Anteroposterior Variation of the Cell Types in the Interrenal Gland of the Male Toad *Rhinella arenarum* (Amphibia, Anura)

Eleonora Regueira,* María Florencia Scaia, María Clara Volonteri, and Nora Raquel Ceballos

Laboratorio de Endocrinología Comparada, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires and Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina

ABSTRACT The interrenal gland of anurans synthesizes the steroids aldosterone and corticosterone, but it is unknown whether these hormones are synthesized by the same cell type. In this work, we aim to elucidate whether there are different steroidogenic cell types and whether they have specific regionalization in the interrenal gland of the male toad *Rhinella arenarum*. We characterized all cell types using histological, immuhistochemical, and histochemical methods as well as transmission electron microscopy. Furthermore, we evaluated the organization of the cell types in the gland and anteroposterior variations in the synthesis of the steroids. We found evidence of five cell types: two morphologically different steroidogenic cells, type 1: polyhedral cells tightly attached to each other that have spherical euchromatic nuclei and type 2: retracted cells loosely attached to each other that have oval heterochromatic nuclei. Cell type 2 is mainly observed in the inner zone of the gland. In addition, we observed two types of chromaffin cells, called type 3 and 4 cells, randomly distributed throughout the interrenal gland, as well as type 5 cells, recognized as summer cells. Morphometric analyses of the cell types in the anterior and posterior zones of the interrenal showed that the ratio "area of type 2 cells/total interrenal area" is significantly lower in the posterior zone. In vitro incubations showed that the posterior portion of the gland produces significantly higher amounts of both corticosterone and aldosterone. Overall, our results suggest that the type 2 cells are less active to synthesize both aldosterone and corticosterone, compared to type 1 cells. Unlike most previous reports on the interrenal gland of anurans, in *R. arenarum* there is a zonation of the steroidogenic cell types, which implies that the organ is not anteroposterior or dorsoventrally homogeneous. J. Morphol. 274:331–343, 2013. © 2012 Wiley Periodicals, Inc.

KEY WORDS: anuran; interrenal gland; steroidogenic cells; histology; transmission electron microscopy; radioimmunoassay

INTRODUCTION

The adrenal/interrenal gland of vertebrates consists of two types of endocrine cells: the steroidogenic and the chromaffin cells. In tetrapods, steroidogenic cells synthesize mainly glucocorticoids and mineralocorticoids that are involved in the modulation of the stress response, reproduction, growth and osmoregulation, among others (Sapolsky et al., 2000; Funder, 2010). On the other hand, chromaffin cells, synthesize catecholamines such as adrenaline and noradrenaline, which are secreted into the circulatory system of animals as a rapid response to acute stress (Perri and Capaldo, 2011). The topography and anatomical relationship of the adrenal/interrenal gland shows considerable variation among vertebrates. Also the arrangement and the degree of intermingling of chromaffin and steroidogenic cells may vary among vertebrate taxa (Grassi Milano and Accordi, 1983; Gallo and Civinini, 2003; Perry and Capaldo, 2011). In anuran amphibians, the interrenal gland is closely associated with the ventral surface of the kidney, surrounding the ventral renal vein, and is organized into anastomosing cell cords separated by large sinusoidal capillaries (Hanke and Weber, 1965; Piezzi and Burgos, 1968; Grassi Milano and Accordi, 1983). Cords are composed of steroidogenic cells intermingled with small groups of chromaffin cells (Grassi Milano and Accordi, 1983). In some species such as *Rana temporaria* and *R. catesbeiana*, a third cell type was also found (Stilling cells) with characteristics similar to mast cells. The function of these cells is discussed controversially (Moorhouse, 1963; Sottovia-Filho, 1974;

Kawamura, 1986; Lesouhaitier et al., 1996). Histological studies in *R. catesbeiana* and *R. ridibunda* described the chromaffin cells as mainly localized in the central region of the gland and that steroidogenic, and Stilling cells constitute the peripheral cords (Nonaka et al., 1995; Süren, 1999). In addition, Hanke and Weber (1965) in *R. temporaria* and Süren (1999) in *R. ridibunda* reported that the more peripheral steroidogenic cells showed signs of greater activity than the most central cells. In both articles, authors interpreted that the differences between steroidogenic cells could be due to the functional status of each cell or to differences in their secretory products. Regarding the latter possibility, only one study in *R. catesbeiana* postulates that corticosterone and aldosterone are synthesized by different cells (Varma, 1977).

Many studies have described the histological organization of the interrenal gland of anurans (Moorhouse, 1963; Hanke and Weber, 1965; Nagatsu et al., 1979; Grassi Milano and Accordi 1983; Delarue et al., 1988; Larcher et al., 1989; Nonaka et al., 1995; Castillo, 2008). However, there is no information describing the histological differences between all cell types or the anteroposterior distribution of cell types in the gland. In this study, we ask if there are different steroidogenic cells specialized in the synthesis of aldosterone and corticosterone in the interrenal gland of *Rhinella arenarum*. We studied the histological and ultrastructural characteristics of the gland, as well as the anteroposterior distribution of the different cellular types.

MATERIALS AND METHODS
Animals

Adult male toads of *Rhinella arenarum* (Hensel, 1867) were collected from a neighboring area of Buenos Aires City, Argentina, from November 2010 to April 2011. Toads were maintained under natural conditions with free access to water and fed crickets and zophobas. Animals were euthanized by immersion in 1% aqueous solution of MS222 (Sigma–Aldrich). The experiments comply with the Guiding Principles for the Care and Use of Research Animals promulgated by the Society for the Study of Reproduction and with the approval of the Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio, Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina.

Histology and Immunohistochemistry

The kidneys and their attached interrenal glands from six toads were rapidly excised, fixed in Bouin's fluid for 12 h at 4°C, dehydrated and embedded in paraffin:histoplast (50:50, %w/w). Serial transverse sections from both interrenal glands from each toad were cut at 7 μm, deparaffinized, hydrated, and used for histology and immunohistochemistry. For the analysis of the cell types and their organization, sections corresponding to the anterior and posterior zones from each specimen were stained with Masson's trichrome stain. In addition, selected sections were tested with the histochemical stain periodic acid-Schiff-hematoxylin (PAS-H; Cook, 1990), specific for neutral carbohydrates, to assess the presence of Stilling cells. To verify the identity of steroidogenic and chromaffin cells

observed with the classic histological technique, other sections of the serial sections from each specimen were submitted to immunohistochemistry. To identify steroidogenic cells an antibody against the 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD/I) enzyme (1:200; rabbit antihuman), kindly provided by Dr. Ian Mason (University of Edinburgh, UK), was employed. This enzyme is involved in the synthesis of both corticosterone and aldosterone; therefore, the antibody recognizes all types of steroidogenic cells. To identify chromaffin cells, an antibody against the tyrosine hydroxylase (TH) enzyme was used (1:1,000; rabbit antirat, Chemicon International, Billerica, MA). For antigen recovery, sections were boiled in 10 mmol L^{-1} sodium citrate at pH 8 for 10 min. The sections were subsequently incubated with 3% H_2O_2 in phosphate buffered saline (PBS) to inactivate endogenous peroxidases. To block unspecific staining, a blocking buffer with 5% nonfat dry milk in PBS was applied for 1 h. Next, primary antibody was applied to the sections overnight at 4°C in a humid chamber. For immunoperoxidase staining, sections were washed with PBS and incubated with biotinylated secondary antibody (1:500; DAKOCytomation, Denmark) for 1 h and then with peroxidase-conjugated streptavidin for 45 min (1:400; GE Healthcare, UK). Immunostaining was revealed with 3,30-diaminobenzidine solution (DAKO North America, Carpinteria, CA), which produces a brown precipitate, and the sections were counterstained with hematoxylin. Sections were examined using a Leica DM2000 microscope, and images were captured with an incorporated Leica ICC50 digital camera.

Western Blot of 3β-HSD/I and TH Enzymes

To assess the specificity of the heterologous antibodies used for the immunohistochemistry, homogenate from one interrenal gland was analyzed by Western blot. Samples from the homogenate were loaded onto 12.5% sodium dodecyl sulfate-polyacrylamide gel and electrophoresed for 140 min at 130 V. Proteins were transferred to polyvinyl difluoride membranes (Hybond LFP, Amersham) at 300 mA for 90 min, and membranes were incubated for 10 min in PBS containing 0.1% Tween-20 (PBS-T) and 3% peroxide hydrogen and blocked for 1 h at room temperature in PBS-T containing 5% nonfat dry milk. Incubations with primary antibodies were performed overnight at 4°C in PBS-T with 3β-HSD/I antibody (1:500) or with TH antibody (1:2,000). Membranes were then incubated with the goat antirabbit IgG secondary antibody coupled to horse radish peroxidase (1:5,000; Chemicon International). Immunopositive bands were visualized by enhanced chemiluminescent detection in a Fujifilm LAS-1000 chemiluminescent detection system.

Transmission Electron Microscopy

Small segments from the anterior region of the interrenals from two toads were fixed in 3% glutaraldehyde in PBS at pH 7.4 at room temperature for 24 h, rinsed in buffer, and postfixed in 1% OsO_4 (2 h, 4°C). Samples were dehydrated through an ethanol/acetone series before embedded in epoxy resin and polymerized. Semithin sections were cut at 2 μm and stained with 1% toluidine blue in 1% Na_2CO_3. Ultrathin sections were cut at 90–150 nm with a glass blade on a Sorvall Porter Blum MT2-B Ultra-Microtome (Ivan Sorvall Inc., Newtown, Conn., USA), stained with uranyl acetate and lead citrate (Reynolds, 1963), and observed with a Philips EM 301 transmission electron microscope a at the Centro de Microscopías Avanzadas (Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina).

Morphometry

For image analysis, low-power micrographs of both interrenal glands from each animal from the anterior and posterior ends, stained with Masson's trichrome stain or immunolabeled for TH

enzyme, were processed for morphometric investigation by a computerized image analysis system with a public domain Java image processing program (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, http://imagej.nih.gov/ij/, 1997–2012). The proportion of the less abundant type of steroidogenic cell in the anterior and posterior ends of the interrenal glands was evaluated in the Masson's trichrome stained sections, because these cells can be properly differentiated with this classical histological technique. The proportional area occupied by the steroidogenic cells was calculated in each section according to the ratio, steroidogenic cells area/total interrenal area. The proportion of chromaffin cells in the anterior and posterior zones was evaluated in immunohistochemically prepared samples and was expressed as the number of TH-immunolabeled cells/total interrenal area. Values were recorded for each animal as the mean of right and left glands.

In Vitro Incubations

Interrenals from eight toads were separated from the kidneys under a binocular stereomicroscope and arbitrarily divided into an anterior, intermediate, and posterior zone to evaluate anteroposterior differences in the synthesis of corticosterone and aldosterone. Fragments from both glands of each toad corresponding to the same region were incubated together in 1 ml of Dulbecco's modified Eagle's medium at pH 7.4, containing 10% (v/v) of charcoal stripped fetal bovine serum and 10 mM Hepes, for 1 h at 28°C. After the incubation, media were stored at −20°C to further evaluate the production of corticosterone and aldosterone by radioimmunoassay. Fragments were weighed in an analytical balance (±0.2 mg).

Radioimmunoassay of Corticosterone and Aldosterone

Steroids in the incubation media were analyzed without extraction. For the assay, serial dilutions of the standard curve, nonspecific binding, maximum binding and samples were incubated with 10,000 dpm of [^{3}H] corticosterone (78.1 Ci/mmol, Perkin-Elmer, Boston, MA) or 10,000 dpm of [^{3}H] aldosterone (76.4 Ci/mmol, Perkin-Elmer, Boston, MA). The incubations with [^{3}H] corticosterone or [^{3}H] aldosterone were carried out in a final volume of 0.5 and 0.3 ml, respectively, at 4°C for 18–22 h. Corticosterone and aldosterone antibodies were kindly provided by Dr. Celso Gómez-Sánchez (University of Mississippi), and employed in a dilution of 1:9,000 and 1:1,000, respectively. The cross-reactivity with aldosterone or corticosterone was less than 0.01% for both antibodies (Gómez-Sánchez et al., 1975, 1987). Buffers for corticosterone and aldosterone assays were 10 mmol L^{-1} sodium borate at pH 8, 1% gelatin, 20 mM ethylenediaminetetraacetic acid (EDTA), and PBS at pH 7.4, 0.5% bovine serum albumin, respectively. Charcoal-dextran method was used to separate bound and free hormones. The sensitivity of corticosterone and aldosterone assays was 50 and 100 pg/ml, respectively. Mean intra-assay variation and interassay variation were 9.3 and 12.2%, respectively, for corticosterone assay and 6.2 and 13.3%, respectively, for aldosterone assay. Radioactivity in the supernatant (bound) was determined by liquid scintillation counting with a Wallac 1409 DSA equipment (Wallac, Turku, Finland). The scintillation cocktail for all samples was OptiPhase-Hi safe 3 (Wallac). Corticosterone and aldosterone production was expressed as media concentrations per milligrams of interrenal.

Statistical Analysis

Results from the morphometric analysis and the in vitro incubation were expressed as means ± standard error. Values from morphometry were analyzed with a one-way analysis of variance (ANOVA) test and a posteriori contrast using Tukey's test. For the in vitro assays, a randomized block design was used to minimize the variability between replicates (each animal). Two-way ANOVAs followed by Tukey's multiple comparisons tests were used to detect significant differences among the synthesis of hormones in the anterior and posterior fragments. For the two-way ANOVAs test, a Log_{10} transformation was used. Differences were considered significant when $P < 0.05$. Before statistical analysis, data were tested for normality and homogeneity using Lilliefors and Bartlett's tests. There was no interaction between the experimental factor (each animal) and the region of the interrenal gland evaluated.

RESULTS
Standard Histology and Immunohistochemistry

The interrenal gland of *R. arenarum* is a vermiform orange organ tightly attached to the ventral side of the kidney (Fig. 1A). Cross sections of the kidney and interrenal showed that the gland is not intermingled with the renal tubules and that the ventral side of the gland is surrounded by a capsule of dense connective tissue (Fig. 1B). Histological studies showed that the gland is organized into anastomosing pluristratified cords separated by sinusoidal capillaries that surround the renal efferent vein (Fig. 1C,D). The sections stained with the Masson's trichrome showed that the cords contain three different cell types: type A cells (the typical steroidogenic cells) that were spherical in shape with a clear and vacuolated cytoplasm and a spherical euchromatic nucleus with conspicuous nucleoli (Fig. 2A; Table 1); type B cells that were dark, basophilic, and slightly columnar in shape with an oval euchromatic nucleus, with darker chromatin than type A cells (Fig. 2B; Table 1); and type C cells that were triangular or slightly oval cells, larger than the types A and B, with an euchromatic nuclei and large nucleoli (Fig. 2C; Table 1). Individual type C cells were usually associated with a cell with an oval nucleus (Fig. 2C). In addition, PAS histochemical stain revealed the presence of solitary cells with PAS-positive cytoplasmic granules and an eccentric nucleus (Fig. 2D), which could correspond to the so called Stilling cells (Moorhouse, 1963; Sottovia-Filho, 1974; Kawamura, 1986; Lesouhaitier et al., 1996) previously reported as absent in this species (Piezzi and Burgos, 1968).

The serial sections of interrenals were also analyzed by immunohistochemistry to differentiate steroidogenic from chromaffin cells. This analysis showed that type A and B cells were immunoreactive for the steroidogenic enzyme 3β-HSD/I (Fig. 3), whereas type C cells were immunoreactive for the TH enzyme, the limiting enzyme in catecholamine synthesis (Fig. 4). When the primary antibody was omitted, no immunoreactivity was observed (Figs. 3E and 4D). In addition, Western blot analysis showed the specificity of the antibodies for the corresponding enzyme of the toad, because they recognized proteins with an apparent molecular weight corresponding to that previously
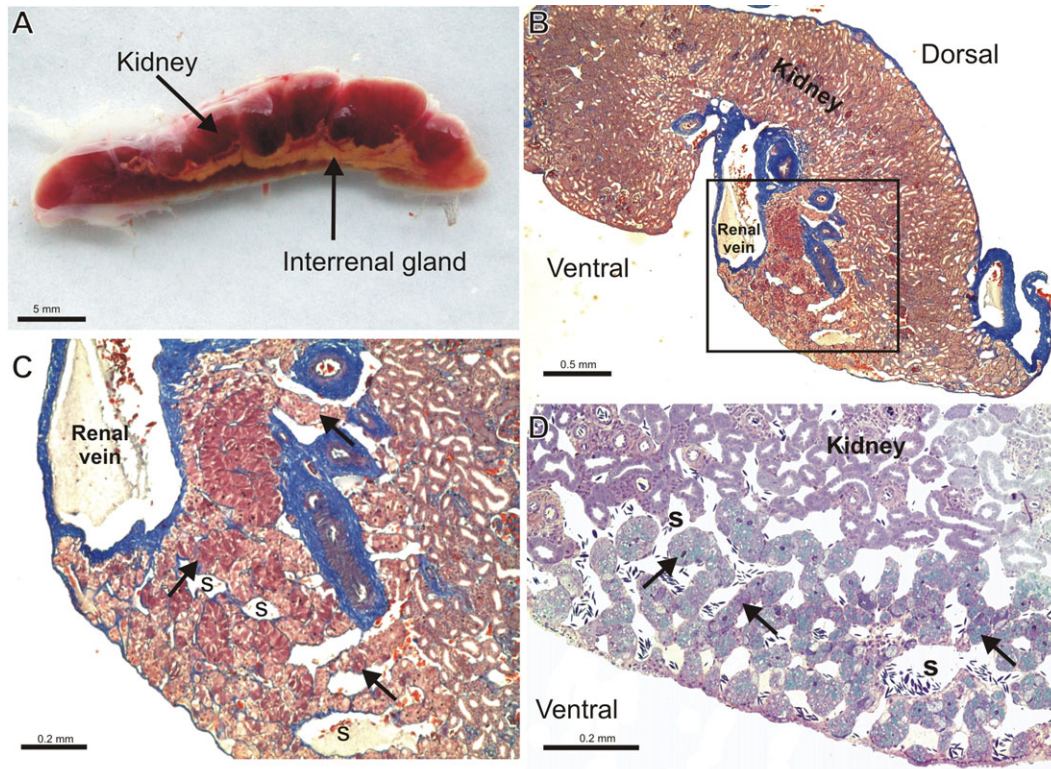
Fig. 1. *Rhinella arenarum*, gross morphology and topography of the interrenal gland in cross sections. (**A**) Ventral view of the kidney and interrenal gland. (**B**) Cross section of paraffin-embedded interrenal and kidney (7 μm) stained with Masson's trichrome. (**C**) Higher magnification of the portion marked with a rectangle in (B). Anastomosing cords separated by sinusoidal capillaries surrounding the renal efferent vein. (**D**) Cross section of epoxy resin-embedded interrenal and kidney (2 μm) stained with toluidine blue. Arrows: anastomosing cords; S: sinusoidal capillaries.

described for the 3β-HSD/I and TH enzymes (42 and 59 KDa, respectively; Arbogast and Voogt, 2002; Thomas et al., 2002; Figs. 3F and 4E). Taken together, these results suggest that type A and B cells are steroidogenic cells, whereas type C cells are chromaffin cells.

Semithin Sections and Transmission Electron Microscopy

To further assess the identity and characteristics of the cell types previously described, interrenals were analyzed by transmission electron microscopy (TEM). The analysis of the semithin sections stained with toluidine blue showed five different cell types. Type 1 and 2 corresponded to two different steroidogenic cells. Type 1 cells were polyhedral, clear, with lipid droplets, blue granules, and spherical nuclei with euchromatin and several nucleoli (Fig. 5A–C). On the other hand, type 2 cells were irregular in shape, darker than type 1 cells, with lipid droplets, blue granules, and oval and dark nuclei with irregular edges (Fig. 5B). Type 3 and 4 cells were less abundant than type 1 and 2, and they were usually found in close contact with each other and were larger than the

steroidogenic cells (Fig. 5C,D). Type 3 cells were polyhedral, elongated, with highly packed small cytoplasmic granules and oval nuclei (Fig. 5C). Type 4 cells were larger than the other types and were oval and elongated (Fig. 5D). Their spherical nuclei had a central position with one large nucleolus, and their cytoplasm had small scattered granules (Fig. 5D). These cells were usually associated with another cell whose nuclei were oval (Fig. 5D). Due to the large size of these cells and to the presence of cytoplasmic prolongations, it was common to observe portions of these cells without nuclei (Fig. 5C). Type 5 cells were spherical and smaller than all the other types, and their cytoplasm had large dark granules (Fig. 5C). These cells were always surrounded by type 1 and 2 cells (Fig. 5C).

Transmission electron microscopy allowed the characterization of the distinct cell types observed in the semithin sections. There were two different cells containing lipid droplets, types 1 and 2, previously described by toluidine-blue staining (Fig. 6A,B). Both types had abundant mitochondria with closely packed tubular cristae (characteristic of steroid producing cells; Fig. 6C–F), an abundant smooth endoplasmic reticulum made up of vesicles
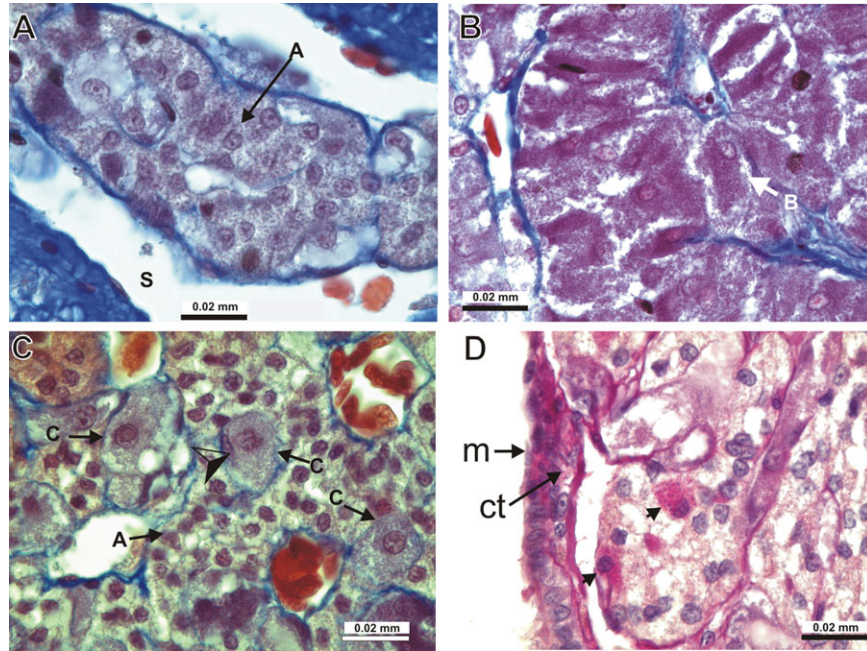
Fig. 2. *Rhinella arenarum*, cell types in the interrenal gland identified with paraffin histology. (**A–C**; 7 μm) Stained with Masson's trichrome. (A) Arrow: type A cells (for details see "Results" section). S: sinusoidal capillaries. (B) Arrow: type B cells. (C) Arrows: type A and C cells. Arrowhead: nucleus of a cell associated with type C cell. (**D**; 7 μm) Stained with the PAS-H. Short arrows: cells with PAS-positive cytoplasmic granules; ct: connective tissue; m: mesothelium.

and short cisternae and Golgi complex (Fig. 6G). Type 1 cells were in close contact and had lipid droplets of up to 5 μm (Fig. 6A,C,H). The nuclei were spherical with regular edges and lax chromatin with one large nucleolus (Fig. 6A,C). In type 2 cells, the plasma membranes of adjacent cells were not tightly attached as in type 1, showing few areas of contact between cells (Fig. 6E,I,J). Lipid droplets of type 2 cells had a diameter of up to 2 μm, and their nuclei were oval, with irregular edges, moderately condensed chromatin, and heterochromatin close to the nuclear membrane (Fig. 6B).

Type 3 and 4 cells were identified as chromaffin cells because of the characteristics of their cytoplasmic granules (Piezzi, 1967; Capaldo et al., 2004b). Type 3 cells had tightly packed granules of variable shape with an electron-dense core (Fig. 7A,B). The granules of type 4 cells had a fine granular core of variable electron density, some of them having a clear halo between the core and the limiting membrane (Fig. 7A,C). According to these characteristics, type 3 and 4 cells could correspond to noradrenaline and adrenaline producing cells, respectively (Piezzi, 1967; Capaldo et al., 2004b). As shown in semithin sections, there were small oval nuclei attached to type 4 cells (Fig. 7A).

Type 5 cells had an approximate size of 9 μm and were characterized by an oval shape with eccentric nuclei and by being surrounded by type 1 cells (Fig. 7D). Their cytoplasm contained numerous granules of variable size, shape, and electron density (Fig. 7E). Ultrastructural characteristics of these cells and their proximity to cells with lipid

TABLE 1. *Cell types in the interrenal gland of R. arenarum*

|	Histological technique						
Masson's trichrome	3β-HSD/I IHQ	TH IHQ	PAS	Semithin section	TEM	Identification of cell type	Distinctive characteristics
A	(+)	(−)	(−)	1	1	Steroidogenic	Polyhedral cell, tightly attached to each other, spherical nucleus with lax chromatin
B	(+)	(−)	(−)	2	2	Steroidogenic	Retracted cell, loosely attached to each other, oval nucleus with dense chromatin
C	(−)	(+)	(−)	3	3	Chromaffin	Noradrenaline granules (?)
				4	4	Chromaffin	Adrenaline granules (?)
NO	NO	NO	(+)	5	5	Stilling	Surrounded by lipid cells, eccentric nucleus, large granules

IHQ: immunohistochemistry; NO: not observed.
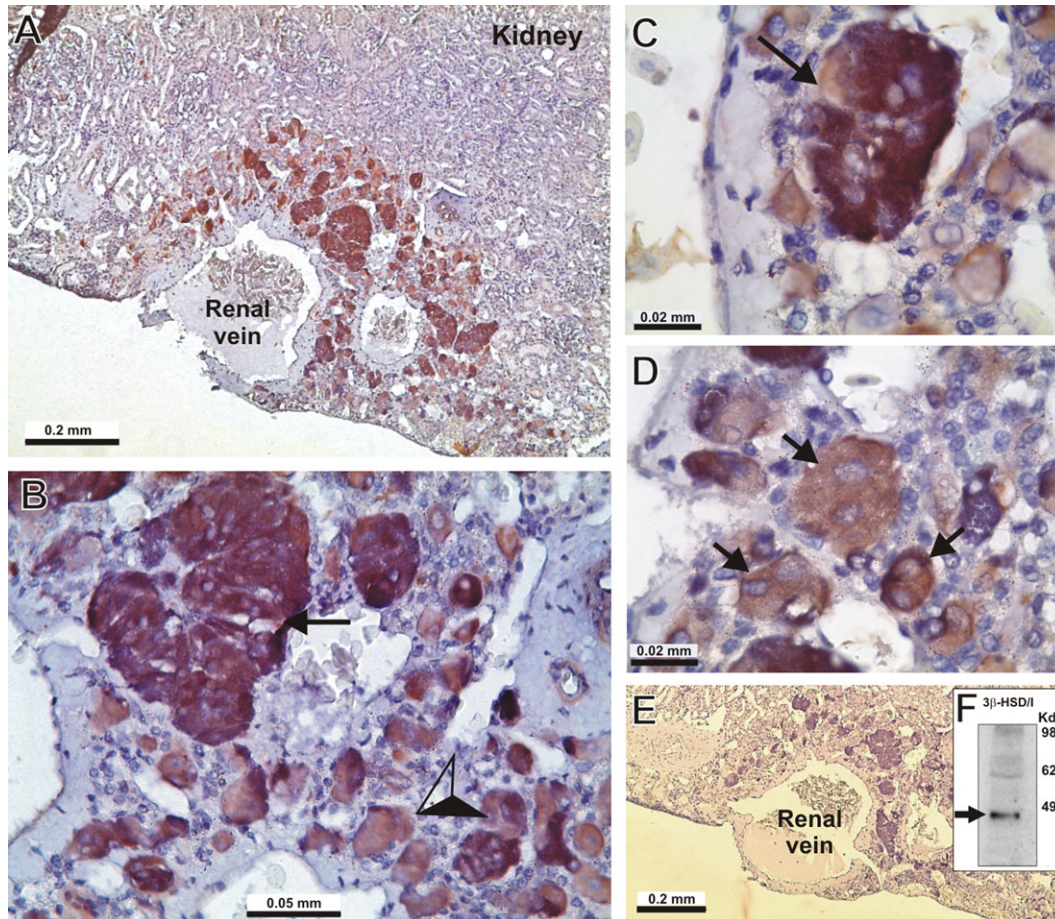
Fig. 3. *Rhinella arenarum*, immunohistochemical detection of steroidogenic cells. (**A**) Topographic view of paraffin-embedded cross section of interrenal gland (7 μm) immunostained with anti-3β-HSD/I antibody. (**B**)–(**D**) are details of the immunostained region shown in (A). (B) Arrowhead: type A cells; arrow: type B cells. (C) Higher magnification of type B cells (arrows). (D) Higher magnification of type A cells (arrow). (**E**) Negative control without the primary antibody. (**F**) Western blot analysis of 3β-HSD/I in homogenate of interrenal.

droplets suggest that they are similar to the Stilling or summer cells (Kawamura, 1986). Table 1 summarizes the distinctive characteristics of the cell types described in the interrenal gland of *Rhinella arenarum*.

Organization of the Different Cell Types

The anastomosing cords of the interrenal glands contained intermingled steroidogenic, chromaffin, and Stilling cells (Figs. 1 and 2). Transverse sections stained with Masson's trichrome showed that the most peripheral steroidogenic cells were mainly type A, whereas type B cells, also steroidogenic, were found mostly in the inner zone of the gland, as amorphous groups that did not seem to form cords (Fig. 8). Morphometric analysis of these cells in the anterior and posterior zones of the gland showed that there is a significant reduction of type B cells in the posterior zone of the glands ($P = 0.003$; Fig. 8, Table 2). However, type 1 ster-

oidogenic cells are the most abundant type, and they constitute almost all cells of the peripheral area of the gland. Therefore, and because there are only few chromaffine cells with a uniform distribution along the gland (Table 2), the area occupied by type 1 cells is almost equivalent to subtracting the "type 2 cells area" from the "total interrenal gland area." Strikingly, in the semithin sections of interrenals, corresponding to the anterior zone, there were no amorphous groups (Fig. 1D), suggesting that these groups could be a technical artifact due to the paraffin embedding procedure.

The immunohistochemistry of TH enzyme allowed the differentiation of the chromaffin cells, showing that these cells were scattered throughout the interrenal gland (Fig. 4A). In addition, semithin sections showed that chromaffin cells were in close contact to the sinusoidal capillaries (Fig. 5C,D). Morphometric analysis of the TH immunoreactive cells in the anterior and posterior zones of
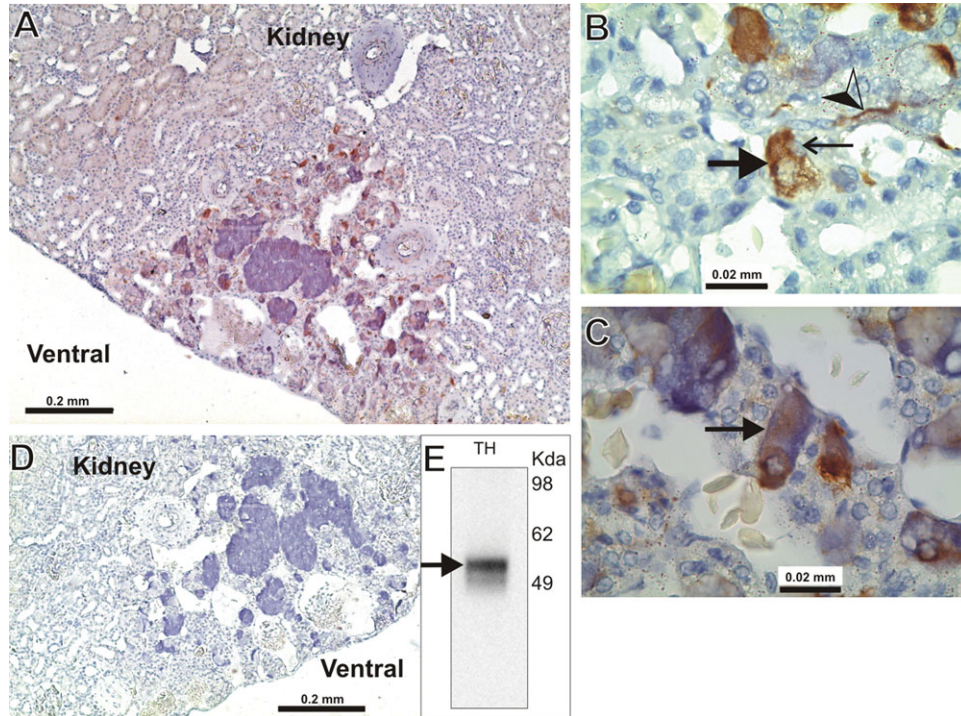
Fig. 4. *Rhinella arenarum*, immunohistochemical detection of chromaffin cells. (**A**) Topographic view of paraffin-embedded cross section of interrenal gland (7 μm) immunostained with anti-TH antibody. (**B**) and (**C**) are details of the immunostained region shown in (A). (B) Thick arrow: type C cell; thin arrow: nucleus of a cell associated to type C cell; arrowhead: cell prolongation. (C) Arrow: type C cell with a more oval shape. (**D**) Negative control without the primary antibody. (**E**) Western blot analysis of TH in homogenates of interrenals.

the interrenal indicated that there were no significant differences between the amount of chromaffin cells in both zones of the gland ($P = 0.753$; Table 2).

Anteroposterior Synthesis of Corticosterone and Aldosterone

To analyze whether regional variation existed in the synthesis of corticosterone and aldosterone, the in vitro biosynthesis of both steroids was analyzed by radioimmunoassay. Figure 9 shows that there was a significant difference in the synthesis of corticosterone and aldosterone in the posterior zone of the interrenal in comparison with the anterior zone ($P < 0.0001$ for corticosterone and $P = 0.005$ for aldosterone).

DISCUSSION

The integrated analysis of data obtained with different histological techniques demonstrated the presence of two types of steroidogenic cells, two types of chromaffin cells and also, as in other anurans, Stilling cells (Table 1). Steroidogenic type A cells, with a vacuolated cytoplasm and immunoreactive for the steroidogenic enzyme 3β-HSD/I, correspond to type 1 cells described in semithin

section and TEM examination (Table 1). In contrast, type B cells have a basophilic and nonvacuolated cytoplasm in paraffin-embedded sections, but they are also immunoreactive for the steroidogenic enzyme 3β-HSD/I. This type corresponds to type 2 cells of semithin section and TEM examination (Table 1). The ultrastructural study of type 2 cells shows that the plasma membranes of adjacent cells are not in close contact with each other, explaining why these cells are not compactly arranged in the middle of the interrenal gland and have a shrunken appearance in paraffin-embedded sections. In contrast, in semithin sections, lipids that remain in the cells avoided the shrinking and the loss of the organization as cords. Hanke and Weber (1965) also observed that in paraffin-embedded sections of the interrenal gland of *Rana temporaria* the steroidogenic cells of the middle of the gland were not compactly arranged.

Piezzi and Burgos (1968) described the fine structure of the interrenal gland of *Rhinella arenarum*, showing that cords are composed of one single type of steroidogenic cell and chromaffin cells. However, this work shows that there are two types of steroidogenic cells coexisting in the interrenal gland and that type 2 cells are mainly located in the middle of the organ with type 1 cells in the peripheral area. The fact that there are two
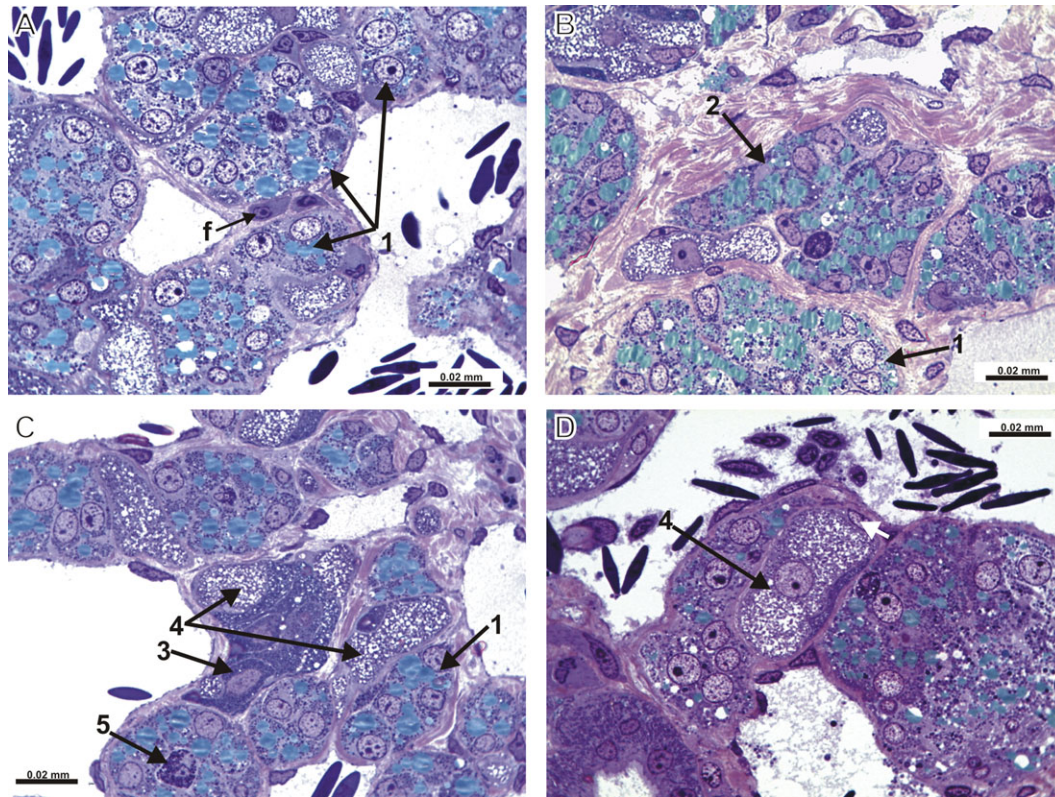
Journal of Morphology

Fig. 5. *Rhinella arenarum*, cell types in epoxy resin-embedded semithin sections (2 μm) of interrenal stained with toluidine blue. (**A**)–(**D**) show cell types 1–5, as indicated by the arrows. For details of the cell types see "Results" section. In (A), f: fibroblast. White arrow in D: nucleus of a cell associated to type 4 cell.

types of steroidogenic cells with different characteristics raises two possibilities: they can be corticosterone and aldosterone producing cells or they are different stages of a single cell type. Many studies described different steroidogenic cell types in the interrenal of anuran amphibians (Moorhouse, 1963; Hanke and Weber, 1965; Varma, 1977; Álvarez, 1992; Süren, 1999). Particularly, Hanke and Weber (1965) and Süren (1999) observed that cells in the peripheral area of the gland have histological characteristics that suggest that they are more active than those located in the central zone. Troyer (1968) and Süren (1999) proposed that the size of the nucleus allows to evaluate the activity of a cell, that is, that the larger the nucleus the more active the cell. In this sense, in the interrenal of *R. arenarum*, type 1 cells could be more active than type 2 cells. Also, the presence of euchromatin in type 1 cells suggests that these cells are more active than the type 2. Particularly in steroidogenic cells, the size of the lipid droplets, their osmiophilia, the density of the mitochondrial matrix and the development of the smooth endoplasmic reticulum are indicative of increased cellular activity (Hanke and Weber, 1965; Álvarez, 1992; Süren, 1999; Capaldo et al., 2004a–c, 2006; Gay et al., 2008). In the interrenal of the toad

R. arenarum, from all these parameters only the size of the lipid droplets differs between type 1 and 2 cells, being larger in the former. However, there is controversy regarding whether a steroidogenic cell with larger lipid droplets is more or less active (e.g., Álvarez, 1992; Capaldo et al., 2004a–c, 2006; Gay et al., 2008; Süren 1999). Capaldo et al. (2006) and Gay et al. (2008) suggested that larger lipid droplets are indicative of a cell preparing for a season of increased synthesis of steroids and that these cells are therefore active. In the toad, both type 1 and 2 cells have characteristics of highly active cells, such as osmiophilic lipid droplets, a well-developed smooth endoplasmic reticulum, and abundant mitochondria with closely packed tubular cristae. Therefore, the only difference between both cell types that suggests that type 2 cells are less active than type 1 is the shrunken nucleus with dense chromatin. On the other hand, the two types could be the morphological evidence of two cells that synthesize different steroids. As mentioned in the "Introduction" section, only one paper proposes that in anurans corticosterone and aldosterone are synthesized by different cell types (Varma, 1977). In this study, specimens of *Rana catesbeiana* maintained in distilled water had many cells with mitochondria
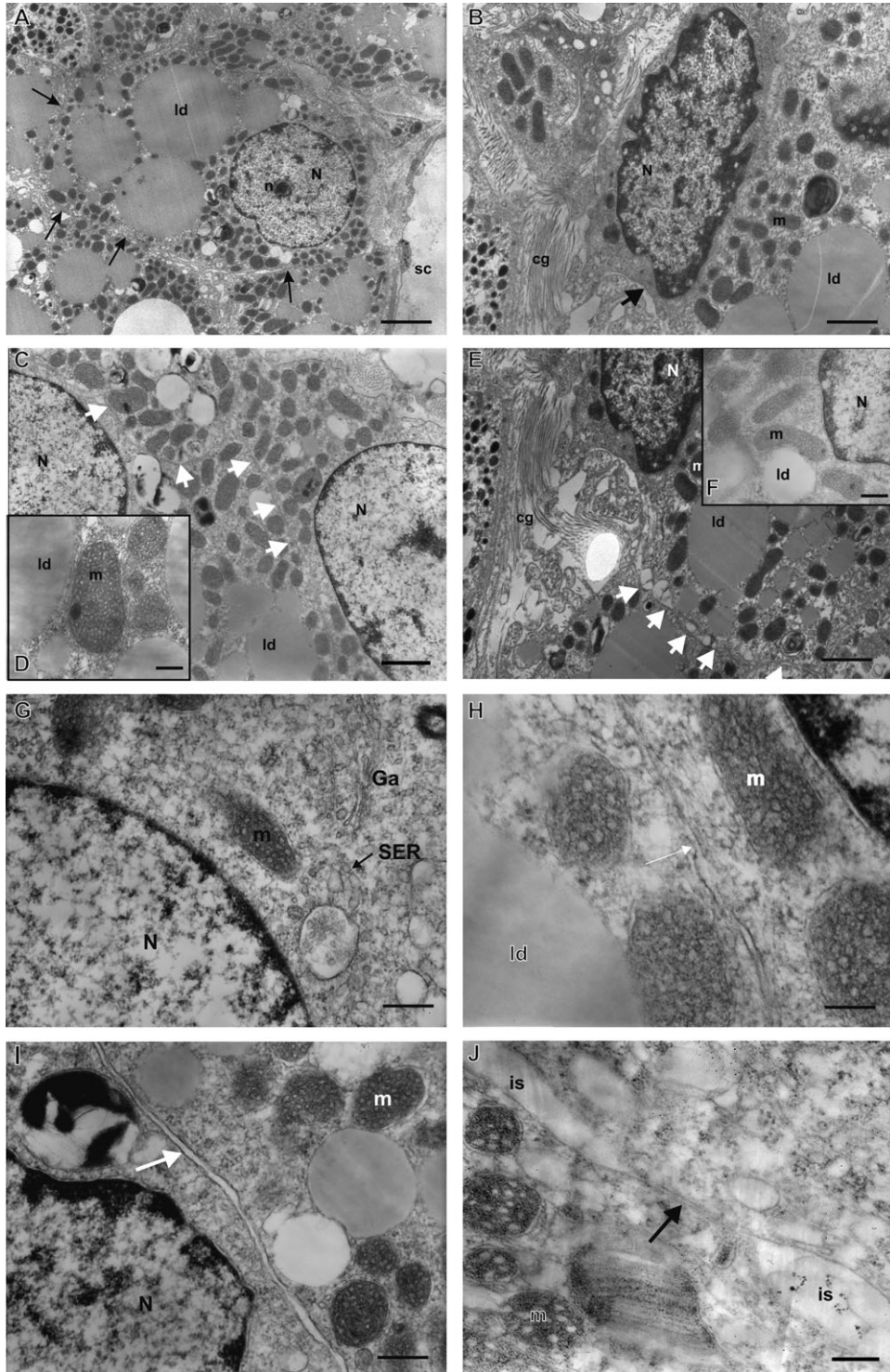
Fig. 6. *Rhinella arenarum*, ultrastructural features of the steroidogenic type 1 and 2 cells. Ultrathin sections (90–150 nm) stained with uranyl acetate and lead citrate. Arrows indicate contact between different cells. (**A**) Type 1 cell surrounded by other cells of the same type (scale bar = 2.5 μm). (**B**) Portion of type 2 cell showing part of the nucleus (scale bar = 1.12 μm). (**C**) Detail of type 1 cells (scale bar = 1 μm). (**D**) Details of lipid droplets and mitochondria of type 1 cell (scale bar = 0.28 μm). (**E**) Detail of type 2 cells (scale bar = 1.12 μm). (**F**) Details of lipid droplets, nucleus, and mitochondria of type 2 cell (scale bar = 0.28 μm). (**G**) Micrograph showing the characteristics of the smooth endoplasmic reticulum, Golgi complex, and mitochondria of steroidogenic cells (scale bar = 0.28 μm). (**H**) Detail of plasma membranes of contiguous type 1 cells (scale bar = 0.14 μm). (**I,J**) Detail of plasma membranes of contiguous type 2 cells (I: scale bar = 0.28 μm and J: scale bar = 0.14 −m). A–J: cg: collagen; Ga: Golgi complex; is: intercellular space; ld: lipid droplet: m: mitochondria; N: nucleus; sc: sinusoidal capillary; SER: smooth endoplasmic reticulum.
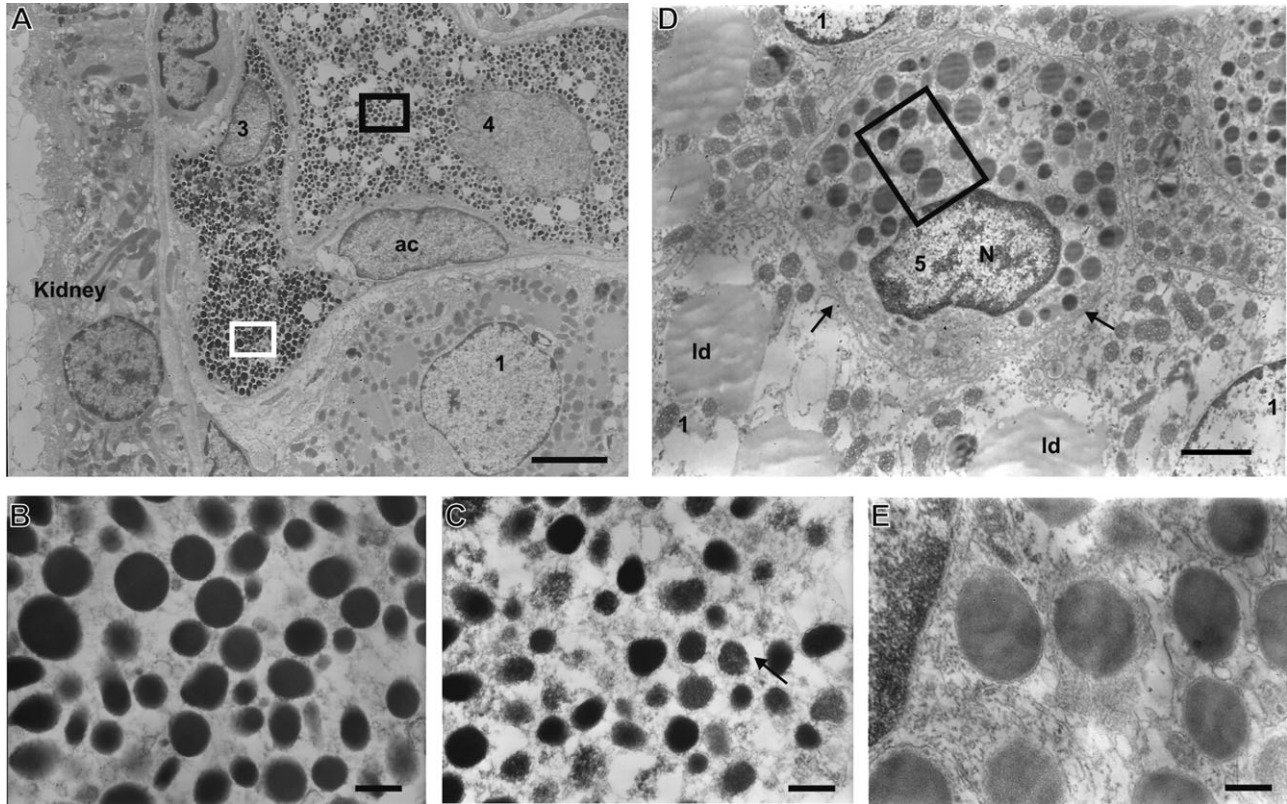
Fig. 7. *Rhinella arenarum*, ultrastructural features of cell types 3–5. Ultrathin sections (90–150 nm) stained with uranyl acetate and lead citrate. (**A**) Micrograph shows type 3 cell in contact with type 4 cell and a nucleus of a cell attached to type 4 cell; ac: attached cell (scale bar = 2.5 μm). Type 1 cell can be seen in the bottom of the image. (**B**) Detail of the granules in the white rectangular area marked in type 3 cell (scale bar = 0.22 μm). (**C**) Detail of the granules in the black rectangular area marked in type 4 cell; arrow: halo between granular core and limiting membrane of the granule (scale bar = 0.22 μm). (**D**) Micrograph shows a type 5 cell in contact with type 1 cells (scale bar = 1 μm). (**E**) Detail of the granules in the rectangular area marked in type 5 cell (scale bar = 0.17 μm).

with lamellar cristae, a mitochondrial characteristic of the aldosterone-producing *zona glomerulosa* of the mammalian adrenal cortex (Shelton and Jones, 1971). Considering that in amphibians, plasma concentration of aldosterone is quite similar to the concentration of corticosterone, being even greater during the summer (Dupont et al., 1976; Pozzi et al., 2002), it was expected that the interrenal gland of untreated toads would have steroidogenic cells with lamellar mitochondrial cristae. However, in *R. arenarum*, none of the steroidogenic cell types had mitochondria with that characteristic.

Two types of chromaffin cells (catecholaminergic cells) were identified according to their ultrastructural characteristics. Piezzi (1965, 1967) also described two types of chromaffin cells in the interrenal gland of *Rhinella arenarum*, one of which would synthesize mostly adrenaline while the other noradrenaline. Here, both types of chromaffin cells were identified based on the characteristics of the cytoplasmic granules described by Piezzi (1967), characteristics that match with those observed in chromaffin cells of other verte-

brates (Wasserman and Tramezzani, 1963; Yoakim and Grizzle, 1980; Geneser, 2000). The study of the distribution of chromaffin cells using TH immunohistochemistry showed that these cells are scattered throughout the interrenal glands and have long processes that contact the steroidogenic cells (Fig. 4B), similar to *Rana temporaria*, *R. catesbeiana*, and *R. ridibunda* (Hanke and Weber, 1965; Nagatsu et al., 1979; Delarue et al., 1988). However, unlike *R. catesbeiana* and *R. ridibunda* (Nonaka et al., 1995; Süren, 1999), in the interrenal of *R. arenarum* there is no zonation in the distribution of the chromaffin cells. In addition, a small nucleus was associated with individual chromaffin type 4 cells, a characteristic similar to the interaction between ganglion cells and satellite cells in the sympathetic ganglion. This interaction suggests that, as proposed by Cocchia and Michetti (1981) in the rat, the functional relationship between glia-like cells and secretory cells of the interrenal would have similar characteristics to that of the glial cells with neurons. Furthermore, and in contrast to a previous report (Piezzi and Burgos, 1968), this work describes the presence of
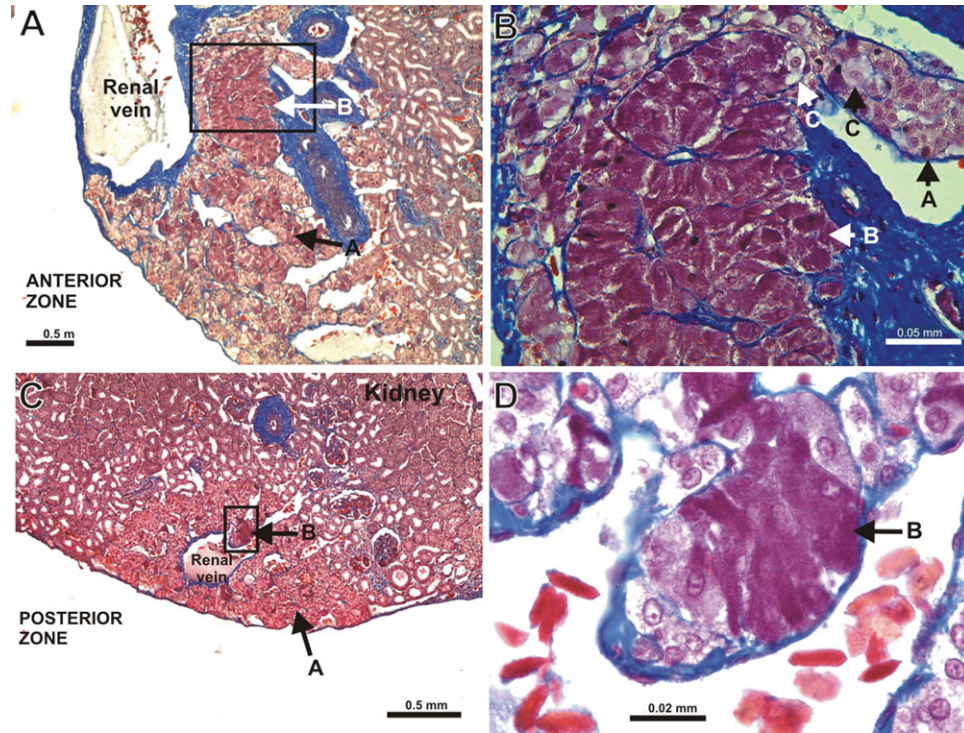
Fig. 8. *Rhinella arenarum*, anteroposterior variation of steroidogenic cell types. (**A**) Cross section (7 μm) of the anterior zone of paraffin-embedded interrenal stained with Masson's trichrome. (**B**) Higher magnification of the portion marked with a rectangle in (A). (**C**) Cross section (7 μm) of the posterior zone of paraffin-embedded interrenal stained with Masson's trichrome. (**D**) Higher magnification of the portion marked with a rectangle in (C). Arrows: cell types A–C.

small cells, surrounded by steroidogenic cells, which have an eccentric nucleus and PAS-positive granules that could correspond to the Stilling or summer cells described in other anurans (Moorhouse, 1963; Sottovia-Fihlo, 1974; Kawamura, 1986; Lesouhaitier et al., 1996). These cells have morphological similarities to mast cells, and some authors suggest that given their proximity to steroidogenic cells they could regulate the synthesis of steroids by a paracrine mechanism (Lesouhaitier et al., 1996).

The analysis of the anteroposterior organization of the cell types showed that the area corresponding to steroidogenic type B cells (or type 2) decreases toward the caudal end of the interrenal

gland with no change in chromaffin cells (Fig. 8; Table 2). In *Rana temporaria*, Hanke and Weber (1965) described that the amount of chromaffin cells decreases from the cranial to the caudal end, but they did not discuss regionality in steroidogenic cells. The evaluation of the anteroposterior variations in the synthesis of aldosterone and corticosterone showed an anteroposterior increase in

TABLE 2. *Morphometric analysis of the cell types in the anterior and posterior zones of the interrenal gland*

Zone	B cells area/total interrenal area	Chromaffin cells/total interrenal area
Anterior	0.17 ± 0.02^a	0.25 ± 0.02^A
Posterior	0.07 ± 0.01^b	0.24 ± 0.02^A

Values are expressed as mean $\pm$ SE; $n = 6$. For details see "Material and Methods" section. Different lower case letters mean significant differences in the relative area of B cells between the anterior and posterior zones with $P < 0.05$. Different upper case letters mean significant differences in the relative number of chromaffin cells between the anterior and posterior zones with $P < 0.05$.
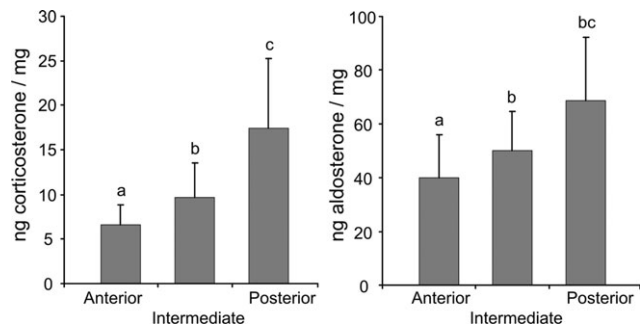

Fig. 9. *Rhinella arenarum*, anteroposterior variation in the synthesis of corticosterone and aldosterone. Fragments of both interrenal gland of each toad corresponding to the anterior, intermediate, and posterior zone were incubated in vitro during 1 h at 28°C. The amount of corticosterone and aldosterone produced in the incubation media was measured by radioimmunoassay. Hormone production is expressed as nanograms of the steroid per milligrams of interrenal incubated, and values are presented as mean $\pm$ SE; $n = 8$. Different letters indicate significant differences between the zones with $P < 0.05$.

the synthesis of both steroids, which suggests that type B cells are a less active stage of the steroidogenic cells in the interrenal. Furthermore, as the anteroposterior pattern of corticosterone and aldosterone secretion is similar, it is possible to speculate that type A and B cells correspond to different stages of steroidogenic cells able to synthesize both steroids. In support of this last possibility, Nonaka et al. (1995) described that in *R. catesbeiana* a single enzyme, the cytochrome P-450 (11β, aldo), is involved in the final steps of corticosterone and aldosterone synthesis. Furthermore, this enzyme is expressed in all the steroidogenic cells of the interrenal of the frog.

In conclusion, this study shows that there is an anteroposterior regionalization of two different steroidogenic cell types. Steroidogenic cells with different histology/ultrastructure seem to be in different stages with different steroidogenic capacity, but it is not possible to conclude that aldosterone and corticosterone are synthesized by distinct cell type. As in other nonmammalian tetrapods, the interrenal gland of anuran amphibians is not organized in a cortex and a medulla but has an anteroposterior and dorsoventral zonation of the steroidogenic cell types, which implies that the organ is not homogeneous.

ACKNOWLEDGMENTS

LITERATURE CITED

Álvarez R. 1992. Functional stages in the interrenal cells of *Rana perezi* (Anura: Ranidae). J Morphol 212:65–70.

Arbogast LA, Voogt JL. 2002. Progesterone induces dephosphorylation and inactivation of tyrosine hydroxylase in rat hypothalamic dopaminergic neurons. Neuroendocrinology 75:273–281.

Capaldo A, Gay F, Valiante S, Laforgia V, Varano L. 2004a. Effects of noradrenaline administration on the interrenal gland of the newt, *Triturus carnifex*: Evidence of intra-adrenal paracrine interactions. J Morphol 259:33–40.

Capaldo A, Gay F, Valiante S, Laforgia V, Varano L. 2004b. Effects of adrenaline administration on the interrenal gland of the newt, *Triturus carnifex*: Evidence of intraadrenal paracrine interactions. J Morphol 261:18–25.

Capaldo A, Gay F, Valiante S, Laforgia V, Varano L. 2004c. Release of aldosterone and catecholamines from the interrenal gland of *Triturus carnifex* in response to adrenocorticotropic hormone (ACTH) administration. J Morphol 262:692–700.

Capaldo A, Gay F, De Falco M, Virgilio F, Laforgia V, Varano L. 2006. The adrenal gland of newt *Triturus carnifex* (Amphibia, Urodela) following *in vivo* betamethasone administration. Anat Embriol 211:577–584.

Castillo SS. 2008. A possible role of insulin-like growth factor-II C-peptide in regulating the function of steroidogenic cells in adult frog adrenal glands. Acta Histochem 110:451–461.

Cocchia D, Michetti F. 1981. S-100 antigen in satellite cells of the adrenal medulla and the superior cervical ganglion of the rat. An immunochemical and immunocytochemical study. Cell Tissue Res 215:103–112.

Cook HC. 1990. Carbohydrates. In: Bancroft JD, Stevens A, editors. Theory and Practice of the Histological Techniques. London: Livingstone Churchill. pp 119–142.

Delarue C, Leboulenger F, Morra M, Héry F, Verhofstad AJ, Bérod A, Denoroy L, Pelletier G, Vaudry H. 1988. Immunohistochemical and biochemical evidence for the presence of serotonin in amphibian adrenal chromaffin cells. Brain Res 459:17–26.

Dupont W, Leboulenger F, Vaudry H, Vaillant R. 1976. Regulation of aldosterone secretion in the frog *Rana esculenta* L. Gen Comp Endocrinol 29:51–60.

Funder JW. 2010. Minireview: Aldosterone and mineralocorticoid receptor, past, present, and future. Endocrinology 151:5098–5102.

Gallo VP, Civinini A. 2003. The development of adrenal homolog of rainbow trout *Oncorhynchus mykiss*: An immunohistochemical and ultrastructural study. Anat Embryol 209:233–242.

Gay F, Laforgia V, Capaldo A. 2008. Human follicle-stimulating hormone modulation of adrenal gland activity in the Italian crested newt, *Triturus carnifex* (Amphibia, Urodela). Comp Biochem Physiol A: Mol Integr Physiol 151:126–132.

Geneser F. 2000. Histología, 3rd ed. Buenos Aires/Madrid: Ed. Médica Panamericana.

Gómez-Sánchez C, Murry BA, Kem DC, Kaplan NM. 1975. A direct radioimmunoassay of corticosterone in rat serum. Endocrinology 96:796–798.

Gómez-Sánchez CE, Foecking MF, Ferris MW, Chavarri MR, Uribe L, Gómez-Sánchez EP. 1987. The production of monoclonal antibodies against aldosterone. Steroids 49:581–587.

Grassi Milano E, Accordi F. 1983. Comparative morphology of the adrenal gland of anuran amphibia. J Anat 136:165–174.

Hanke W, Weber K. 1965. Histophysiological investigation on the zonation, activity, and mode of secretion of the adrenal gland of the frog, *Rana temporaria* Linnaeus. Gen Comp Endocrinol 5:444–455.

Kawamura K. 1986. Occurrence and release of histamine-containing granules in summer cells in adrenal glands of the frog *Rana catesbeiana*. J Anat 148:111–119.

Larcher A, Delarue C, Idres S, Lefebvre H, Feuilloley M, Vandesande F, Pelletier G, Vaudry H. 1989. Identification of vasotocin-like immunoreactivity in chromaffin cells of the frog adrenal gland: effect of vasotocin on corticosteroid secretion. Endocrinology 125:2691–2700.

Lesouhaitier O, Feuilloley M, Lihrmann I, Ugo I, Fasolo A, Tonon MC, Vaudry H. 1996. Localization of diazepam-binding inhibitor-related peptides and peripheral type benzodiazepine receptors in the frog adrenal gland. Cell Tissue Res 283:403–412.

Moorhouse DE. 1963. A study of the adrenal tissue of the male African bullfrog, *Rana adspersa*. Quart J Microsc Sci 104:51–56.

Nagatsu I, Karasawa N, Kondo Y, Inagaki S. 1979. Immunocytochemical localization of tyrosine hydroxylase, dopamine-beta-hydroxylase and phenylethanolamine-*N*-methyltransferase in the adrenal glands of the frog and rat by a peroxidase–antiperoxidase method. Histochemistry 64:131–144.

Nonaka Y, Takemori H, Halder SK, Sun T, Ohta M, Hatano O, Takakusu A, Okamoto M. 1995. Frog cytochrome P-450 (11 β,aldo), a single enzyme involved in the final steps of glucocorticoid and mineralocorticoid biosynthesis. Eur J Biochem 229:249–256.

Perri SF, Capaldo A. 2011. The autonomic nervous system and chromaffin tissue: Neuroendocrine regulation of catecholamine secretion in non-mammalian vertebrates. Auton Neurosci: Basic Clin 165:54–66.

Piezzi RS. 1965. Two types of chromaffin cells in the adrenal gland of the *Bufo arenarum* Hensel. Acta Physiol Latinoam 16:282–285.

Piezzi RS. 1967. Chromaffin tissue in the adrenal gland of the toad, *Bufo arenarum* Hensel. Gen Comp Endocrinol 9:143–153.

Piezzi RS, Burgos MH. 1968. The toad adrenal gland. I. Cortical cells during summer and winter. Gen Comp Endocrinol 1:344–354.

Pozzi AG, Lantos CP, Ceballos NR. 2002. Effect of salt acclimatization on 3 β-hydroxysteroid dehydrogenase/isomerase activity in the interrenal of *Bufo arenarum*. Gen Comp Endocrinol 126:68–74.

Reynolds ES. 1963. The use of lead citrate of high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17:208–212.

Sapolsky RM, Romero LM, Munck AU. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev 21:55–89.

Shelton JH, Jones AL. 1971. The fine structure of the mouse adrenal cortex and the ultrastructural changes in the *zona glomerulosa* with low and high sodium diets. Anat Rec 170:147–182.

Sottovia-Filho D. 1974. Morphological and histochemical study of mast cells in lizards and frogs, with special reference to the so-called "summer cells" or "Stilling cells" of Amphibians. J Herpetol 8:305–309.

Süren S. 1999. The functional relationships between the neurosecretory material and the adrenal gland of *Rana ridibunda* (Amphibia-Anura). Turk J Zool 23:305–311.

Thomas JL, Mason JI, Brandt S, Spencer BR Jr, Norris W. 2002. Structure/function relationships responsible for the kinetic differences between human type 1 and type 2 3β-hydroxysteroid dehydrogenase and for the catalysis of the type 1 activity. J Biol Chem 277:42795–42801.

Troyer JR. 1968. Neurosecretory material in the supraoptic-hypophyseal tract of the bat throughout the hibernating and summer periods. Anat Rec 162:407–415.

Varma MM. 1977. Ultrastructural evidence for aldosterone- and corticosterone-secreting cells in the adrenocortical tissue of the American Bullfrog (*Rana catesbeiana*). Gen Comp Endocrinol 33:61–75.

Wasserman GF, Tramezzani JH. 1963. Separate distribution of adrenaline and noradrenaline-secreting cells in the adrenal of snakes. Gen Comp Endocrinol 3:480–489.

Yoakim EG, Grizzle JM. 1980. Histological, histochemical and ultrastructural studies on the interrenal and chromaffin cells of the fathead minnow, *Pimephales promelas*, Rafinesque. J Fish Biol 17:477–494.