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Original Article

Differentiation of endocrine myocardiocytes in the developing heart of the toad *(Bufo arenarum Hensel)*

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ABSTRACT The differentiation of endocrine myocardiocytes was investigated in the heart of developing toad *Bufo arenarum Hensel*, combining ultrastructural and immunocytochemical procedures. The distribution of immuno-reactive atrial natriuretic peptide (ANP) in the whole heart was appraised by light microscopy, applying biotin-streptavidin and immunofluorescence techniques. With the latter procedures ANP was first recognized at embryonic stage 22, in both atrium and ventricle. In the ensuing stages the ANP-reactivity became stronger in the atrium, while it became dimmer in the ventricle. At the end of the larval prometamorphic stage, atrial myocardiocytes acquired almost all the features of adult myoendocrine cells. At electron microscope level, small inclusions, about 110-120 nm in diameter, resembling secretory granules were found in myoendocrine cells beginning at embryonic stage 22. However, no immunogold labeling of ANP occurred until stage 25. The number of secretory granules diminished in the ventricles and increased in the atrium of the larval heart and at the end of the prometamorphic stage the atrial myoendocrine cells presented the ultrastructural characteristics of active secretory cells. The synthesis of ANP in larvae is enhanced at a critical period of development when the developing toad switches from an aquatic environment to terrestrial life. The cardiac hormones seem to play a key role in the regulation of the osmolarity of body fluids at this developmental stage.

KEY WORDS: *toad, ANP, development, immunocytochemistry*

Introduction

The atrium of the mammalian heart contains special myocardiocytes which exhibit the characteristics of both cardiac muscle cells and endocrine cells (see Forssmann, 1988; Genest and Cantin 1988; Inagami, 1989, for reviews). These myoendocrine cells synthesize and secrete a family of hormonal peptides currently known as Atrial Natriuretic Peptides (ANP).

ANP-related peptides have been found in a wide range of species in the zoological scale. In lower vertebrates, both the atrium and ventricles may produce cardiac hormones (Reinecke *et al.*, 1985, 1987; Netchitailo *et al.*, 1986, 1988). In the toad *Bufo arenarum* and the fish *Hypostomus cordovae*, the concentrations of cardiac hormones are exposed to broad seasonal variations (Aoki *et al.*, 1988; Casco, 1992; Maldonado *et at.*, 1992). In spite of the numerous reports published on this subject over the last few years, little is known about the physiological role played by these hormones in cold-blooded animals.

In the present study we have investigated the ontogeny of myoendocrine cells in the heart of the toad *Bufo arenarum Hensel* applying combined ultrastructural and immunocytochemical techniques. The development of the toad heart provides an excellent model for studying the ANP activity in early stages of amphibian differentiation.

Results

Embryonic stages

The ultrastructure of the embryonic heart at the first heartbeat stage (stage 19) reveals differentiating cardiac cells characterized by an amoeboid profile and interconnected cytoplasmic processes.

Abbreviations used in this paper: ANP, atrial natriuretic peptide; BSA, bovine serum albumin; PBS, phosphate buffer saline.

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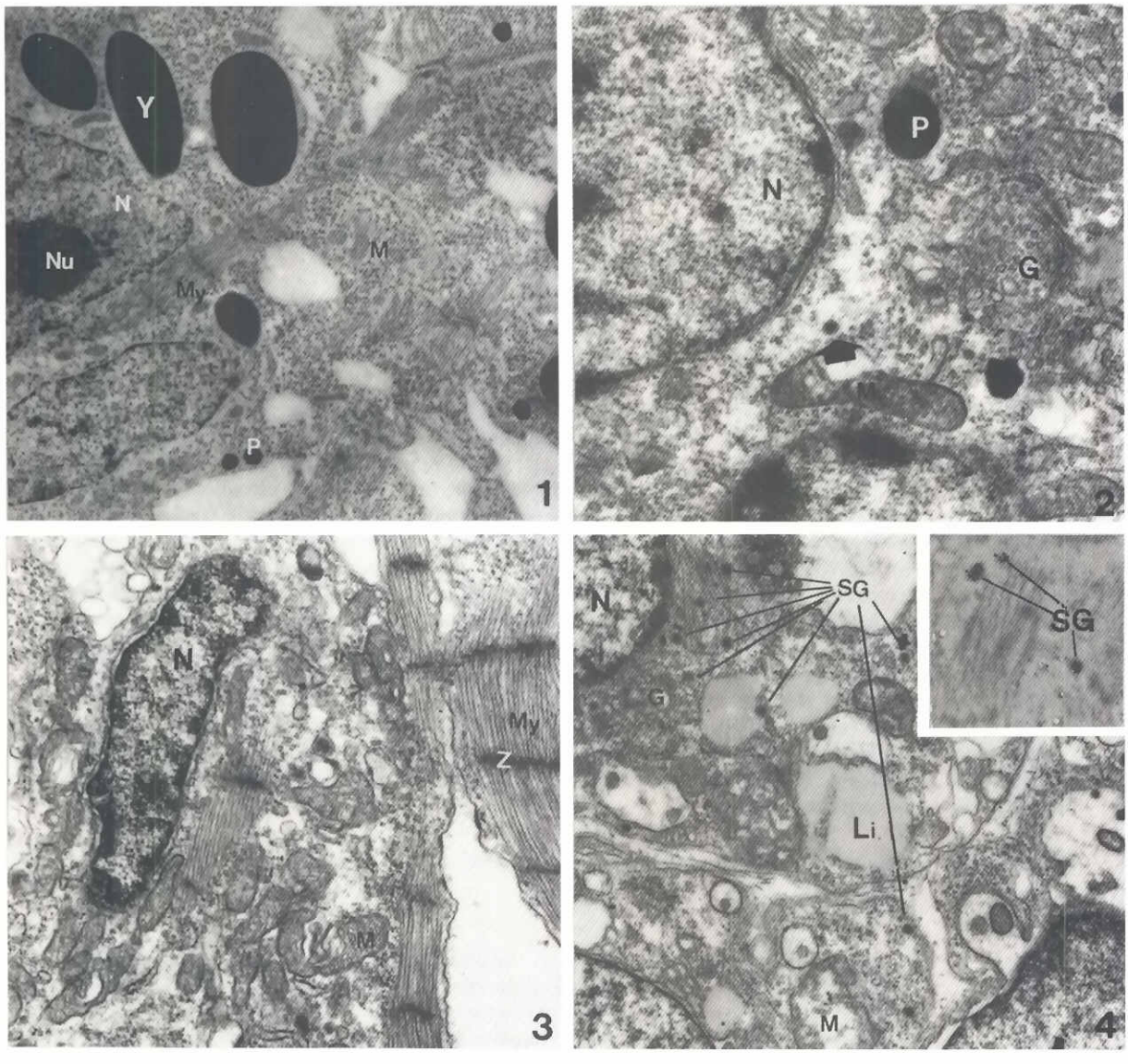


Fig. 1. Electron micrograph of an atrial cardiocyte from a stage 19 embryo. *This cell is characterized by a high number of large yolk platelets (Y) and numerous pigment bodies (P). Note the scarce development of myofibrils (My). N: nucleus, Nu: nucleolus. X4,000.*

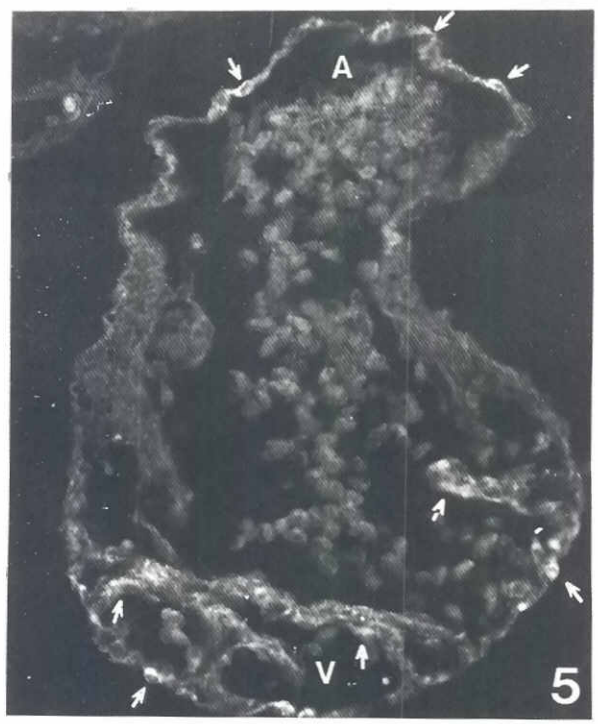
Fig. 2. Perinuclear area of a stage-19 embryo atrial myocardiocyte, showing well-developed Golgi Complex (G). *At this stage no immunostaining can be demonstrated; however, some structures resembling secretory granules are seen (big arrow). P: pigment body; N: nucleus; M: mitochondria. X10,500.*

Fig. 3. Electron micrograph of atrial myocardiocyte from stage 22 embryo. *Note a well-developed sarcomere in subsarcolemal myofibrils (My). Z: Z band, M: mitochondria; N: nucleus. X10,000.*

Fig. 4. Electron micrograph of an atrial myoendocrine cell from stage 25 embryo. *Note the numerous small secretory granules (SG). N: nucleolus; M: mitochondria; Li: lipid droplets; G: Golgi complex. X10,000. Insert: immunoelectron-microscopy of an atrial myoendocrine cell from a stage 25 embryo showing small secretory granules (SG) strongly labeled with gold particles. X12,500.*

The cytoplasm contains a number of yolk platelets of various sizes, lipids droplets and pigment inclusions. Few myofibrils with a rudimentary organization appear at this stage. While bands A, I and Z can be recognized, H zones and M lines are absent. Differentiating sarcomeres occur predominantly beneath and parallel to the plasma membrane (Fig. 1). Some structures resembling secretory granules are present in the cytoplasmic matrix, but no ANP-immunoreactive material is detected (Fig. 2).

At stage 22 (tail-fin circulation), cardiac cells display a more complex organization and a depletion of yolk platelets and lipids droplets (Fig. 3). Mitochondria become polymorphic with a denser matrix and abundant cristae. The sarcomeres attain dissimilar degrees of differentiation and no structure with immunoreactive ANP is detected at electron microscopic level. In contrast, the hearts processed for light microscopic immunocytochemistry display a weak but specific labeling of ANP in both atrial and ventricular



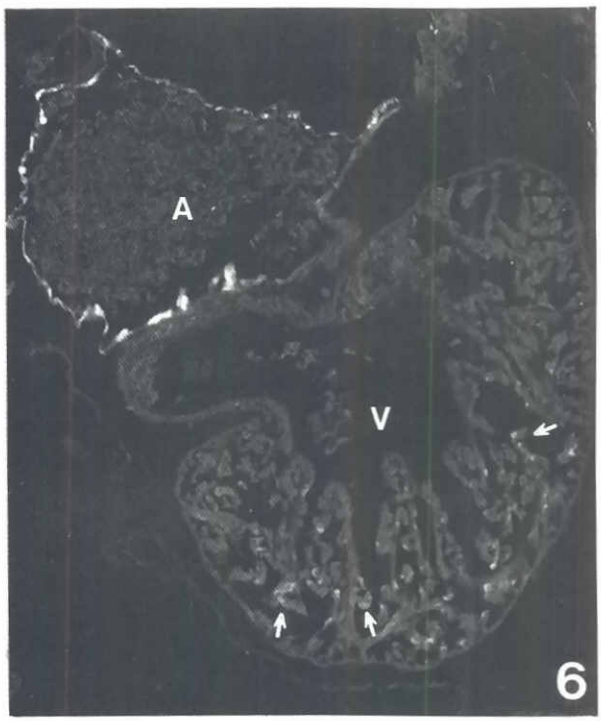


Fig. 5. Heart of a stage 22 embryo. *Immunofluorescence staining in the atrium (arrows) and in the developing ventricular trabeculae (arrows). A light autofluorescence of red blood cells was detected. Sagittal sections. X300.*

Fig. 6. Heart of a stage 25 embryo. *Strong immunofluorescence at the atrial level (A). The ventricle (V) exhibits a more complex organization than the previous stages. The staining traces the areas of trabeculae (arrows). X100.*

myocardiocytes (Fig. 5). In control sections these areas are not immuno-stained. Other structures, including pericardial and endocardial membranes and myocardial cells of the truncus arteriosus and sinus venosus are also negative to ANP immunolabeling.

At stage 25 (complete operculum), myocardiocytes maintain a heterogeneous degree of differentiation; however, the majority exhibit a remarkable depletion of yolk platelets and lipid droplets (Fig. 4 and insert). The cardiac cells present a new ultrastructural feature — the appearance of small ANP-immunoreactive secretory granules in both atrial and ventricular myocardiocytes (Figs. 4). The immunostaining of light microscopic preparations is stronger at this stage, particularly in atrial and trabecular myocardiocytes (Fig. 6). The ANP-immunoreactive material appears scattered within individual myocardiocytes and there is a diffuse staining of myofibrils.

Larval stages

In premetamorphic larvae (stages 1 to 9), the cardiac cells contain scarce lipid droplets and a modest development of the Golgi complex and rough endoplasmic reticulum (Figs. 7 and 8). The mitochondria are more numerous and complex. The myofibrils of atrial myocardiocytes exhibit the striations typical of adult cells but their morphological organization is still rudimentary in comparison to ventricular myocardiocytes. Secretory granules, about 110-120 nm in diameter, occur evenly distributed in both atria and ventricles (Fig. 11). They have a strong affinity to the colloidal gold-ANP antiserum complex.

At prometamorphic stages (stages 10 to 15) the myocardiocytes retain the majority of the features described in the previous stage. Secretory granules are present in both atria and ventricle; however, at the end of stage 15, their numbers decrease gradually in the ventricle, while they accumulate massively in the atria (Figs. 12 and 13). The majority of atrial and ventricular myocardiocytes reveal an intense ANP-immunoreactivity (Fig. 14). In cells of the ventricular trabeculae, the staining is stronger in perinuclear regions.

At metamorphic climax (stage 16 to 20), the myoendocrine cells acquire an advanced degree of specialization as judged by the differentiation attained by the muscle fibers and the secretory organelles. The secretory granules are more numerous in the atrium and show a tendency to aggregate at perinuclear areas of the cytoplasm. In contrast, the myoendocrine cells of the ventricle are seen less frequently and the number of secretory granules occurring in their cytoplasm decreases remarkably.

Discussion

The development of the heart of the toad *Bufo arenarum* constitutes an excellent model for studying the differentiation of myocardial cells (Paz and Pisanó, 1990). In many respects the embryology of amphibian heart is similar to that of higher vertebrates (Fox, 1984); however, the developing cardiac cells of amphibia contain yolk platelets and lipid droplets in amounts large enough to sustain the development of the heart well beyond heartbeat initiation stage (Lemanski, 1973).

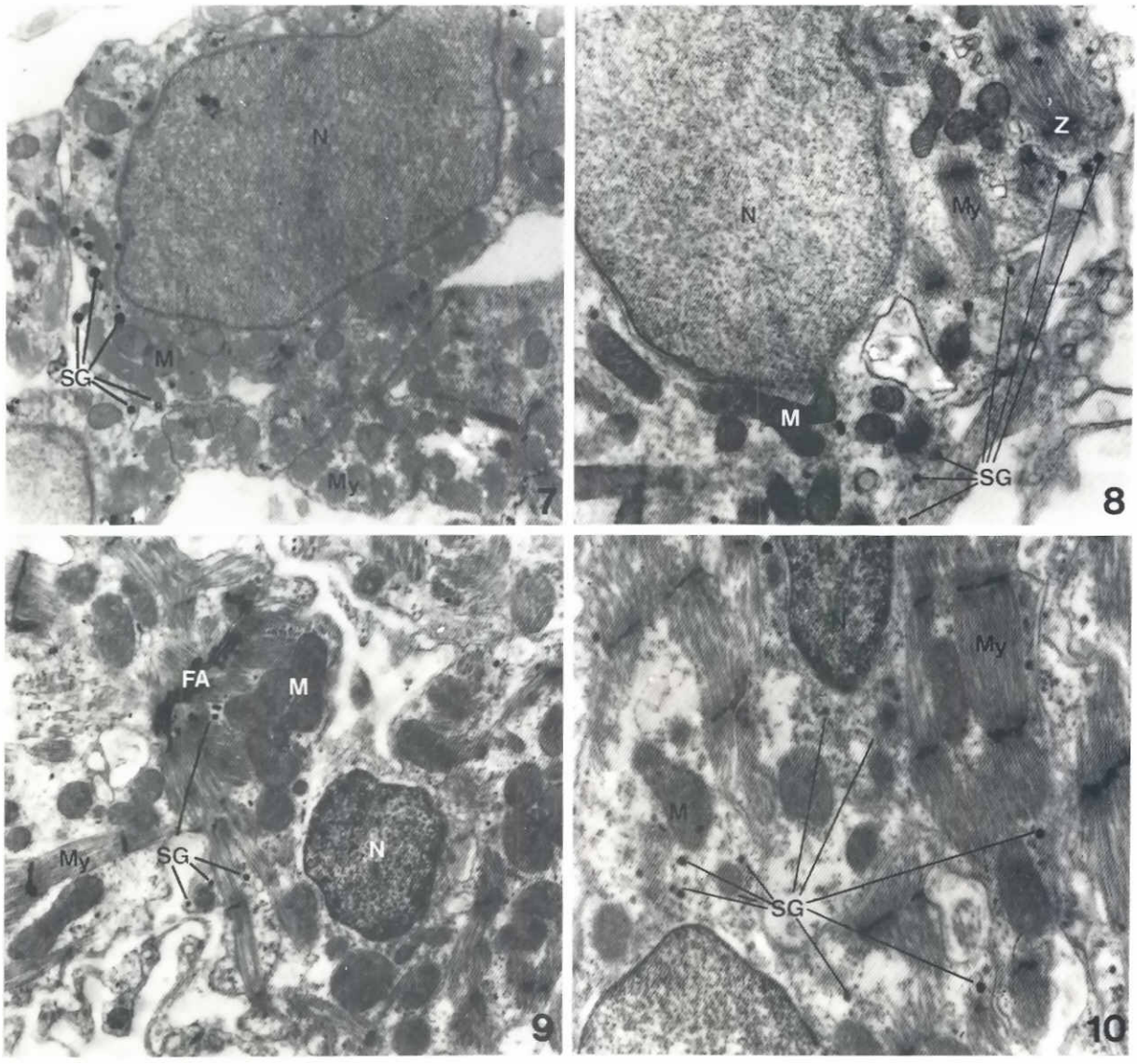


Fig. 7. Electron micrograph of an atrial myoendocrine cell from a premetamorphic larva (stage II). *SG: secretory granules; M: mitochondria; My: myofibrils. X7,500.*

Fig. 8. Electron micrograph of a ventricular myoendocrine cell from a stage II premetamorphic larva. *SG: secretory granules; M: mitochondria; My: myofibrils. X10,000.*

Fig. 9. Electron micrograph of an atrial myoendocrine cell from a stage 15 prometamorphic larva. *SG: secretory granules; FA: fascia adherens; N: Nucleus; My: Myofibrils. X10,000.*

Fig. 10. Electron micrograph of a ventricular myoendocrine cell from a stage 15 prometamorphic larvae. *There is a gradual increase in the complexity of the myofibrillar contractile apparatus with respect to previous stages. SG: secretory granules; M: mitochondria; Z: Z band; My: myofibrils. X9,000.*

Immunocytochemistry reveals that there are no ANP-containing secretory granules before stage 22. Concurrent with the start of feeding on foreign material (stage 25), the developing heart acquires a strong immunofluorescence to ANP in atrial myocardiocytes. In contrast, the immunoreactivity of the trabecular cells of the ventricle becomes progressively fainter. At the electron microscope, few cytoplasmic inclusions resembling secretory granules were detected between stages 19 and 22; however, no ANP was labeled with the immunogold technique until stage 25. These observations suggest that ontogenetically, cardiac hormones appear to play a key role at this critical period of development when the myocardiocytes attain an advanced degree of differentiation and the toad shifts from aquatic life to a terrestrial environment. Also occurring at stage 25 are other important events, such as thyroid gland activation, which seems to be critical to metamorphosis (Etkin, 1963; Fox, 1984).

In more advanced stages of development, the premetamorphic

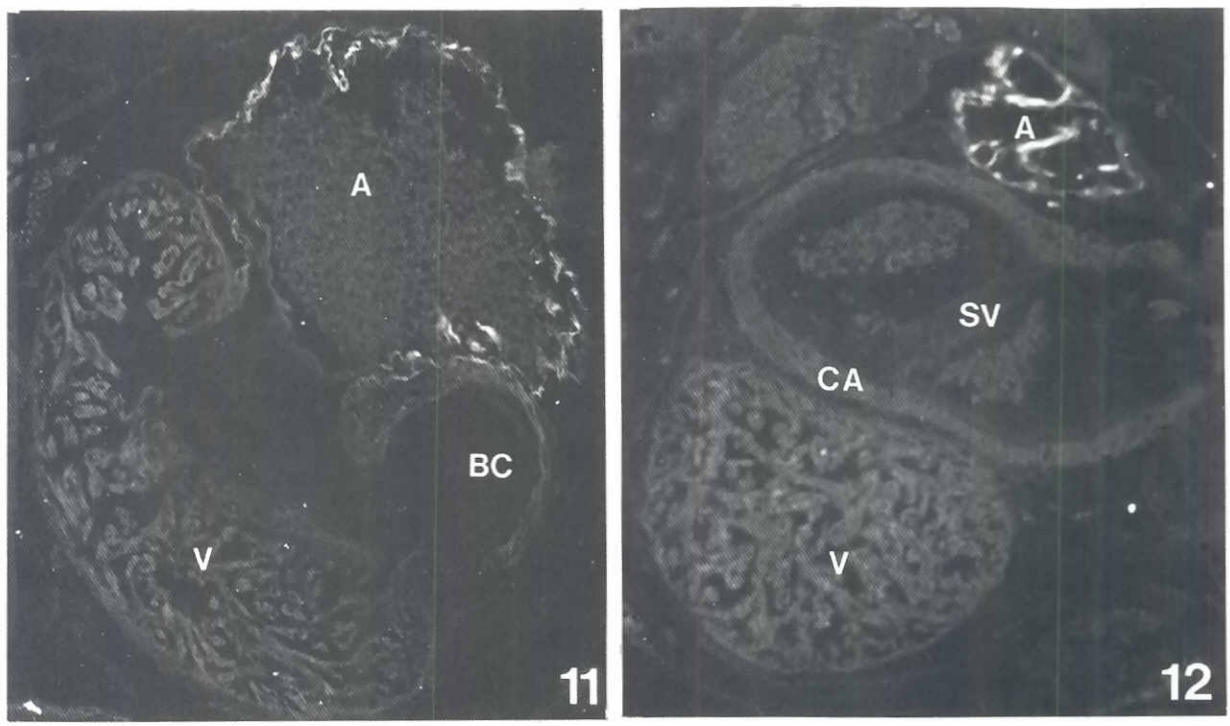


Fig. 11. Immunofluorescence labeling of premetamorphic larvae at stage 9. *Note a strong staining (arrows) at atrial (A) level and diffuse staining in ventricular trabeculae (arrows). The bulbus cordis is not labeled . X100.*

Fig. 12. Light micrograph from a stage 18 prometamorphic larva *reveals a strong immunoreactivity in auricle (A), a very light staining in ventricle (V) and no labeling of conus arteriosus (CA) and spiral valve (SV). X70.*

larvae of *Bufo arenarum* present an ANP immunoreactivity comparable to that described at light microscopic level in *Bufo japonicus formosus* (Hirohama *et al.*, 1989). An important modification observed at this period is the strong and homogeneous immunofluorescence observed in both atria and ventricle, which can be correlated with the ultrastructural morphology. In prometamorphic stages there is a gradual decrease in the ANP-immunostaining in ventricular muscle cells. This observation is comparable to the remodeling occurring in the developing heart of the rat, whose ANP-labeling of ventricular myocardiocytes declines at about the day of birth (Scott and Jennes, 1988, 1989).

In amphibia there is an important exchange of body fluids through the skin and the switching from an aquatic to a terrestrial environment might have a direct influence on the osmoregulatory mechanisms in which ANP could be an important physiological factor. The biological activity of the ANP in amphibia is not as well established as in mammals (Kaltenbach, 1988). In the frog, the ANP did not modify blood pressure, glomerular filtration or the excretion of sodium and chloride (Frick and Toygar, 1988). However, the presence of ANP receptors in the median eminence, the pars nervosa and the adenohypophysis of the frog (Netchitailo *et al.*, 1988) suggests that this hormone may play a regulatory role in secretion of pituitary hormones involved in the control of the osmolarity of body fluids.

The findings described in the present report suggest that cardiac hormones exert important physiological effects on the water-electrolyte balance during the ontogenic development of *Bufo arenarum*.

Materials and Methods

Animals

Female toads collected in the surroundings of the city of Buenos Aires, Argentina, were injected with homologous pituitary glands to induce ovulation (Pisanó, 1957). Ovocyte strings were removed from the ovisac and impregnated with sperm of the same species (Paz and Pisanó, 1990). Different embryonic and larval stages according to Del Conte and Sirlin (1952) and De Martin *et al.* (1985) were processed for immunocytochemistry and electron microscopy.

Immunohistochemistry

Immunocytochemistry of the whole heart was performed in embryos and larvae fixed in cold acetone, dehydrated, and embedded in paraffin (Mukai *et al.*, 1989). Six-micron thick sections were cut and stored at 4°C until staining. For immunofluorescence, the sections were briefly exposed to phosphate buffered saline (PBS) containing 0.5% triton X-100 and bovine serum albumin (BSA), followed by incubation in a drop of rabbit anti-human ANP serum, diluted 1:200, for 18 h, at 4°C. The excess of antiserum was removed and the sections washed twice in PBS and then incubated in sheep anti-rabbit IgG conjugated with fluorescein (diluted 1:4 in PBS). The slides were washed twice in PBS and mounted in 50% glycerol. Control sections were performed following a similar protocol but specific antiserum was replaced by non-immune serum or incubated with the secondary antibody alone. The stained sections were dehydrated in graded ethanol, cleared in xylene, and mounted with Permount.

Electron microscopy

For ultrastructural studies, embryonic and larval hearts were fixed in a mixture of 3% glutaraldehyde, 3% formaldehyde, 1% picric acid and 0.1 M phosphate buffer, pH 7.2, for 24 h at 4°C. After fixation the tissues were

diced and treated with 1% osmium tetroxide for 1 h at room temperature and stained *en bloc* with 2% aqueous uranyl acetate for 2 h. After dehydration in acetone, the tissues were embedded in araldite and sectioned with a Jeol Jum-7 ultramicrotome. The sections mounted on copper grids were contrasted with uranyl acetate and lead citrate and examined in a Siemens Elmiskop 101 electron microscope.

Electron microscopic immunocytochemistry

The hearts of embryos and larvae at different developmental stages were fixed as above but the embedment was in LR-White (London Resin Co., Hampshire, UK). Thin sections were mounted on nickel grids and incubated onto a drop of primary antiserum (diluted 1:1000 to 1:4000) in a moist chamber, at 4°C, for 24 h. After a short rinse in PBS, the grids were transferred to a drop of protein A-gold complex (Maldonado and Aoki, 1983) for 30 min at room temperature. The sections were then stained with uranyl acetate and lead citrate and examined in a Zeiss EM 109 electron microscope.

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