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An Ultrastructural Study on the Endocrine Pancreas of *Caiman latirostris*, with Special Reference to Pancreatic Motilin Cells*

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Summary. **Pancreatic endocrine cells of *Caiman latirostris* were investigated by electron microscopy using conventional and immunocytochemical methods. Ultrastructurally, four types of endocrine cells were classified according to the morphology of their secretory granules. Three types of endocrine cells were identified as either glucagon, insulin or somatostatin cells by the presence of such characteristic granules well established in mammals. The remaining endocrine cell type could not be classified by its ultrastructural features alone.**

Immunocytochemical observations confirmed the ultrastructural classification of glucagon, insulin and somatostatin cells. In addition, endocrine cells immunoreactive for either pancreatic polypeptide (PP) or motilin were identified. Morphometric analysis of PP- and motilin-immunoreactive granules demonstrated that they were the most polymorphous and smallest granules among the pancreatic endocrine cell granules. Although both PP and motilin granules closely resemble each other, motilin granules were smaller in size and more spherical in shape than PP granules.

Crocodiles are regarded as belonging to that order of reptiles most closely related evolutionarily to the stock from which birds were derived. In view of this unique phylogenetic position, crocodiles are of considerable interest with regard to comparative endocrinology. Despite this, there are only several studies on pancreatic endocrine cells in crocodiles. MILLER (1962) described the structure of pancreatic islets in *Alligator mississippiensis* and GABE (1969), in *Crocodylus niloticus* and *Caiman crocodilus*. MILLER and LAGIOS (1970) and GRILLO et al. (1977) reported the ultrastructural features of islet tissue in *Alligator mississippiensis* and *Osteolaemus tetraspis*, respectively. TITLBACH (1981) demonstrated both the light and electron microscopic features of crocodile islets in *Alligator mississippiensis* and *Caiman niger*.

Four major pancreatic hormone producing cells (glucagon, insulin, somatostatin and pancreatic polypeptide), have been identified immunocytochemically in the endocrine pancreas of the American alligator *Alligator mississippiensis*, (BUCHAN et al., 1982) and in the Nile crocodile, *Crocodilus niloticus*, (RHOTEN, 1987). In addition to the four major pancreatic endocrine cells, motilin-immunoreactive cells have been identified at the light microscopic level in the pancreas of the caiman, *Caiman latirostris* (YAMADA et al., 1986) and echidna, *Tachyglossus aculeatus* (YAMADA et al., 1990). Since motilin-immunoreactivity was not co-localized in any of the other four endocrine cell types, motilin-immunoreactive cells were reported as a new cell type independent of established pancreatic endocrine cell types.

In the present study, the caiman pancreas was examined at the ultrastructural level using conventional, immunocytochemical and morphometric methods. The purpose of this study was to establish ultrastructural criteria for glucagon-, insulin-, somatostatin-, pancreatic polypeptide- and motilin-immunoreactive cells in the caiman pancreas.

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MATERIALS AND METHODS

A young female *Caiman latirostris*, weighing 850 g in body weight and measuring 54 cm in total body length, was captured in August near Botucatu, Brazil. After decapitation, a laparotomy was performed and the pancreas was removed, cut into small pieces and separated into three groups prior to being placed in a suitable fixative. Tissues in the first group were prefixed with 3.0% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, for 2 h at 4℃ and then postfixed with 1.0% OsO_4 in the same buffer for 1 h at room temperature. The second group was prefixed with Karnovsky's fixative (KARNOVSKY, 1965) for 2 h at 4℃ and then postfixed with 1.0% OsO_4 for 1 h. The third group was fixed with Karnovsky's fixative without postfixation in OsO_4. After fixation, specimens were processed routinely and embedded in araldite. For ultrastructural evaluation, thin sections of the first group were stained routinely with uranyl acetate and lead citrate prior to examination in a Hitachi H-600 Electron Microscope.

For immunocytochemical localization of peptides at the ultrastructural level, the gold-labelled avidin-biotin method (COGGI et al., 1986) was applied to the specimens fixed with Karnovsky's fixative and OsO_4 or Karnovsky's fixative only. Before incubation with the specific antiserum, nickel grids supporting thin sections were pretreated with 3.0% H_2O_2 for 10 min at room temperature and followed by incubation with non-immunized goat serum for 1 h at room temperature. The grids were incubated with the primary antisera overnight at 4℃. The primary antisera used in this study were anti-porcine glucagon (donated by N. YANAIHARA, Shizuoka, GL-5, 1:1,000), anti-beef-pork insulin (Immuno Nuclear Corp., Stillwater, Lot. 47291, 1:200), anti-human somatostatin (donated by S. ITO, Niigata, 1:500), anti-avian pancreatic polypeptide (donated by J. R. KIMMEL, Kansas City, Lance-10/5/81 Bleed, 1:1,000) and anti-porcine motilin (donated by N. YANAIHARA, R-1104, 1:500). These were incubated with biotinilated second antisera (anti-guinea pig IgG, Vector Lab. Inc., Burlingame, 40605, 1:100 or anti-rabbit IgG, Vector Lab. Inc., 70209, 1:200) followed by incubation in colloidal gold (15 nm in diameter)-labelled streptavidin (Amersham International plc., Buckinghamshire, RPN. 1215) for 1 h at room temperature. Following incubation, the grids were thoroughly washed in phosphate buffered saline to remove unbound antibodies and gold particles. The immunostained grids were then counterstained with uranyl acetate and lead citrate prior to ultrastructural examination.

Photomicrographs of each cell type including those stained immunocytochemically were taken and printed at a final magnification of 16,000×. Major and minor axes of endocrine granules were measured using a computerized image analyzer (Spicca, Nippon Avionics, Tokyo). The mean granule diameter (d) and standard deviation (S.D.) of the granules were calculated. The corrected diameters (D) based on the formula, $D=4/\pi\times d$ (BAETENS et al., 1976) were computed to allow for sectioning artifacts. The degree of circularity (DC), an index of the shape of granules, was estimated from the perimeter (PM) and the area (A) of granules by the formula, $DC=4\pi\times(A\text{-}PM/2)/0.9PM^2$. The more the granular shape approaches a perfect circle, the more DC of the granule approaches 1.0. The data values obtained for each cell type were analyzed by the Welch test (WELCH, 1956) for significant differences.

RESULTS

Ultrastructure

Although endocrine cells were found in small groups, true islets of a mammalian type characterized by a clear separation from the exocrine pancreas by a distinct capsule of connective tissue were not present in the caiman. Instead, groups of endocrine cells were found scattered within the exocrine portion in relation to blood vessels. Each cell type contained a nucleus, profiles of rough and smooth endoplasmic reticulum, scattered thin, cylindrical mitochondria, Golgi complexes and various cytoplasmic granules. In specimens fixed with glutaraldehyde and OsO_4, four types of endocrine cell were identified based upon the ultrastructural appearance of the secretory (endocrine) granules. Morphometric data concerning the secretory granules for each cell type are shown in Table 1.

Type I cell: Granules of this type were generally round in profile and were larger than those of any other cell type observed (Fig. 1a-c). They often had an inner, highly electron-dense core and an outer zone

Fig. 1. The micrographs of the left column (×6,000) show the whole cell of each type, and those of the right column (×30,000) show ultrastructures of endocrine granules in each cell type. **a-c:** Type I, **d** and **e:** Type II, **f** and **g:** Type III, **h** and **i:** Type IV cells.

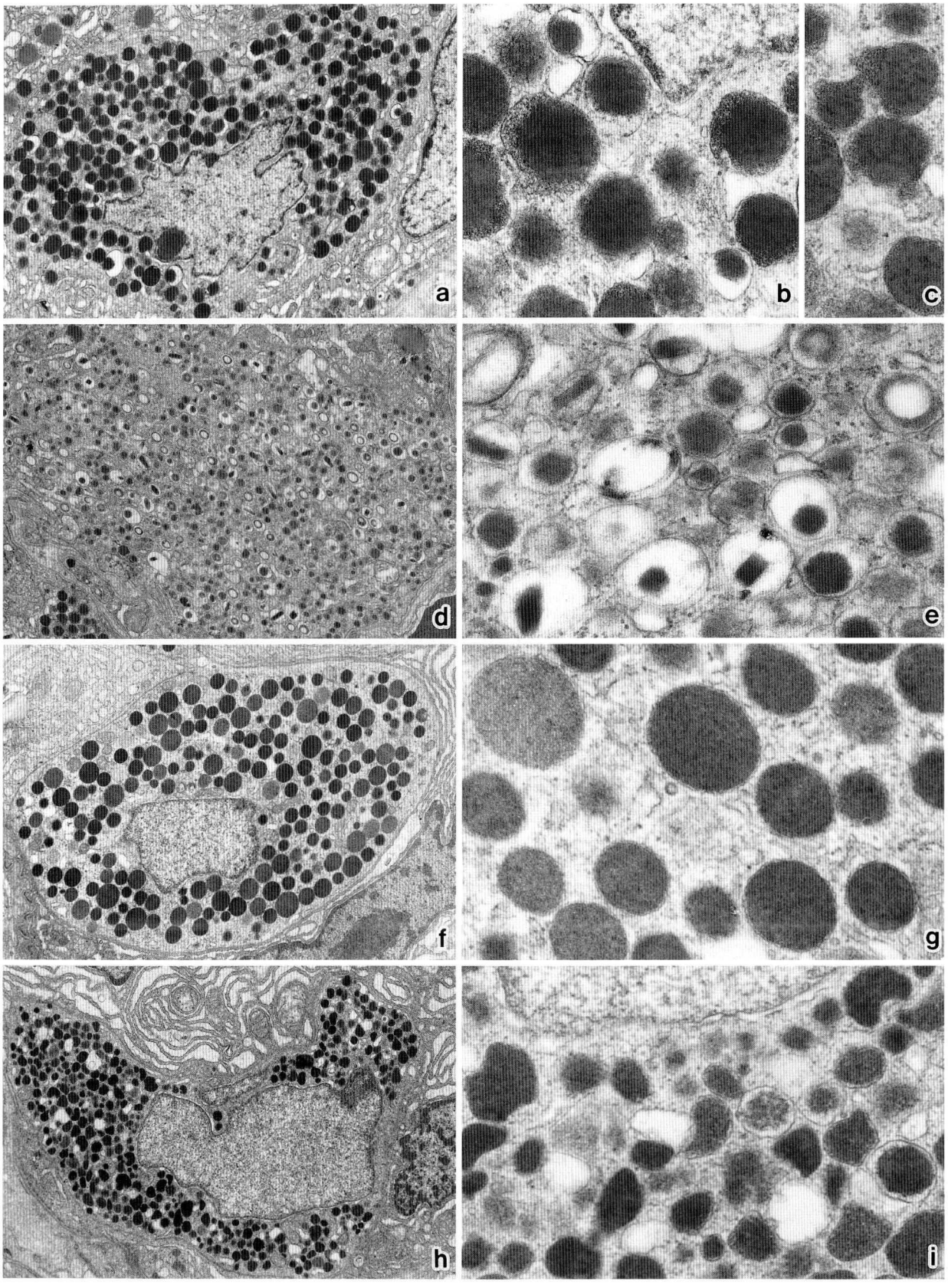


Fig. 1. Legend on the opposite page.

consisting of moderately electron-dense material. A narrow electron-lucent rim separated the core and the limiting membrane of some granules; in some cases, this electron-lucent rim was of considerable width. In addition to these, a protuberance or extension of secretory granule matrix together with the limiting membrane was observed at one pole of some granules (Fig. 1c). The mean diameter of the major axis and the degree of circularity (DC) of 1,558 granules from 15 cells was 448±103 nm (corrected diameter, D=570 nm) and 0.92±0.04, respectively.

Type II cell: This cell type was characterized by the presence of round or ovoid granules with a moderately electron-dense, often irregularly shaped crystalline core. The contents of granules were separated from the limiting membrane by a distinct, and at times rather wide, electron-lucent zone (Fig. 1d, e). The core was extremely variabe in size, shape and density, even in granules of the same cell. Most cores were rod-, rhomboid- or ring-shaped. The mean diameter of the major axis and DC of 1,456 granules from 17 cells was 368±74 nm (D=469 nm) and 0.91±0.05, respectively.

Table 1. Diameter and degree of circularity (DC) of endocrine granules of each cell type in the pancreas of *Caiman latirostris*.

Cell type	Axis granule diameters[1] (nm)		DC[1]	N[2]
	Major axis	Minor axis		
Type I[a]	448±103	383±85	0.92±0.04	1,558
Glucagon[b]	355±83	300±68	0.93±0.05	433
Type II[a]	363±74	290±54	0.91±0.05	1,456
Insulin[b]	359±82	276±55	0.92±0.04	324
Type III[a]	401±89	348±77	0.92±0.05	1,457
Somatostatin[b]	388±80	324±69	0.95±0.02	368
Type IV[a]	311±87	238±64	0.85±0.08	2,467
APP[b]	279±74	183±46	0.86±0.08	726
APP[c]	299±63	226±52	0.87±0.06	421
Motilin[c]	218±53	173±42	0.90±0.06	686

[1]mean±S.D., [2]N=number of granules measured, [a]Samples fixed with glutaraldehyde and OsO_4, [b]Samples fixed with Karnovsky's fixative and OsO_4, [c]Samples fixed with Karnovsky's fixative only.

Type III cell: Although granules of this type were similar in shape to those of Type I cells, their contents were homogeneous and the limiting membrane was tightly bound to their exterior (Fig. 1f, g). The mean diameter of the major axis and DC of 1,457 granules from 19 cells was 401±89 nm (D=511 nm) and 0.92±0.05, respectively.

Type IV cell: The Type IV cell was characterized by the presence of small, irregularly shaped granules (Fig. 1h, i) (Table 1). The granules appeared either round, oval, ellipsoidal (often comma-like), or kidney-shaped. Most exhibited a homogeneous, highly electron-dense material limited by a membrane. A halo between the contents of the granule and the limiting membrane was a rare observation. The mean diameter of the major axis and DC of 2,467 granules from 31 cells was 311±87 nm (D=396 nm) and 0.85 ± 0.08, respectively.

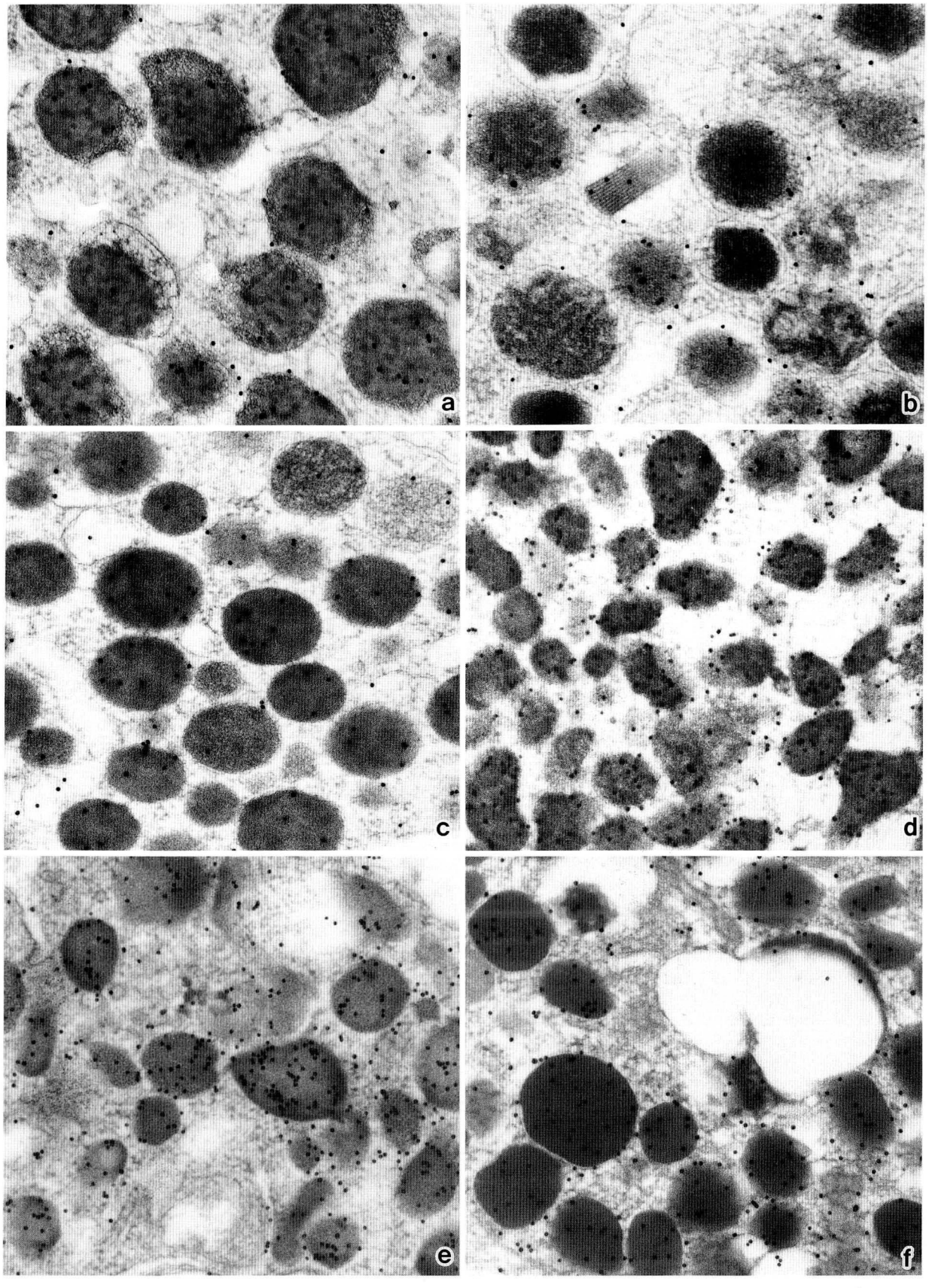
Statistically, the mean diameters of the major axes of the granules from each cell type were significantly different ($p<0.01$) each other. The Type I cell granules were the largest of all granules, while the Type IV cell granules were the smallest of all granules examined in this study. The DC of the granules of the four cell types showed significantly differences ($p<0.01$) from each other except for between those of the Type I and Type III cells. In particular, the DC of Type IV cell granules was significantly smaller ($p<0.001$) than those of the other cell types, suggesting that the Type IV granules are most irregular in shape among the granule types examined in this study.

Immunocytochemistry

Five types of endocrine cells immunoreactive for either glucagon, insulin, somatostatin, avian pancreatic polypeptide (APP) or motilin at the ultrastructural level were identified. Gold particles labelled for each antiserum were localized exclusively on the endocrine granules of the corresponding cell type. Morphometric data concerning the gold-labelled granules are shown in Table 1.

Glucagon-immunoreactive cells were characterized by spherical endocrine granules having a dense core. Some of these granules exhibited a protuberance or extension of the granular matrix and limiting membrane at one location on the granule (Fig. 2a). Granules of glucagon-immunoreactive cells were very similar in morphology to those described for Type I

Fig. 2. Electron micrographs illustrating the ultrastructure of immunoreactive endocrine granules for glucagon (**a**), insulin (**b**), somatostatin (**c**), avian pancreatic polypeptide (**d**) and motilin (**e** and **f**). All specimens were fixed with Karnovsky's fixative and OsO_4 except for the specimen in micrograph **f** fixed with Karnovsky's fixative only. ×50,000



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cells. The mean diameter of the major axis and DC of 433 granules from 5 cells was 355±83 nm (D=452 nm) and 0.93±0.05, respectively.

Insulin-immunoreactive cells exhibited a number of crystalline granules identical to those observed in Type II cells (Fig. 2b). The mean diameter of the major axis and DC of 324 granules from 5 cells was 359±82 nm (D=457 nm) and 0.92±0.04, respectively.

Somatostatin-immunoreactive cells had slightly ovoid granules that varied in consistency from electron opaque to electron dense. Their contents were closely bound by a limiting membrane (Fig. 2c). The mean diameter of the major axis and DC of 368 granuls from 5 cells was 388±80 nm (D=494 nm) and 0.95±0.02, respectively. Somatostatin-immunoreactive granules showed a remarkable resemblance to those observed in Type III cells.

Both APP- and motilin-immunoreactive cells were characterized by the presence of small, highly electron-dense and irregularly shaped granules (Fig. 2d-f). Ultrastructurally, these immunoreactive cells closely resembled the Type IV cell. They were observed in tissues fixed either with Karnovsky's fixative followed by OsO_4 or Karnovsky's fixative only. Since only a few motilin-immunoreactive cells were observed in tissues treated with OsO_4, a comparison between APP- and motilin-immunoreactive cells was done in tissues prepared with Karnovsky's fixative only. The mean diameter of the major axis and DC of 726 APP-immunoreactive granules from 9 cells fixed with Karnovsky's fixative and OsO_4 was 279±74 nm (D=355 nm) and 0.86±0.08, respectively. In tissues fixed with Karnovsky's fixative only, the mean diameter of the major axis and DC of 421 APP-immunoreactive granules from 5 cells was 299±63 nm (D=381 nm) and 0.87±0.06, respectively. In contrast, the mean diameter of the major axis and DC of 688 motilin-immunoreactive granules from 7 cells fixed with Karnovsky's fixative only was 218±53 nm (D=278 nm) and 0.90±0.06, respectively.

Statistically, the mean diameters of the major axis and the DC of all immunoreactive granules examined were significantly different ($p<0.01$) from each other except for those of glucagon- and insulin-immunoreactive granules. The mean diameter of the major axis of somatostatin-immunoreactive granules was significantly larger ($p<0.01$) than those of the other immunoreactive granules, while those of APP-and motilin-immunoreactive granules were significantly smaller ($p<0.001$) than those of other immunoreactive granules. Furthermore, the mean diameter of the major axis of motilin-immunoreactive granules was smaller ($p<0.01$) than that of APP-immunoreactive granules, being the smallest of all immunoreactive granules examined in this study. The DC of APP- and motilin-immunoreactive granules was significantly smaller ($p<0.001$) than those of other immunoreactive granules. Furthermore the DC of motilin-immunoreactive granules was significantly larger ($p<0.01$) than that of APP-immunoreactive granules (Table 1), suggesting that motilin-immunoreactive granules are not so irregular in shape as APP-immunoreactive granules. The DC of APP-immunoreactive granules was the smallest of all immunoreactive granules studied, confirming the observation that APP-immunoreactive granules have the most irregular shape of the immunoreactive granules studied.

DISCUSSION

Pancreatic hormones of the American alligator are reported to be more closely related to avian than mammalian species (BUCHAN et al., 1982; LANCE et al., 1984). Alligator PP cells are detected by avian PP antiserum only (BUCHAN et al., 1982). It is well established that bombesin- or gastrin releasing peptide (GRP)-immunoreactivity is demonstrated only in the intramural nerve elements in mammals. In the American alligator, both types of immunoreactivities were identified in endocrine cells of the fundic glands as well as in the intramural nerves, as also holds true in the case of birds (BUCHAN et al., 1983). These observations offer the evolutional evidence for the development of birds and certain crocodilians from a common branch of the reptilian group. In our previous studies on the caiman, however, PP cells were detected by both avian and bovine PP antisera (YAMADA et al., 1986) and GRP-immunoreactivity was demonstrated only in the intramural nerves (YAMADA et al., 1987). Therefore it was suggested that the caiman may be a type of crocodile more closely aligned to mammalian forms, whereas the alligator type of crocodilian more closely aligned to avian forms (YAMADA et al., 1987). In this unique species, the pancreatic endocrine cells were examined ultrastructurally and immunocytochemically in the present study.

In the family crocodilidae (*Alligator mississippiensis* and *Caiman niger*), pancreatic endocrine cells have been classified ultrastructurally as either A-, B-, D-, EC- or PP-cells (TITLBACH, 1981). Recently, four cell types immunoreactive for one of the major pancreatic hormones (glucagon, insulin, somatostatin and pancreatic polypeptide) have been visualized at the light and electron microscopic levels in the American alligator (BUCHAN et al., 1982) and Nile croco-

dile (RHOTEN, 1987). In addition to the established four types of pancreatic endocrine cells, motilin-immunoreactive cells also have been demonstrated in the pancreas of *Caiman latirostris* at the light microscopic level (YAMADA et al., 1986). In the present study, cells immunoreactive for glucagon, insulin, somatostatin, avian pancreatic polypeptide (APP) and motilin were identified ultrastructurally and immunocytochemically in the pancreas of the caiman, *Caiman latirostris*.

The glucagon-immunoreactive A cells had characteristic large, electron dense granules. The matrix of the granules was of moderate density and separated by a halo of variable width from the limiting membrane. The protuberances or extensions observed in some A-granules in this study have been described previously in the Nile crocodile and other animals (RHOTEN, 1973, 1987). The protuberances may have a significant, but as yet undefined, role in pancreatic endocrine cells. The insulin-immunoreactive B cells were the most easily identified cell type due to the presence of crystalline granules seen by routine electron microscopy, and have been described previously in various vertebrates including crocodiles (TITLBACH, 1981). The variable appearance of the B-granules from round, dense cored types to crystalline types is typical for these secretory granules in the Crocodilia (TITLBACH, 1981; BUCHAN et al., 1982; RHOTEN, 1987). In the American alligator (BUCHAN et al., 1982), somatostatin-immunoreactive D cells were reported to contain large, electron-dense secretory granules. In contrast, D cells of the caiman contained smaller secretory granules which varied from being moderately to highly electron dense. These characteristics are similar to those described in the Nile crocodile (RHOTEN, 1987).

Type IV cells contained two cell types immunoreactive for either APP or motilin. Although it was difficult to distinguish between these two cell types by routine electron microscopy, ultrastructural immunostaining made it possible to distinguish PP- from motilin-immunoreactive cells. In the Nile crocodile (RHOTEN, 1987), the PP-granules were quite similar in appearance to D-granules, although each could be identified on a quantitative basis, with the PP-granules being larger. The similarities between PP-granules and D-granules have also been noted in mammals and other reptiles (LARSSON et al., 1974; GREIDER et al., 1978; RHOTEN and HALL, 1981; RHOTEN, 1984). In the caiman, however, the PP-granules were relatively small in size and similar to those reported in the American alligator (BUCHAN et al., 1982). In the American alligator, PP-immunoreactive cells were identified using anti-avian PP serum but not anti-mammalian PP serum (BUCHAN et al., 1982). In the caiman (YAMADA et al., 1986) and Nile crocodile (RHOTEN, 1987), antisera for both avian and mammalian PP revealed endocrine cells in the pancreas. The present results suggest that differences exist concerning the ultrastructure of PP-granules in various species of crocodile.

When granules of Types I, II, III and IV cells were compared with corresponding immunoreactive granules, glucagon-, insulin-, somatostatin- and APP-immunoreactive granules, respectively, the former granules fixed with glutaraldehyde and OsO_4 were larger than the latter granules fixed with Karnovsky's fixative and OsO_4. Fixation with Karnovsky's fixative followed by OsO_4 may have reduced their granular sizes. This reducing may be caused by the hyperosmoticity of Karnovsky's fixative.

In the present study, the ultrastructure of motilin-immunoreactive cells in the caiman pancreas was demonstrated. Motilin, a 22 amino acid peptide, originally isolated from porcine duodenal mucosa (BROWN et al., 1971), stimulates enteric smooth muscle locally (BROWN et al., 1971; ITOH, 1976; CHRISTOFIDES et al., 1979). It has been well established that motilin-immunoreactive cells are one of the major endocrine cell types in the proximal small intestine of mammals. The presence of motilin-immunoreactive cells also has been reported in the caiman pyloric stomach (pyloric cecum) (YAMADA et al., 1987) and in the pyloric region of domestic pigeon, *Columba livia* var *domestica* (SAITO et al., 1989). The presence of motilin-immunoreactive cells was reported in the caiman pancreas at the light microscopic level and described as an independent pancreatic endocrine cell type (YAMADA et al., 1986). The present study confirmed the presence of motilin-immunoreactive cells in the caiman pancreas at the ultrastructural level and distinguished them from other established pancreatic endocrine cell types. Motilin- and APP-immunoreactive endocrine granules were very similar in appearance and indistinguishable from each other without immunolabelling. Morphometric analysis of the immunoreactive granules demonstrated that motilin-immunoreactive granules were more spherical in shape and smaller in diameter than granules of APP-immunoreactive cells ($p<0.01$).

In the intestine, it is a matter of controversy as to whether or not motilin-immunoreactive cells contain serotonin (5-hydroxytryptamin) and are classifiable as an enterochromaffin (EC) cell type (POLAK et al., 1975; FORSSMAN et al., 1976; PEARSE, 1976; HEITZ et al., 1978; HELMSTAEDTER et al., 1979; KOBAYASHI et al., 1980). Serotonin-immunoreactivity also was observed in the caiman pancreas but was co-localized

exclusively in glucagon-immunoreactive endocrine cells and not in motilin-immunoreactive cells (YAMADA et al., 1986). Ultrastructurally, motilin-immunoreactive granules in human gut endocrine cells are characterized by small, round, solid granules with a closely bound limiting membrane. Those of the dog are slightly larger (approximately 200 nm in diameter) in size and fairly irregular in shape (USELLINI et al., 1984). Motilin-immunoreactive granules of caiman pancreatic endocrine cells closely resemble those observed in motilin-immunoreactive cell in the gut of the dog.

In the caiman, motilin-immunoreactive cells have been reported in the pyloric stomach, duodenum and pancreas (YAMADA et al., 1986, 1987). Comparative ultrastructural studies of motilin-immunoreactive cells from these regions are needed to further elucidate the role of the motilin-immunoreactive cells in this species.

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