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Characterization of Pancreatic Endocrine Cells of the European Common Frog *Rana temporaria*

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To characterize the endocrine cell types of the pancreas of *Rana temporaria*, conventional staining, silver impregnation, and immunocytochemical methods for light and electron microscopy have been applied to paraffin, thin and semithin sections, many of them serial pairs. Quantitative data on the frequency and distribution (insular, extrainsular among the exocrine cells, or within the pancreatic ducts) of each endocrine cell type are also reported. Four distinct endocrine cell types have been identified: insulin (B) cells, which are also immunoreactive for [Met]enkephalin; glucagon/PP (A/PP) cells, also immunoreactive for GLP1; somatostatin (D) cells; and a fourth endocrine-like cell type (X cells) of unknown content and function. X cells display characteristic ultrastructure and tinctorial traits but are nonimmunoreactive for all of the 37 antisera tested. The presence of [Met]enkephalin in amphibian pancreatic endocrine cells is now reported for the first time. Almost half (44.9 ± 7.9) of the total endocrine cell population lies outside the islets, mainly spread among the exocrine cells. Approximately 37.2 ± 4.6% of the total endocrine cell population was immunoreactive for insulin, 48.8 ± 6.9% was immunoreactive for glucagon/PP, and 14.0 ± 4.9% was immunoreactive for somatostatin; 79.2 ± 6.4% of glucagon/PP cells are found within the exocrine parenchyma, representing the majority (86.4 ± 4.3%) of extrainsular endocrine component. On the contrary, most B cells (94.2 ± 2.1%) are located within the islets; 30.8 ± 12.9% of D cells are found outside the islets.

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Careful identification of hormonal and prohormonal peptides is a very important tool for the classification of the endocrine cells, which are scattered in various epithelia and form the diffuse endocrine system (DES). Structural description at light and electron microscopic levels, topography, response to functional stimuli, detection of specific receptors, and localization of biogenic amines, individual enzymes, and structural proteins are also important tools for precise cell characterization. Pancreatic endocrine cells in amphibians have not been fully characterized using these criteria. Until now, insulin, glucagon, oxyntomodulin, glucagon-like peptide 1 (GLP1), glucagon-like peptide 2 (GLP2), and pancreatic polypeptide (PP) have been purified and sequenced from amphibian pancreas (Pollock *et al.*, 1988; McKay *et al.*, 1990; Conlon *et al.*, 1998a,b,c).

In amphibians, endocrine A, B, and D cells were first characterized by conventional light and/or electron microscopic techniques. During the 1980s, the introduction of immunocytochemistry permitted the demonstration of peptides analogous to the four main mammalian pancreatic peptides in the amphibian pancreas: glucagon, insulin, somatostatin, and PP (Kaung and Elde, 1980; Tomita and Pollock, 1981; El-Salhy *et al.*, 1982; Buchan, 1985; Putti *et al.*, 1990). Frequently, glucagon and PP were shown to colocalize in the same

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cells. Since then, some efforts have been made to correlate the immunocytochemical, tinctorial, and/or ultrastructural features of the pancreatic endocrine cells in amphibians (Tomita and Pollock, 1981; Ortiz de Zárate *et al.*, 1991; Oikawa *et al.*, 1992; Trandaburu *et al.*, 1995; Accordi *et al.*, 1998). On the other hand, some other neuroendocrine peptides and markers (ACTH, insulin-like growth factors IGF-I and IGF-II, NPY, oxytocin, pancreastatin, PHI, PYY, VIP, adrenomedullin, and chromogranins) have been detected in the endocrine pancreas of amphibians (Hansen *et al.*, 1989; Ding *et al.*, 1991, 1997; Reinecke *et al.*, 1991, 1995; Putti *et al.*, 1995, 1997; Mulder *et al.*, 1997; Maake *et al.*, 1998; López *et al.*, 1999).

The aim of the present article is to give a more complete description of the pancreatic endocrine cells of adult *Rana temporaria.* A detailed immunocytochemical and ultrastructural study has been performed using serial semithin and thin sections. In addition to classic pancreatic peptides, other antisera have been used to sort out whether other regulatory peptides and neuroendocrine markers that have been already localized in mammalian endocrine pancreatic cells are also present in the amphibian pancreas. A semiquantitative estimation of the distribution of each endocrine cell type within the pancreas has also been carried out.

METHODS

Twenty-five adult specimens of *Rana temporaria* were used in this study. Animals were collected during the spawning season, between February and April. The frogs were kept in the laboratory and fed with earthworms until dissection. For dissection the animals were anesthetized with ether.

Fixation and Embedding

Fixation was carried out by immersion. Nine pancreases were fixed in Bouin's fluid for 24 h, cleared in 70% ethanol, dehydrated through graded ethanol, and embedded in paraffin. The remaining 16 pancreases were sliced in small pieces (1 mm^3) and distributed in three lots for different fixation procedures for electron microscopy. Pieces of lot 1 were fixed for 5 h in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and postfixed for 2½ h in 1% OsO_4 in phosphate buffer. Samples from lot 2 were fixed for 2 h in a mixture of 1% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer. In that case postfixation with OsO_4 was reduced to 1 h. The third lot was fixed in Zamboni's fixative (Stefanini *et al.*, 1967) for 24 h without postfixation. All specimens were dehydrated with ethanol, washed with propylene oxide, and embedded in Epon 812 (Luft, 1961).

Sections (5 µm) from paraffin-embedded tissue were used for immunocytochemical detection. Semithin sections (1 µm) from lot 1 were stained with silver impregnation for argyrophilia (López *et al.*, 1983). Semithin sections from lot 3 were used for Giemsa staining (Díaz de Rada *et al.*, 1986) and for immunocytochemical procedures. Lot 1 was used for conventional ultrastructural microscopy. Thin sections were placed on grids, double stained with uranyl acetate and lead hydroxide, and observed with a Zeiss EM 10CR electron microscope. For ultrastructural immunolabeling thin sections from lot 2 were used.

Immunocytochemistry

After paraffin sections were dewaxed with xylene, blockade of endogenous peroxidase was achieved by treatment with 3% H_2O_2 in methanol. Sections were rehydrated through alcohols of decreasing grades and then placed in 0.55 M Tris-buffered saline (TBS) at pH 7.4. Unspecific binding sites were blocked with 5% normal swine serum in TBS. Sections were incubated overnight at 4°C with the primary antiserum diluted in TBS (see Table 1). After three rinses in TBS (5 min), sections were incubated with biotinylated swine serum directed against rabbit immunoglobulins (E353, Dakopatts, Glostrup, Denmark) for 30 min at room temperature. After rinsing in TBS, sections were treated with avidin–biotin–peroxidase complexes (ABC) (K533, Dakopatts) prepared 30 min in advance. After additional washes, peroxidase was demonstrated by the diaminobenzidine/H_2O_2 method (D-5637, Sigma Chemical Co, St. Louis, MO, U.S.A.). Occasionally, sections were counterstained with Harris hematoxylin.

For immunocytochemistry on semithin sections the ABC technique was also used. After removal of the epon with ethanol (Lane and Europa, 1965) and rehydration through graded alcohols, sections were incubated with 5% normal swine serum for 30 min at room temperature. Before the first incubation, when osmi-

TABLE 1
List of Antisera Used[a]

Antisera raised against	Source / Cod. No	Dilution
Porcine insulin	MILAB / B-39-100	1:10,000
Porcine pancreatic glucagon	MILAB / B-31-100	1:5,000
Synthetic somatostatin (Som 14)	INCSTAR / 20H2T	1:2,000
SRIF (Som 14)	RPMS[b] / 1460	1:2,000
Human PP	MILES / 64-711-1	1:2,000
Synthetic human GLP1 (1–19)	RPMS / 1642	1:2,000
Synthetic hamster GLP1	RPMS / 1167	1:2,000
GLP2 (Varndell *et al.*, 1985)	RPMS / 1482	1:1,000
GLP2	RPMS / 1650	1:1,000
Secretin (Kanamori *et al.*, 1989)	MILAB / B-33-100	1:1,000
Porcine VIP	RPMS / 925	1:1,000
VIP	RPMS / 652	1:500
Porcine GIP	RPMS / 924	1:1,000
Synthetic porcine PYY	RPMS / 1234	1:1,000
Synthetic porcine NPY	RPMS / 1101	1:1,000
[Met]ENK	RPMS / 1657	1:8,000
[Met]ENK	INCSTAR / 18H2T	1:5,000
[Leu]ENK	RPMS / 774	1:1,000
Dynorphin	RPMS / 785	1:1,000
ACTH (1–24)	RPMS / 1371	1:1,000
αMSH	RPMS / 1463	1:1,000
γMSH	RPMS / 1380	1:500
βEndorphin	RPMS / 395	1:500
Serotonin	INCSTAR / 43H2TR	1:1,000
Bovine neurotensin (Flucher *et al.*, 1988)	MILAB / B-44-100	1:2,000
Substance P (Rindi *et al.*, 1986)	INCSTAR / 17H2T	1:1,000
Synthetic bombesin	INCSTAR / 30H2T	1:1,000
Sauvagin	RPMS / 725	1:500
Gastrin	RPMS / 1199	1:500
Neuromedin B	RPMS / 1196	1:1,000
Synthetic porcine neuromedin U	RPMS / 1487	1:1,000
Human calcitonin	RPMS / 272	1:1,000
CGRP	RPMS / 1210	1:1,000
Cerulein	RPMS / 935	1:1,000
Porcine motilin	RPMS / 932	1:1,000
Human NSE	RPMS / 1735	1:500
IAPP	RPMS / 2131	1:500

[a] Abbreviations: GLP, glucagon-like peptide; VIP, vasoactive intestinal peptide; GIP, gastric-inhibitory polypeptide; SRIF, somatotropin release-inhibiting factor; PP, pancreatic polypeptide; PYY, tyrosine-tyrosine polypeptide; NPY, neuropeptide Y; ENK, enkephalin; ACTH, corticotropin; MSH, melanotropin; CGRP, calcitonin gene-related peptide; NSE, neuron-specific enolase; IAPP, islet-amyloid polypeptide.

[b] Gift from Prof. Julia M. Polak, Royal Postgraduate Medical School (London), Department of Histochemistry.

cated sections were used, a 10-min treatment with 3% H_2O_2 (Bendayan and Zollinger, 1983) was applied. Incubation with primary antibodies lasted for 48 h. Second-layer and ABC incubations took 1 h, respectively. All other steps were carried out as described for paraffin sections.

A total of 37 antisera against neuroendocrine regulatory peptides and markers were tested (Table 1). All primary antisera used were raised in rabbits, with the exception of the insulin antibodies, which were raised in guinea pigs. In the latter case, after incubation with primary antibody, sections were incubated with rabbit anti-guinea-pig IgG conjugated with peroxidase (P141, Dakopatts). Peroxidase was then demonstrated as mentioned above.

When there were positive results, absorption controls were carried out by incubating the primary antibody with an excess of the antigen (10 nmol peptide per milliliter of optimally diluted antibody). The peptides used as antigen were insulin (I3505), glucagon (G4250), somatostatin (S9129), PP (P9903), [Met]ENK (M6638), and GLP1 (G3265), all six purchased from Sigma. Negative controls (omission of any of the layers of the immunocytochemical protocol and use of nonimmune goat serum as a first layer) were also performed.

Ultrastructural Immunolabeling

Ultrastructural immunolabeling was carried out according to De Mey (De Mey *et al.*, 1981). Lot 2 thin sections were mounted on nickel grids and incubated with 3% H_2O_2 for 10 min. After three washes in double-distilled water, sections were placed in filtered 0.55 M TBS at pH 7.4. Afterward, sections were incubated with 2% normal goat serum in TBS at room temperature for 30 min. Incubation with primary antibody diluted in TBS, usually 10 times more concentrated than in light immunocytochemistry, was carried out overnight at 4°C. Subsequent steps, all at room temperature, included rinses in 0.1% bovine seroalbumin in TBS (BSA-TBS) and incubation with goat anti-rabbit IgG colloidal gold particles of 15 nm (Cod. 640017EM, Peninsula, St. Helens, Merseyside, England) for 1 h and cleared with BSA-TBS and then with double-distilled water. Finally, preparations were stained for 5 min in aqueous uranyl acetate and later in lead hydroxide.

Quantitative Studies

For ultrastructural characterization of endocrine cell types, the size of secretory granules (mean diameter ± SD) was calculated according to Martínez (Martínez *et al.*, 1989) on electron micrographs magnified 33,000 times.

To estimate the proportion (mean percentage ± SD) and the preferred distribution of the endocrine cell types, semiquantitative studies were carried out with samples from seven different frogs. The whole pancreas of each frog was totally cut in 5-μm serial sections; three serial sections were randomly selected and immunocytochemically stained for insulin, glucagon, and somatostatin respectively. Positive cells distributed in the endocrine islets, scattered (extrainsular) throughout the exocrine parenchyma or included in the ductal epithelium, were counted separately. Given the difficulty of distinguishing between a polar section of an islet and scattered extrainsular cells within the exocrine tissue, we considered endocrine cells as extrainsular component only when they appeared isolated or in small groups (≤3). Only endocrine cells in which the nucleus was visible were counted; 13,901 endocrine cells were counted (1,000 to 2,563 from each frog): 5,143 were B cells (424 to 1,037), 6,744 were A / PP cells (456 to 1,239), and 2,014 were D cells (120 to 639).

RESULTS

With Giemsa staining, four endocrine cell types are distinguished in semithin sections of nonosmicated samples of the pancreas of *Rana temporaria* (Fig. 1). Ultrastructural features characterized four different endocrine cell types (Figs. 2–5). Hormonal products of these cell types have also been determined (except for the X cell type) by immunocytochemistry in serial semithin sections.

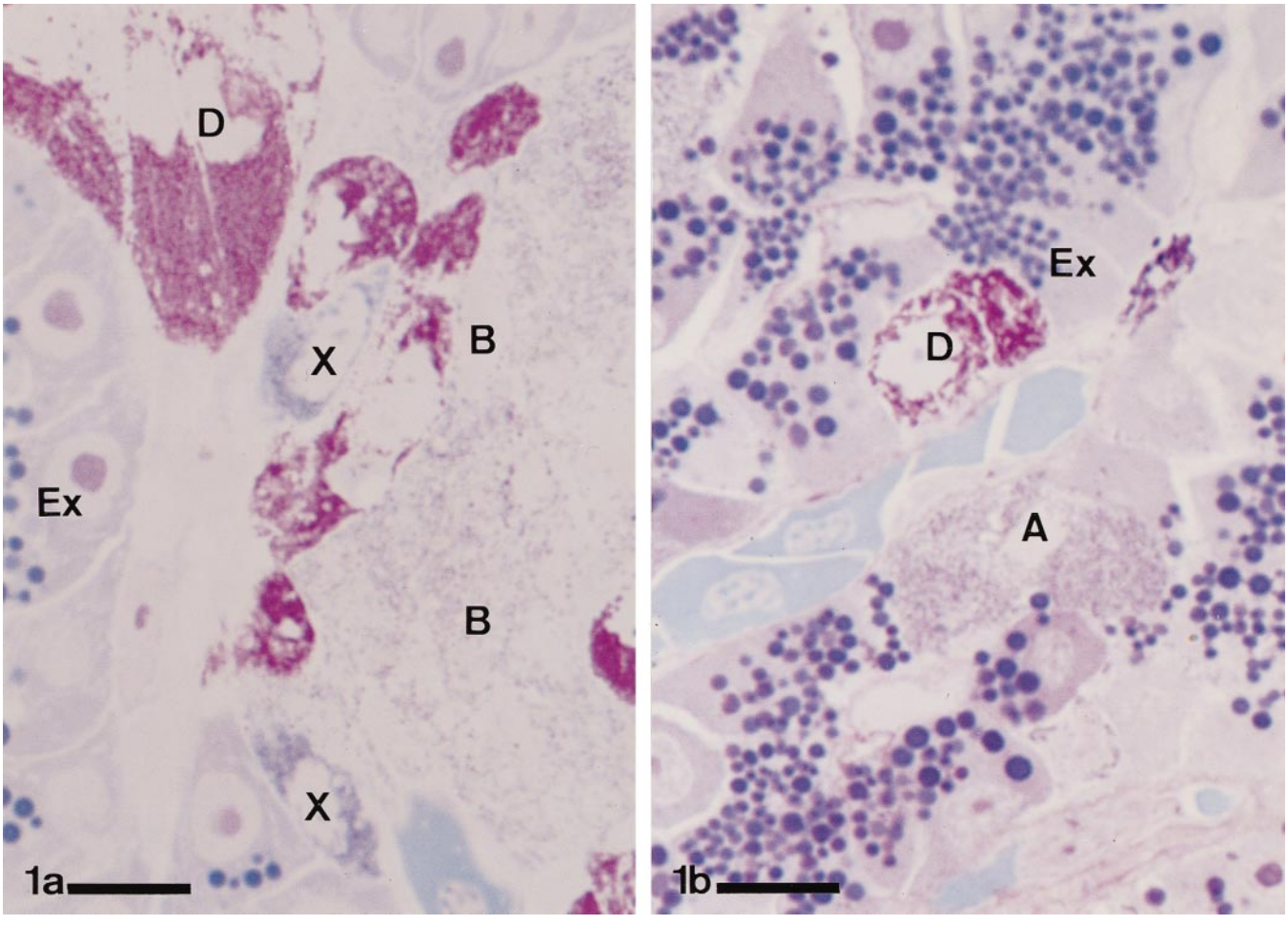
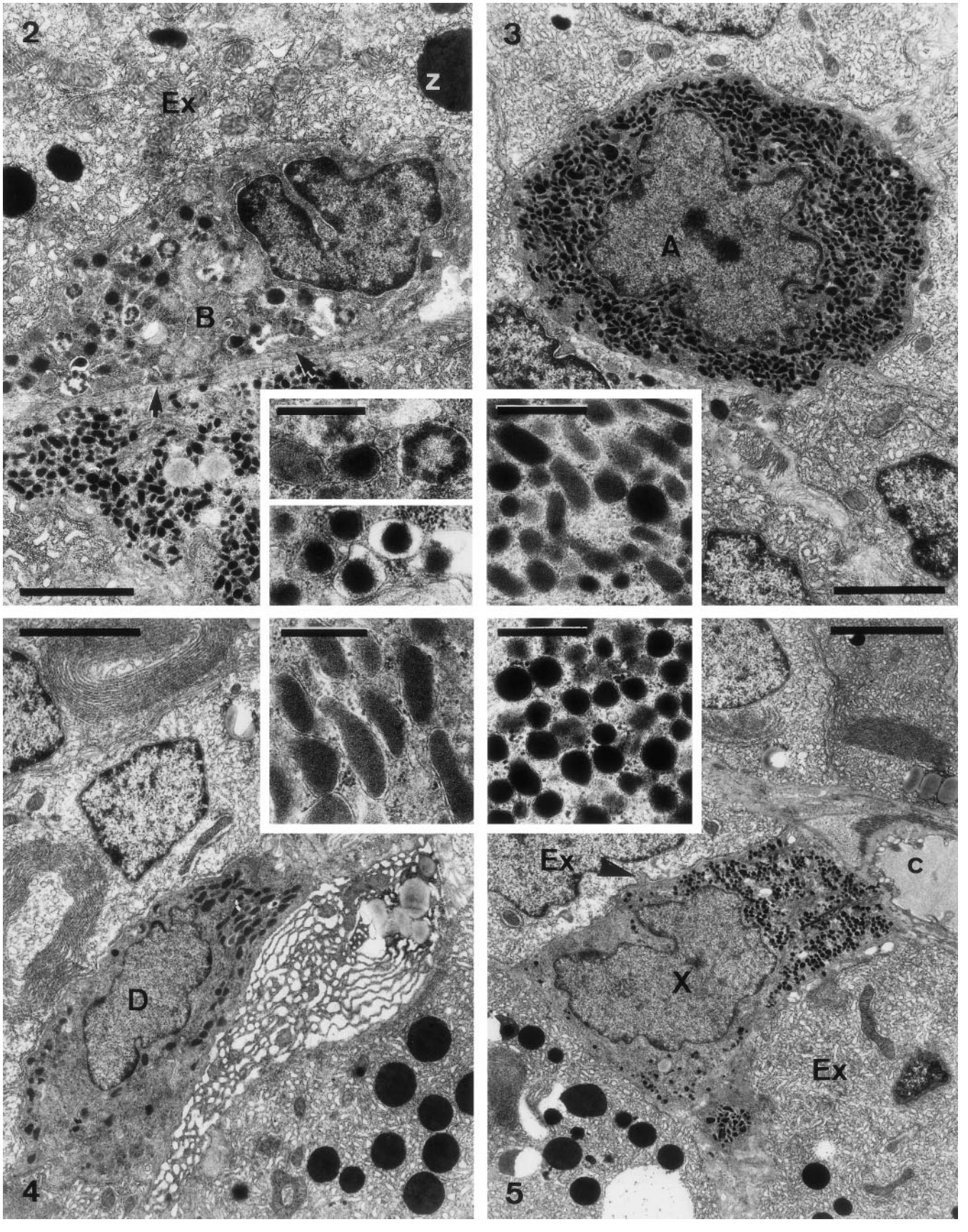


FIG. 1. Giemsa stain applied to semithin sections of pancreas. Four pancreatic endocrine cell types are easily recognized. B, insulin cells (pale color); A, glucagon / PP cell (rose-blue); D, somatostatin cells (strong purple); X, X cells (blue); Ex, exocrine cells; Original magnification ×1700; bars, 10 μm.


2
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3
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Insulin (B) Cells

Insulin (B) cells are columnar in shape and appear mainly in the central region of the endocrine islets, adjacent to the blood vessels (Fig. 6). Secretory granules are often grouped in the cytoplasmic region closer to the blood vessel (Fig. 6b). These cells possess spherical granules (diameter 358 ± 10 nm) with characteristic granular content (Fig. 2): most of them have a central core of electron-dense material, while in some other granules the dense content appears in a beads-on-a-string fashion or homogeneously filling the whole granule volume. The frequency of these types of granules varies between cells and between animals. B cells have low affinity for the Giemsa stain (Figs. 1a and 6b). The insulin content of the cells has been demonstrated by immunocytochemistry, both in semithin and thin sections (Figs. 7a and 8). Insulin cells are also immunoreactive for [Met]ENK (Figs. 7b and 9).

Absorption controls were carried out preincubating both anti-insulin and anti-[Met]ENK with both insulin or [Met]ENK separately. The immunoreaction was blocked only when each antiserum was preincubated with its specific antigen (Fig. 9).

Glucagon/PP (A/PP) Cells

Glucagon / PP (A / PP) cells bear characteristic bacilliform granules, with an average length of 378 ± 6 nm and diameter of 208 ± 8 nm (Fig. 3). The cytoplasm is usually almost entirely filled with electron-dense secretory granules of a homogeneous content. These cells are immunoreactive for glucagon, GLP1, and PP (Figs. 10 and 11). They show a slight rose-blue color when Giemsa stain is applied (Figs. 1 and 12) and are also argyrophilic (Fig. 13a). These cells are usually round and possess a central nucleus, but it is common to observe cells with cytoplasmic processes toward the neighboring cells when they are scattered within the exocrine tissue (Fig. 11). None of the cells localized in the exocrine tubules or acini have been shown reaching the lumen. In the islets, glucagon / PP cells usually appear at the periphery, surrounding the rest of the insular cells.

Somatostatin (D) Cells

Somatostatin (D) cells (Fig. 14) are easily recognized at the ultrastructural level because they contain the largest secretory granules (length 517 ± 19 nm and diameter 323 ± 9 nm) among the pancreatic endocrine cells (Fig. 4). Their granules are usually bacilliform. Irregular, polymorph granules are also observed. The content of these granules is homogeneous, occupies the whole granule, and is less electron-dense than the content of the granules of the other cell types (Fig. 4 inset). Somatostatin cells (Fig. 14) can be easily localized on Giemsa staining due to their strong purple cytoplasm (Fig. 1). Somatostatin-immunoreactive cells have been identified both in serial semithin–thin pairs (Figs. 14b,c) and in immunogold-labeled sections (Fig. 15). None of the other antisera tested immunoreacted with somatostatin cells. Inthe islets, somatostatin-immunoreactive cells appeared mainly at the periphery, adjacent to glucagon / PP cells. Somatostatin cells scattered within the exocrine tissue normally exhibit long cytoplasmic processes.

X Cells

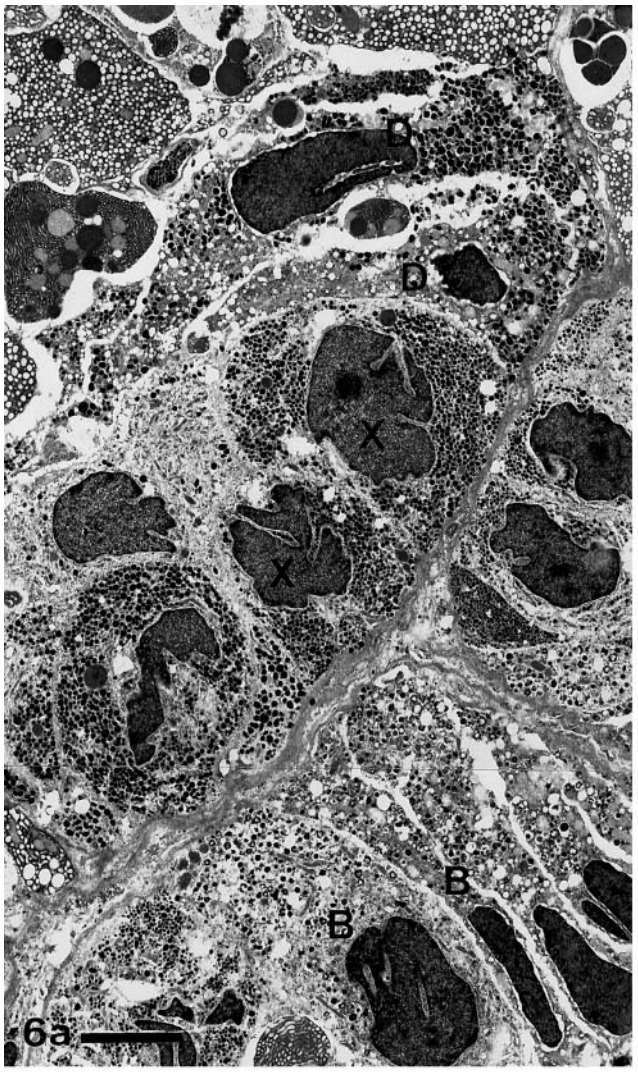
Another endocrine cell type, X cells, are different from insulin, glucagon / PP, and somatostatin cells and have been characterized ultrastructurally (Fig. 5) and tinctorially with Giemsa staining (Fig. 1a). These cells display secretory granules clearly different from the

FIG. 2. Ultrastructure of an insulin cell (B); Original magnification ×10,700; bar, 2 μm. This is one of the few insulin cells localized within exocrine (Ex) glands. Z, zymogen granules. Arrows indicate basal lamina. The insets show the detail of insulin granules; Original magnification ×33,000; bar, 0.5 μm.

FIG. 3. Glucagon / PP cell (A); Original magnification ×6,850; bar, 3 μm. The inset illustrates the ultrastructure of A granules; Original magnification ×33,000; bar, 0.5 μm.

FIG. 4. Somatostatin cell (D). Original magnification ×5,500; bar, 4 μm. The inset shows the large polymorphic secretory granules; Original magnification ×33,000; bar, 0.5 μm.

FIG. 5. Ultrastructure of an X cell (X) located within the exocrine tissue (Ex) and adjacent to a capillary (c). Arrow indicates interdigitating process with exocrine cell; Original magnification ×4,350; bar, 5 μm. The inset shows detail of the granules; Original magnification ×33,000; bar, 0.5 μm.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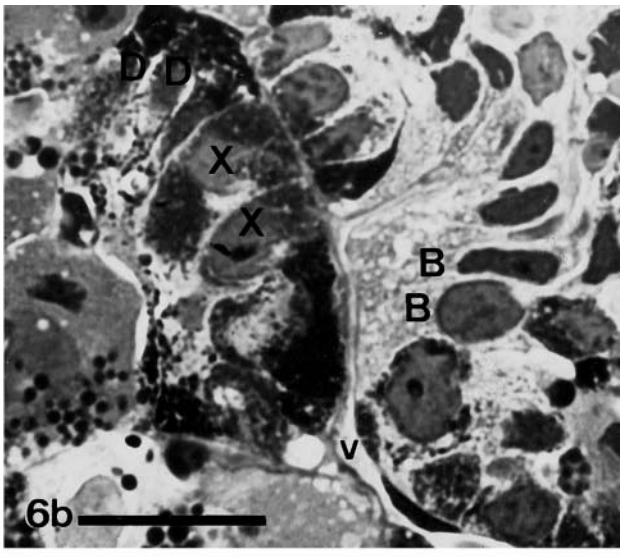
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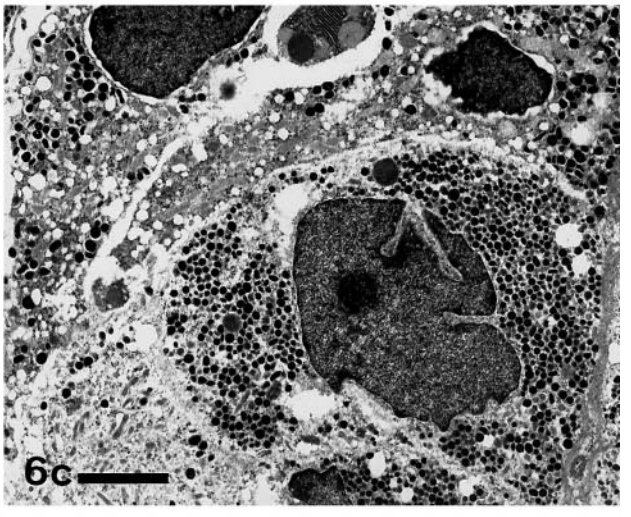
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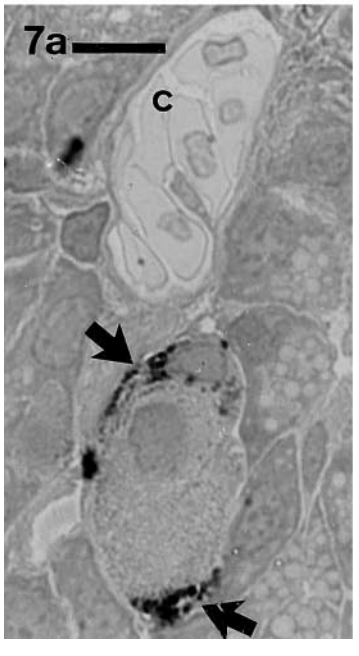
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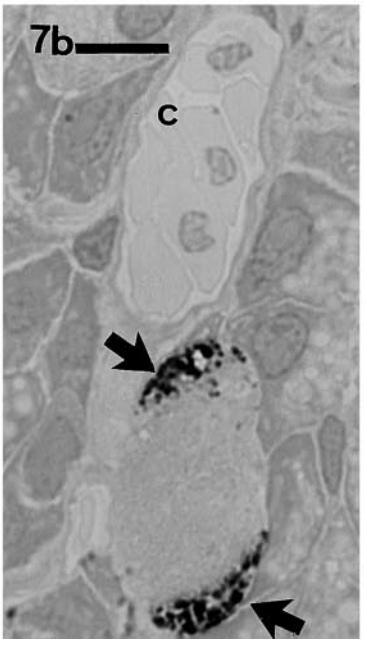
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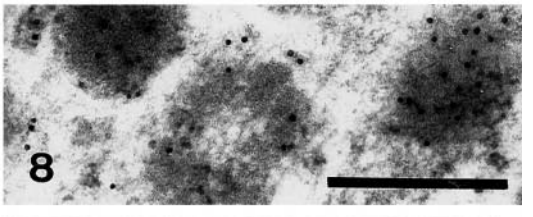
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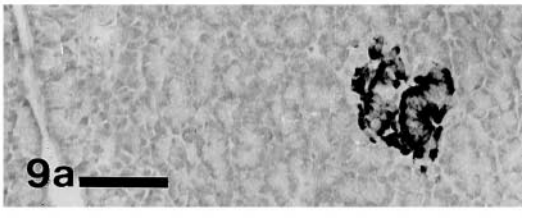
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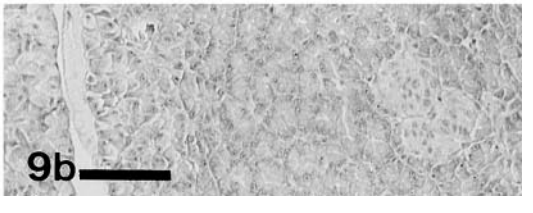

9b

granules described above: they are regular in size, smaller (diameter 215 ± 3 nm), and spherical in shape (Fig. 5 inset; Fig. 6c). The highly electron-dense granular content is distributed homogeneously throughout the granule. These cells are also distinguished in semithin sections stained with Giemsa by their patent blue-colored cytoplasm (Figs. 1a and 6b). X cells have not shown immunoreactivity for any of the antisera that we have tested. X cells are usually found in the islets, near the blood vessels, alternating with insulin cells.

Mixed Cells

We have often observed endocrine–exocrine cells containing organelles characteristic of exocrine cells, e.g., zymogen granules, and at the same time containing numerous secretory endocrine granules. Some exocrine acini are composed of normal exocrine cells and some adjacent mixed cells. Among the heterogeneous endocrine granules of these cells it is easy to recognize some granules with their content aligned in a beads-on-a-string fashion, just as in the insulin cells. In addition, in semithin sections immunostained with anti-[Met]ENK we have found that in some areas the cytoplasm of exocrine cells shows a disperse spotted immunolabeling, probably corresponding to the endocrine granules of some mixed cells.

Morphometric Studies

Each endocrine cell type shows a different distribution within the pancreas (Fig. 16). The percentages of insulin, glucagon / PP, and somatostatin cells are 37.2 ± 4.6, 48.8 ± 6.9, and 14.0 ± 4.9, respectively (Fig. 17). The extrainsular endocrine cell population represents 44.9 ± 7.9%, while the insular cells represent 54.2 ± 7.9% of the total counted cells. Insulin cells are almost always located within islets (94.2 ± 2.1%). On the contrary, A cells are found mainly scattered within the exocrine glands (79.2 ± 6.4% of total glucagon / PP cells) and represent the majority of the extrainsular endocrine cell population (86.4 ± 4.3%). The ratio of insular to extrainsular somatostatin cells is 3:1. Finally, we have found cells representing all four endocrine cell types within the epithelium of the pancreatic ducts, mainly glucagon / PP cells.

DISCUSSION

In the present study, four endocrine cell types have been identified in the pancreas of *Rana temporaria* adult specimens by applying ultrastructural, immunocytochemical, silver impregnation, and Giemsa staining techniques: insulin, glucagon / PP, somatostatin cells, and a fourth cellular type, X cells, of unknown peptide(s) content. A great amount of extrainsular cells, especially of the glucagon / PP type, have been found.

Characterization of Rana temporaria Endocrine Cells

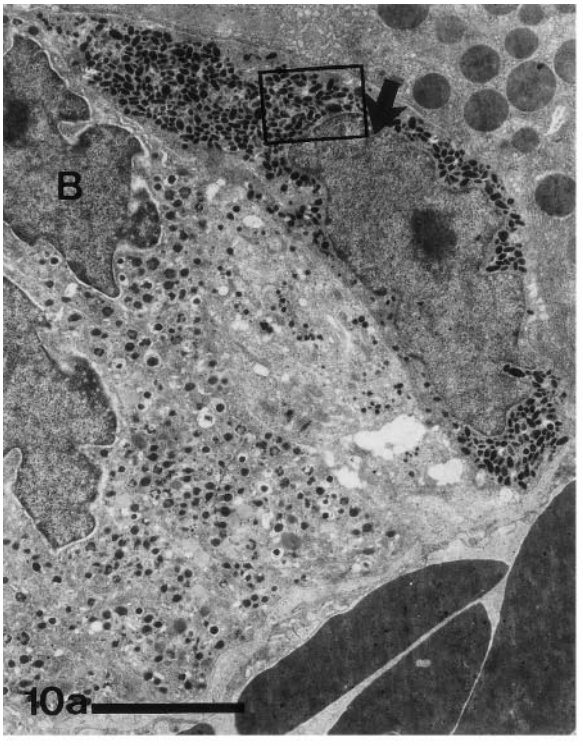
Insulin (B) cells. The morphological features of B cells in the adult frog correlate well with those described for such cells in tadpoles of this same species (Ortiz de Zárate *et al.,* 1991). Both larval and adult B cells have low affinity for the colorants, are not labeled with silver impregnations, and show similar ultrastructural traits. Crystalline inclusions in secretory granules of B cells have also been previously described in other amphibian species (Prieto Díaz *et al.,* 1967; Kobayashi, 1969; Tomita and Pollock, 1981; Accordi *et al.,* 1998)

FIG. 6. Serial thin (a) and semithin sections (b) of an islet where B, D, and X cells, are easily distinguished by their ultrastructural characteristics (a) and by their response to Giemsa stain (b). v, blood vessel. (c), detail of the upper X cell in (a). Original magnifications: (a) ×2750; bar, 5 µm; (b) ×1300; bar, 20 µm; (c) ×6250; bar, 2 µm.

FIG. 7. Serial semithin sections of an endocrine cluster where two cells (arrows) are simultaneously immunoreactive for both insulin (a) and [Met]enkephalin (b) antisera; c, capillary; Original magnification ×1300; bars, 10 µm.

FIG. 8. Insulin secretory granules immunolabeled with insulin antibodies by the colloidal gold technique; Original magnification ×50,000; bar, 0.5 µm.

FIG. 9. Absorption control in serial sections. (a) Immunostaining for anti-[Met]enkephalin. (b) Immunostaining is abolished when the antiserum has been preabsorbed with [Met]enkephalin; Original magnification ×125; bars, 100 µm.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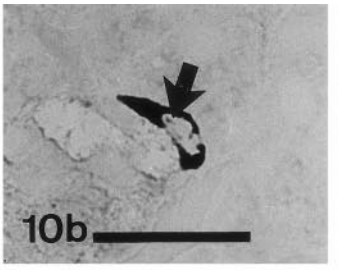
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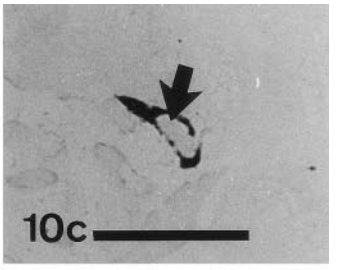
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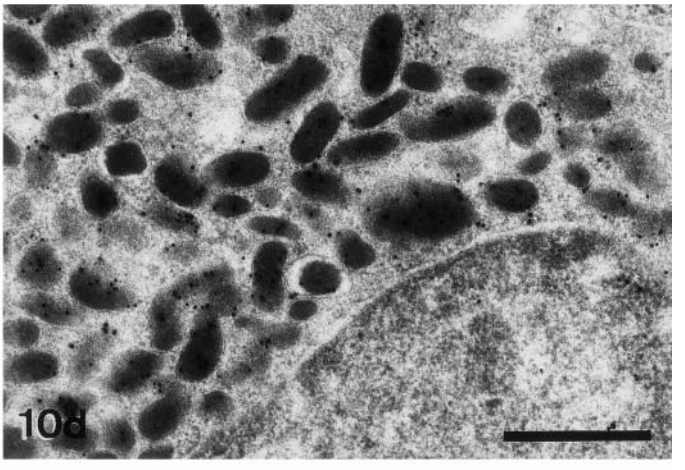
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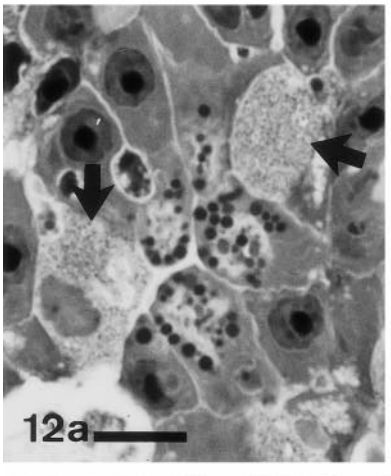
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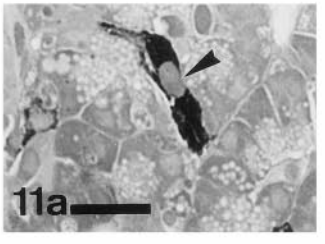
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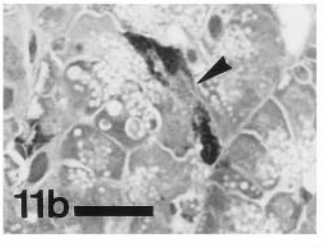
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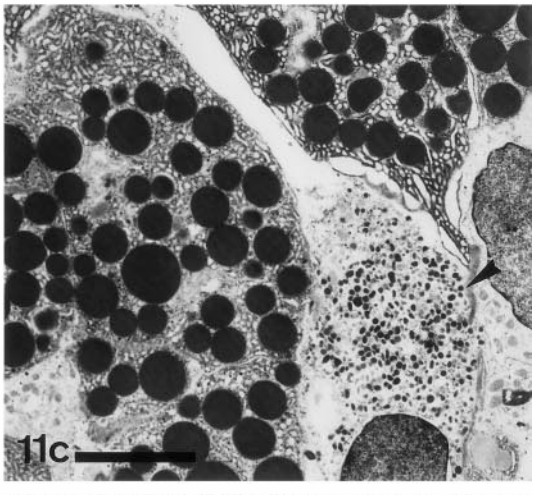
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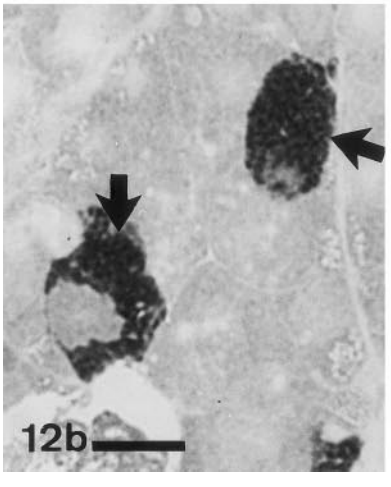
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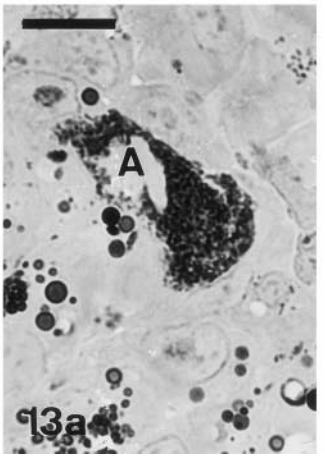
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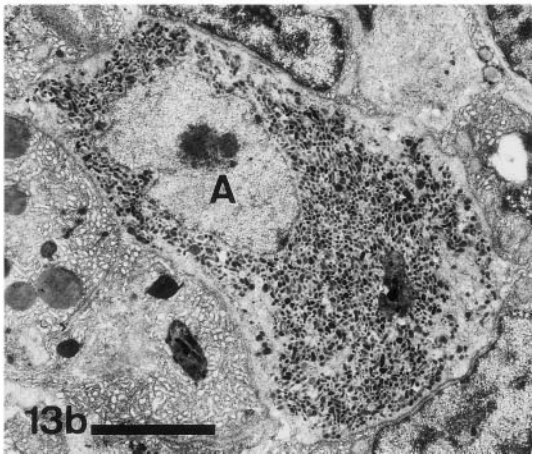
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and in nonamphibian vertebrates (Pelletier, 1977; López *et al.*, 1988). Although different types of granules were found, we did not have sufficient evidence to support the existence of more than one type of insulin cell, as proposed in the toad *Bufo bufo* (Accordi *et al.*, 1998).

A new finding now reported is the colocalization of [Met]ENK immunoreactivity in B cells of the frog pancreas. This is the first time that [Met]ENK-immunoreactivity is observed in amphibian pancreatic endocrine cells. We have not observed immunoreactivity for any of the other antisera tested that are specific for peptides derived from the three known opioid precursors (pro-enkephalin, pro-dynorphin, and pro-opiomelanocortin). In the B cells of guinea pig [Met-]ENK-immunoreactivity has already been reported and considered as a marker for the presence of a pro-enkephalin in these cells (Cetin, 1990). The presence of the prohormone might also be true for amphibian pancreas.

Glucagon/PP (A/PP) cells. The ultrastructural features of A / PP cells in adult frogs are also coincident with those observed in tadpoles (Ortiz de Zárate *et al.*, 1991). Nevertheless, our observations and previously reported studies (Prieto Díaz *et al.*, 1967; Trandaburu and Trandaburu, 1968; Kobayashi, 1969; Tomita and Pollock, 1981; Oikawa *et al.*, 1992; Accordi *et al.*, 1998) suggest that there exist species-specific differences in the ultrastructure of the secretory granules of the A / PP cells in amphibians.

In the present work these cells have always been found to be immunoreactive for both glucagon and PP, as reported previously in the same species (El-Salhy *et al.*, 1982; Buchan, 1985; Putti *et al.*, 1997). Colocalization of the two peptides is a frequent feature, especially in amphibians, both in urodeles and anurans (see also Kaung and Elde, 1980; Putti *et al.*, 1990; Oikawa *et al.*, 1992; Putti *et al.*, 1995; Reinecke *et al.*, 1995; Maake *et al.*, 1998). However, this is not a general feature of the amphibian pancreas. In some species the four classic endocrine cell types have been reported separately, i.e., in *Rana catesbeiana* (Tomita and Pollock, 1981) and *Bufo bufo* (Grassi Milano and Chimenti, 1995; Accordi *et al.*, 1998). At the same time, different patterns of colocalization have been reported: complete glucagon / PP colocalization—*Triturus* and some red frogs (Putti *et al.*, 1990)—or A / PP cells coexisting with occasional A and PP cells—*R. pipiens* and *Xenopus* (Reinecke *et al.*, 1995; Maake *et al.*, 1998). The observations made by El-Salhy (El-Salhy *et al.*, 1982), who found that different patterns of colocalization result from different methodological approaches, are noteworthy. Finally, it has been suggested that glucagon / PP cells could be identified with the amphiphil cells (Epple, 1967; Trandaburu *et al.*, 1969; Trandaburu, 1970; Putti *et al.*, 1990), transient cells with double hormonal potentialities and more abundant in the spring specimens.

GLP1—but not GLP2—immunoreactivity has been detected in *Rana temporaria* glucagon / PP cells. In amphibians, two glucagon-like peptides have been sequenced and found to be structurally similar to human GLP1 (*Bufo;* Conlon *et al.*, 1998a) or human GLP1 and GLP2 respectively (bullfrog; Pollock *et al.*, 1988). Therefore, GLPs of *R. temporaria* could resemble those of *Bufo.* Finally, in contrast with descriptions reported by Putti and co-workers (1995, 1997), we have not localized PYY or NPY immunoreactivity in A / PP cells of the frog pancreas. The different antigenic recognition of the antisera might explain these differences in the results.

Somatostatin (D) cells. In the present work the somatatostatin cells have been clearly identified not only ultrastructurally and immunocytochemically, but

FIG. 10. An A cell (arrows) in serial thin (a) and semithin sections immunostained for glucagon (b) and GLP1 (c). B, insulin cell. (d) Enlargement of rectangle in (a) shows glucagon-immunolabeling of A granules at the ultrastructural level. Original magnifications: (a) ×4350, bar, 5 μm; (b and c) ×550, bars, 40 μm; (d) ×20,600; bar, 1 μm.

FIG. 11. Serial semithin sections of two cells immunostained for glucagon (a) and PP (b). The larger cell (right, arrowheads) extends a paracrine process among the exocrine cells; original magnification: ×550; bars, 20 μm. (c) Electronography of this cell observed in a serial thin section; original magnification: ×3450; bar, 5 μm.

FIG. 12. Scattered glucagon / PP cells (arrows) in serial semithin sections. (a) Giemsa stain. (b) Immunostaining for PP. Original magnification (a and b): ×1300; bars, 10 μm.

FIG. 13. Serial semithin (a) and thin (b) sections of a glucagon / PP cell; (A). (a) Grimelius silver impregnation. Original magnifications: (a) ×1300, bar, 10 μm; (b) ×3450, bar, 5 μm.

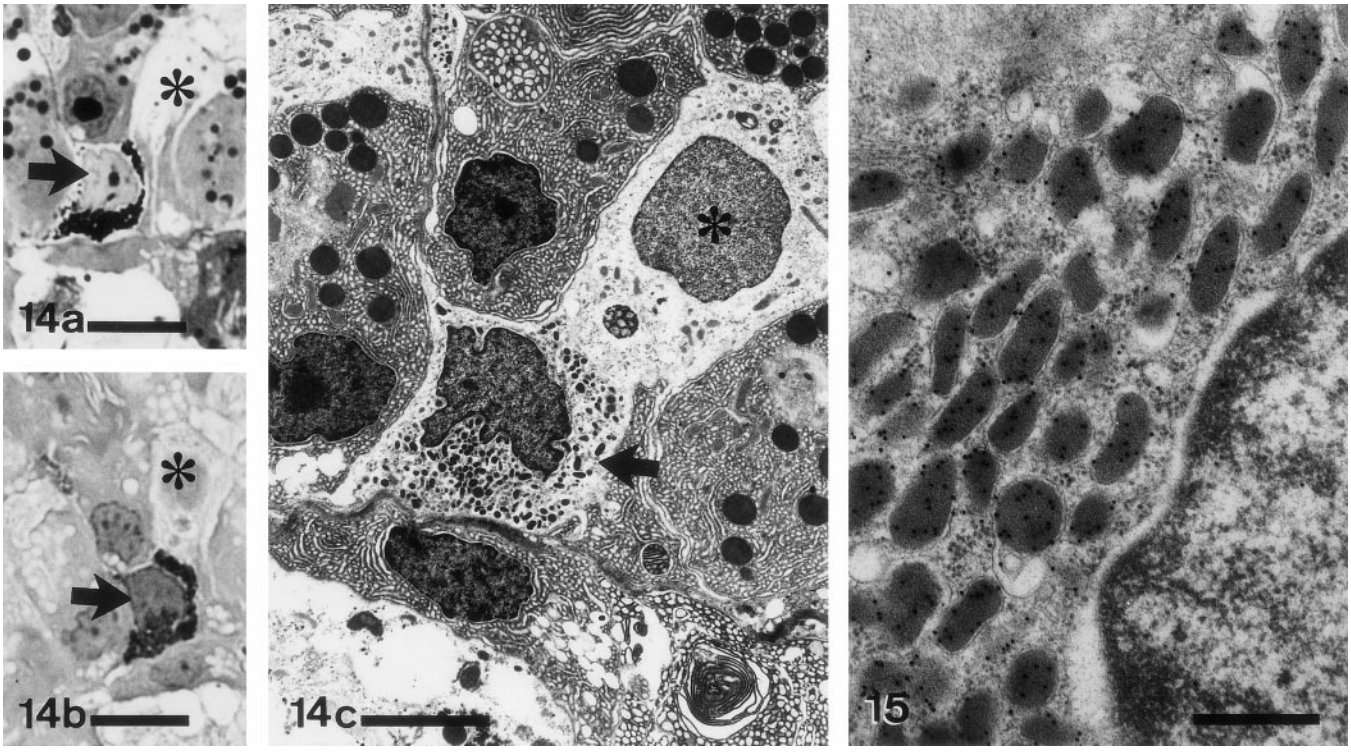

FIG. 14. Somatostatin cell (arrows) in serial semithin (a and b) and thin (c) sections. Asterisks indicate centroacinar cell. (a) Giemsa stain. (b) Immunostaining for somatostatin. Original magnifications: (a and b) ×1300; bars, 10 μm; (c) ×3450, bar, 5 μm.

FIG. 15. Detail of D granules immunolabeled at the ultrastructural level with somatostatin antibodies; Original magnification ×33,000; bar, 0.5 μm.

also by their characteristic deep purple color resulting from Giemsa staining applied to semithin sections. This latter technique has also been useful in distinguishing D cells in the pancreas of other nonmammalian vertebrate species, including fishes (Donoso, 1985) and reptiles (López *et al.*, 1988). Although there are some differences in average size, their secretory granules are comparable to those of the somatostatin cells of the tadpoles of the same species (Ortiz de Zárate *et al.*, 1991) and of other amphibians (Trandaburu and Trandaburu, 1968; Kobayashi, 1969; Tomita and Pollock, 1981).

X cells. X cells have been classified as endocrine cells based on their characteristic small, dense secretory granules. In addition, most of X cells are distributed within islets. X cells have been characterized as a fourth, different endocrine cell type because their secretory granules show a different ultrastructure and its cytoplasm exhibits a unique blue color after Giemsa staining. Furthermore, none of the six antisera which have rendered positive results in B, A/PP, or D cells have stained X cells. According to consulted literature, there are endocrine cells in the pancreas of some amphibian species different from the four classic pancreatic endocrine cell types and immunoreactive for other peptides, e.g., ACTH, oxytocin, PHI, VIP (Putti *et al.*, 1995, 1997), and adrenomedullin (López *et al.*, 1999). Hence, all these substances are good candidates to be stored in X cells, especially those described in nonclassic endocrine cells with a distribution pattern similar to that of *R. temporaria* X cells (mainly insular). This is the case with VIP-immunoreactive cells found in *R. temporaria* and other frogs (Putti *et al.*, 1997) and adrenomedullin-immunoreactive cells found in urodeles and anurans (López *et al.*, 1999). In spite of the fact we have not obtained immunoreactivity for VIP and ACTH, we cannot rule out their presence. Further studies are needed to clarify this issue.

Mixed cells. The presence of mixed endocrine–exocrine cells described here has also been observed in

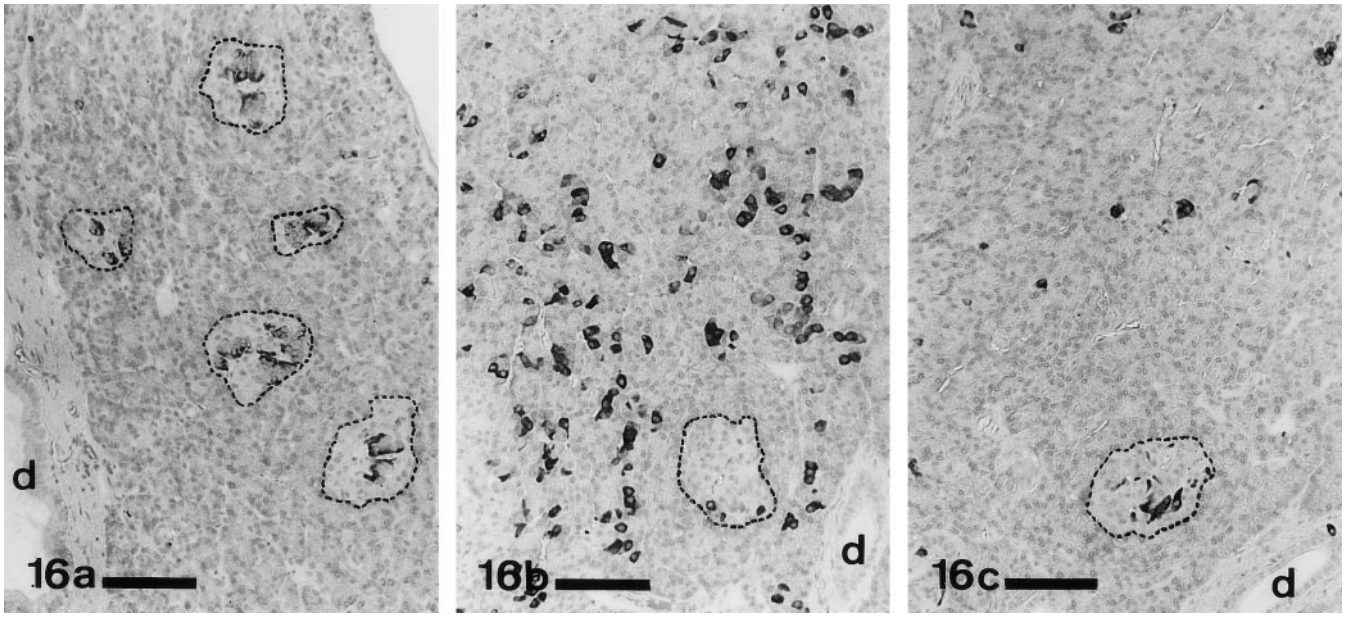

FIG. 16. Panoramic micrographs of *Rana temporaria* pancreas immunostained for insulin (a), glucagon (b), and somatostatin (c) showing the distribution of endocrine cells. B and D cells are mainly located within the islets, while A / PP cells are mainly scattered among exocrine tissue. Endocrine islets are delimited with broken lines; d, exocrine ducts; Original magnification ×125; bars, 100 µm.

the developing pancreas in *Rana temporaria* (Ortiz de Zárate *et al.*, 1991), in other amphibian species (Kobayashi, 1969), and in other vertebrates as well (Forssmann, 1976; Rombout *et al.*, 1979; Titlbach, 1981). The type of endocrine granules that they contain depends on the species, but β granules have frequently been shown. The features of some of the endocrine granules we have observed and their [Met]ENK immunoreactivity, which is only found in insulin cells, supports the hypothesis that mixed cells are mainly of the β type, and thus the mixed cells may secrete insulin. The origin and functional significance of mixed cells is still unknown (Kobayashi, 1969; Theret *et al.*, 1975; Forssmann, 1976; Zafirova *et al.*, 1992).

Distribution of Pancreatic Endocrine Cells

It is generally accepted that cytoarchitectural distribution of the different types of endocrine cells within the pancreas is important for their mutual functional interactions. There are very few data concerning angioarchitecture of amphibian endocrine islets and its relationship with the different endocrine cell types (Syed Ali, 1989). In rats, it has been strongly suggested that the order of islet perfusion, both in the physiological pancreatic condition (Bonner Weir and Orci, 1982; Samols *et al.*, 1988) and in islet isografts (Menger *et al.*, 1994), is from the B-cell core to the mantle of A and D cells (B → A → D). This would facilitate the role of B cells inhibiting A-cell secretion and of A cells stimulat-

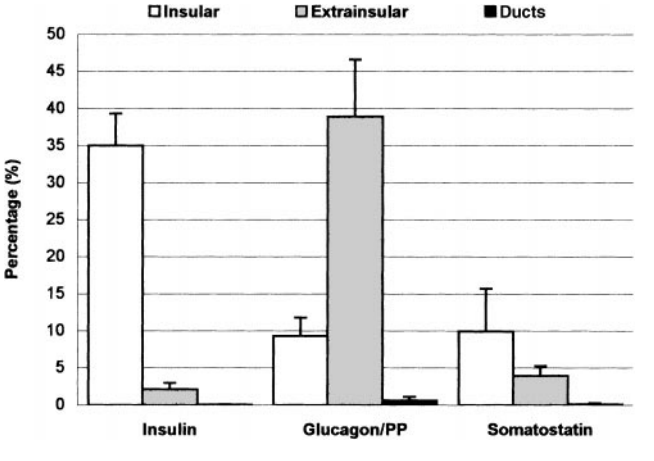

FIG. 17. Quantification and distribution of the endocrine cell types in the pancreas of *Rana temporaria.* Estimations are expressed as a percentage of the total number of insulin, glucagon / PP, and somatostatin endocrine cells (X cells are not included). Exact percentages are specified in the text. Bars represent SD.

ing D-cell secretion. On the other hand, cytoarchitectural distribution of endocrine cells can also be very influential on the neighboring exocrine parenchyma, e.g., via the postulated antagonistic effect of glucagon and insulin in the regulation of exocrine pancreatic secretion (Schönfeld and Müller, 1994).

Morphometric studies of frog pancreas. The volumetric estimation of the endocrine component of the amphibian pancreas has been previously calculated for different species (Kaung and Elde, 1980; El-Salhy *et al.*, 1982; Francini *et al.*, 1996) ranging from 2% (*Rana pipiens*) to 3.3% (*R. temporaria*) of the total pancreas. These reports also provide the relative volume occupied by B, A, D, and PP cells. For example, in *R. temporaria* 30% of the endocrine cells are B cells, 30% A, 9% D, and 30% PP cells (El-Salhy *et al.*, 1982). Taking into account that glucagon and PP immunoreactivity colocalize, the data could be corrected to 44, 44, and 11% B, A/PP, and D cells, respectively. In contrast, the present study shows that the A/PP cells are more abundant than B cells (48 and 38%, respectively). Some differences in the methodological approach could explain the difference from the earlier study. While El-Salhy and co-workers reported a proportion based in volumetric estimations, our data are based in cellular counts. Our study also provides new quantitative data regarding the pattern of distribution for each endocrine cell type, which was not contemplated in previous volumetric reports. For instance, we showed that almost half of the pancreatic endocrine component is extrainsular. This distribution of endocrine cells has also been reported in the toad (Francini *et al.*, 1996). In both species more than 80% of the extrainsular component is constituted by A/PP cells. Also, in both species B cells are almost restricted (92–94% of these cells) to the insular compartment. Although no quantitative data have been reported, some other authors have also underlined the importance of the extrainsular endocrine component of the pancreas of amphibians (Brinn and Epple, 1990; Putti *et al.*, 1990, 1997).

Amphibian extrainsular endocrine component. The ontogenetic and physiological significance of the extrainsular distribution of the endocrine cells, especially A/PP cells, has already been widely reviewed and discussed. As we have proposed in earlier reports (Ortiz de Zárate *et al.*, 1991), scattered endocrine cells might originate from *in situ* differentiation of stem cells within the tubular acini or might correspond to endocrine cells that remain in the ducts or acini when, in tadpoles, the endocrine clusters ''pinch off'' from the exocrine system to form the endocrine islets. It has also been postulated that they might arise from high cell-proliferative activity of preexisting endocrine cells (Putti *et al.*, 1997). Based on studies carried out in mammals, it has been suggested that the number of A/PP cells in the diffuse component is so great because in the developing pancreas they might be the precursor of all the endocrine cell types (Putti *et al.*, 1997). These totipotential cells, initially secreting almost all the pancreatic hormones, would lose this potentiality during development, completely in mammals and partially in some lower vertebrates, where multiple colocalization can be found in the same cell in adults. Nevertheless, neither in *Xenopus* pancreas nor in *Rana* has colocalization been described at any stage of development (Maake *et al.*, 1998).

From the physiological point of view, the presence of scattered cells first suggests that the target organ of their hormones might be the exocrine pancreas. The communication between endocrine and acinar cells might occur by paracrine secretion (for review see Putti *et al.*, 1997). In this way, scattered A/PP cells might modulate the exocrine secretion, since their secretory products, mainly PP, essentially have an inhibitory action on pancreatic secretion.

In summary, this study has shown the existence of three well-characterized endocrine cell types in the pancreas of *Rana temporaria*, namely insulin cells, glucagon/PP cells, somatostatin cells, and a fourth cell type whose content and function remain unknown. Not only classical hormones but other endocrine substances have been shown to be produced by these cells. Thus [Met]ENK immunoreactivity in amphibian pancreatic endocrine cells is reported for the first time. Also, the predominance, at least from the quantitative point of view, of the extrainsular endocrine component of the amphibian pancreas, mainly of glucagon/PP cells, is demonstrated.

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