Belg. J. Zool. — Volume 121 (1991) — issue 2 — pp. 235-245 — Brussels 1991

(Manuscript received on 7 January 1991)

IMMUNOREACTIVITY OF *BUFO MARINUS* HEART FOR ATRIAL NATRIURETIC FACTOR

by

JACQUES GILLOTEAUX
Department of Anatomy
Northeastern Ohio Universities College of Medicine
4209, State Route 44
Rootstown, Ohio 44272 U.S.A.

SUMMARY

This communication shows that the toad *Bufo marinus* L. heart contains immunoreactive-atrial natriuretic factor (IR-ANF) which is largely present in the atrial myofibers and in several layers of cardiomyocytes of the ventricles. Atrial myocytes of the truncus arteriosus wall also show IR-ANF content. Functional aspects of *Bufo marinus* body fluid homeostasis related to these observations are discussed.

Key words : heart, Amphibian, atrial natriuretic factor, immunohistochemistry, body water regulation.

INTRODUCTION

In 1956, KISCH showed that the sarcoplasm of atrial myocytes contains dense cored vesicles or granules. Since these granules were exclusively observed in the cardiac myofibers, they were named « specific » granules (JAMIESON and PALADE, 1964). Because the number of granules was altered with changes in bodily water-salt balance (BENCOSME and BERGER, 1971), it was suggested that an endocrine factor was contained in the heart and secreted upon demand from the heart to facilitate excretory functions (DE BOLD, 1979, 1989 ; DE BOLD *et al.*, 1981). Biochemical analysis of the atrial granules (FLYNN and DAVIES, 1983) demonstrated that these physiological effects were caused by a polypeptide which was contained within these atrial granules. A family of peptides was isolated and sequenced, and among these one was named atrial natriuretic factor [ANF] (FLYNN and DAVIES, 1983). ANF is produced as a portion of a larger precursor polypeptide molecule (126 amino acids in length or proANF) which represents the major storage form of the peptide inside the secretory granules (FLYNN *et al.*, 1983 ; GELLER *et al.*, 1984). In several reports we have shown that the cardiac endothelial linings of the endocardium, the blood vessels, and the epicardium were structural barriers which could control and

regulate the transport of ANF towards the circulation (GILLOTEAUX and LINZ, 1990 ; GILLOTEAUX *et al.*, 1988, 1991). We have also suggested that these endothelia could also be important routes to activate proANF before its release into the circulation as ANF (1-28). In addition, based on our observations and other reports, we have proposed that the integrity of such endothelia was critical to specifically activate ANF before or at the site of release into the circulation (GILLOTEAUX, 1990 ; GILLOTEAUX *et al.*, 1991).

Comparative developmental, biochemical, and immunohistochemical studies originating from lower Vertebrates are still scarce even though they could provide precious information about the physiology of the cardiac-kidney-adrenal cortex axis and other organs associated with sodium and water excretion (skin, gills, etc.) (FORSSMANN *et al.*, 1989). New information obtained from comparative work could also shed light into morpho-functional characteristics of this cardiac peptide in all Vertebrates. This would be most valuable from studies investigating species adapted to peculiar or specialized ecological niches. More morphological and molecular information is currently being collected from a range of several species (fishes, amphibians) in our laboratory. Some of the preliminary observations of ANF immunolocalizations detected in cardiac tissues of the toad are described in this report.

MATERIAL AND METHODS

Organ used : Four hearts from male toads (*Bufo marinus* L.), kept at constant temperature (8 ° C) under running water with a 12-hr light, 12-hr dark cycle in the animal care facility of the Physiology laboratory of the Free University of Brussels, were obtained from the Laboratory of Physiology of the Free University of Brussels during sabbatical leave in the Neuropathology and Electron Microscopy Laboratories in the Erasme-Anderlecht Campus.

LM immunohistochemistry : Hearts were fixed either by 4 % buffered formaldehyde (0.1 *M* phosphate) or by Bouin-Hollande sublimated-trichloracetic mixture for a maximum of 5 hours by immersion before being embedded in paraffin. Samples fixed in the Bouin-Hollande fixative were washed for 3 hours in running tap water before dehydration and embedding in paraffin. Both techniques gave identical results but a denser immunoreactivity was observed after Bouin-Hollande fixation. Seven-µm thick sections were cut and,following deparaffinization, STERBERGER's (1979) indirect immunohistochemical method, using peroxidase-antiperoxidase (P.A.P.), was employed to visualize ANF immunoreactivity sites of the rabbit anti-ANF serum # 10-5 antibodies in serial sections of toad hearts following the procedure previously described in GILLOTEAUX *et al.* (1991). Immunostained patterns were then observed and photographed in a Zeiss photomicroscope. Alternate sections were stained by hematoxylin-eosin (H & E). Specificity control tests included preabsorption of the ANF antiserum with somatostatin, CCK, or NPY did not reduce the intensity of immunostaining ; omission of the primary antiserum, incubation with preimmune serum (1/1000), and

preabsorption of the primary antibody with 10^{-6} *M* synthetic atriopeptin III(5-25) or α-human-ANF(1-28) [Bachem AG. and Peninsula Co.] gave negative results.

ANF antiserum # 10-5 : this anti-ANF serum was a gift from Dr L. Jennes (Wright State University, Dayton, OH). It was obtained by immunization of a rabbit with synthetic atrial natriuretic peptide (rat 5-28) which was coupled to keyhole limpet hemocyanin via 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide-HCl (JENNES and STUMPF, 1983). The antiserum recognizes atrial natriuretic peptide (5-15), ANF(1-28), and beta-rANF(17-48). These data show that the antiserum should bind to free active ANF and to ANF as a portion of the precursor molecule. Since the antibody does not recognize the ANF fragment (18-28) it is suggested that (a) an intact cystine bridge is required for binding or (b) the portion of the peptide in close vicinity with the N-terminus is the site which is recognized by the antibody.

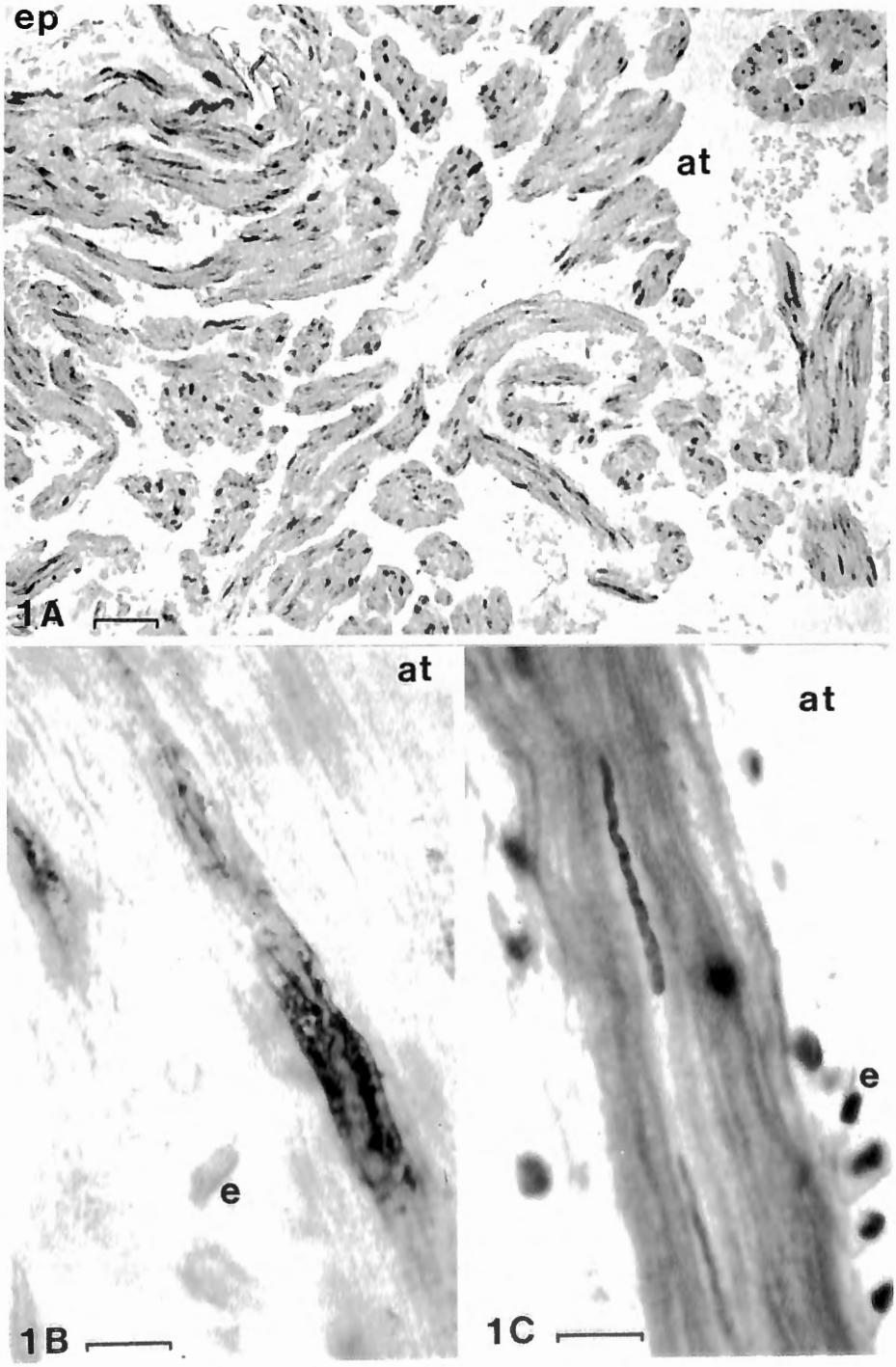
RESULTS

As in mammalian hearts, the atrial walls of this amphibian are less thick (0.15-0.58 mm) than the ventricular wall (0.28-0.83 mm). A loosely, delicate, trabeculated network of atrial myocytes bundles ranging from 25 to 150 µm in diameter originate from the thin atrial walls and two distinct chambers are clearly separated. The right atrial wall is almost twice as large as the left one. The ventricle extends 15 mm in height from the atrial-ventricular junction and is about 13 mm in its widest diameter. It shows only a small open cavity (less than half the radius of the ventricle) in its upper region. This muscular chamber, although made of trabeculated muscular bundles, appears more compact than both atrial chambers and communicates with the open cavity via a large number of small fissures between trabeculated strands of myocardial bundles. A narrow endocardial lining of 5 to 12 µm covers the cardiac luminal surfaces and an epicardial layer, 25-100 µm in thickness, covers the myocardium (Pl. I, 1A and Pl. II, 2).

In this study the respective measurement of atrial versus ventricular myocyte lengths and width are not attempted as our attention focused on the description of immunostaining patterns. The atrial cardiomyocytes attain 5-16 µm in width. They are spindle-shaped, branching, and are often binucleated (Figs 1B-C). The ventricular myocytes are typically branching and their diameter ranges between 12 and 20 µm. After H & E staining, both atrial and ventricular myocytes show a narrow, pale perinuclear zone suggestive for the locations of the RER, Golgi apparatus, and ANF-containing granules.

As expected, the immunostaining patterns of the atrial (Pl. I, 1B) and ventricular (Pl. II, 2) myocytes are localized in the same aforementioned perinuclear cell regions. All the myocytes of the atrial walls show an intense and positive granularity in a 5-8 µm thick perinuclear sarcoplasmic region which corresponded to the most medial zone of each myofiber (PL. I, 1B). Following a careful examination of the immunohistochemical localizations, immunostained structures can be detected throughout the sarcoplasm of the muscle fibers, but with a decreased

PLATE I



intensity from the perinuclear region toward the fiber peripheral regions (Pl. I, 1B compared with Pl. I, 1C).

Interestingly enough, a subepicardial layer of 8-14 immunostained cardiomyocytes is detected in the ventricle. Furthermore, scattered stained myocytes can also be observed within deeper, thick trabeculae of the ventricular wall (Pl. II 2 where immunoreactive sites are exemplified by arrows). ANF-immunoreactivity appears restricted to the perinuclear sarcoplasm, since a dispersed and only faint staining is shown in the more distal regions of the ventricular myocytes (Pl. II, 2 inset). Finally, a narrow layer of immunostained myocytes is observed in cells adjacent to the endothelium of the lumen of the truncus arteriosus. These myocytes measure not more than 7 μm in diameter (Pl. II, 3). Immunoreactive sites for ANF products in the toad hearts are summarized in Fig. 1.

DISCUSSION

Comparisons between immunoreactive staining observations and hematoxylin-eosin stained sections show that all the toad myocytes detected show a similar ANF-IR staining localization as the mammalian myocytes where similar rabbit antibodies raised against synthetic rat ANF (Arg 101-Tyr 126) produced immunochemical staining in all the toad atrial myocytes. It is not surprising to find that ANF-like peptides can be detected in the walls of the amphibian ventricle since a small number of authors (CANTIN *et al.*, 1987 ; CHAPEAU *et al.*, 1985 ; NETCHITAILO *et al.*, 1986, 1988 ; TRILLO *et al.*, 1986) have already reported comparable observations in frog heart, but without illustrating their descriptions about the ventricular wall.

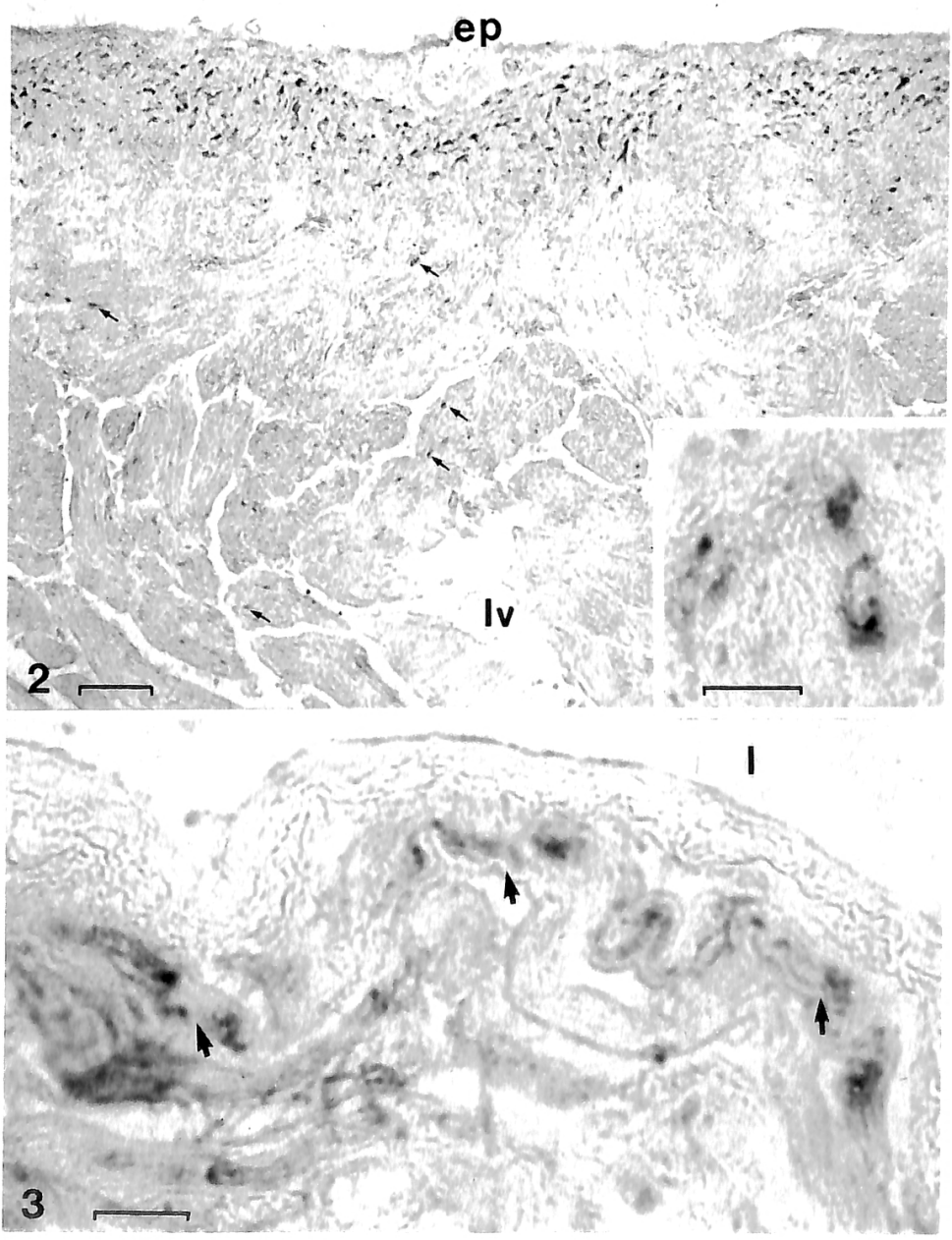
An ultrastructural survey of the cardiac tissues of this type of vertebrate is not described in this report because tissues were given after they were fixed by Bouin or formaldehyde. However, using the same antiserum and immunoelectron gold labeling technique used with success in other investigations, it was demonstrated that the atrial granules in the Syrian hamster (GILLOTEAUX *et al.*, 1991 ; GILLOTEAUX and LINZ, 1990) and in the carp (*Cyprinus carpio* L.) (GILLOTEAUX and XU, in preparation) contain ANF-like product. Consequently, it is possible to

---

PLATE I

1 A-C - *Bufo marinus* right atrium : at : atrium chamber ; e :erythrocytes ; ep : epicardial layer. In A : Region of the atrial wall and trabeculae which shows dense ANF immunoreactivity localized in the perinuclear regions of each atrial myocyte. An enlarged myocyte is illustrated in B. PAP indirect method using anti-ANF # 10-5 diluted 1 :4000 [v/v], no counterstain. In C : Detailed view of nuclear region of an atrial myocyte depicting a pale, circumnuclear zone following hematoxylin-eosin stain. Scales : in A is 100 μm ; in B and C is 10 μm.

PLATE II



suggest that the immunostained areas correspond to the presence of sarcoplasmic granules containing a peptide of close composition and stereochemistry to ANF. The positive cross-reaction allows the identification of the sites of production and storage of ANF-like compound probably contained in atrial and ventricular granules in the toad. The cross-reactivity of similar ANF antiserum with the frog atrial tissues indicates that an ANF-like compound or a molecular precursor is well conserved among all vertebrates (GILLES *et al.*, 1990 ; KIM *et al.*, 1989 ; LAZURE *et al.*, 1988 ; SAKATA *et al.*, 1988).

Though the immunoreactivity, at first, appears weaker in the ventricular wall, the large number of myocytes and the large size of the ventricle suggest that there is likely to be more ANF produced in the ventricle than the atria. Meanwhile, it is not yet demonstrated whether the mode of secretion is continuous or regulated in a similar way to that which BLOCH *et al.* (1986) showed in the mammalian ventricular tissues. There it was found that the content is released continuously while the atrial myocytes secrete ANF, following a regulated mode of transport. ANF is also largely expressed in the ventricle in human hypertrophic cardiomyopathies (KAWAMURA *et al.*, 1991), during late fetal development, and in the perinatal age (RASCHER *et al.*, 1987 ; SMITH *et al.*, 1989). There, ANF is distributed in the same pattern as detected in these lower vertebrates and provides a high plasma level of ANF. It is appropriate to speculate that cardiac stress or specific myopathic defects can favor the production and release in the circulation of large amounts of ANF needed to facilitate the vasodilation of blood vessels and to compensate the compromised cardiac output. During pregnancy, the mammalian fetus lives in an uterine cavity provided with an unlimited amount of fluid whereas in the first few days following delivery an important and necessary diuresis and natriuresis occur. After a week of age the neonate heart decreases its ANF production (SMITH *et al.*, 1989 ; TULASSAY, 1988). This phenomenon is certainly a response to potential dehydration and to maintain survival in a « dry » environment.

The functional significance of a larger number of ANF sites of production and release in the toad could relate to the fact that this toad, like other Amphibians, was kept in an aquarium instead of a terrarium ; in the aquarium, the abundant water intake needs to be excreted continuously and diluted urine is formed, as in

---

PLATE II

2 - Ventricular wall demonstrating ANF immunohistochemical staining in a wide subepicardial layer of myocytes and in interspersed myocytes of the myocardium (examples are arrowed). e : epicardial layer ; lv : left ventricle chamber. Scale is 100 µm. Insert shows oblique sections of three immunostained myocytes ; scale is 10 µm. PAP indirect method anti-ANF ▷10-5 diluted 1 :4000 [v :v], no counterstain.

3 - Subluminal region of truncus arteriosus wall where ANF immunostaining is detected in a small number of atrial myocytes (examples are arrowed) ; l : lumen. Scale is 10 µm.

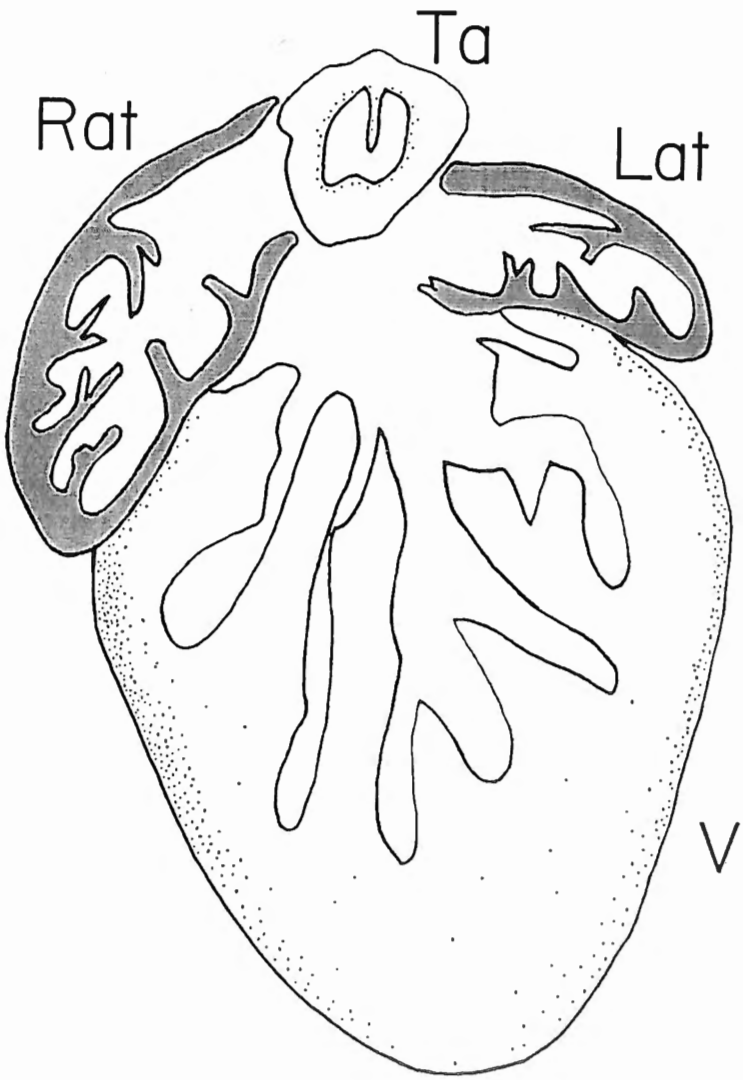


Fig. 1. — Diagrammatic representation of ANF-immunostaining pattern in *Bufo marinus* heart. Atrial cells contain ANF and are depicted by shading of the atria. The ventricular wall immunoreactivity is less intense and its distribution is represented by a stippled pattern. Lat : left atrium, Rat : right atrium, Ta : truncus arteriosus, V : ventricle.

frogs. Anurans are able to osmoregulate between hypo- to isotonicity whereas *Bufo marinus* is adapted as osmoconformer (SHOEMAKER, 1977). These adaptations meet large changes in environmental salinity and are probably related to the production

and secretion of large quantities of ANF, since a large supplemental sodium and water intake is continuously provided through the skin (DEYRUP, 1964 ; DUELLMAN and TRUEB, 1986). Skin and kidneys are the main sites for water regulation in an amphibian, especially in this terrestrial anuran. As water is 70-80 % of the total body weight of most amphibians, a critical function of the kidneys is to continually excrete an hypotonic urine, even in the case of strongly dehydrated toads (LEAF *et al.*, 1958). However, *B. marinus*, a brakish-adapted amphibian, would contain less IR-ANF in the heart ventricles than the frog, because the maintenance of their osmoregulatory homeostatic mechanisms is less critical than those of amphibians whose habitat and ecological niche is quasi restricted to freshwater (GILLES *et al.*, 1990). This assumption is being investigated in our laboratory. While altering the external milieu, we are measuring ANF mRNA production in the three cardiac regions where ANF immunoreactivity is described in this report.

Finally, the water-electrolyte balance in *Bufo* is dependent on blood circulation. The lymphatic system is an important, often neglected, component of an extensive system for electrolyte and metabolite exchanges in these vertebrates (CARTER, 1979). Lymph heart contractions and lymph flow can be modulated and controlled by aldosterone, produced in abundant interrenal tissues (CRABBÉ and DE WEER, 1964 ; ZEIDEL, 1990). In addition, it is possible that these supplemental regions of production of ANF, antagonistic to mineralocorticoids, would certainly grant that additional, production sites of ANF-like compounds originate from these poorly-studied lymphatic hearts. Their secretion (probably made of ANF-like compounds) could counterbalance the large amounts of antidiuretic arginine-vasotocin and aldosterone-secreting structures (RUMYANTSEV and KRYLOVA, 1990 ; SHOEMAKER, 1977).

ACKNOWLEDGEMENTS

This study was supported in large part by the Research Challenge fund granted by the Board of Regents of the State of Ohio in 1988 to the Northeastern Ohio Universities College of Medicine and awarded to the author following a competitive selection.

REFERENCES

BENCOSME S. A. and J. M. BERGER (1971) — Specific granules in mammalian and non-mammalian vertebrate cardiocytes, in : *Methods and Achievements in Experimental Biology*, vol. 5, Eds. E. BAJUSZ and G. JASMIN, p. 173-213, Karger, Basel.

BLOCH K. D., J. G. SEIDMAN, J. D. NAFTILAN, J. T. FALLON and C. E. SEIDMAN (1986) — Neonatal atria and ventricles secrete atrial natriuretic factor via hormone-specific secretory pathways. *Cell*, **47** : 695-702.

CANTIN M., J. DING, G. THIBAULT, J. GUTKOWSKA, L. SALMI, R. GARCIA and J. GENEST (1987) — Immunoreactive atrial natriuretic factor is present in both atria and ventricles. *Mol. Cell. Endocrinol.*, **52** : 105-113.

CARTER, D. B. (1979) — Structure and function of the subcutaneous lymph sacs in the anura (Amphibia). *J. Herpetology,* **13** : 321-325.

CHAPEAU C., J. GUTKOWSKA, P. W. SCHILLER, R. W. MILNE, G. THIBAULT, R. GARCIA, J. GENEST and J. CANTIN (1985) — Localization of immunoreactive synthetic atrial natriuretic factor (ANF) in the heart of various animal species. *J. Histochem. Cytochem.,* **33** : 541-550.

CRABBÉ J. and P. DE WEER (1964) — Action of aldosterone on the bladder and skin of the toad. *Nature (Lond.),* **202** : 278-279.

DE BOLD A. J. (1979) — Heart atrial granularity effects of changes in water electrolyte balance. *Proc. Soc. Exp. Biol. Med.,* **161** : 508-511.

DE BOLD A. J. (1989) — On the shoulders of giants : the discovery of atrial natriuretic factor. *Can. J. Physiol. Pharmacol.,* **65** : 2007-2012.

DE BOLD A. J., H. B. BORENSTEIN, A. T. VERESS and H. SONNENBERG (1981) — A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. *Life Sci.,* **28** : 89-94.

DEYRUP I. J. (1964) — Water balance and kidney, in : *Physiology of the Amphibia,* Ed. J. E. MOORE, Acad. Press, New York, pp. 251-328.

DUELLMAN W. E. and L. TRUEB (1986) — *Biology of Amphibians,* McGraw-Hill Book Co. New York, pp. 197-227.

FLYNN T. G. and P. L. DAVIES (1983) — The biochemistry and molecular biology of atrial natriuretic factor. *Biochem. J.,* **232** : 313-321.

FLYNN T. G., M. L. DE BOLD and A. J. DE BOLD (1983) — The amino acid sequence of an atrial peptide with potent diuretic and natriuretic properties. *Biochem. Biophys. Res. Comm.,* **117** : 859-865.

FORSSMANN W.-G., K. NOKIHARA, M. GAGELMANN, D. HOCK, S. FELLER, P. SCHULZ-KNAPPE and F. HERBST (1989) — The heart is the center of a new endocrine, paracrine, and neuroendocrine system. *Arch. Histol. Cytol.,* **52** suppl : 293-315.

GELLER D. M., M. C. CURRIE, K. WIKITANI, B. R. COLE, S. P. ADAMS, K. F. FORK, N. R. SIEGEL, S. R. EUBANKS, G. R. GALLUPI, and P. NEEDLEMAN (1984) — Atriopeptins : a family of potent biologically active peptides derived from mammalian atria. *Biochem. Biophys. Res. Commun.,* **120** : 333-338.

GILLES J., P. NETCHITAILLO, F. LEBOULENGER, M. CANTIN, G. PELLETIER and H. VAUDRY (1990) — Localization and characterization of the N-terminal fragment of atrial natriuretic factor (ANF) precursor in the frog heart. *Peptides,* **11** : 199-204

GILLOTEAUX J. (1990) — Endocardial endothelium damage in congestive heart failure : pathophysiological implications. *J. Mol. Cell. Cardiol.,* **22** (suppl. III) : S 93.

GILLOTEAUX J. and D. LINZ (1990) — Endocardial surface changes during hamster atrium development. *Annals. N. Y. Acad. Sci.,* **588** : 373-376.

GILLOTEAUX J., L. JENNES, R. MENU and J.-J. VANDERHAEGHEN (1991) — Ultrastructural immunolocalization of the atrial natriuretic factor pathways in fetal, neonatal, and adult Syrian hamsters : from the atrial cardiomyocytes to the circulation via the endocardium, atrial capillaries and epicardium. *J. Submicr. Cytol. Pathol.,* **23** : 75-91.

JAMIESON J. D. and G. PALADE (1964) — Specific granules in atrial muscle cell. *J. Cell Biol.,* **23** : 151-172.

JENNES L. and W. E. STUMPF (1983) — Preparation and use of specific antibodies for immunochemistry of neuropeptides. In : *Methods in Enzymology,* P. M. CONN, ed., Academic Press, New York, **103** : 448-459.

KAWAMURA A., M. ISHIDA, M. KIDA, T. UEGAITO, M. TANAKA, A. MATSUMORI, I. FUJIWARA, H. IMURA and C. KAWAI (1991) — Excpression and distribution of atrial natriuretic peptide in human hypertensive hearts and hearts with hypertrophic cardiomypathy. *Circulation,* **83** : 181-190.

KIM S. H., K. W. CHO, G. Y. KOH, K. H. SEUL, J. N. SO and H. RYU (1989) — Phylogenetic study on the immunoreactive atrial natriuretic peptide in the heart. *Gen. Com. Endocrinol.,* **74** : 127-135.

KISCH B. (1956) — Electron microscopy of the atrium of the heart. I. Guinea pig. *Exp. Med. Surg.,* **14** : 99-112.

LAZURE C., H. ONG, N. MCNICHOLL, P. NETCHITAILLO, M. CHRETIEN, A. DE LEAN and H. VAUDRY (1988) — The amino acid sequences of frog heart atrial natriuretic-like peptide and mammalian ANF are closely related. *FEBS Letters,* **238** : 300-306.

LEAF A., J. ANDERSON and L.B. PAGE (1958) — Active sodium transport by the isolated toad bladder. *J. Gen. Physiol.,* **41** : 657-668.

NETCHITAILO P., M. FEUILLOLEY, G. PELLETIER, M. CANTIN, A. DELEAN, F. LEBOULENGER and H. VAUDRY (1986) — Localization and characterization of atrial natriuretic factor (ANF)-like peptide in the frog atrium. *Peptides,* **7** : 573-579.

NETCHITAILO P., L. M. FEUILLOLEY, G. PELLETIER, A. DE LEAN, H. ONG, M. CANTIN, J. GUTKOWSKA, F. LEBOULENGER and H. VAUDRY (1988) — Localization and identification of immunoreactive atrial natriuretic factor (ANF) in the frog ventricle. *Peptides,* **9** : 1-6.

RASCHER W., M. BALD, J. KREIS, T. TULASSAY, U. HEINRICH, K. SCHÄRER (1987) — Atrial natriuretic peptide in infants and children. *Horm. Res.,* **28** : 58-63.

RUMYANTSEV P. P. and M. I. KRYLOVA (1990) — Ultrastructure of myofibers and cells synthesizing DNA in the developing and regenerating lymph-heart muscles. *Int. Rev. Cytol.,* **120** : 1-52.

SAKATA J. I., K. KANGAWA, and H. MATSUO (1988) — Identification of new atrial natriuretic peptide in frog heart. *Biochem. Biophys. Res. Commun.,* **165** : 1338-1345.

SHOEMAKER V. H. (1977) — Osmoregulation in Amphibians and Reptiles. *Ann. Rev. Physiol.,* **39** : 449-471.

SMITH F. G., T. SATO, V. A. VARILLE and J. E. ROBILLARD (1989) — Atrial natriuretic factor during fetal and postnatal life : a review. *J. Devel. Physiol.,* **12** : 55-62.

STERNBERGER L. A. (1979) — *Immunocytochemistry*, 2nd ed., Wiley & Sons, New York.

TRILLO A. Y., A. Y. MARTINEZ-PALOMO and S.A. BENCOSME (1986) — Estudio ultrastructural del musculo cardiaco en relacion a los granulos especificos. II. Estudio comparativo. *Arch. Inst. Cardiol. Mex.,* **36** : 45-57.

TULASSAY T. (1988) — Role of atrial natriuretic peptide in the postnatal adaptation of term and preterm infants. *Contr. Nephrol.,* **67** : 138-144.

ZEIDEL M. L. (1990) — Renal actions of atrial natriuretic peptide : regulation of collecting duct sodium and water transport. *Ann. Rev. Physiol.,* **52** : 747-759.