The Fine Structure of the Pancreatic Endocrine Cells of Salamander, *Hinobius nebulosus*

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The pancreatic endocrine cell of adult salamanders, *Hynobius nebulosus*, was studied with light and electron microscopy. The following results were obtained.

1. The endocrine cells were distributed individually or in small groups, islets, and were located either in the acinus or in the duct epithelium.

2. Two types of endocrine cell group, the A cell islet and B cell islet, were observed. Each islet was light-microscopically composed of two to five cells, but electron-microscopically, only two or three cells were in direct contact with each other.

3. These islets were not always composed of the same cell type, but frequently were mingled with other cell types.

4. A and B cells were easily distinguished on the basis of the morphology of secretory granules.

5. In the B cells, the secretory granule core did not show a typical crystalline pattern. Crystalloid structures originated from mitochondria were occasionally encountered and intracytoplasmic filaments were most prominent.

6. D cells were identified by the correlative observation of thin sections with an adjacent semithin section stained with silver impregnation by HELLMAN and HELLERSTRÖM. The D cell secretory granules were electron low dense to moderately dense and showed varying numbers of elongate profiles besides round ones.

7. F-like cells contain electron dense, elliptic secretory granules except for spherical ones.

8. Another type of cells was distinguished. They had a scanty cytoplasm which contained extremely dense, secretory granules with different shapes.

9. The average long and short diameters of secretory granules of each cell type were measured using Reiz-ASM image analyzers.
INTRODUCTION

Investigations on the ultrastructure of the pancreatic islet cell have been made in a variety of animal species. Especially, until 1971 amphibian islet cells were widely studied in Ambystomatoidea (SATO et al., 1966; GROSSNER, 1967) and Salamandroidea (SATO et al., 1966; HERMAN & SATO, 1970) of urodela as well as in various species of anura (KOBAYASHI, 1966, 1967; LANGE, 1967, 1968; WELSCH & STORCH, 1971), but thereafter no further studies appear. Unfortunately, in these early studies the identification of islet cell types is not always agreeable among each author and several discrepancies remain unsettled.

The present work concerns the fine structure of pancreatic endocrine cells in the salamander, Hynobius nebulosus, belonging to Cryptobranchoidea whose islet cells have not yet been elucidated, and the results are compared with the previous reports on amphibian endocrine pancreas.

MATERIALS AND METHODS

Adult male salamanders, Hynobius nebulosus, ranging from 10 to 12.5 cm in total length, were collected from mountain-stream of Nagasaki City in their breeding season from late February to early March and kept in an aquarium at natural temperature (10–17°C) for two weeks before sacrifice. During this period they took no food. The pancreatic tissue was removed under anesthesia and fixed in a 3 % cold glutaraldehyde in phosphate buffer adjusted to pH 7.4 for 2 hrs. The tissue was then washed overnight in buffer containing sucrose and postfixed in 1 % osmium tetroxide in Millonig buffer for 1.5 hrs. Following dehydration in graded concentrations of ethanol, the tissue was embedded in Epon 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under the JEM-T8 electron microscope. Using Leitz-ASM image analyzers, the long and short diameters of secretory granules in each cell type were measured on the electron micrographs at a final magnification of 17,000.

For light microscopic observation, a part of the tissue was fixed in Bouin or Levi fluid and processed for paraffin section. Sections were stained with GOMORI’s aldehyde fuchsin–trichrome dyes (1950) as well as with silver impregnation (HELLMAN and HELLMAN, 1960).

In addition, for a comparison of the electron and light microscopic images, thin sections and their adjacent semithin sections (1μ) were cut from the same Epon block on the ultramicrotome. After the resin was removed with saturated solution of sodium hydroxide in absolute ethanol, the semithin sections were impregnated with silver nitrate solution of Hellman Hellerström (LÓPEZ et al., 1983) for the demonstration of D cell.
RESULTS

Light microscopic observations show that the pancreatic islet cells of adult *Hynobius nebulosus* differ from those of mammals and birds in that they are distributed singly or in small clusters of two to six cells within the basal portion of the exocrine pancreatic acinus as well as among the basal epithelial cells of the small secretory duct. In aldehyde fuchsin–trichrome staining preparations, according to the staining properties of secretory granules, two types of endocrine cell or cell group, A cells or A cell islets and B cells or B cell islets, are easily distinguished: the A cells are mainly cylindrical or fusiform and stained red with orange G; the B cells are usually rounded or fusiform and tinctured violet with aldehyde fuchsin (Fig. 1). In the acinus or duct epithelium, these both types of cells or cell islets usually lie along the capillary. On the other hand, D cells are stained green with trichrome or blackend with silver impregnation, and show round cylindrical, or fusiform outlines, being sparcely distributed in some acini or duct epithelia (Fig. 2).

![Fig. 1. A part of the pancreas tissue of a salamander, *Hynobius nebulosus*. A cells (A) are stained red, B cells (B) violet, and D cells (D) green. Fixation in Bouin, aldehyde fuchsin–trichrome by GOMORI. × 560.](image1)

![Fig. 2. D cells (arrows) in the pancreas tissue of a salamander, *Hynobius nebulosus*. Fixation in Bouin, silver impregnation by HELLMAN and HELLERSTRÖM. × 120.](image2)
ratio of A, B and D cell number per tissue section was about 6:6:1.

Electron microscopically, part of the cytoplasm of each endocrine cell, irrespective of its location in the duct epithelium or in the acinus, usually rests on the basal lamina facing the interlobular or perivascular connecting tissue (Figs. 3,4). In each islet, however, only two or three cells are in direct contact with each other, though the length of contact is variable, and other cells usually seem to be separated from the cell cluster by a cytoplasmic process of the adjacent acinar or duct epithelial cells (Figs. 3,4). These islets are not always composed of the same cell type, but frequently mingled with other cell types (Fig. 3). In addition, each islet cell and its surrounding acinar or duct epithelial cells are in places connected by desmosomes, whose smaller types are also found among the islet cells: their adjacent plasma membranes are either closely interdigitated, or run almost straightly, or show dilated intercellular spaces containing microvillous projections.

On the basis of the ultrastructural morphology of the pancreatic islet cells reported up to date in different animal species, we could identify a few D and F-like cells as well as abundant A and B cells. The present authors would describe at first the fine structure of secretory granules characteristic for each cell type and then other features in common to each type such as cell organelles and inclusions. Additionally, in each cell type the average long and short diameters of secretory granules and the average ratio of the short diameter to the long diameter of secretory granules were summerized in table 1.

The A cells are also located singly in the cell islets as well as among the acinar or duct epithelial cells, besides the A cell islets (Fig 3). Secretory granules of the A cells, α granules, are round or oval bodies, ranging from 170 to 200 nm in long diameter and from 140 to 160 nm in short diameter. They are membrane-bounded and usually have a narrow clear space, a halo, between the limiting membrane and the homogeneous content (Fig. 5). The content of most A granules is electron dense, but that of a few granules show a moderate density. In most A cells, the granules are closely distributed throughout the cytoplasm.

The B cells are either concentrated into the B cell islets (Fig. 4), or situated

<table>
<thead>
<tr>
<th>Cell type</th>
<th>long diameter (μm)</th>
<th>short diameter (μm)</th>
<th>short diameter to long diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.1891±0.0055</td>
<td>0.1559±0.0075</td>
<td>0.8249±0.0105</td>
</tr>
<tr>
<td>B</td>
<td>0.2621±0.0357</td>
<td>0.2098±0.0290</td>
<td>0.8000±0.0129</td>
</tr>
<tr>
<td>D</td>
<td>0.2441±0.0360</td>
<td>0.1752±0.0398</td>
<td>0.7135±0.0622</td>
</tr>
<tr>
<td>(F)</td>
<td>0.1808±0.0222</td>
<td>0.1292±0.0187</td>
<td>0.7134±0.0322</td>
</tr>
</tbody>
</table>
Fig. 3. The A cell islet in the secretory duct epithelium. A: A cell, B: B cell, L: duct lumen × 4,500.

Fig. 4. The B cell islet in the exocrine acinus. B: B cell, F: F-like cell, C: capillary lumen. × 4,500.
individually within the A cell islets (Fig. 3) as well as among the acinar or duct epithelial cells. Secretory granules unique for the B cells, β granules, are membrane-limited, round or ovoid, larger bodies measuring 200 to 350 nm in long diameter and 160 to 280 nm in short diameter; they have a clear space of different width between the limiting membrane and the content (Fig. 7). The content consists of an electron dense material, and its shape is variable: some granules have a round dense core as do the α granules; other have a polygonal or irregular-shaped core; still others have fragments of

![Fig. 5. A part of the A cell cytoplasm. G: Golgi apparatus × 20,000.](image)

![Fig. 6. A part of the D cell cytoplasm. L: lysosomal body, M: mitochondria, N: nucleus, × 20,000.](image)
Fig. 7. A part of the B cell cytoplasm. Note various shapes of secretory granule cores and exocytotic invagination (arrow) of the plasma membrane. C: capillary lumen, L: lipid droplet. × 20,000. Inset shows a secretory granule concentrically surrounded by filaments. × 20,000.

Fig. 8. Crystalloid structure bounded by double membranes near the nucleus (N). × 20,000. Inset shows an immature crystalloid structure developing in the mitochondrial matrix. × 20,000.
crystallloid platelet (Fig. 7). In the latter two cases, the granules are larger and have a wide space beneath the limiting membrane. The frequency of such different types of granule cores differ from cell to cell, but their electron density usually is as follows: a round core > a polygonal or irregular-shaped core > fragments of platelet. In general, the distribution density of secretory granules in the B cell seem to be lower than that in the A cell (Fig. 3). The invaginated plasma membrane suggesting the exocytosis of secretory granules was sometimes encountered (Fig. 7).

Correlative observation of thin sections with semithin sections stained with silver impregnation reveals the following characteristics of D cells: 1) the silver impregnation method by HELLMAN & HELLERSTROM applied to the present Epon sections blackens not only the cells identified as D cells but also the A cells; 2) the D cells are singly located among duct-epithelial cells as well as among excretory acinar cells; 3) secretory granules of the D cells, \( \delta \) granules, show spherical, oval, or ellipsoidal profiles with moderately dense to low dense content and measure 170 to 340 nm in long diameter and 120 to 250 nm in short diameter; 4) the granules have a closely applied, limiting membrane or a narrow clear space between the limiting membrane and the granule content; 5) in most D cells, secretory granules are densely distributed in the entire cytoplasm. Some cells were closely packed with low dense granules, most of which exhibited oval or elliptic outlines (Fig. 9). However, such cells were also impregnated in dark brown with HELLMAN & HELLERSTROM method (Fig. 9 inset).

The cells which are tentatively termed here 'F-like' cells are individually seen among acinar cells and within the duct epithelium. The secretory granules of this type cell resemble \( \alpha \) granules in size and electron density (Fig. 10). However, they differ from the latter in that they show elliptic or trigonal profiles besides round or oval ones and give an impression of angular form. They usually are closely packed in the cytoplasm. The granules have long diameters of 140 to 210 nm and short diameters of 100 to 150 nm, but seem to have a large variety of size among each cell. They have a distinct clear halo between the limiting membrane and the dense core.

The pancreatic endocrine cells of each type mentioned above show light-microscopically a variety of profiles such as round, oval, elliptic, columnar, or fusiform outlines, but electron-microscopically some A, D and F-like cells located within the acini are elongated and sometimes extend a long tapering process filled with secretory granules between the acinar cells, while most of the B cells, irrespective of their location, show a round or oval shape. The nuclei are round or ovoid and usually have slight incisions; in addition, the nuclei of some B and D cells are deeply indented and indicate an irregular outline.

In the cytoplasm, the Golgi apparatus is located usually in the juxtanuclear area and occasionally in the peripheral cytoplasm. It consists of three to six layers of flattened cisternae and associated vacuoles and vesicles; some of the dilated cisternae or vacuoles frequently contain a dense core presumably considered to be a prosecretory granule (Fig. 11A). In the B cells, the Golgi cisternae are possessed of a GERL-Like dark lamella
Fig. 9. A part of the D cell cytoplasm, in which most of the secretory granules show oval or elliptic profiles with a low to moderate, electron density. This electron micrograph demonstrates the fine structure within the rectangle (arrow) of Inset. N: nucleus. × 20,000. Inset shows a semithin section stained with silver impregnation method by Hellman and Hellerstrom. A cells (A) as well as a D cell (D) are stained dark brown. C: capillary lumen. × 560.

Fig. 10. A part of the cytoplasm of the cell type tentatively termed 'F-like'. G: Golgi apparatus. × 20,000.
Fig. 11. The golgi area of each cell type (A, B, D and F-like cell). Arrow indicates a GERL-like structure. \( \times 20,000 \).

Fig. 12. A part of the cytoplasm of another type cell. Note extremely dense secretory granules of variable shapes. M: mitochondria, N: nucleus. \( \times 20,000 \).
coated with bristles at their trans side (Fig. 11B). The mitochondria are small and show round, oval, or rod-like profiles, having mostly transversely- and occasionally longitudinally-placed lamellar cristae throughout almost all the cell types. They are sparcely scattered in all parts of the cytoplasm.

Cisternae of the granular endoplasmic reticulum and free ribosomes, singly or in polysomal clusters, are dispersed among secretory granules in the cytoplasm. In general, they are densely distributed when secretory granules are loosely dispersed, and vice versa. Small aggregates of glycogen particles are also noticed intermingled with the free ribosomes, especially they are prominent in the B cells. In some of the A and F-like cells, concentric lamellar array of the granular cisternae can be seen in a restricted area near the nucleus. In the A, B and F-like cells, bundles of filaments are sparsely distributed in the cytoplasm, in particular in the periphery and around the nucleus: in the B cells, these filaments are best developed and in some areas around the nucleus they enclose each of B granules concentrically (Fig. 7 inset). Lysosomal dense bodies and lipid droplets are usually detected among secretory granules in all the cell types. Especially in the B cells, the former often contain parallel tubular structures and/or small lipid-like dense droplets in the homogeneous, moderately dense matrix. Relatively large lipid droplets also occur in the B cells (Fig. 4).

Rod-like, dense crystalloids, about 0.5 μ in width and to 3.5μ in length, are occasionally encountered in the B cell cytoplasm (Fig. 8). At higher magnification, it is noticed that the crystalloids are composed of parallel straight dense lines embedded into a moderately dense matrix and surrounded by loose fitting, double limiting membranes. In some immature crystalloids, the double membranes are continuous with the inner and outer mitochondrial limiting membranes (Fig. 8 inset).

Sometimes, another unusual type cells were encountered among the acinar cells (Fig. 12). They are rounded cells and characterized by containing a few number of smaller, extremely dense homogeneous granules (100–180 nm) with round, oval, elliptic, or crescent profiles. The mitochondria are frequently swollen and the granular endoplasmic reticulum is moderately developed. A few intracytoplasmic filaments are also present. The nucleus is irregular in shape and has deep indentations. In addition, the cells of this type have a scanty cytoplasm and show a high nucleo-cytoplasmic ratio to other cell types: their outlines are rather irregular and display short cytoplasmic processes.

DISCUSSION

The previous cytology of urodèle pancreatic islets has shown that in Ambystoma tigrinum and Diemictylus viridescens the islet consists of two to ten cells (SATO et al., 1966), while in Amphiuma tridactylum it varies from a few cells to groups containing over 100 cells (HERMAN & SATO, 1970). In the present observation on the pancreas of Hy- nobius nebulosus, the endocrine cell groups were composed of only two to five cells in section, and these small groups occurred not only in the exocrine acinus but also in the
small duct epithelium. Such differences of organization of pancreatic endocrine cells into islets may reflect the species difference between urodela, and it is suggested that the islet of *Hynobius nebulosus* may be at a more primitive stage of islet evolution than that of other urodela. In addition, the presence of endocrine cell groups within the pancreatic small duct epithelium has not yet been reported in the urodela mentioned above, but light-microscopically in toad (KOBAYASHI, 1966, 1969), axolotl and salamander (EPPEL, 1966), as well as electron-microscopically in *Ichtyophis kohtaoensis* (WELSCH & STORCH, 1971).

It is generally accepted in mammalian pancreatic islets that A cells, glucagon-producing cells, contain $\alpha$ granules, which are membrane-bounded, dense, spherical bodies with a narrow clear halo, and that such $\alpha$ granules are common in shape and electron density to all species. In *Hynobius nebulosus*, however, most of the $\alpha$ granules appear to be of two types, dense granules and moderately dense granules, though a few intermediate granules are also present. Such a variability in the electron density of $\alpha$ granules has been also described in *Ambystoma tigrinum* and *Diemictylus viridescens* (SATO et al., 1966), and it may represent maturation stages of secretory granules: probably, $\alpha$ granules may be decreased in density with maturation, since immature secretory granules in the Golgi area have a dense content enclosed by a loosely fitted limiting membrane (Fig. 11A).

$\beta$ granules, secretory granules of insulin-producing cells, have been reported to exhibit fine structures characteristic of each animal species (SATO et al., 1966; FUJI-TA, 1968). In *Hynobius nebulosus*, their content shows prominent variations in shape, such as a round core, a polygonal or irregular-shaped core, or fragments of platelet. Of these granule contents, the round core is constantly observed and the fragments of platelet do not indicate a typical crystalline pattern consisting of alternating dark and light parallel straight lines as shown in some urodela (SATO et al., 1966; GROSSNER, 1967). Therefore, the $\beta$ granules of *Hynobius nebulosus* seem to resemble in appearance those of *Diemictylus viridescens*, most of which have spherical to ellipsoidal cores, rather than those of *Ambystoma tigrinum*, *Amphiuma means* (SATO et al., 1966) as well as axolotl (GROSSNER, 1967), which exhibit plate-like, distinct crystals.

Such morphological variations of the $\beta$ granule core among urodele species may reflect differences in molecular structure of either insulin or its associated binding protein, as suggested by LACY (1957) in mammalian islet cells.

Recent morphological studies have suggested that pleomorphic cores of the $\beta$ granules in the same cell may indicate a series of maturation stage of secretory granules (WATANABE et al., 1975). In the present study also, immature granules in the Golgi area had a dense spherical content similar to the round core in the $\beta$ granule (Fig. 11B). Accordingly, it was conceivable that in *Hynobius nebulosus* the content of the $\beta$ granules may probably alter from a round core via a polygonal or irregular-shaped core ultimately into fragments of platelet.

In the B cells of *Hynobius nebulosus*, rod-like crystalloids consisting of parallel straight dense lines, occasionally appeared in the cytoplasm: they were enclosed by lo-
osely applied, double limiting membranes. Similar crystalloid bodies have been reported in the B cell of the toad islet and supposed to be derived from mitochondria with close-
ly packed longitudinal cristae (KOBAYASHI, 1966). The present authors suggest that the crystalloids in the B cells of Hynobius nebulosus may originate from the mitochondrial matrix, since the double membranes surrounding some crystalloids were continous with the inner and outer mitochondrial limiting membranes. These crystalloids might be as-
sociated with a substance accumulated into the mitochondrial matrix during cellular me-
tabolism.

D cells have been light-microscopically demonstrated to correspond to argyrophil
cells (EPPLÉ, 1964; FUJITA, 1964) as well as metachromatic cells (SOLCIA & SAMPIE-
tro, 1965. FUJITA, 1968). Electron-microscopically, they are characterized by having round secretory granules with low density (FUJITA, 1968). In the present study, the D cell granules of Hynobius nebulosus showed oval or elliptic profiles except for round ones. Especially in some cells, elongate granules occupied the majority of secretory gran-
ules.

In other amphibian pancreas, the granules of the D cells have been indentified as spherical bodies in Ambystoma tigrinum, Diemictylius viridescens, and Amphiuma means (SATO et al., 1966) as well as in axolotol (GROSSNER, 1967), whereas in Ichtyophis kohlaeensis (Gymnophona) they are described as elongated form (WELSCH and STORCH, 1972). In addition, type IV cells classified by KOBAYASHI (1966, 1969) in toad and Xenopus laevis contain many less dense, elongate secretory granules and seem to resemble the D cells observed in the present study. However, it is unclear at the present time why the form of D cell granules is different between amphibian species. Recent immunohistochemical studies have demonstrated that D cells may be somatostatin-produc-
ing cells (PELLETIER et al., 1975; ORCI et al., 1975).

In the present study the endocrine cells which contain many dense ellipsoidal or trigonal granules besides round or oval ones were tentatively described as F-like cells. Similar islet cells were first found in the uncinate process of dog pancreas and termed 'F cells' by MUNGER et al. (1965). Later F cells have also been reported in mice (DEHOYOS-GUEVARA, 1969), Guinea pigs and bats (WATARI, 1973). The function of F cells is not known, though their secretory granules were pointed out to resemble in shape those of some enterochromaffin cells distributed in the intestinal mucosa (WATARI, 1973).

Recently, pancreatic polypeptide cells (PP-cells) have been electron-microscopically demonstrated in the teleost fish (Xiphophorus helleri, H.) with correlative immunohisto-
chemical and electron microscopical studies and it has been confirmed that the secretory granules of the PP-cells show a striking analogy in morphology with those of the glucagon-
producing A cells in this teleost (KLEIN and VAN NOORDEN, 1980). In the present study the F-like cells seem somewhat to resemble in shape, size, and electron density of granules the A cells except for that they contain numerous ellipsoidal granules besides round ones. Therefore, we can not completely eliminate the possibility that the F-like
cells in *Hynobius nebulosus* might correspond to the PP-cells.

In the hynobius pancreas, like some mammals (HERMAN et al., 1963; LEDUC & JONES, 1968) and birds (BJÖRKMAN & HELLMAN, 1964; MIKAMI & MUTHO, 1971) as well as anura (KOBAYASHI, 1966, 1969), so-called acinar–islet cells which have cell organelles as well as secretory granules, characteristic of both acinar and endocrine cells, were occasionally encountered within the acini. Such cells frequently contained fragments of apposed two plasma membranes in their cytoplasm. Therefore, we agree to the opinion of KOBAYASHI (1966, 1969) that acinar–islet cells may probably be caused by fusion of adjacent exocrine and endocrine cells.

Recently, the relation between endocrine cells and nervous elements has attracted a histologist’s attention in connection with APUD (Amine Precursor Uptake and Decarboxylation) theory (PEARSE, 1969) or paraneuron concept (FUJITA & KOBAYASHI, 1973). In the pancreatic islet cells their close contact with nerve fibers (axones) or Schwann cells has been reported in mammals (BENCOSME, 1959; WINBORN, 1963; LEGG, 1967; WATARI, 1968; KOBAYASHI & FUJITA, 1969) and fishes (WATARI, 1972; KUDO & TAKAHASHI, 1973). In *Hynobius nebulosus*, however, we could not confirm such an intimate relationship between endocrine cells and nervous elements. Additionally, all the endocrine cells observed did not stretch their apex to the lumen of the acinus or duct.

ACKNOWLEDGEMENTS

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