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Pancreatic endocrine cells in *Bufo bufo*: immunocytochemistry and ultrastructure

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ABSTRACT

The endocrine pancreas of the toad consists of rounded islets of various sizes embedded in the exocrine tissue. Isolated cells are also present. At least 4 types of endocrine cell are distinguishable by shape, size and electrondensity of the secretory granules as well as by their immunoreactivity with different antisera: insulin, somatostatin, pancreatic polypeptide, and glucagon cells. Insulin cells can be divided into 2 types according to their cytoplasmic electrondensity. Colocalisation of different hormones in the same cell is rarely observed. The close contact between endocrine and exocrine cells and the scarcity of nerve supply is indicative of a paracrine control of hormone secretion.

Key words: Amphibia; toad; insulin; somatostatin; glucagon; pancreatic polypeptide.

INTRODUCTION

The endocrine pancreas of amphibians consists of islets located within the exocrine tissue. The islets are composed of at least 4 types of cell. Not many studies have dealt with the morphology of endopancreatic cells in adult amphibians. The anatomy of the pancreas in several species of amphibians and reptiles was described by Epple (1966), Penhos & Ramey (1973), Hellman & Hellerström (1962), Lange (1973), Lange et al. (1975), and Khanna & Kumar (1973). A histological description and demonstration of immunoreactivity by different cell types was made by Kaung & Elde (1980) in *Rana pipiens*, by Buchan (1985) in several species of anurans and urodeles, Kowan et al. (1991) in *Xenopus laevis*, El-Salhy et al. (1982) in several anurans, and Putti et al. in *Triturus* (1990) and in *Rana arvalis* (1995). An ultrastructural description of the islet cells in amphibians is still lacking, except for those by Tomita & Pollock (1981) in *Rana catesbeiana* and Trandaburu et al. (1995) describing the somatostatin-immunoreactive-cells in *Rana esculenta*.

The aim of this work is the immunocytochemical identification of endocrine cells of the toad *Bufo bufo* by means of the immunogold labelling reaction which allows a precise identification of the different cells together with their fine structural description.

MATERIALS AND METHODS

Five adult specimens of the toad *Bufo bufo* L. of each sex were collected from ponds in the surroundings of Rome, killed by fast decapitation and dissected to remove the pancreas. This was fixed in a mixture of 1% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.3), after being divided into 5 regions: central body, gastric lobe, hepatic lobe, splenic lobe and duodenal lobe. Following 2 h fixation, the specimens were rinsed in cacodylate buffer and postfixed in 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.4), for 1 h, then dehydrated in an ascending series of ethanol and embedded in Araldite resin.

Ultrathin sections for conventional electron microscopy were collected on copper grids and stained with uranyl acetate and lead citrate. Ultrathin sections for immuno-electronmicroscopy collected on nickel grids were processed with the immunogold method as described by Van Bergen en Henegouwen & Leunissen (1986), with slight modifications. Sections were etched in a saturated solution of sodium metaperiodate for 45 min, rinsed with bidistilled water and treated with phosphate buffered saline-glycine (PBS-glycine) 50 mM pH 8.4, for 10 min, followed by 3 successive rinses of phosphate buffered saline (PBS) containing 10% normal goat serum and 1% bovine serum

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Table *Antisera, dilution and incubation times used for immunocytochemical reaction*

Primary antisera	Source	Code	Dilution	Incubation time
Guinea pig antiporcine insulin	Chemicon	AB942	1:100	24 h at 4 °C
Rabbit antiporcine glucagon	Dako	A565	1:50	24 h at 4 °C
Rabbit antihuman somatostatin$_{14}$	Dako	A566	1:100	24 h at 4 °C
Rabbit antihuman synthetic pancreatic polypeptide	Chemicon	AB939	1:100	24 h at 4 °C

albumin (PBG). The grids were then incubated in primary antiserum (dilution and source indicated in the Table) for 24 h at 4 °C, rinsed 6 times in PBG, then incubated for 1 h at room temperature with one of the secondary antisera: goat antirabbit IgG, or goat antiguinea pig IgG, conjugated with 10 or 20 nm gold parties (Biocell Research Laboratories, Cardiff, UK) diluted 1:25 in PBG, rinsed 6 times on drops of PBG, then 3 times on drops of PBS-glycine, and 4 times on drops of bidistilled water. Double immunostaining of the same section was performed following the method of Wang & Larsson (1985): after the first immunoreaction the grids were exposed to paraformaldehyde vapour at 80 °C for 1 h, then submitted to a second immunoreaction by using another primary antiserum and a gold probe of different diameter. The grids were eventually contrasted with uranyl acetate (2% aqueous solution) and lead citrate (Venable & Coggeshall, 1965).

The specificity of immunostaining was determined by omission of the first antiserum and by parallel incubation with antisera preabsorbed 24 h with 10 nmol/ml of their corresponding antigens, which were obtained commercially. Positive controls were performed on tissues known to contain the peptide in question.

RESULTS

The whole pancreas can be divided into 5 regions: central body, splenic lobe, gastric lobe, hepatic lobe, duodenal lobe.

Endocrine pancreatic islets are round or oval-shaped and variously sized (50–150 µm), embedded in the exocrine tissue (Fig.1) and occasionally lined by a thin layer of connective tissue. Isolated endocrine cells were also observed. Four types of endocrine cells were identified, according to their immunoreaction (I-R) with different antisera.

Insulin I-R cells (I) are variously shaped with frequent cytoplasmic projections. The nucleus is rounded or lobed, secretory granules show an irregular, polymorphous, crystalloid or star-shaped dark core, separated by a clear halo from the limiting membrane, 150–300 nm in width (Figs 2, 3). The cytoplasm also contains small rounded mitochondria with tubular cristae and other cytoplasmic organelles, such as portions of smooth and rough endoplasmic reticulum (RER), Golgi complex, glycogen granules and a few lysosomes. I-cells are arranged in large groups, in contact with exocrine cells or other endocrine cells. In several pancreatic islets 2 types of insulin cells were observed, which could be divided into dark and light cells, according to their cytoplasmic electrondensity (Fig. 4). Dark cells show dense cytoplasm and large lobed nuclei with abundant condensed chromatin. Light cells show electronlucent cytoplasm and round nuclei with scarce chromatin. Both types of cell show strong immunoreactivity. Insulin cells are often found in groups, which may be variously located in the islet: in the centre, often in contact with glucagon cells, or externally, in contact with the exocrine tissue. They are evenly distributed in all pancreatic regions.

Somatostatin 14 I-R cells (S) are elliptical with long cytoplasmic projections. The nucleus is lobed and displays masses of condensed chromatin at the periphery. Secretory granules are evenly diffused in the cytoplasm: they are elliptical in shape; transverse diameters average 40 and 150 nm respectively. Granule content is homogeneous and moderately electrondense (Fig. 5). A few elliptical or elongated mitochondria with parallel cristae and some lysosomes are present in the cytoplasm. These cells are found mainly in the central pancreatic body and in the splenic lobe, isolated or arranged in groups, in contact with glucagon or pancreatic polypeptide cells.

Glucagon I-R cells (G) are variously shaped with round or elliptical nuclei. Secretory granules are evenly distributed in the cytoplasm and are mainly round, although sometimes drop or pear-shaped, and are strongly electrondense (Figs 6, 7). Their diameter ranges from 140 to 300 nm. Small round or elongated mitochondria with dark matrices are scattered in the cytoplasm. The cells are in contact with S or I cells and are mainly found in hepatic and gastric lobe. The

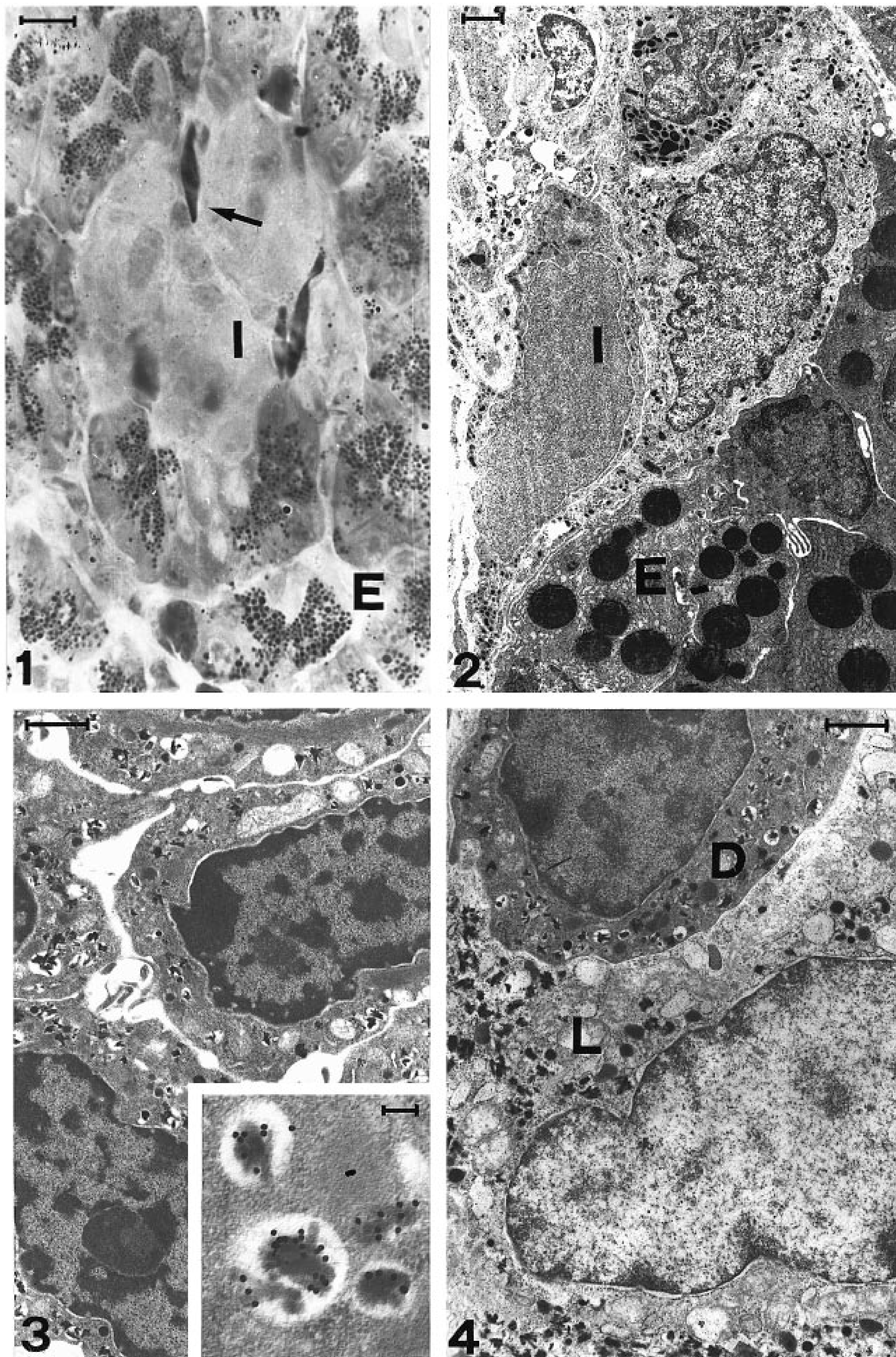

Fig. 1. Semithin section showing that endocrine pancreatic islets (I) are round or oval-shaped, embedded in the exocrine tissue (E). Blood supply is assured by a net of capillary vessels (arrow). Light micrograph of semithin section; methylene blue stain. Bar, 10 µm.

Fig. 2. Electron micrograph showing that contact between endocrine islets (I) and exocrine tissue (E) is very close. Different types of endocrine cells can be observed within an islet. Bar, 1 µm.

Fig. 3. Electron micrograph showing a group of insulin I-R cells with lobed nuclei and dark cytoplasm. Secretory granules have an irregular polymorphous crystalloid core, surrounded by a clear halo. Bar, 1 µm. Insert: electron micrograph of secretory granules immunolabelled for insulin, gold particle size 20 nm. Bar, 0.1 µm.

Fig. 4. According to their cytoplasmic electrondensity, 2 types of insulin cells can be observed: dark cells (D), have dense cytoplasm and large lobed nuclei with abundant condensed chromatin. Light cells (L) have electronlucent cytoplasm and round nuclei with scarce chromatin. Both types of cell show strong immunoreactivity. Electron micrograph. Bar, 1 µm.

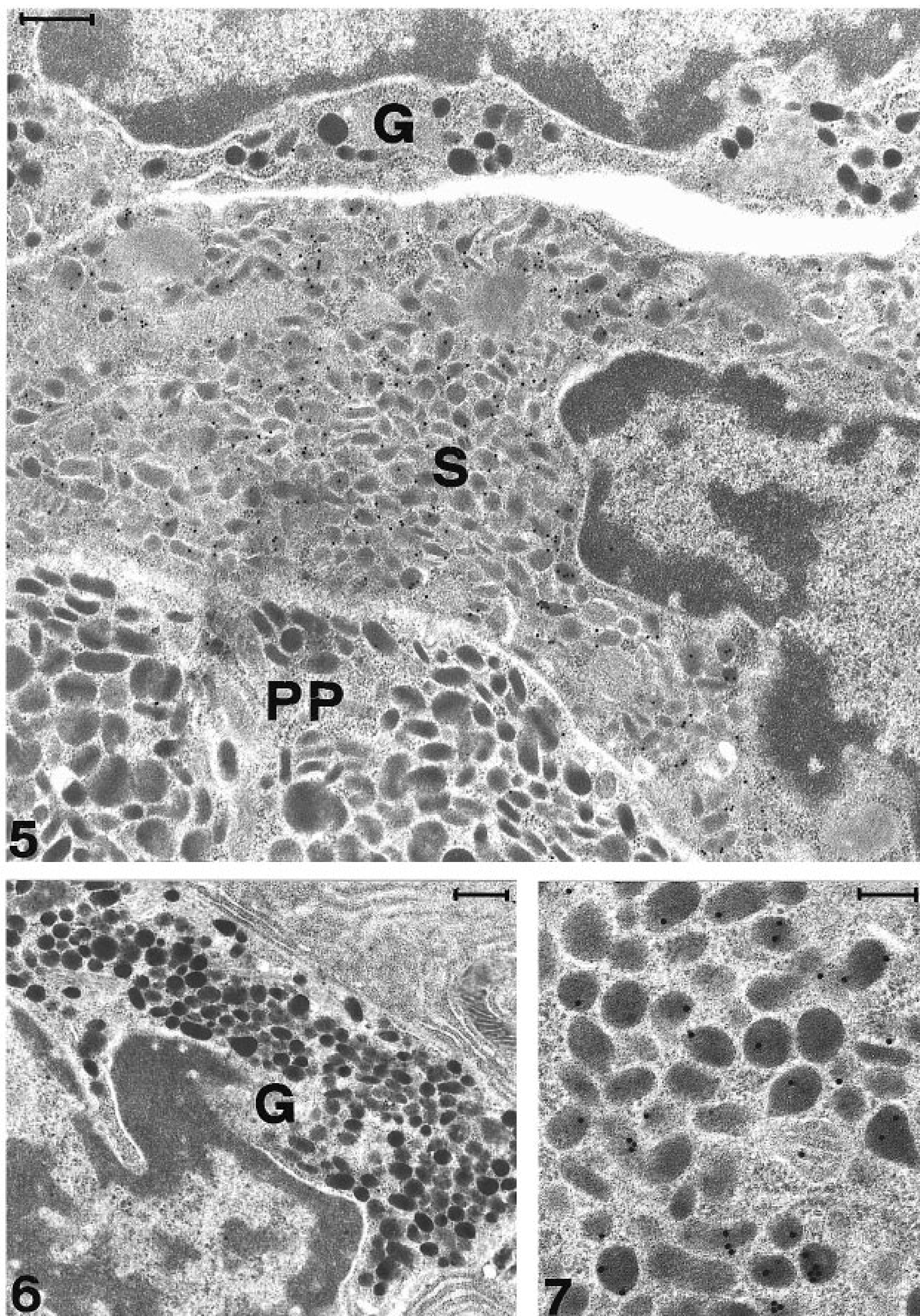

Fig. 5. Electron micrograph of a somatostatin 14 I-R cell (S), in contact with glucagon I-R (G) and pancreatic polypeptide I-R (PP) cells. Secretory granules are elliptical, evenly diffused in the cytoplasm. Their content is homogeneous and moderately electrondense. Anti-somatostatin immunolabelling, gold particle size 20 nm. Bar, 0.5 μm.

Fig. 6. Electron micrograph of a glucagon I-R cell (G) showing strongly electrondense secretory granules evenly distributed in the cytoplasm. Bar, 0.5 μm.

Fig. 7. Electron micrograph of glucagon I-R secretory granules which are mainly round, sometimes drop or pear-shaped and strongly electrondense. Antiglucagon immunolabelling, gold particle size 20 nm. Bar, 0.2 μm.

immunoreactivity of these cells is weaker than that of the other cell types.

Pancreatic polypeptide I-R cells (PP) are variously shaped with a lobed, deeply indented nucleus, showing chromatin masses clumped against the nuclear envelope. Cytoplasmic secretory granules are rounded (mean diameter 150 nm) or elliptical in shape: transverse diameters are 100 and 300 nm, respectively. They are strongly electrondense, and evenly distributed in the cytoplasm. The 2 types of granules are mainly segregated in different cells, both showing immunoreaction (Figs 8–10). Small elongated mitochondria with parallel cristae, lysosomes and glycogen granules occur in the cytoplasm. The cells are often

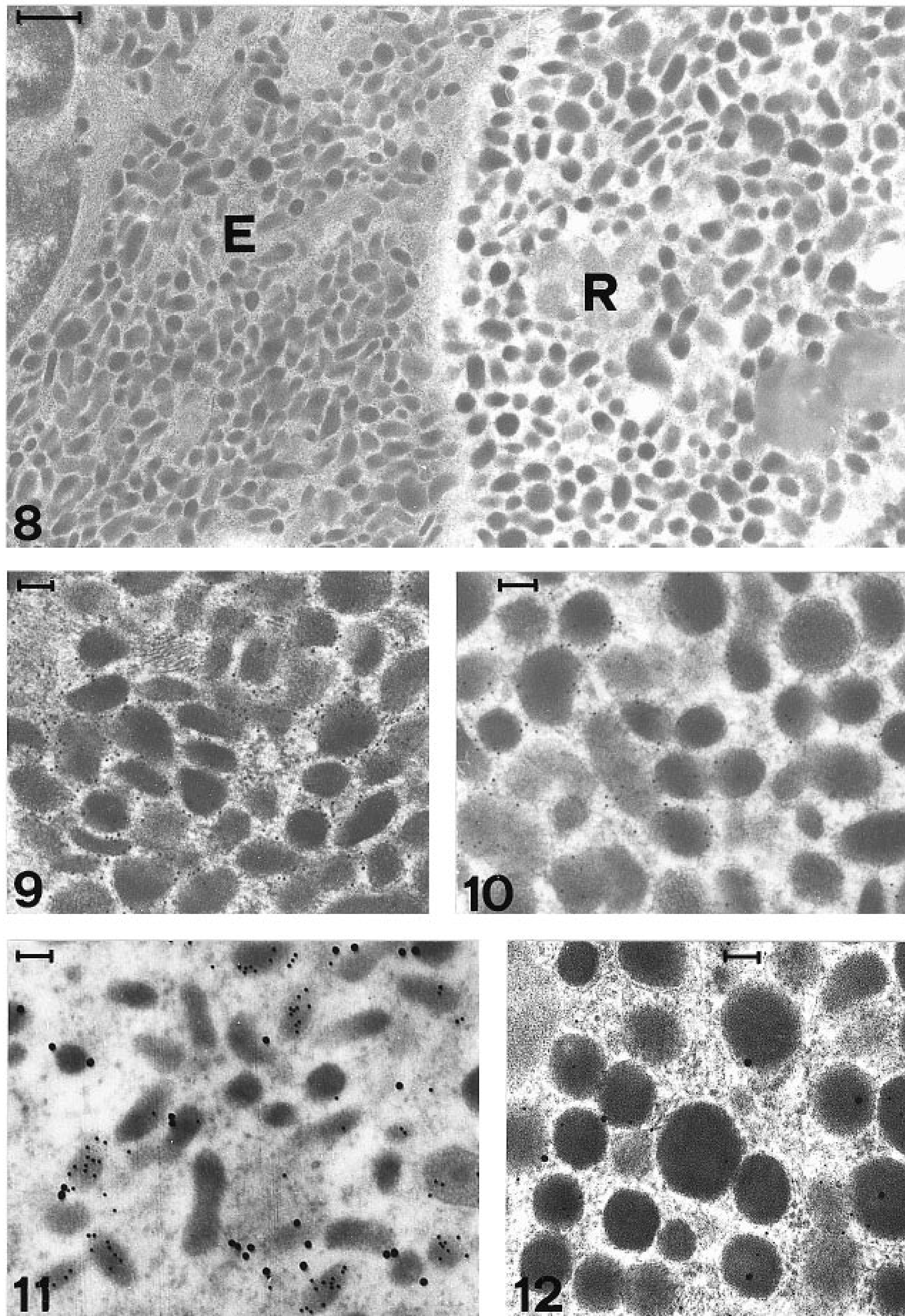

Fig. 8. Electron micrograph showing 2 types of cells reacting to pancreatic polypeptide antiserum; they contain different shaped granules, elliptical (E) or rounded (R), evenly distributed in the cytoplasm. Bar, 0.5 μm.

Fig. 9. Electron micrograph of a pancreatic polypeptide I-R cell with elliptical granules. Anti-PP immunolabelling, gold particle size 10 nm. Bar, 0.1 μm.

Fig. 10. Electron micrograph of a pancreatic polypeptide I-R cell with rounded granules. Anti-PP immunolabelling, gold particle size 10 nm. Bar, 0.1 μm.

Fig. 11. Electron micrograph showing the coexistence of S and PP I-R granules in the same cell demonstrated by double immunolabelling. Antisomatostatin immunolabelling: gold particle size 10 nm; anti-PP immunolabelling: gold particle size 20 nm. Bar, 0.1 μm.

Fig. 12. Electron micrograph showing the coexistence of G and PP I-R granules in the same cell which was occasionally observed by double immunolabelling. Antiglucagon immunolabelling: gold particle size 10 nm; anti-PP immunolabelling: gold particle size 20 nm. Bar, 0.1 μm.

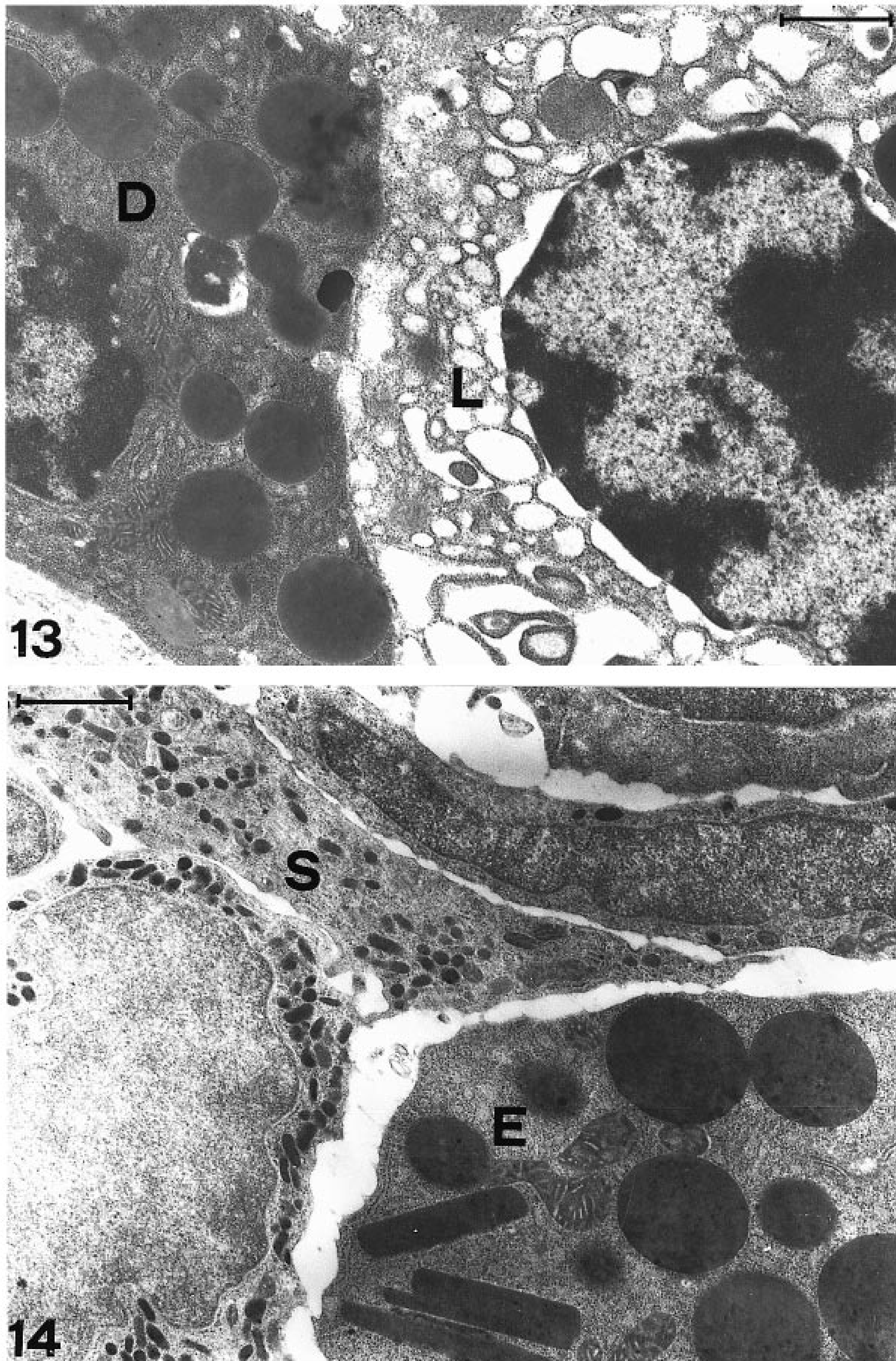

Fig. 13. Electron micrograph of ultrathin section of exocrine pancreatic cells with different cytoplasmic electrondensity: dark cells (D) contain a large amount of RER, arranged in tightly packed vesicles and cisternae, and many large, evenly diffused, zymogen granules; light cells (L), contain loosely arranged SER and a smaller number of zymogen granules. Bar, 1 μm.

Fig. 14. Electron micrograph showing somatostatin I-R cells (S), without a surrounding layer of connective tissue, in close contact with an exocrine cell (E) with a large number of zymogen granules. Bar, 1 μm.

located at the periphery of the islet, isolated or in groups, in contact with S or G cells. They are mainly located in the central pancreatic body and the splenic and hepatic lobes.

Colocalisation was investigated by means of double immunoreaction in the same section. The coexistence of S and PP-granules in the same cell was occasionally observed using the double immunostaining technique (Fig. 11). The shape of secretory granules was oval and their density was intermediate between that of S and PP cells. Coexistence of G and PP granules in the same cell rarely occurred (Fig. 12).

Exocrine pancreatic cells are rounded or polygonal in shape, with a round or lobed nucleus. The

cytoplasm contains a large amount of RER, many zymogen granules and, occasionally, dark elongated crystals. Mitochondria are elongated with parallel cristae. On the basis of the cytoplasmic electrondensity and of the amount of organelles, exocrine cells can be divided into 2 main types (Fig. 13): (1) dark cells, whose dense cytoplasm contains a large amount of RER, arranged in tightly packed vesicles and cisternae, and many large evenly diffused zymogen granules; the nucleus contains much chromatin; and (2) light cells, whose cytoplasm contains loosely arranged RER and a smaller number of zymogen granules. The nucleus is oval with sparse chromatin masses. Dark cells are more numerous and more frequently in contact with endocrine cells than light cells.

As to the relationships between endocrine and exocrine tissue, different arrangement patterns were observed: large endocrine islets are lined by a thin layer of connective tissue. The number of zymogen granules in the exocrine tissue surrounding the islets was variable. Mainly around the somatostatin cells (Fig. 14), which often appear isolated, without any layer of connective tissue, the exocrine cells contain a large number of zymogen granules.

Endocrine islets are abundantly vascularised by a net of small capillary vessels. The nerve supply is scarce, assured by unmyelinated nerve fibres and nerve terminals of the cholinergic type.

DISCUSSION

The endocrine pancreatic cells of *Bufo* are isolated or arranged in islets. Larger islets are found mainly in the central body, whereas the smaller ones as well as the isolated cells mainly occur in the other regions. Islet size can be compared with that in different vertebrates. Kowan et al. (1991) reported islet size of *Xenopus*, the newt and mammals. Our results in *Bufo* demonstrate that islet size is more similar to that of *Xenopus* than of other vertebrates, in which it is larger.

Endocrine islet composition in *Bufo* revealed that the 4 cell types (I, S, PP and G) occur together in the central pancreatic body, whereas only some of them coexist in the different regions with I-cells, which are always present. These last are the most abundant in the islets, forming large groups. The relative abundance of I-cells in comparison with the other cell types was noted in other anurans, as well as in urodeles (Epple & Brinn, 1975).

Antisera used in immunolabelling reactions did not show the same sensitivity. In particular the rabbit antiglucagon antiserum showed, notwithstanding the high concentration used, a weak reactivity that might be due to incomplete affinity. Antisera commercially obtained were all raised in mammals; the phyletic distance between amphibians and mammals, and the consequent structural difference in aminoacid sequence or conformation of hormones could account for the weak immunoreaction.

The comparison of ultrastructural features of endopancreatic cells of other amphibians reveals that I-cells are similar to those described in *R. catesbeiana* by Tomita & Pollock (1981) as to the shape and size of secretory granules. Two types of I-cells were observed in *Bufo*, differing by cytoplasmic electrondensity and nuclear shape. Similar features were never described in amphibians but were observed in teleosts by Aguillero et al. (1993) and were regarded as stages of involution or degeneration. In *Bufo* both types of I-cells showed strong immunolabelling in the presence of well preserved cytoplasmic organelles, suggesting that the 2 types might represent different functional stages. The other cell types described by Tomita & Pollock (1981) differ slightly from those of *Bufo*, as to the electrondensity of S-cells, stronger in *R. catesbeiana*, and particularly for the shape of granules of PP cells, which are more abundant and polymorphous in *Bufo*, where, in addition, 2 types were described containing respectively round or oval granules, both being PP immunoreactive. Further investigations should be necessary to ascertain whether some of these cells contain other hormones of the peptide family (NPY, PYY). Several studies have been conducted on the molecular evolution of the PP family (Conlon et al. 1986; Cheung et al. 1991); PP-like peptides have a similar molecular structure and seem to be derived from a single ancestral gene. Somatostatin-IR-cells described by Trandaburu et al. (1995) in *Rana esculenta* show the same long cytoplasmic projections as in *Bufo*, but differ in the shape of the granules, which are rounded in *Rana* and elongated in *Bufo*.

No evidence of exocytosis was observed in endocrine pancreatic cells of *Bufo*. The discharge mechanism of secretory granules is probably undetectable in normal conditions and might be visible only on stimulation.

Colocalisation of different hormones in the same cell (mostly PP and G) was reported in the pancreas of Teleosts by Aguillero et al. (1993), in *Rana pipiens* by Kaung & Elde (1980), in several amphibians by Buchan (1985), in *Rana arvalis* by Putti et al. (1995) and in human fetal pancreas by De Krijger et al. (1992). This aspect of hormonal secretion appears to be related to the early appearance of hormones in

lower vertebrates or to their early production during development. The coexistence of PP and G, cited by several authors, was rarely detected in *Bufo*. A small number of endocrine pancreatic cells showed coexistence of PP and S. Such coexistence was described only in the Japanese newt by Oikawa et al. (1992), but not in other vertebrates. Different cell populations were also observed during the development of the endocrine pancreas of *Bufo*, as described by Grassi Milano & Chimenti (1995).

The relationships between endocrine and exocrine tissue in *Bufo* are very close, as the layer of connective tissue surrounding the islet is very thin or even lacking around small islets or single cells. The occurrence of a larger amount of zymogen granules was observed mainly around S-cells. This association is described in several vertebrates, and the effects of endocrine cells on exocrine pancreas are reported as the presence of 'zymogen mantel' in peri-insular exocrine cells (Epple & Brinn, 1980). The close contact between endocrine and exocrine tissue and the scarcity of a nerve supply may be indicative of a paracrine control of hormone secretion. The blood supply, assured by a net of small capillary vessels, may also contribute to this function.

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