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Immunoreactive Atrial Natriuretic Peptide in Frog Tadpoles, *Rana amurensis*, at Different Stages of the Life Cycle

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The biochemical characteristics of immunorecative atrial natriuretic peptide (irANP) and the changes in the levels of irANP in the heart were investigated during the metamorphosis of frog tadpoles. Immunohistochemical localization of pro-ANP in cardiocytes and the presence of irANP in the peritoneal fluid of metamorphosing tadpoles were also examined. The major form of irANP in the cardiocytes of tadpoles (*Rana amurensis*) was high molecular weight on gel filtration chromatography and reverse-phase HPLC. The levels of irANP in the atrium of tadpoles were five to seven times higher than those in the ventricle. In metamorphosing tadpoles the levels of irANP in the atrium increased at stage XX, the climax of metamorphosis, and decreased at stage XXV ($P < 0.05$), the completion of metamorphosis. When the levels of irANP was expressed as a function of body weight of tadpoles, a continuous increase in the levels of irANP was observed from pre- to postmetamorphosis ($P < 0.05$). The levels of irANP in the ventricle were found to be higher in the adult frogs than in tadpoles (*R. amurensis*) ($P < 0.01$). Pro-ANP (31-67) immunoreactivity was detected in the ventricle as well as in the atrium of tadpoles (*R. nigromaculata*). The peritoneal fluid was also found to contain low molecular weight of irANP and the levels of irANP were 55.4 ± 9.1 pg/ml.

Changes of the level of irANP at different stages of the life cycle suggest that ANP may play a role in the regulation of body fluid homeostasis of frog tadpoles during the metamorphosis.

KEY WORDS: irANP, tadpoles, metamorphosis, immunohistochemistry

Atrial natriuretic peptide (ANP) with potent diuretic, natriuretic and vasorelaxant activities was first discovered in mammalian atrial myocytes (De Bold *et al.*, 1981). Subsequent studies revealed that ANP is also present in the submammalian cardiocytes (De Bold and Salerno, 1983; Chapeau *et al.*, 1985; Reinecke *et al.*, 1985; Cho *et al.*, 1988a; Kim *et al.*, 1989; Takei *et al.*, 1989). As a consequence, the heart of vertebrates has been considered as a novel endocrine organ. The biochemical characteristics and physiological roles of ANP have been intensively studied. ANP is stored as a prohormone and processed into a series of biologically active small fragments by site specific proteolytic enzymes (Bloch *et al.*, 1987) and both C- and N-terminal fragments have been observed to exhibit biological activities (Vesely *et al.*, 1987; Martin *et al.*, 1990). It has been reported that ANP has a potent vasodepressor activity and inhibitory action in arterial contraction in submam-

mals (Cho *et al.*, 1988; Takei *et al.*, 1989; Olson and Meisheri, 1989). ANP also causes an inhibition of aldosterone (De Lean *et al.*, 1984) and renin release (Obana et al., 1985) in mammals.

Considering the physiological function for ANP in mammals and the characteristics of life cycle and habitat of amphibia, it is of interest to disclose the roles for ANP in amphibian frogs. Accordingly, the presence and the characteristics of ANP were investigated in the atrium (Netchitailo *et al.*, 1986a; Kim *et al.*, 1989), ventricle (Netchitailo *et al.*, 1988), lymph heart (Ryu *et al.*, 1992), interrenal gland (Lihrmann *et al.*, 1988) and central nervous system (Netchitailo *et al.*, 1987) of the frog. The primary amino acid sequence of frog ANP has also been determined in two species of frogs and indicated to have a homology to mammalian ANP (Lazure *et al.*, 1988; Sakada *et al.*, 1988).

In view of the dramatic life cycle of amphibia, questions about the presence of ANP and the changes during the life cycle in the level of ANP in the heart of frog tadpoles are raised. The purpose of the present study was therefore to investigate the biochemical characteristics and the changes in the levels of ANP in the heart of metamorphosing frog tadpoles. In addition, the presence of ANP in the peritoneal fluid of tadpoles and the immunohistochemical localization of ANP in cardiocytes of metamorphosing frog tadpoles were identified.

Materials and Methods

Animals

Frog tadpoles, *Rana amurensis* and *R. nigromaculata,* were captured from farmlands in the area of Jeonju city from April to June and maintained in aerated aquaria at 18°C. In order to collect frog tadpoles of the desired size and stages in natural state, we undertook field work at intervals of two or three days by checking the development of tadpoles. The table for frog tadpole development by Taylor and Kollros (1946) was used to determine the stages of tadpoles. Three stages of tadpoles, XV (premetamorphosis), XX (climax of metamorphosis), and XXV (postmetamorphosis) were used. We repeated experiments for two years with same protocols. Adult frogs, *R. amurensis* and *R. nigromaculata,* were also captured in a farmland. They were kept in a tank and wetted with running tapwater for one or two days before being sacrificed.

Preparation of samples

Tissue samples were prepared as previously described elsewhere (Kim *et al.*, 1989). Tadpoles (*R. amurensis*) were pithed and the heart was rapidly removed under a stereomicroscope. Atria and ventricles were gently separated. After weighing, tissues were boiled in 0.1 N acetic acid containing 200 kallikrein inhibitory units (KIU)/ml aprotinin, 10 mM ethylenediaminetetraacetic acid (EDTA), 50 benzoyl arginine ethyl ester units (BAEE)/ml soybean trypsin inhibitor (SBTI), and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 10 min and homogenized with Polytron homogenizer at 4°C. Samples were centrifuged at 10,000 xg for 10 min and the supernatant was collected. To measure the levels of irANP in the heart of tadpoles, the extracts were diluted and assayed directly. To investigate the molecular profiles of irANP, the extracts were prefiltered on Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA).

In order to provide evidence for the occurence of irANP in the extracellular fluid of frog tadpoles (*R. amurensis*), blood sampling was attempted by heart puncture. But it was difficult to obtain enough samples. Therefore, the presence of irANP in the extracellular compartment of tadpoles was only investigated in the peritoneal fluid. Identification of circulating irANP in the extracellular fluid of tadpoles was determined in the peritoneal fluid. The peritoneum of tadpoles was gently punctured with fine-tipped glass carpillary tube connected with a suction apparatus. Peritoneal fluid was collected into the prechilled vial containing protease inhibitors as described above. Twenty to 30 microliters of peritoneal fluid was obtained from a single tadpole at stage XV. Peritoneal samples were centrifuged at 10,000 xg for 10 min at 4°C, and the supernatant was subjected to an extraction using a Sep-Pak. Fifty to 80 and 300 to 500 tadpoles were used for one measurement of levels of irANP and molecular profiles of irANP in the peritoneal fluid, respectively.

Blood samples of adult frog (*R. amurensis*) were collected by heart puncture into prechilled tubes containing EDTA and protease inhibitors mixture as described above. Blood samples were processed as described elsewhere (Baeyens *et al.*, 1989) with modification. The blood obtained was immediately centrifuged at 10,000 xg for 5 min. Following the centrifugation, plasma was extracted with 100% acetonitrile (1:1 dilution), mixed, and allowed to stand at 4°C. for 10 min and centrifuged again. The supernatants were taken and dried. The samples were then ready for radioimmunoassay. One hundred microliter of blood was collected from each adult frog and 300 microliter of plasma were used for a single assay.

Radioimmunoassay (RIA) of ANP

IrANP was determined by radioimmunoassay (RIA) as previously described elsewhere (Cho *et al.*, 1988a, b). Briefly, anti-ANP antibody was raised against synthetic rat atriopeptin III (AP III, ANP (101-126)) (Novabiochem, Laufelfingen, Switzerland) in New Zealand White rabbits and the cross-reactivity of the anti-AP III antisera was determined with ANP analogues and their fragments (Cho *et al.*, 1988b; Kim *et al.*, 1989). Synthetic AP III (Peninsula Laboratories, Belmont, CA) was iodinated by using chloramine-T method. The measurement of irANP in heart extracts, gel filtrates, plasma and peritoneal fluid was performed with disequilibrated RIA condition. The lyophilized or dried samples were reconstituted with Tris-acetate buffer (0.1M, pH 7.4) and incubated with anti-AP III antibody for 24 hrs at 4°C. Following the additional incubation with [125 I]-AP III for 24 hrs at 4°C, the bound form was separated from free form by dextran-coated charcoal suspension. In the assay of peritoneal fluid of tadpoles the separation of bound form was achieved by double antibody technique.

Gel filtration chromatography and high performance liquid chromatography (HPLC)

Gel chromatography and HPLC were performed as reported previously (Kim *et al.*, 1989). To determine the apparent molecular mass of irANP, the lyophilized heart extract was reconstituted with 500 ml of 0.1 N acetic acid, centrifuged at 10,000 xg for 10 min. The supernatant was withdrawn and applied to a Sephadex G-50 (Pharmacia, Uppsala, Sweden) column (1.5 × 30 cm, Bio-Rad, Richmond, CA). The elution buffer was 0.1 N acetic acid solution. The flow rate was 0.2 ml/min and the fraction volume was 2 ml. Each fraction was dried and the concentration of ANP was determined by RIA. Blue dextran, cytochrome C and synthetic AP III were used as molecular weight markers. The pro-ANP was partially purified from the rat (Wistar) by the method of Trippodo *et al.* (1983) and used as a proANP marker. Reverse-phase HPLC was performed on a μBondapak C_{18} (Waters Associates, Milford, MA) column. The reconstituted sample with 100 μl of 0.1 % trifluoroacetic acid (TFA) was centrifuged and the supernatant was subjected to a column. Elution was performed with the linear gradient of 20% to 60% acetonitrile in 0.1% TFA for 40 min at a flow rate of 1 ml/min. Gel permeation HPLC was also performed on a TSK-GEL G 2,000 SW column (7.5 x 300 mm, Toyo Soda, Tokyo, Japan) and eluted with 30% acetonitrile in 0.1% TFA. The flow rate was 0.3 ml/min. The fractionated samples were dried and irANP content was determined by RIA.

Immunohistochemistry

IrANP in the cardiocytes of frog tadpoles (*R. nigromaculata*) was visualized using the avidin-biotinylated peroxidase method employing an ABC kit (Vectastain ABC kits, Vector Lab. Inc., Burlingame, CA) (Kim *et al.*, 1992). Briefly, tissue sections mounted on glass slides were deparaffinized, rehydrated, and then exposed to 3% H_2O_2 solution in methanol for 20 min followed by several rinses in 0.02 M phosphate buffered saline (PBS, pH 7.4) containing 0.01% bovine serum albumin, 0.1% gelatin, 0.1% Triton X-100, and 0.1% Tween 20. Slides were then incubated with 10% normal goat serum for 30 min at room temperature, drained, and incubated with an anti pro-ANP (31-67) antibody (Peninsula Laboratories, Belmont, CA) and an anti α-ANP (99-126) antibody (Peninsula Laboratories, Belmont, CA) at a dilution of 1:200 for 16 hrs at 4°C, respectively. After rinsing in PBS, slides were incubated successively with biotinylated goat anti-rabbit IgG (1:1,

000) for 30 min at room temperature. The slides were then rinsed again in PBS and treated with avidin-biotinylated peroxidase complex for 30 min. After rinsing in PBS, the location of the peroxidase labelling on sections was developed with a solution containing 0.025% DAB (3,3'-diaminobenzidine tetrachloride, Sigma Chemical Co., St Louis, MO) in PBS and 0.003% hydrogen peroxide. Sections were then counterstained with hematoxylin, dehydrated and coverslips were mounted with Canada balsam. To test the specificity of the immunohistochemical staining, primary antiserum omitted procedures were undergone.

Chemical analysis

Protein concentrations in the peritoneal fluid and plasma were measured by Bradford method (Bradford, 1976). The levels of Na^+ and K^+ were measured by flame photometry (Beckman, Fullerton, CA).

Statistical analysis

Statistical significance was tested using Student's t-test or ANOVA test and was defined as a P value of less than 0.05. The results were given as MEAN ± S.E.M.

Results

Radioimmunoassay

The radioimmunoassay (RIA) routinely yielded lower limit of 10 pg per assay tube and the intra- and inter-assay coefficients of variation were 10.7 ± 3.6% (n = 7) and 10.5 ± 1.7% (n = 7) respectively.

Atrial and ventricular extracts (*R. amurensis*) competitively displaced ^{125}I-AP III bound to antibody and the slope of dilution curves was relatively paralleled to the standard curve of AP III (Fig. 1). The right shiftness of dilution curve of ventricular extract means the difference of irANP concentration in the extract.

Molecular profiles of irANP in the heart extract of the frog tadpole, *R. amurensis*

Two distinct peaks of irANP corresponding to high and intermediate molecular weights were

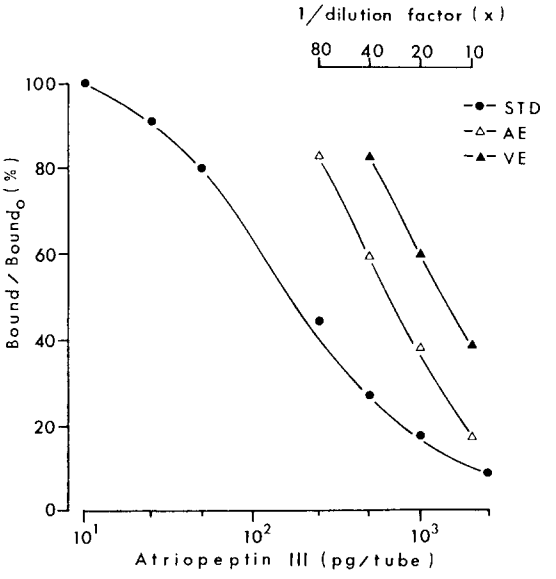


Fig. 1. A representative standard curve (STD) of atriopeptin (AP) III and inhibitory effect of different dilutions of the atrial (AE) and ventricular (VE) extracts of frog tadpoles, *Rana amurensis*. B_0, ^{125}I-labelled tracer bound at zero-dose; B, ^{125}I-labelled tracer bound at dose shown on abscissa. B_0 and B values are corrected for non-specific binding.

observed on Sephadex G-50 gel filtration chromatography in tadpoles at stage XV (Fig. 2A). At stage XXV mainly the high molecular weight of irANP was shown (Fig. 2B). Elution profiles of the heart extract of tadpoles at stage XV on reverse phase (RP)-HPLC were also determined (Fig. 3). Major peak of irANP was corresponded to rat pro-ANP (Fig. 3).

Presence of irANP in the peritoneal fluid of frog tadpoles, *R. amurensis*

The serial dilution curve of the concentrated peritoneal fluid yielded a competition curve paralleled to the standard curve (Fig. 4A). Low molecular weight of irANP emerged at the retention time corresponding to AP III on gel permeation-HPLC (Fig. 4B) and RP-HPLC (Fig. 4C). The amount of irANP in the peritoneal fluid (55.4 ± 9.1 pg/ml, n = 5) was significantly lower than that in the the plasma (354.7 ± 74.5 pg/ml, n = 6) of adult frog (Table 1).

Changes of the level of irANP in metamor-

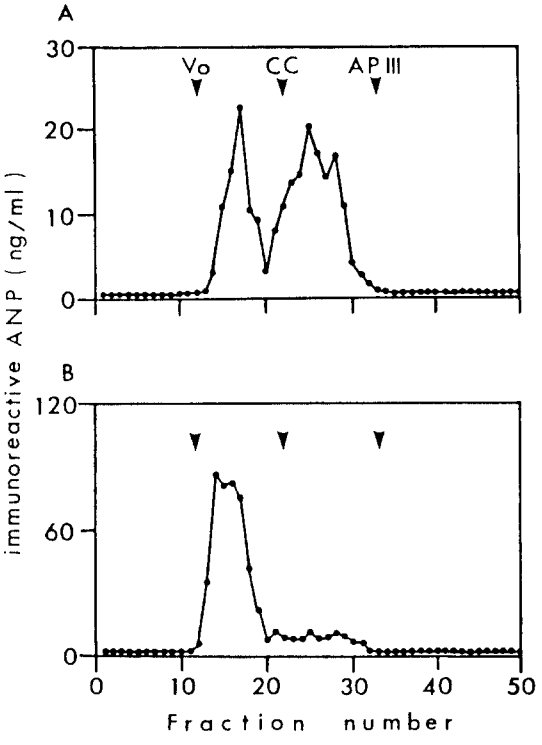


Fig. 2. Gel (Sephadex G-50) filtration chromatography of irANP in the tadpole heart extract, *R. amurensis*, during metamorphosis. Identification of tadpoles at stages XV (A) and XXV (B) was based on Taylor and Kollos' stage (1946). Arrowheads indicate the elution peaks of molecular markers, blue dextran (Vo), cytochrome C (CC), and AP III, respectively.

phosing frog tadpoles, *R. amurensis*

In all stages, the levels of irANP were found to be five to seven times higher in the atrium than in the ventricle (Fig. 5A). The highest level of irANP in the atrium was observed at stage XX, climax of metamorphosis, and decreased at stage XXV ($P < 0.05$), postmetamorphosis. No significant changes in the level of irANP were found in the ventricle of tadpoles during the development (Fig. 5A). The levels of irANP in the adult frog ventricle were higher than that in the tadpoles ($P < 0.01$).

Dramatic changes in the body weight of tadpoles were measured during the development (Table 2). When the levels of irANP were expressed as a function of body weight, the continuous increase in irANP content of the whole heart was observed from pre- to postmetamorphosis (Fig. 5B).

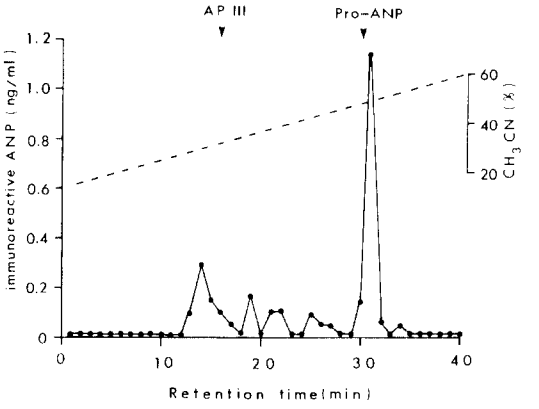


Fig. 3. RP-HPLC profile of irANP in the tadpole heart extract at stage XV, *R. amurensis*, on a μBondapak C_{18} column. The first arrow indicates the retention time of AP III and the second one indicates the retention time of rat pro-ANP.

Immunohistochemical localization of irANP in frog tadpoles, *R. nigromaculata*

ANP immunoreactivity against the anti pro-ANP (31-67) antibody as well as against the anti α-ANP (99-126) antibody were strongly observed both in the atrium and the ventricle of tadpoles from three developmental stages XV, XX and XXV (Table 3; Fig. 6, 7). Neither the smooth muscle nor the connective tissue showed positive staining. Intensity of the irANP staining in the atrium of tadpoles in three stages was stronger than in the ventricle.

Discussion

Biochemical characteristics of irANP in the tadpole heart

In the two developmental stages of tadpoles, gel filtration chromatography of heart extracts showed one or two peaks of irANP. The major immunoreactive peak was observed at the corresponding fraction to high molecular weight (above 13 K) while the other immunoreactive peptide was eluted between the cytochrome C and atriopeptin (AP) III. In previous works, the intermediate molecule which was more hydrophobic than synthetic AP III (101-126) was found in the adult frog

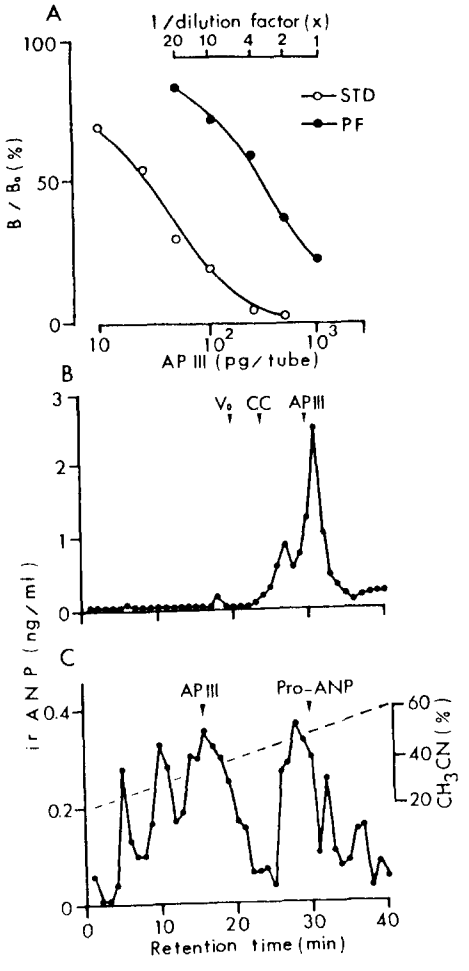


Fig. 4. Standard curve of AP III (STD) and inhibitory effect of different dilutions of the peritoneal fluid (PF) of tadpoles, *R. amurensis*, at stage XV (A). Gel permeation HPLC (B) and RP-HPLC (C) profiles of irANP in the peritoneal fluid of tadpoles.

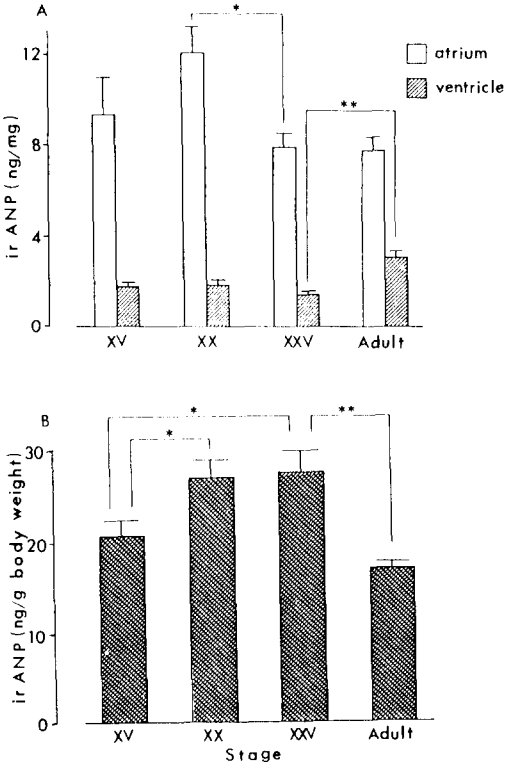


Fig. 5. A Changes in the levels of irANP in the atrium and ventricle of tadpoles during metamorphosis and of adult frogs, *R. amurensis*. XV (n = 7), XX (n = 7) XXV (n = 9), and adult frog (n = 12). The levels of irANP was calculated by atrial or ventricular irANP content per wet weight of tissues. B. Changes in the levels of irANP in the whole heart per gram body weight of tadpoles and adult frogs. The data were originated from Table 2 and Fig 5A. The levels of irANP in the whole heart was calculated by the addition of atrial and ventricular irANP content which was multiplied by total tissue weight. Stage XV (n = 12), XX (n = 11), XXV (n = 12), and adult frog (n = 12). *, $P < 0.05$; **, $P < 0.01$.

Table 1. The levels of irANP, protein and electrolytes in the peritoneal fluid of frog tadpoles at stage XV and the plasma of adult frogs, *Rana amurensis*.

	IrANP levels (pg/ml)	Protein (ug/ml)	Electrolytes (mEq/L)	
			Na	K
Peritoneal fluid	55.4 ± 9.1 (n = 5)	808.1 ± 264.4 (n = 9)	78.0 ± 8.4 (n = 5)	2.2 ± 0.2 (n = 5)
Frog plasma	354.7 ± 74.5 (n = 6)	2751.9 ± 363.7 (n = 4)	101.3 ± 4.2 (n = 11)	4.8 ± 1.6 (n = 11)

Values are the mean ± S.E.M.

Table 2. Changes in the levels of irANP in the whole heart and in the body weight of tadpoles during metamorphosis and adult frogs, *R. amurensis*.

	Stages of tadpoles			Adult frogs
	XV	XX	XXV	
irANP levels (ng/mg)	4.4 ± 0.4 (n = 20)	5.6 ± 0.4* (n = 19)	4.7 ± 0.3 (n= 18)	5.2 ± 0.3 (n = 12)
Body weight (mg or g)	210.0 ± 9.0 (n = 20)	176.0 ± 5.0** (n = 20)	143.0 ± 5.0** (n = 20)	2.9 ± 0.6 (n = 12)

Values are the mean ± S.E.M. The levels of irANP was calculated by whole heart ANP content per wet weight of heart. Body weight in mg and g are for tadpoles and adult frogs, respectively. Significantly different from the stage XV at the levels of *, $P < 0.05$; **,. $P < 0.01$.

Table 3. ANP immunoreactivity against the anti pro-ANP (31-67) antibody and the anti α-ANP (99-126) antibody in the atrium and ventricle of frog tadpoles and adult frogs, *R. nigromaculata*.

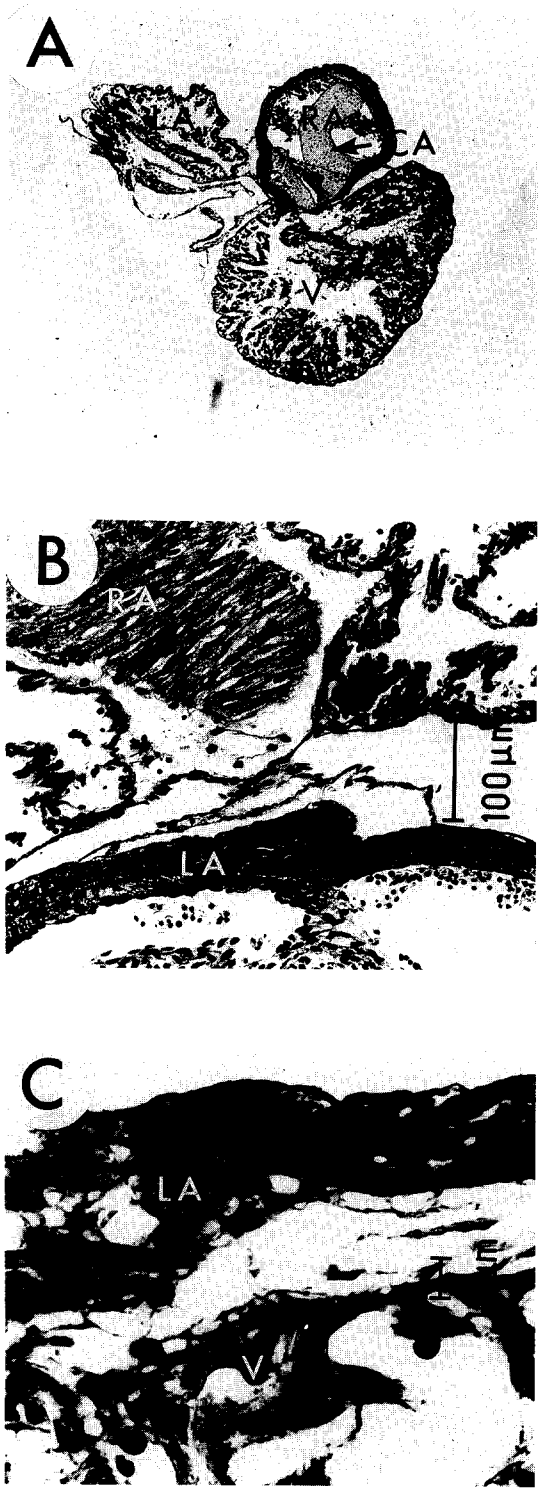
		Antibody	Stages of tadpoles			Adult frogs
			XV	XX	XXV	
Atrium	Right	pro-ANP	++	++	++	++
		α-ANP	+++	+++	+++	++
	Left	pro-ANP	++	++	++	++
		α-ANP	++	+++	++	++
Ventricle		pro-ANP	+	+	+	+
		α-ANP	+	+	+	+

Immunoreactivity; +, moderate; ++, strong; +++, very strong. The grade was scored by two persons.

ventricle (*R. ridibunda*) and it was suggested that it might represent an N-terminal extended form (Netchitailo *et al.*, 1988). Considering the predominant storage of high molecular weight peptide in the heart (Vuolteenaho *et al.*, 1985) and the release of pro-ANP from the primary neonatal cardiocyte cultures (Glembotski *et al.*, 1985), it is likely that the occurence of small peptides may be artifactual. Reverse-phase HPLC of tadpole heart extracts resolved one clear elution peak. This immunoreactive peptide showed the same retention time as the rat pro-ANP. These data strongly suggest that the tadpole heart may mainly contains the high molecular weight ANP.

Changes of the Level of irANP in metamorphosing tadpoles

In the present study, we found that the levels of irANP in the atrium reaches maximum at climax of metamorphosis (stage XX) and shows a decline at postmetamorphosis (stage XXV). According to the previous report (Hirohama *et al.*, 1989), the number of atrial irANP granules in toad tadpoles were found to be increased during the developmental process. They showed that the granules were much higher in the number at postmetamorphosis. Although the species and stage of tadpoles in which the increase in the number of irANP granules was observed is not exactly accorded with this study, our and their results suggest that the changes in the levels of irANP may be linked with the developmental process of amphibian tadpoles.



In frog tadpoles, both the progressive increase in water content during early development and the decrease in water content as well as in body size during metamorphosis has been described (Brown *et al.*, 1988). Changes during the metamorphosis in the regulation of water and electrolyte balance have also been reported (Just *et al.*, 1977). Considering the increase in the levels of irANP and the changes of water and salt balance during amphibian metamorphosis and the physiological roles of ANP in mammals, it is suggested that ANP in frog tadpoles may be involved in the regulation of the fluid and electrolytes homeostasis during the metamorphosis.

Presence of irANP in the peritoneal fluid of tadpoles

The present study identified the existance of irANP in the peritoneal fluid of frog tadpoles, *R. amurensis*. The concentration of irANP in the peritoneal fluid of tadpoles was six times lower than the plasma levels of irANP in adult frogs. Gel permeation chromatography showed that the major form of irANP in the peritoneal fluid was low molecular weight corresponding to AP III. The primary amino acid sequence of C-terminal ANPs in R. catesbeiana (Sakada *et al.*, 1988) and R. ridibunda (Lazure *et al.*, 1988) was reported. They observed that C-terminal frog ANPs were composed of 21 or 24 amino acids (Sakada *et al.*, 1988) and 25 to 26 amino acids (Lazure *et al.*, 1988), and they were highly homologous to known mammalian ANP sequences. Our result (Fig.4) suggests that the circulating form of tadpole irANP may be similar to mammalian ANP.

Immunohistochemical localization of proANP in the tadpole heart

Fig. 6. Coronal section and immunohistochemical localization of irANP in the heart of tadpoles, *R. nigromaculata*. Whole heart of a tadpole at stage XXV was immunostained with the anti pro-ANP (31-67) antibody (A, × 20). Differences of ANP immunoreactivity between the right and left atria (B, × 200) and the left atrium and the ventricle (C, × 200) are observed. LA, left atrium; RA, right atrium; V, ventricle; CA, conus arteriosus (arrow indicated).

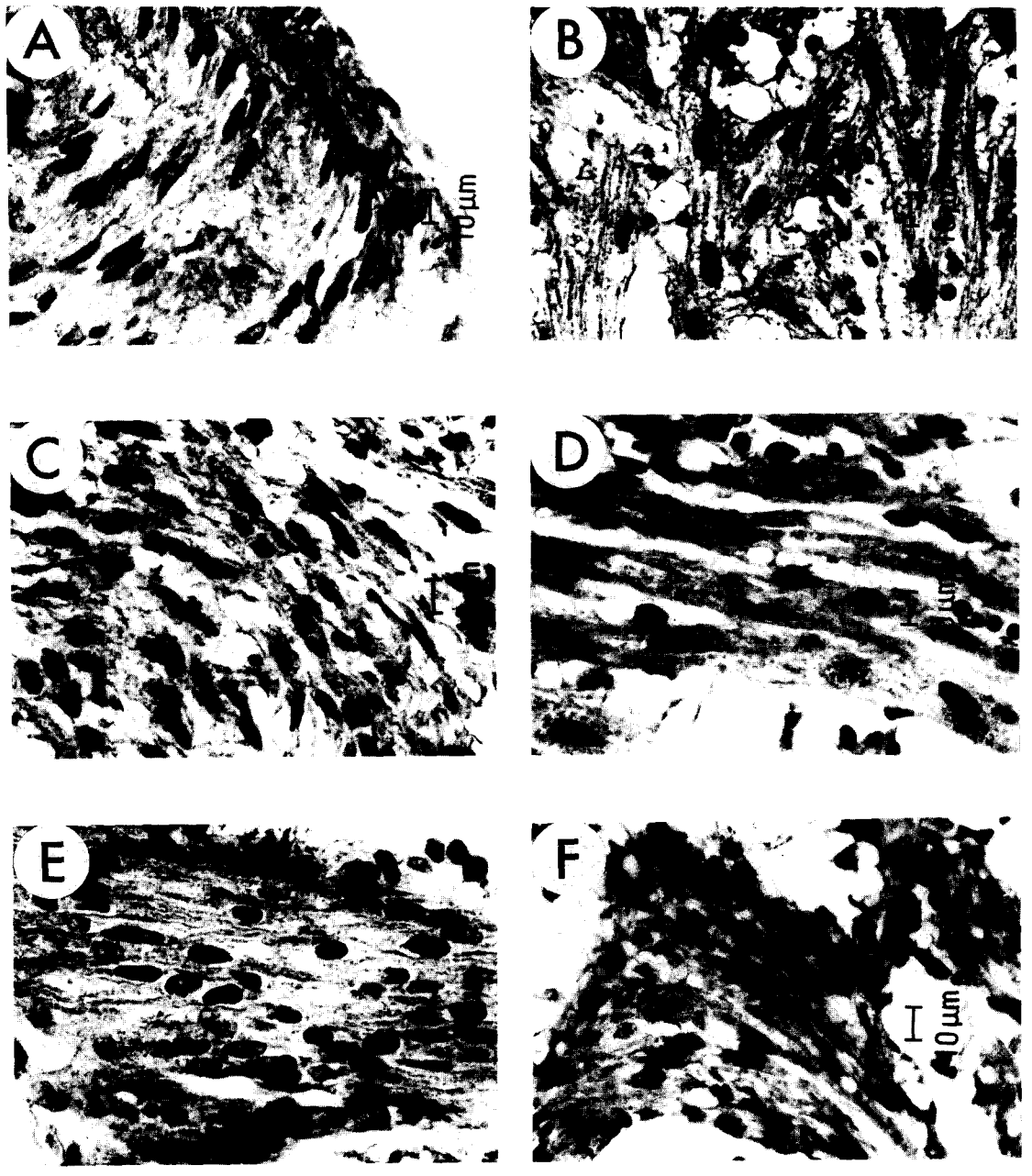


Fig. 7. Immunohistochemical localization of pro-ANP in the atrium and the ventricle of tadpoles, *R. nigromaculata*, during metamorphosis. All the atrial (A, C, E, × 400) and the ventricular (B, D, F, × 400) cardiocytes at stage XV, XX, and XXV, were positively immunostained.

The presence of pro-ANP in the tadpole heart (*R. nigromaculata*) was again supported by the immunohistochemical study using an anti pro-ANP (31-67) antibody. This observation also clearly provided the difference of ANP immunoreactivity between the atrium and the ventricle of

tadpoles, while the density change in the atrium during the metamorphosis was not distinguished exactly under a light microscope. In tadpoles, the pattern of irANP distribution in the heart was similar to that in adult frogs. It was interestingly shown in adult toads that the number of ANP immunoreactive ventricular cells was increased during the breeding season (Hirohama *et al.*, 1989). In the present study, it is also suggested that the increased levels of irANP in the adult frog ventricle may be due to the adaptation of frog tadpoles into the terrestrial life which may cause an augmented ventricular activity and hypertrophy.

In summary, irANP was stored as a prohormone in the atrium and the ventricle of tadpoles and observed as a low molecular weight form in the extracellular compartment. The atrial levels of irANP in tadpoles increased significantly during the metamorphosis. Further study should be carried out to elucidate the physiological roles for ANP in the metamorphosis of frog tadpoles.

Acknowledgments

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개구리 유생(*Rana amurensis*)의 생활사에 따른 immunoreactive Atrial Natriuretic Peptide의 함량 변동

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개구리 유생의 변태과정중에 심장조직내 immunoreactive Atrial Natriuretic Peptide (irANP: 심방이뇨호르몬)의 생화학적 특성 및 함량변동을 조사하였다. Pro-ANP의 존재를 면역조직화학법으로 알아보고 유생의 복강액내 irANP의 존재도 확인하였다. 유생(*Rana amurensis*)의 심장조직내 irANP는 주로 고분자량 이었으며, 심방내 irANP 의 농도는 심실보다 5-7배 더 높았다. 변태과정중 유생의 심방내 irANP 의 농도는 XX 단계에서 증가하였으며, XXV 단계에서는 감소하였다($P < 0.05$). 심장조직내 irANP 의 농도를 유생의 체중과의 함수관계로 나타낼 경우, irANP 의 농도는 변태과정의 전기부터 말기에 이르는 동안 증가함을 보였다($P < 0.05$). 심실내 irANP 의 농도는 유생에서 보다도 성체개구리에서 증가되어 나타났다($P < 0.01$). 면역조직화학법에 의하여 pro-ANP는 유생(*Rana nigromaculata*)의 심방 뿐만아니라 심실에서도 관찰되었다. 유생의 복강액에서 관찰된 irANP는 저분자량 이었으며, 농도는 55.4 ± 9.1g/ml 이었다.

이상의 결과로서 개구리 유생의 변태과정중 irANP 함량변동은 ANP가 유생의 체액의 항상성 조절에 역할 할 것임을 시사하고 있다.