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CHARACTERIZATION OF THE ATRIAL NATRIURETIC FACTOR SYSTEM IN LUNGS OF THE TOAD *BUFO PARACNEMIS*

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Summary

Blood pressure in the amphibian pulmonary circulation is relatively high because a single ventricle serves both the systemic and pulmonary circulation, creating a high degree of plasma filtration from pulmonary capillaries. Previous studies have shown that lung atrial natriuretic factor (ANF) may have an important physiological function in preventing edema in mammals. In this study, we report the presence of the complete ANF system in the lungs of the toad *Bufo paracnemis*. Radioimmunoassay of tissue homogenates revealed that toad lung ANF concentration was approximately twice as high (928.5±83.0 pg mg^{-1} protein) as that of lung tissue in mammals of a similar size. The amount of ANF was significantly higher in the left than in the right atrium (15.0±1.2 *versus* 1.9±0.8 ng mg^{-1} protein; N=4, P<0.001), while the ventricle contained 488.3±41.8 pg mg^{-1} protein. In extracts of both lungs and atria, high-performance liquid chromatography revealed two forms of the peptide; prohormone and a carboxy-terminal peptide of low molecular mass, which is the biologically active form of peptide. The presence of the prohormone suggests that ANF is synthesized in toad lungs and atria. Characterization of toad lung receptors by a competitive binding assay demonstrated three different subtypes of ANF receptors: the guanylyl cyclase (GC) receptors, GC-A and GC-B, as well as clearance (C) receptors. We conclude that the toad *Bufo paracnemis* has a well-developed complete ANF system in the lung, suggesting that it has a role in toad lung physiology.

Key words: Atrial natriuretic factor (ANF), lungs, receptors, toad, *Bufo paracnemis*.

Introduction

DeBold *et al.* (1981) and DeBold and Salerno (1983) were the first to report that atria of various animal species produce a hormone involved in the regulation of extracellular fluid volume and electrolyte balance. This atrial natriuretic factor (ANF) is capable of inducing diuresis, natriuresis and vasorelaxation (Brenner *et al.* 1990). Further studies documented the presence of ANF in a number of non-atrial tissues, including the central nervous system, chromaffin cells of the adrenal medulla and lungs (for a review, see Gutkowska and Nemer, 1989). In humans, ANF is translated as a 152 amino acid pre-prohormone which is processed into a 126 amino acid prohormone (Asn^{1}–Tyr^{126}), the storage form in atrial granules. Further processing of the prohormone by membrane-bound enzymes yields the biologically active 28 amino acid peptide (Ser^{99}–Tyr^{126}) that is released into the circulation. Important sequence homologies between the C-terminal regions of mammalian and amphibian ANF have been suggested by Netchitailo *et al.* (1987), who reported that the heart of frogs contains a peptide immunologically related to mammalian ANF (Arg^{101}–Tyr^{126}).

Since its discovery, several publications have appeared on the systemic, hormonal, renal, cardiac, neurological and pulmonary effects of ANF (for a review, see Perreault and Gutkowska, 1995), its mechanisms of action and its role in physiological and pathological conditions in mammals. However, the ANF system has not been thoroughly investigated in other vertebrate classes. Comparative data indicate that the ANF system developed at an early point of evolution, since ANF-like peptides have been found in a single-celled organism *Paramecium multimicronuclatum* (Vesely and Giordano, 1992). Previous studies from this laboratory have demonstrated the presence of ANF in the amphibian heart (Chapeau *et al.* 1985) and this has been confirmed by others (Gilles *et al.* 1990; Lazure *et al.* 1988;

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Netchitailo *et al.* 1986, 1988; Bruno and Coviello, 1992). Nevertheless, the presence of ANF in amphibian lung has not been investigated despite the fact that the lung is the organ at highest risk of damage from hypertension (Smits *et al.* 1986). Blood pressure in the amphibian pulmonary circulation is relatively high because the systemic and pulmonary circulations share a single ventricle (Shelton and Boutilier, 1982; Shelton, 1985). The high pressure leads to a very high degree of plasma filtration from pulmonary capillaries (Smits *et al.* 1986). Therefore, protection against pulmonary edema is critical, and a pulmonary ANF system could be an important factor in such protection (cf. Gutkowska and Nemer, 1989). In mammals, the lung is a target organ for ANF actions, and it is also a site for the synthesis and release of the hormone (Perreault and Gutkowska, 1995). In the present study, we investigated the ANF system in the lungs of the toad *Bufo paracnemis.*

Materials and methods

Experimental animals

Toads, *Bufo paracnemis* (Lutz), weighing 90–300 g were collected close to Ribeirão Preto (São Paulo State, Brazil) a few weeks before use. The experiments were performed in July, corresponding to the dry season during which the toads are inactive. They were kept in containers equipped with dry areas and running water. Temperature was maintained at approximately 25 °C.

Tissue preparation and ANF extraction

The toads were pithed, after which the heart and lungs were quickly removed and frozen in liquid nitrogen, then stored at −70 °C. Tissues were thawed and homogenized (Brinkman Polytron) on ice in 0.1 mol l^{-1} acetic acid (1 ml g^{-1}) containing the three proteinase inhibitors EDTA (10^{-3} mol l^{-1}), phenylmethylsulfonyl fluoride (PMSF, 10^{-5} mol l^{-1}) and pepstatin-A (0.5×10^{-5} mol l^{-1}). The tissue homogenates were centrifuged for 20 min at 30 000 ***g*** at 4 °C. The pellet was rehomogenized in half of the original volume and centrifuged. Both supernatants were combined and stored at −70 °C. Protein content was determined spectrophotometrically using bovine serum albumin (BSA) as a standard (Spector, 1978). ANF levels were measured in lungs, atria and ventricles by direct radioimmunoassay (RIA) (Gutkowska, 1987) on at least three serial dilutions of homogenates.

Characterisation of the molecular forms of lung ANF was performed on extracted lung homogenates as described previously by Gutkowska *et al.* (1986) for human plasma. The homogenates were passed twice through Sep-Pak C18 cartridges (Waters Associates, Milford, MA, USA) preactivated with 8 ml of acetonitrile, followed by 8 ml of 0.2 % ammonium acetate buffer (pH 7.4). After sample application, the Sep-Pak cartridges were washed with 5 ml of 0.2 % ammonium acetate buffer (pH 7.4). The ANF was eluted with 3 ml of 60 % acetonitrile in 0.2 % ammonium acetate (pH 7.4) (v/v), and the eluates were evaporated in a Speed Vac. ANF extraction was monitored by the addition of ^{125}I-labeled rat ANF (rANF; 20 000 cts min^{-1}) to samples of homogenates. The average recovery was 65 %.

Reverse-phase-high-performance chromatography (RP-HPLC)

RP-HPLC was performed with an LKB system (Bromma, Sweden) and an ultraviolet detector at 210 nm in an analytical C18 μBondapak column (Waters Associates). Reconstituted lung extracts were injected through a 1 ml sample loop *via* a Rheodyne valve. Elution was conducted with an acetonitrile gradient of 15 % to 50 % in 0.1 % trifluoroacetic acid at a flow rate of 1 ml min^{-1}. ANF was detected by direct RIA performed on 50 and 100 μl samples of each HPLC fraction. The eluate was equilibrated prior to injection of the sample, and a blank run was also measured by RIA. The synthetic 28-amino-acid carboxy-terminal peptide (Ser^{99}–Tyr^{126}) and ANF prohormone (Asn^{1}–Tyr^{126}), purified from rat atrial granules (Thibault *et al.* 1987), were chromatographed under identical conditions and served as standards.

Radioimmunoassay

ANF was quantified in tissue homogenates and HPLC fractions by RIA (Gutkowska, 1987). Antibodies were generated against synthetic rat ANF (Arg^{101}–Tyr^{126}) to recognize the carboxy terminus of the circulating ANF (Ser^{99}–Tyr^{126}) as well as the 126-amino-acid prohormone. Rat ANF was labeled with carrier-free ^{125}I-Na (Amersham Canada, Oakville, Ontario) by the lactoperoxidase method, as described previously (Gutkowska, 1987). The iodinated mixture was purified by HPLC on a C18 μBondapak column, and mono-iodinated rat ANF ([^{125}I]rANF) was used for the assay. The sensitivity of this assay was below 2 pg.

Lung membrane preparation

Lung tissue was homogenized at 4 °C in 2 ml of Tris–HCl buffer containing 0.25 mol l^{-1} sucrose, 3 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} EDTA and 5 mmol l^{-1} Trizma base, adjusted to pH 7.5 with HCl. The homogenates were centrifuged at 5000 ***g*** for 20 min. The supernatant was removed and centrifuged at 100 000 ***g*** for 90 min. The resulting pellet was resuspended in 50 mmol l^{-1} Tris–HCl buffer (pH 7.5) and recentrifuged at 100 000 ***g*** for 90 min. The membrane pellet was resuspended in 1 ml of 50 mmol l^{-1} Tris–HCl buffer (pH 7.4), separated into samples and stored at −70 °C.

Saturation and competition binding assays

Saturation studies were performed using lung membranes (5 μg of protein) incubated with increasing concentrations of [^{125}I]rANF (12.5–800 pmol l^{-1}) in the presence or absence of 10^{-6} mol l^{-1} rANF for 2.5 h at room temperature. Kinetic variables were calculated using Scatchard analysis.

Competitive binding assays were performed essentially as described by Gutkowska *et al.* (1988) for rat kidney glomerular membranes. Optimal incubation conditions (time and protein concentration) were determined from these (see Fig. 3A,B).

Lung membranes (5 μg of protein) were incubated with 35 pmol l^{-1} [^{25}I]rANF for 2 h at room temperature (20–23 °C) in a final volume of 0.2 ml of 50 mmol l^{-1} Tris–HCl buffer (pH 7.4) containing 0.1 % bacitracin, 25 mmol l^{-1} $MnCl_2$, 0.5 mmol l^{-1} PMSF, 5 mmol l^{-1} $MgCl_2$, 0.4 % BSA and 1 mmol l^{-1} EDTA in the presence of increasing concentrations (10^{-12} to 10^{-6} mol l^{-1}) of inhibiting peptides: rat ANF (Ser^{99}–Tyr^{126}), rat brain natriuretic peptide (BNP-32), C-type natriuretic peptide (CNP) and C-ANF [des-(Gln^{116},Ser^{117}, Gly^{118},Leu^{119},Gly^{120}) $ANF_{102–121}$], which is a five-amino-acid ring-deleted analog of ANF (Peninsula Laboratories, Inc., Belmont, CA, USA) and shows high specificity for clearance (C) receptors. Binding in the presence of 1 μmol l^{-1} rANF was considered nonspecific. At the end of the incubation period, the mixture was rapidly filtered through 1 % polyethyleneimine-treated Whatman GF/C filters. After rinsing twice with 3 ml of Tris–HCl buffer (pH 7.4), radioactivity trapped on the filters was measured in a gamma counter. The equilibrium dissociation constant (K_d) and maximum binding capacity (B_{max}) for the ligands used in the competitive binding radioreceptor assays were calculated using the LIGAND computer program (Elsevier-Biosoft, Cambridge, UK) with non-linear square regression curve fitting.

Data are presented as mean values ± S.E.M. The statistical significance between means was assessed using Student's *t*-test. $P<0.05$ was considered significant.

Results

Serial dilution of toad lung extracts paralleled the RIA standard curve (Fig. 1), indicating that the extracts possessed a peptide immunologically indistinguishable from synthetic ANF. The concentration of ANF measured in lung tissues from four toads averaged 928.5±83 pg mg^{-1} protein. The amount of ANF present was higher in the left than in the right atrium (15.0±1.2 *versus* 1.9±0.8 ng mg^{-1} protein; $P<0.001$), whereas ventricular ANF content was 488.3±41.8 pg mg^{-1} protein.

The molecular forms of ANF in lung homogenates were characterized by RP-HPLC and compared with elution positions of synthetic rANF (Ser^{99}–Tyr^{126}) and proANF (Asn^1–Tyr^{126}). Sep-Pak-extracted lung homogenates purified by RP-HPLC (Fig. 2A) showed three immunoreactive peaks. Peak 1 corresponded to the low-molecular-mass carboxy-terminal circulating ANF and peak 3 had the same retention

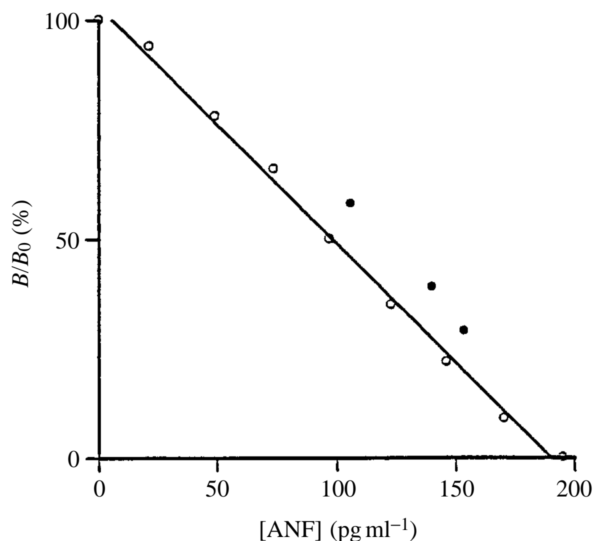

Fig. 1. Linearized [percentage of logit B/B_0 plotted *versus* log(ANF concentration)] standard curve for ANF radioimmunoassay (open circles). *B* represents radioactivity bound to the antibody in presence of increasing concentrations of ANF standards, B_0 represents radioactivity bound in the absence of unlabeled ANF. Three serial dilutions of toad lung homogenates (filled circles) show parallelism to the RIA standard curve.

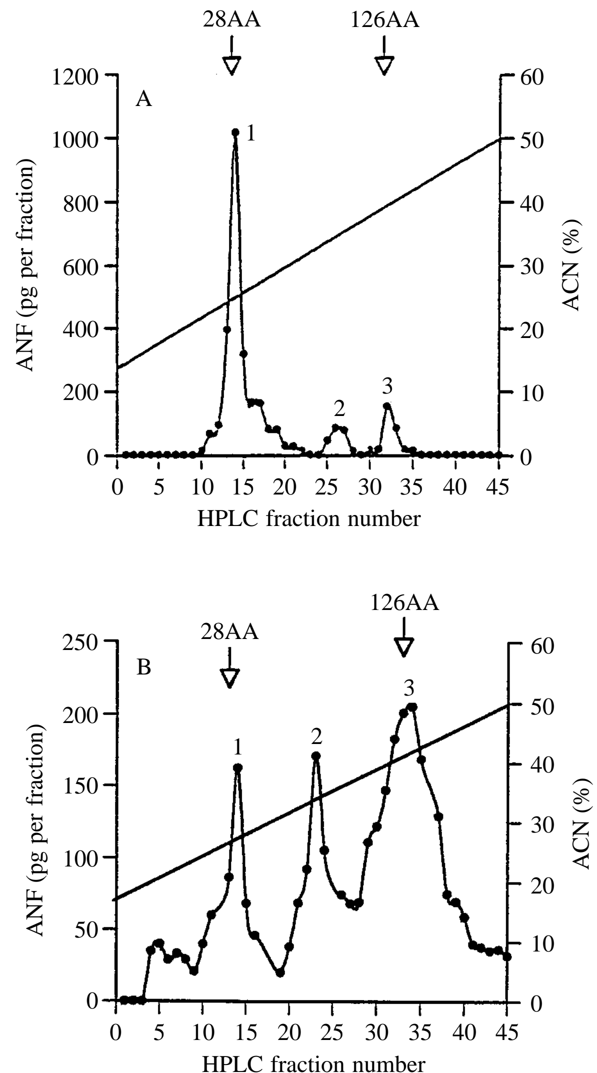

Fig. 2. RP-HPLC elution profile of ANF immunoreactivity in (A) toad lung and (B) atrial extracts. Dried tissue extracts reconstituted in 15 % acetonitrile (ACN) were applied to a C18 μBondapak column and eluted with an acetonitrile gradient (15 % to 50 %) in 0.1 % trifluoroacetic acid and collected in 45 fractions. Three peaks were eluted from lung and atrial extracts. The position of peak 1 corresponds to circulating rat ANF and that of peak 3 to ANF prohormone, which were used to standardize the column. The arrows show the elution positions of rat ANF (28AA) and prohormone (126AA).

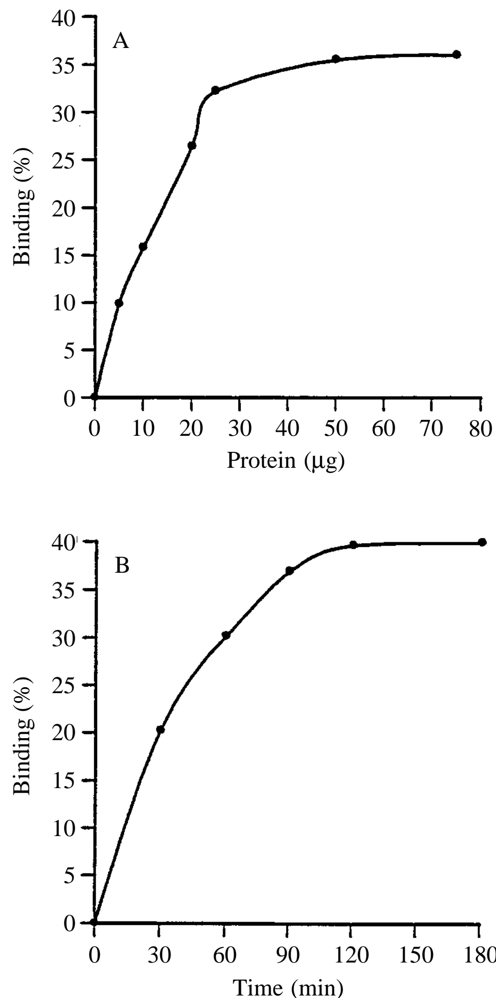

Fig. 3. (A) Binding of [^{125}I]rANF to toad lung membranes increased with increasing protein concentrations up to 30 µg per tube. (B) Binding of [^{125}I]rANF to toad lung membranes increased with time but was stable between 2 and 3 h of incubation at room temperature.

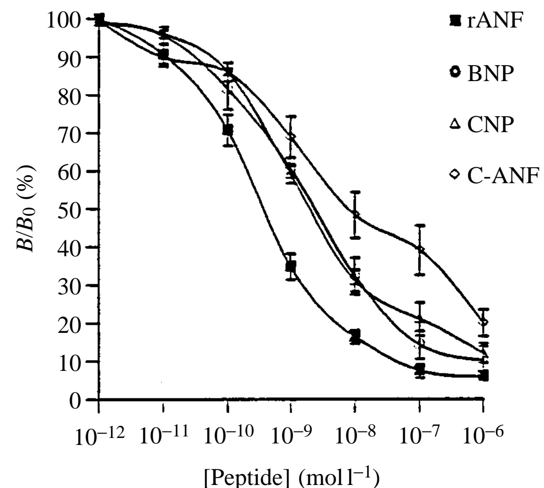

Fig. 4. Competitive binding curves obtained by binding of [^{125}I]rANF to toad lung membranes and inhibition by rat ANF (rANF), C-type natriuretic peptide (CNP) and brain natriuretic peptide (BNP) as well as synthetic C-ANF [des-(Gln^{116},Ser^{117},Gly^{118},Leu^{119},Gly^{120}) ANF$_{102–121}$]. Values are reported as per cent B/B_0, where B and B_0 represent binding in the presence and absence of inhibiting peptides. Each point represents the mean ± S.E.M. of six determinations performed in duplicate.

Table 1. *Kinetic parameters of toad lung natriuretic peptides binding sites obtained from competitive binding radioreceptor assays.*

	K_d (nmol l^{-1})	B_{max} (fmol mg^{-1} protein)
ANF	0.27±0.02	1279±280
BNP	1.15±0.08	892±104
CNP	0.96±0.09	1138±114
C-ANF	38.04±4.08	1018±91

K_d, dissociation constant; B_{max}, maximum binding capacity.
Values are mean ± S.E.M. (N=6).
ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; C-ANF, an analog of ANF (see Materials and methods).

time as proANF. Similarly, Fig. 2B demonstrates that atrial extracts contained three ANF peaks with peak 1 corresponding to circulating ANF and peak 3 to prohormone. However, peak 2 could not be identified because none of the available standards showed a similar retention time. The presence of ANF prohormone suggests that ANF is synthesized in the lungs and atria of toads.

Specific [^{125}I]rANF binding to toad lung membranes increased linearly with membrane protein concentration up to 30 µg per tube (Fig. 3A). Maximum binding occurred at 1.5 h and was stable for at least two additional hours of incubation at room temperature (Fig. 3B). Consequently, competitive binding assays were performed by incubating 5 µg of lung membrane protein with [^{125}I]rANF for 2 h at room temperature.

Fig. 4 represents curves obtained from competitive binding assays of toad lung membranes, plotted as a percentage of B/B_0, where B represents binding with, and B_0 binding without, unlabeled peptides. Increasing concentrations of unlabeled rANF, BNP, CNP and C-ANF progressively inhibited [^{125}I]rANF binding to lung membranes. Inhibition of [^{125}I]rANF binding to toad lung membranes by rANF, BNP and CNP indicated the presence of GC-A and GC-B receptors, both subtypes of guanylyl cyclase receptors. Inhibition of binding with C-ANF, although of a lesser magnitude, indicated the presence of C receptors. Kinetic variables (Table 1) calculated from six competition curves showed that B_{max} for total ANF receptors was 1279±280 fmol mg^{-1} protein and K_d was 0.27±0.02 nmol l^{-1}. Selective inhibition using CNP and C-ANF revealed that B_{max} of GC-B receptors was 1138±114 fmol mg^{-1} protein and B_{max} of C receptors 1018±91 fmol mg^{-1} protein. However, K_d was higher for C receptors (38.04±4.08 *versus* 0.96±0.09 nmol l^{-1}), indicating a

lower affinity for clearance receptors. Scatchard analysis of the saturation curves yielded similar affinities to those obtained by competitive binding assay but lower ANF binding sites (B_{max} 575±105 fmol mg^{-1} protein and K_d 0.21±0.04 nmol l^{-1}). The discrepancy may have arisen from the different methods of calculation used (Hall, 1992).

Discussion

This study demonstrates that toad lungs possess a prominent ANF system. The lungs synthesize and contain immunoreactive ANF and also exhibit the various ANF receptor subtypes.

Lung and atrial immunoreactive ANF was determined in the present study using an antibody raised against the carboxy terminus of rat ANF. In our early work (Chapeau *et al.* 1985), a strong positive reaction with antibodies produced against rat carboxy-terminal Arg^{101}–Tyr^{126} was obtained in atrial tissue of a toad (*Bufo marinus*) and a frog (*Rana catesbeiana*) but a much weaker reaction was observed in ventricular tissue. Furthermore, the presence of the N-terminal fragment of ANF precursor has been demonstrated in both atrial and ventricular tissue from the adult male frog *Rana ridibunda* (Gilles *et al.* 1990; Netchitailo *et al.* 1988). Both observations indicate a great degree of homology between rat and frog ANF, suggesting that the structure of ANF has been well-conserved during evolution. Indeed, it appears that the amino acid sequence of frog atrial ANF is closely related to that of mammalian ANF. Lazure *et al.* (1988) purified ANF from the atria of the frog *Rana ridibunda* and determined its primary structure, showing it to have a remarkable homology with rat ANF (Ser^{99}–Tyr^{126}). The disulfide bridge that is a condition of expression of biological activity is entirely conserved in both species. The carboxy-terminal ANF peptide has also been isolated and characterized in another frog species, *Rana catesbeiana* (Sakata *et al.* 1988), and similar homology with rat ANF has been found.

The amount of ANF found in the toad lung was twice as high as the levels reported for the lung tissue of similarly sized mammals (Gutkowska *et al.* 1989). Moreover, in contrast to mammals, we found that amphibians have a higher concentration of ANF in the left atrium than in the right atrium, an observation that has also been reported by others (Netchitalio *et al.* 1986).

Molecular cloning has identified three types of natriuretic peptide receptors. Two of these are the guanylyl cyclase ANF receptors, GC-A and GC-B, which are glycoproteins of about 120 kDa forming an integral part of the guanylyl cyclase molecule (Aranjaniyil *et al.* 1987). These receptors mediate the physiological effects of ANF by regulating the accumulation of intracellular guanosine 3′,5′-cyclic monophosphate (cyclic GMP). The third type, the C receptor, exists as a disulfide-bridged dimers that, under reducing conditions, appears as a protein with a molecular mass of 66 kDa. This receptor has a shorter intracellular tail that lacks the cyclase domain, but it may be coupled to the adenylyl cyclase/cyclic AMP system through an inhibitory Gi protein (Anand-Srivastava and Trachte, 1993). In addition, C receptors may clear ANF from the plasma by a process involving binding, internalization and degradation in the cytosol. Blockade of ANF clearance receptors increases plasma ANF levels and potentiates the biological activity of ANF (Maack *et al.* 1987). Discrimination between the receptors is based on their binding affinities to natriuretic peptides. Binding studies show that, of the guanylyl cyclase receptors, GC-A binds ANF⩾BNP>CNP, while GC-B binds CNP>ANF⩾BNP. The C receptor is less specific in its binding requirements since it is able to bind all natriuretic peptides and ANF synthetic analogs. Thus, the five-amino-acid ring-deleted ANF analog C-ANF, which shows high selectivity for clearance receptors, can be used to demonstrate their presence (Maack *et al.* 1987).

The present study shows that all the ANF receptor subtypes are found in toad lung. Whereas the guanylyl cyclase receptors mediate the known biological actions of ANF, the role of C receptors is still being debated. However, there is evidence that the C receptors are involved in removing ANF from the circulation. Radiolabeled ANF, injected as a bolus, is removed from the rainbow trout (*Oncorhynchus mykiss*) circulation within 2 min. The highest levels of uptake of iodinated ANF are found in all four arches of the gill. During a single passage through the gills, 60 % of [^{125}I]ANF is extracted. Extraction of [^{125}I]ANF by the isolated perfused gill is 53 %, a value similar to that observed *in vivo* (Evans *et al.* 1989).

The well-developed pulmonary ANF system of *Bufo paracnemis* could prevent or alleviate edema, as suggested by studies of ANF function in mammalian lungs (Imamura *et al.* 1988). In mammals and birds, the ventricular septum permits division into a low-pressure pulmonary circulation and high-pressure systemic circulation. A ventricular septum is absent in amphibians and non-crocodilian reptiles, and the pulmonary artery is perfused at a high pressure, which leads to substantial plasma filtration within the pulmonary capillary bed (Burggren, 1982; Shelton, 1985; Smits, 1989). Thus, the amount of plasma leaving the pulmonary capillaries of *Bufo marinus* is about 100 times greater than in similarly sized mammals (Smits *et al.* 1986; Smits, 1989). Anuran amphibians possess a range of mechanisms to prevent the development of pulmonary edema. The lymphatic systems of the lung appear to be highly effective in *Bufo marinus* (Smits, 1989). Moreover, toads possess baroreceptors at the pulmocutaneous artery which supply both the lungs and part of the skin. The position of these baroreceptors led Smits *et al.* (1986) to suggest that baroreceptor control in the toad is closely linked with a high risk of pulmonary edema. Anuran amphibians are equipped with sphincters distal to the point at which the pulmocutaneous artery branches (de Saint-Oubain and Wingstrand, 1979). This arrangement means that the relative distribution of pulmonary and cutaneous blood flow can be controlled, depending upon the availability of O_2 at the skin or lung surfaces (Boutilier *et al.* 1986). The reduction of pulmonary blood flow could provide an effective defence against edema, but this aspect needs further study. The present

data add the ANF system to the above list of possible edema-reducing mechanisms in toad lungs.

It should be mentioned that hibernation may have an effect on the ANF system since it is known to considerably alter osmotic regulation in toads (Barker-Jørgensen *et al.* 1978). Seasonal effects were not addressed in the present study, although the dry season correlates with burrowing and physiological changes in *Bufo marinus* (Boutilier *et al.* 1979). We are carrying out further studies to investigate whether there are seasonal changes in the ANF system of *Bufo paracnemis*. Seasonal effects have been demonstrated in mammals, with plasma ANF levels having been shown to decrease significantly during hibernation in marmots (Zatzman and Thornhill, 1989).

In conclusion, we report that the toad *Bufo paracnemis* has a well-developed pulmonary ANF system, in addition to that of the atria and ventricles. The high pulmonary perfusion pressure characteristic of anuran amphibians suggests a possible role for ANF in protecting the lungs against edema.

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