuclear cytoplasm (Figs. 2, 6 and 7). The EC cell rests on a basal lamina which is contiguous with that of neighboring epithelial cells, except where cytoplasmic extensions penetrate it. The basal cytoplasmic extensions, traced to their termini, coursed for some distance beneath epithelial cells (Fig. 2), or occasionally extended into spaces directly underlying the EC cell.

Intestinal A Cell Ultrastructure

Intestinal A cells were also observed in the epithelium of the intestinal glands; however their occurrence here is much more infrequent than the EC cell. Like the EC cell, the intestinal A cells were identified by the electron dense spherical granules in their cytoplasm (Figs. 8 and 9). The cores of the granules are 135 nm diameter (maximum); they are bounded by a loosely fitting membrane, forming a 40 nm wide halo around the dense core and making the entire granule-containing vesicle a 175 nm diameter sphere (maximum). Average dimensions of the majority of granules observed in thin sections was between 115 and 130 nm. The granules are concentrated primarily in the infranuclear regions of the cell and are not observed in the cytoplasm of basal cellular extensions. Smaller, electron lucent vesicles (50 to 60 nm in diameter) were observed in the intestinal A cells and these vesicles were also observed in the cytoplasmic extensions (Fig. 10).

The intestinal A cell is slightly elongated and, like the EC cell, has a less electron dense cytoplasm than the non-endocrine epithelial

cells which border on it (Fig. 8). None of the A cells observed bordered on the lumen of the intestinal crypt; instead the apical border appeared rounded and was separated from the lumen by neighboring epithelial cells.

No extensive regions of intercellular contact like the junctional complex in the EC cell are present; discrete desmosomes are infrequently observed along the lateral and apical borders of the cell. The rough endoplasmic reticulum of the intestinal A cell is much less extensive than that of the EC cell and consists of short saccules scattered primarily throughout the apical cytoplasm. Golgi complexes also are less evident than in the EC cell (serial thin sections were essential to show the Golgi complexes in the A cells observed). Lack of microtubules and less extensive distribution of microfilaments are also noted (compare Fig. 8 with Fig. 2). No centrioles or phagosomes are observed in the intestinal A cells.

The most distinctive feature of the intestinal A cell is its singular basal cytoplasmic extension. Unlike the EC cells, having several cytoplasmic extensions, the extension of the A cell is directed through the basal lamina to contact another cell (as yet unidentified, Fig. 10).

Subepithelial Plexus

Fibers of the subepithelial nerve plexus are observed in connective tissue of the lamina propria and directly beneath the epithelial lining of the crypts (Figs. 7, 9 and 11). In cross section these fibers are bundled together in small fasciculi, partially or completely enclosed in Schwann cell cytoplasm (Fig. 12). Six to eleven single fibers have been seen in cross sections of fasciculi approximately 2.0 μm in diameter (including the Schwann cell). Individual fibers, ranging from 0.5 μm

to 0.1 μm in diameter, are irregularly shaped. In longitudinal views near the crypt epithelium, the bundles of fibers lack Schwann cell cytoplasm and have fewer individual fibers (generally three to six). The fibers closest to the epithelium are more uniform in size, averaging about 0.1 μm in diameter.

Swellings of the subepithelial fibers occur within 0.9 μm of EC cells (Fig. 7) and 0.5 μm of intestinal A cells (Fig. 9). The swellings are 0.7 μm in diameter and contain both clear and dense-cored vesicles (Fig. 11). The clear vesicles are uniformly 40 nm in diameter; the dense-cored vesicles, with 80 to 90 nm diameter cores surrounded by a limiting membrane, are 110 to 115 nm in diameter. The *en passant* type swellings also contain mitochondria and neurotubules.

Direct connections between swellings of the subepithelial plexus and enteroendocrine cells have not been observed in this study; axo-axonal synapses however are present within the plexus. The putative neurotransmitter-containing vesicles at those synapses are either clear (Fig. 9) or dense-cored (Fig. 11). The axo-axonal synapses are as close as 1.0 μm from the bases of enteroendocrine cells.

One intestinal A cell has a basal cytoplasmic extension penetrating the basal lamina and contacting the process of an unidentified cell (Fig. 10). The unidentified cell process contains numerous flattened, vesicle-like structures, measuring 70 nm by 25 to 30 nm, with slightly biconcave shapes. The membranes of both the A cell and the unidentified cell process show an increased cytoplasmic density at the point of their closest contact, which is approximately 25 nm (Fig. 10).

PLATE I

Fig. 1

Light micrograph showing cross section of mouse duodenum with a lightly stained basally-granulated enteroendocrine cell (bg) in an intestinal crypt (C). Apical zymogen granules (zg) are seen in Paneth cells. Surrounding the crypts is the loosely arranged connective tissue of the lamina propria (LP) outside of which lies the irregular, more dense connective tissue of the submucosa (SM), the inner circular (CM) and outer longitudinal (LM) layers of the muscularis externa and the outermost serosa (S). Basic fuchsin, alkaline methylene blue stain. X 1,300.

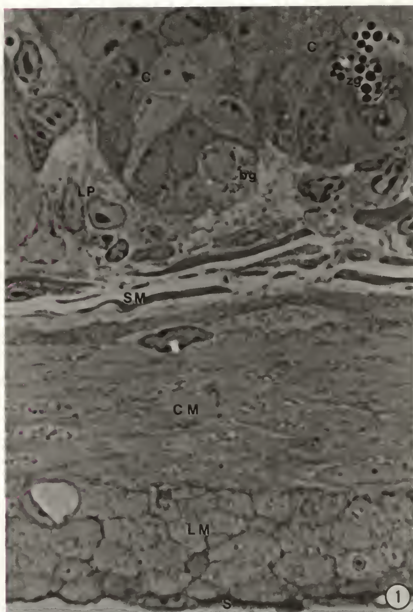

C
C
zg
bg
LP
SM
CM
LM
S
1

PLATE II

Fig. 2

EC cell (EC) between enterocytes (EN) in adult mouse duodenum showing microvilli (mv) at apex of cell, open to crypt lumen (l), and basal cytoplasmic extensions (x), near basal lamina (bl). Note large ovoid nucleus (n), perinuclear rough endoplasmic reticulum (er), apical Golgi complexes (go), phagosome (p) and polymorphic electron dense granules (g). Underlying tissue components include fibroblasts (F), nerve fibers (nf), a fenestrated capillary (c), and a large lymph vessel (lv). X 7,400.

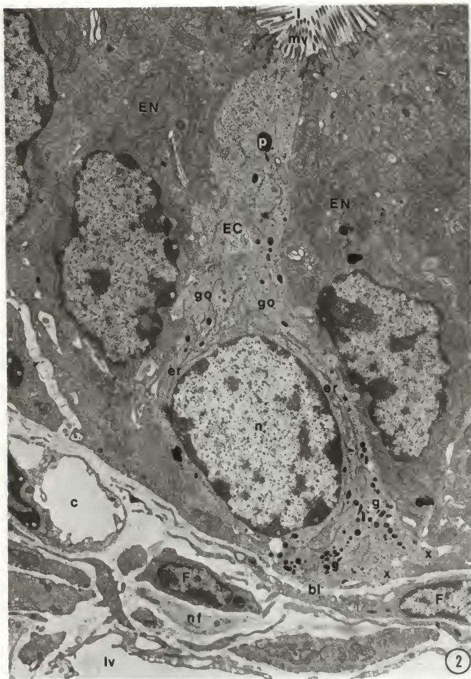

l
mv
EN
p
EN
EC
go
go
er
er
n
c
g
g
x
x
F
bl
F
nf
lv
2

PLATE III

Fig. 3

Junctional complex between apex of EC cell and neighboring enterocyte (EN), composed of zonula occludens (zo), zonula adherens (za), and macula adherens (ma). Microvilli (mv) and associated microtubules (mt), extending into apical cytoplasm, are also seen. X 46,800.

Fig. 4

Supranuclear region of EC cell showing phagosome (p), electron dense granule (g) and centriole (ce). X 29,200.

Fig. 5

Supranuclear region of EC cell showing well developed Golgi complex (go), rough endoplasmic reticulum (er), free ribosomes (r), mitochondria (m) and microfilaments (mf). X 46,800.

Fig. 6

Infranuclear region of EC cell showing basal accumulation of polymorphic electron dense granules (g), basal cytoplasmic extension (x) and basal lamina (bl). X 26,400.

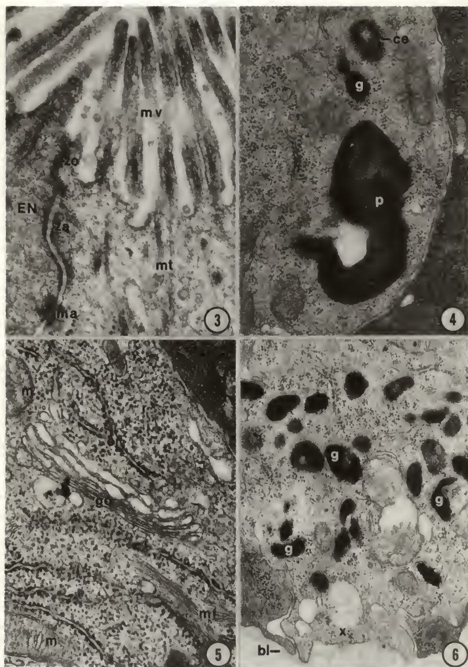

mv
zo
EN
za
mt
ma
3
ce
g
p
4
m
go
mt
m
5
g
g
g
x
bl
6

PLATE IV

Fig. 7

Basal portion of EC cell showing nucleus (n), electron dense granules (g) and mitochondria (m). Beneath the EC cell are fibroblasts (F) and nerve fibers (nf) of the subepithelial plexus with swellings containing clear vesicles (cv), dense-cored vesicles (dcv) and mitochondria (m). X 26,800.

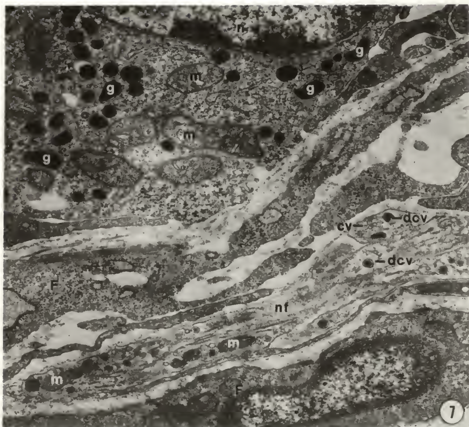

n
g
m
g
g
m
g
cv
dcv
dcv
F
nf
m
m
F
7

PLATE V

Fig. 8

Intestinal A cell (AC) among neighboring enterocytes (EN) in the adult mouse duodenum showing closed nature of rounded apical border (ap) and basal cytoplasmic extension (x) near the basal lamina (bl). Note rounded nucleus (n), round dense granules (g), mitochondria (m) and fragmented rough endoplasmic reticulum (er). Underlying tissue components include nerve fibers (nf) of the subepithelial plexus and collagen fibrils (co). X 26,000.

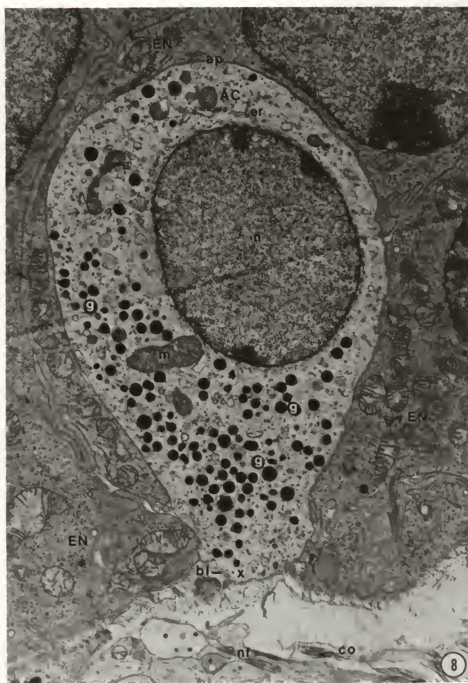

EN
ap
AC
er
n
g
m
g
EN
g
EN
bl
x
nf
co
8

PLATE VI

Fig. 9

A higher magnification of Fig. 7 showing bundle of subepithelial axonal swellings (s) containing clear vesicles (cv) and dense-cored vesicles (dcv). Note also mitochondria (m) and microtubules (mt) contained in the nerve fibers and swellings. X 39,000.

Fig. 10

Post-silver impregnated preparation showing intestinal A cell with highly reactive dense secretory granules (g) and a basal cytoplasmic extension (x) contacting an unidentified cell process (pr) packed with vesicle-like organelles (v). Note basal lamina (bl) separating crypt epithelium from underlying cells and collagen (co). X 35,000.

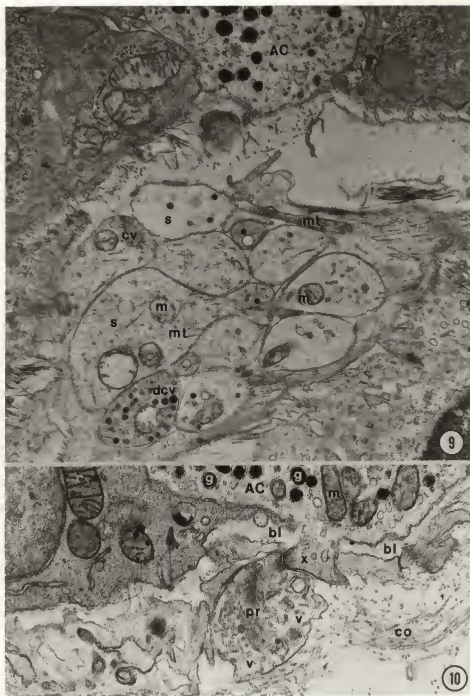

AC
s
mt
cv
m
m
s
mt
dcv
9
g
AC
g
m
bl
bl
x
pr
v
v
co
10

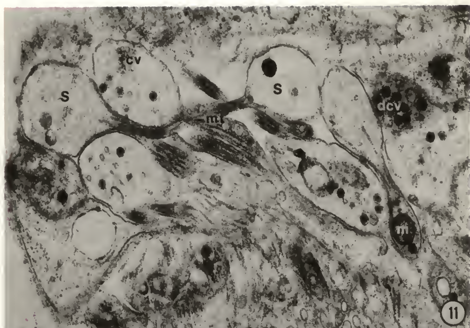
PLATE VII

Fig. 11

Oblique section through subepithelial nerve plexus showing large axonal swellings containing clear vesicles (cv) and dense-cored vesicles (dcv). Two *en passant* swellings (s) of the same nerve fiber are seen in longitudinal plane to be connected by narrow regions with microtubules (mt). X 29,400.

Fig. 12

Cross section through subepithelial nerve fasciculus showing nonmyelinated axons containing clear vesicles (cv), dense-cored vesicles (dcv), microtubules (mt) and mitochondria (m). Note Schwann cell (SC) cytoplasm partially enveloping the bundle of axons. X 43,700.


cv
S
S
mt
dcv
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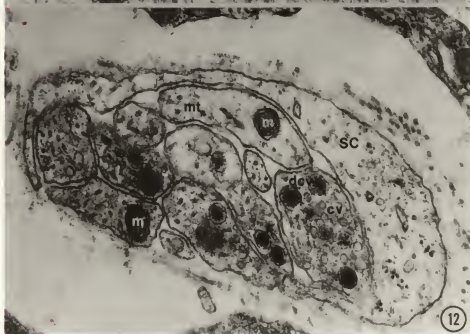
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DISCUSSION

The results of this study add to our knowledge of two basic types of enteroendocrine cells in the small intestine of the adult mouse. The two types of cells are: those which reach the lumen, referred to as "open" cells, and those which do not reach the lumen and are said to be "closed" cells. This study, 1) has confirmed that EC cells are open to the intestinal lumen in the duodenum; 2) it has extended the ultra-structural observations on EC cells by describing junctional complexes seen at the apical poles of these cells; and 3) has shown that: a) in-testinal A cells are closed cells, b) intestinal A cells do not have junctional complexes and c) both open and closed cells have basal cyto-plasmic extensions which penetrate the basal lamina.

A primary objective of this study was to demonstrate, if possible, direct innervation of enteroendocrine cells in the adult mouse intestine or, alternatively, evidence for close enteroendocrine-capillary associa-tion. The approach was by careful serial studies, and in one case a complete three dimensional reconstruction was done. The results showed that direct innervation of EC cells is not a common feature of this cell type in the adult mouse. By contrast, closed cells, in this case the intestinal A cells, were also examined carefully and were shown for the first time not only to be very closely related to nerve fiber swellings of the subepithelial plexus but, in one instance, were in direct contact with an as yet unidentified cellular process. While the vesicular con-tent of this unidentified process is highly suggestive of synaptic ves-icles, further studies will be required to determine if this process is indeed neural.

Direct innervation and/or neurosecretory relationships have been demonstrated in most of the cells currently considered to be paraneurons. Even though enteroendocrine cells have not been demonstrated to be directly innervated they are still classified as paraneurons. Because of the classification of enteroendocrine cells as paraneurons, the results of this study are discussed in relation to the question of whether or not EC and intestinal A cells can be classified as paraneurons.

Can EC and intestinal A cells be classified as paraneurons?

Fujita (1976) defines a paraneuron as a cell which 1) produces substances that are neurotransmitters and protein/polypeptide hormones, 2) packages these products into synaptic vesicle-like and/or neurosecretory-like granules, 3) serves a recepto-secretory function (i.e., releases its secretory products in response to an adequate stimulus) and 4) has a neuroectodermal origin. The two types of gastrointestinal endocrine cells discussed in this work are included in the system of paraneurons described by Fujita. The question which needs to be resolved here is whether or not EC and A cells of the adult mouse gut fit all the criteria for a paraneuron, especially in regard to the relationship between these two cells and the enteric nerve plexus.

Secretory Products. The products manufactured and secreted by enteroendocrine cells include both known neurotransmitters (e.g., 5-HT) and polypeptide substances which have not yet been identified biochemically (e.g., enteroglucagon). More is known about the products of the EC cell than any of the other enteroendocrine cells; the EC cell produces both an amine (5-HT) and a polypeptide (Substance P) (Erspamer and Aspero, 1952; Pearse and Polak, 1975; Heitz et al, 1976). The intestinal A cell

has been shown to be the source of enteroglucagon, a glucagon-like immunoreactive peptide (Polak et al., 1971); the amine moiety of the intestinal A cell has not been determined. These facts support the classification of the EC and intestinal A cells as paraneurons based on their secretory products.

Neurosecretory granules and/or synaptic transmitter vesicles. The several types (ten or more) of enteroendocrine cells all contain secretory granules not unlike those found in endocrine cells elsewhere. Each cell type is supposedly identifiable by the characteristic secretory granules contained in its cytoplasm; however, the need for specific identification of enteroendocrine cells, based on criteria other than granule morphology alone, is evidenced by the confusion in the literature as to the exact number of different enteroendocrine cell types (Forssmann, 1970; Capella et al., 1976). Lack of morphological uniformity in secretory granules of the same cell type has been discussed by others (Lange and Klein, 1976) and is supported by this study which shows intestinal A cells containing spherical granules identical to those in the rat intestine (Forssmann et al., 1969). The granules are reactive to the Grimelius silver method as in other species (Vassallo et al., 1971) but are smaller (175 nm) than those reported in other species (anywhere from 200-700 nm).

Synaptic vesicle-like organelles are found in a majority of the other paraneurons which Fujita lists; however, these organelles have not been reported in enteroendocrine cells by others and were not found in either EC or intestinal A cells in this study. Based on ultrastructural characteristics, such as those reported here, enteroendocrine cells seem to be equipped more for a neurosecretory than a neurotransmitter function.

The presence of neurosecretory-like granules in these cells does, however, fulfill another of the criteria for calling enteroendocrine cells paraneurons.

Recepto-secretory nature. The third criterion for calling enteroendocrine cells paraneurons is that they serve a receptosecretory function. Some confusion has been introduced by Fujita, with respect to the occurrence of open and closed enteroendocrine cells in the mammalian gut. Fujita has stated that closed cells occur only in the oxyntic area of the stomach where they might be able to "recognize thermal and mechanical stimuli" (Fujita, 1976). Whether or not this statement is meant to rule out the possibility of enteroendocrine cells in the intestine which are able to recognize non-chemical stimuli is not clear; it does however ignore previous physiological evidence of mechanosensitive components in the intestinal mucosa and morphological studies of endocrine cells in the gut which do not reach the lumen (Bülbring and Crema, 1958; Orci et al., 1968). This study has shown that there are closed enteroendocrine cells in the small intestine of the mouse and that the intestinal A cell is such a cell, consistant with observations of the intestinal A cell in the rat (Orci et al., 1968).

With respect to the open enteroendocrine cells, Fujita has called them the "taste cells" of the gut and hypothesizes that these cells receive chemical stimuli from the lumen. Recent studies are lending strong support to this hypothesis as it has been shown that adenylate cyclase, an enzyme thought to play some role in chemical stimulus reception, is present on the microvilli of both gustatory cells of taste buds and EC cells (Yamamoto and Ozawa, 1977). The present study has shown ultrastructural evidence that the site for 'sampling' luminal contents is most likely on the microvilli since a junctional complex

completely surrounds the apical pole of open cells, at least in the case of EC cells. These types of junctional complexes have been shown to limit the passage of substance across epithelial linings via intercellular (i.e., along the lateral borders of cells) routes (Farquhar and Palade, 1963).

Although very little firm physiological evidence exists to prove recepto-secretory functioning of enteroendocrine cells, morphological evidence such as discussed here supports the hypothesis that enteroendocrine cells, both open and closed, serve recepto-secretory functions and as such fulfill this criterion of paraneurons. Morphological data are discussed below concerning possible mechanisms by which this function is served.

Neural crest origin. There are two schools of thought concerning the origin of enteroendocrine cells; one proposes a neurogenic origin (Danisch, 1924) while the other proposes that enteroendocrine cells are derivatives of mesenchymal or epithelial cells (Singh, 1964; Pentillä and Lempinen, 1968). Developmental studies give solid evidence for the neuroectodermal origin of enteroendocrine cells (Pearse and Polak, 1971) and are gaining wide support (Osaka and Kobayashi, 1976; Fujita, 1976). The observations in this study of adult animals can neither support nor refute the neuroectodermal origin of enteroendocrine cells; however, the consensus of existing literature supports a neuroectodermal origin of enteroendocrine cells which would meet the developmental criterion of paraneurons.

What are the nerve-enteroendocrine cell relationships?

The four criteria of paraneurons have been discussed in light of the observations of this study. As stated above, paraneurons are, by definition, receptosecretory cells. Since enteroendocrine cells of the small intestine are now classified as paraneurons, morphological evidence for receptosecretory function has been examined in this study. The following discussion presents three types of relationships between enteroendocrine cells and nerve tissue relative to possible functional mechanisms.

Direct innervation. Although virtually all other paraneurons have been shown to be directly innervated (e.g., gustatory, bronchial, carotid body, and pancreatic islet cells), the gastrointestinal endocrine cells have not been shown to be directly innervated (Fujita, 1976); they have been demonstrated to be closely associated with nerve fibers (Solcia, et al., 1970) and are classified as paraneurons as discussed above. Direct contact of basally-granulated cells with nerve terminals in the small intestine of the human fetus (Osaka and Kobayashi, 1976), and direct innervation of the pancreatic A cell (Stach, 1974) led, in this study, to the search for similar direct innervation of both open and closed enteroendocrine cells in the intestine of adult mice. The observations of the present work indicate that EC cells are not directly innervated, but have shown possible evidence (i.e., the direct intercellular contact between an intestinal A cell and the vesicle-filled process of another cell) that intestinal A cells might be directly innervated. Direct innervation of intestinal A cells, if demonstrated by further study to exist, would be consistent with the direct innervation of closely related pancreatic A

cells, where secretomotor innervation is thought to control the release of glucagon (Esterhuizen et al., 1968).

Indirect innervation. Observations of basal cytoplasmic extensions of EC cells in the vicinity of axo-axonal contacts in the subepithelial plexus, as shown in this study, suggests an indirect yet probably functional relationship. Recent evidence shows that iontophoretic application of 5-HT in the vicinity of enteric ganglia has a marked effect on the activity of the enteric nerve fibers (Mayer and Wood, 1978). It is possible that the subepithelial plexus is similarly effected by 5-HT released from EC cells in the adult mouse. If chemical stimuli from the intestinal lumen are shown to cause 5-HT release from EC cells, an indirect afferent innervation could be one mechanism by which this cell communicates with the enteric nerve plexus and serves a recepto-secretory function as postulated (Fujita, 1976; Osaka and Kobayashi, 1976).

Neurosecretion. Most evidence concerning the release of secretory products from the basally-granulated cells in the intestine is limited to demonstration of exocytotic figures which appear very much like those reported originally in the chromaffin cells of the adrenal medulla (the concept is reviewed by Douglas, 1968). This has led to the hypothesis that all enteroendocrine cells release their products in a similar emiocytotic fashion (Fujita et al., 1974); however the fate of products released from enteroendocrine cells is not at all understood at this time. Morphological evidence of fenestrated capillaries near the base of enteroendocrine cells such as those observed in this study suggests that biogenic amines and polypeptide hormones could be released from these cells into the blood-vascular space and the general circulation.

Physiological data supporting the hypothesis that EC cells release 5-HT into intestinal vasculature has been offered along with the suggestion that this 5-HT is picked up by blood platelets and carried throughout the body (Burks and Long, 1966). The functional significance of this theory was questioned after further studies revealed that most of the 5-HT is metabolized in the lung and liver, preventing most of it from reaching the arterial circulation (Page, 1968).

Although neuroendocrinological evidence indicates that most endocrine glands which release kinetic hormones do so under neural control, the effect of neural stimuli on release of enteroendocrine cell products is not known at this time.

CONCLUSIONS

This study has shown that there are two basic types of entero-endocrine cells in the mouse duodenum: open cells, which reach the intestinal lumen, and closed cells, which do not reach the lumen. Ultrastructural evidence suggests that EC cells probably are able to receive luminal stimuli and that intestinal A cells probably receive neural stimuli. Based on this evidence, the enteroendocrine para-neurons of the mouse duodenum may be thought of as either recepto-secretory or neurosecretory cells.

It is obvious that much more investigation will be needed to determine the precise function(s) of each of the more than ten diff-erent kinds of enteroendocrine cells. A logical approach would be to combine electrophysiologic and electron microscopic techniques to correlate activity of the subepithelial nerve plexus with morphological changes in enteroendocrine cells following luminal or neural stimulation.

APPENDIX

Modified Grimelius Silver Impregnation for Enteroendocrine Cell Granules

The method for silver impregnation used in this study has been previously described (Vassalo, et al., 1971); the method for obtaining tissue for this particular histochemical reaction differed slightly from that used for routine electron microscopy. The following procedures immediately precede silver impregnation:

1. Fix the intestinal tissue as indicated in the Materials and Methods, p. 8.
2. Cut the pieces of intestine into 0.5 cm lengths.
3. Embed these 0.5 cm lengths of intestine in a 7% agar solution on a 5 cm diameter filter paper and cool for 0.5 minute at $4^{o}C$.
4. Cut the 0.5 cm lengths into fourths, lengthwise.
5. Place the narrow strips of intestine onto 5 cm diameter filter paper (one per paper) and cover with 7% agar gel; cool again.
6. Place the filter paper onto the plastic stage disc of a Smith and Farquhar Tissue Sectioner (Ivan Sorvall Co.) and cut the strip of intestine into 125 μm thick slices.
7. Transfer the slices of intestine into fresh cacodylate buffer and rinse three times, (5 minutes each) in cacodylate buffer or until the agar is removed from the tissue.
8. At this point follow the remaining steps described by Vassallo, et al. (1971) to complete the silver impregnation.

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THE ULTRASTRUCTURE OF TWO TYPES OF ENTEROENDOCRINE PARANEURONS IN THE MOUSE DUODENUM

by

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ABSTRACT

Two types of basal-granulated enteroendocrine cells in the mouse duodenum were examined with the electron microscope. Analysis of serial thin sections has confirmed that some of these cells reach the intestinal lumen (open cells) and shown that others do not (closed cells). Representative open and closed cell types were chosen and identified specifically as enterochromaffin (EC) cells and intestinal A cells, respectively. EC cells were identified by characteristic polymorphous granules while silver impregnation histochemistry was used to distinguish spherical intestinal A cell granules from spherical granules of other enteroendocrine cells.

EC and intestinal A cells are included in a system of paraneurons, which are receptosecretory cells closely related to neurons and/or neurosecretory cells. The presence of a junctional complex surrounding the apex of EC cells, previously not described, lends ultrastructural evidence suggesting that if the EC cell receives chemical stimuli from luminal contents it does so via its apical microvilli. Multiple basal cytoplasmic extensions of EC cells often pass through the basal lamina and may therefore disperse secretory products over wide interstitial area, possibly allowing for modification of neural activity by release of serotonin and substance P near axo-axonal synapses in the subepithelial plexus which are observed in this study. The intestinal A cell, a closed cell, is shown to lack apical microvilli and junctional complexes but does exhibit a single basal extension through the basal lamina near subepithelial nerve fiber swellings. One example of an intestinal A cell basal extension contacting a vesicle-filled extension of another cell was observed, suggesting a direct neural connectivity;

however, the hypothesis that secretomotor innervation is a mechanism for stimulus and release of intestinal A cell products remains to be proven by further study.