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The amino acid sequences of frog heart atrial natriuretic-like peptide and mammalian ANF are closely related

C. Lazure, H. Ong*†, N. McNicoll*, P. Netchitailo+, M. Chrétien, A. De Léan* and H. Vaudry+

*The J.A. de Sève Laboratory of Molecular Neuroendocrinology, *Laboratory of Molecular Pharmacology, Clinical Research Institute of Montreal, 110 Pine Ave. West, Montreal, †Faculty of Pharmacy, University of Montreal, Canada* and *+Groupe de Recherche en Endocrinologie Moléculaire, UA CNRS 650, Unité alliée à l'INSERM Faculté des Sciences, Université de Rouen, 76130 Mont-St-Aignan, France*

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Despite few studies conducted in non-mammalian species, there has been a number of reports pertaining to the occurrence of a natriuretic-like substance in lower organisms. Thus, an immunoreactive substance reacting with antibodies directed against mammalian atrial natriuretic factor has previously been detected both in heart atria and ventricles of a chordate, the frog. This substance was isolated and purified from frog heart atria and its amino acid sequence established. The sequence, Ala-Pro-Arg-Ser-Ser-Asp-Cys-Phe-Gly-Ser-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Met-Gly-Cys-Gly-Arg-(Phe), is highly homologous to known mammalian ANF sequences. However, when aligned with the complete mammalian ANF precursor sequence at positions 121 to 151, it exhibits a single amino acid insertion at position 129 and other substitutions at positions 121, 125, 133, 135, 144, 147 and 148. Some evidence is also presented concerning the occurrence of uncleaved frog pronatriodilatin, the precursor form of ANF. This study represents the first report pertaining to the structure of a non-mammalian ANF and its precursor.

Atrial natriuretic factor; Amphibian atrium; Peptide evolution; Amino acid sequence; (Frog)

1. INTRODUCTION

In recent years, atrial natriuretic factor (ANF) or atriopeptin (ANP) has been shown to be a potent, diuretic, natriuretic and vasorelaxant peptide. Since its biological action has been reported upon injection of heart atria extract [1], numerous studies have been conducted on its localization, its biological properties and its structure (reviewed in [2]). Thus, in mammals, its major but not exclusive site of synthesis and secretion has been shown to be heart cardiocytes. Recently, the widespread occurrence of ANF and ANF-like peptide in a variety of other tissues have stirred considerable interest since these new locations could be in favor of other biological activities apart from those already documented. Furthermore, its highly conserved primary structure, in particular with respect to its intramolecular disulfide bridge, has been exemplified in a great number of species albeit all belonging to the same phylum. Thus, the amino acid sequence of ANF and sometimes, also, of its precursor termed pro-ANF or pronatriodilatin, is known for rat, human, bovine, porcine, dog and rabbit [2]. Furthermore, the structure of rat, human, mouse and bovine genes are all known thus confirming yet again that not only are the amino acid sequences extremely homologous but also the structural organization of the genes.

Even though there have been reports pertaining to the presence of a peptide akin to mammalian ANF in lower species, no structural data are as yet available. Nevertheless, ANF-like substances have been detected in the hearts of various species mostly through the use of immunological methods such

Correspondence address: C. Lazure, J.A. de Sève Laboratory of Molecular Neuroendocrinology, Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, Quebec H2W 1R7, Canada

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as radioimmunoassays and immunocytochemistry. Chromatographic behavior, electron microscopy and bioassays, general methods relying on a priori mammalian ANF properties, were also used. Thus, antibodies directed against the ANF bioactive core (position 101–126 of pro-ANF) were able to produce a positive reaction in the atria of toad, frog, snake, hen and fish [3]; this study thereby confirmed results previously obtained by DeBold and Salerno [4] in frog hearts. Similar results were described by Reinecke et al. [5] which confirmed that cardiac hormones are not confined to mammals but can be found in avian, reptiles, amphibian and bony fishes. More precisely, ANF-like peptides together with the precursor forms were described in both frog atria [6] and, interestingly albeit at a much reduced level, in ventricles [7]. Finally, the heart atria and ventricles as well as cerebral and esophageal ganglia of a snail (*Helix pomatia*) [8], the hemolymph as well as numerous organs including ganglia of a marine snail (*Aplysia californica*) [9], and the eyestalks of a crustacean (*Homarus gammarus*) [10] were all recently shown to contain ANF-like immunoreactivities. In these species, it is largely recognized that such entities belong to the neuroendocrine system where, in addition to a purely endocrine function, they might play a role as neurotransmitter or neuromodulator. The recent demonstration that, in mammals, ANF is largely distributed at various locations in the central nervous system (CNS) and that, in addition, it exists in brain, at least in porcine, in two molecular forms, ANF and brain natriuretic peptide (BNP) [11], can be taken as evidence in favor of such a dual role.

In the present study, we report the purification and chemical characterization of ANF from acid extracts of frog (*Rana ridibunda*) heart atria. This represents the first report concerning the primary structure of this biologically important peptide and its precursor in a non-mammalian species and thus confirms results obtained by immunological methods.

2. MATERIALS AND METHODS

2.1. *Peptide extraction and crude purification*

Deep frozen frog heart atrias (49 g, from Mont Saint-Aignan, France) were homogenized using a Polytron in 500 ml of 1 M acetic acid containing 1 mM EDTA and the homogenate was centrifuged at 40000 × *g* for 30 min. The resulting supernatant was directly applied for a SP-Sephadex-C25 column (1.5 × 12 cm) at a flow rate of 2.5 ml/min. After application of the sample, the column was washed successively with (i) 40 ml of 1 M acetic acid, (ii) 40 ml of 150 mM ammonium acetate buffer, pH 6.0, (iii) 40 ml of 150 mM ammonium acetate buffer, pH 7.4, and (iv) finally, the retained material was eluted with 1.5 M ammonium acetate buffer, pH 7.4. The pH of the eluate was immediately adjusted to 4–5 by addition of 2.5 ml of glacial acetic acid and 150 μl of trifluoroacetic acid (TFA).

The acidified fraction was then loaded onto a μBondapak-C_{18} semi-preparative column (1 × 15 cm) connected to a Waters 600 liquid chromatographic system using a flow rate of 5 ml/min. The elution was performed using a linear gradient (10–70%) of acetonitrile containing 0.1% TFA at a flow rate of 5 ml/min over a period of 120 min. The eluted fractions were monitored by measuring absorbance at 280 nm. The ANF-containing fractions identified by radioimmunoassay (RIA) and radioreceptor assay (RRA) were pooled and labeled fraction A, B and C following their order of elution from the column. Fractions A and C were further purified by HPLC.

2.2. *Purification of ANF-fraction A*

Fraction A was further purified on a Vydac-C_{18} column (0.46 × 25 cm) in three steps using various gradients or ion-pairing agents.

It was first eluted from the column using a linear gradient (13.5–40.5%) of acetonitrile containing 0.1% TFA using a flow rate of 1 ml/min over 60 min. The ANF-immunoreactive peak A was resolved into two peaks eluting at 22% and 23.4% acetonitrile denoted A1 and A2, respectively. Fraction A1 was rechromatographed using a linear gradient (18–45%) of acetonitrile and 0.05% heptafluorobutyric acid (HFBA) at 1 ml/min for 60 min. During this step, A1 was separated into two fractions denoted A1a and A1b which eluted at 30.6% and 32.4% acetonitrile, respectively. Each fraction was rechromatographed using the same conditions and collected manually. Similarly, fraction A2 was purified using a linear gradient (18–45%) of acetonitrile and 0.05% HFBA at 1 ml/min for 60 min and yielded two immunoreactive fractions denoted A2a and A2b, and eluting at 31% and 33% acetonitrile, respectively. Both fractions were rechromatographed using a linear gradient (10–40%) of acetonitrile and an aqueous phase composed of 50 mM potassium phosphate and 0.1% triethylamine, pH 3.5 (TEAP buffer), at 1 ml/min for 60 min. The fractions were then subsequently desalted using a linear gradient (15–45%) of acetonitrile and 0.1% TFA at 1 ml/min over a period of 30 min. Both fractions A2a and A2b eluted at 25% acetonitrile and were manually collected. All purifications were followed by monitoring UV-absorbance at 214 nm.

2.3. *Purification of ANF-fraction C*

The fraction C was submitted to a linear gradient (30–60%) of acetonitrile and 0.1% TFA at 1 ml/min for 60 min. The resulting immunoreactive peak was further purified using a linear gradient (15–45%) of isopropanol and TEAP buffer at 1 ml/min for a period of 60 min. The single ANF-like peak, eluting at 34.5% isopropanol, was finally desalted using a linear gradient (20–50%) of acetonitrile and 0.1% TFA at 1 ml/min in 30 min.

2.4. *ANF detection procedures*

The ANF content of all HPLC-derived fractions was constantly monitored using two previously described procedures. The first one relies on RIA using a rabbit antiserum developed against rat ANF (99–126) coupled to bovine serum albumin using a soluble carbodiimide; cross-reactivity of this antiserum with synthetic ANF sequences (atriopeptin I, II, III and rat ANF (101–126)) was greater than 90% [12]. The second one relied on the measurement of ANF-like reactivity using RRA as previously described [13]. Both assays detect the biologically active and circulating form of ANF (99–126) but the cross-reactivity of the precursor form (1–126) in the RRA is only 5–10% of that obtained with RIA.

2.5. *Amino acid and sequence analysis*

The HPLC-purified peptides were hydrolyzed in their native or performic acid oxidized state in vacuo using 5.7 N HCl at 110°C for 24 h. The amino acids were separated and quantitated on a modified Beckman 120C autoanalyser equipped with a Variant DS604 plotter/integrator; detection was carried out using post-column ninhydrin.

The amino acid sequence determination was carried out on two automatic sequenators. The liquid-phase Beckman 890M and the gas-phase Applied Biosystem model 470A sequenators were operated as described [14]. The released thiazolinones, after conversion into PTH-derivatives, were separated and quantitated by HPLC as described [15].

3. RESULTS

In order to gain some insight on the behavior of ANF-like molecules in frog atria, a down scaled isolation scheme was attempted. Enough material was obtained so that an amino acid composition of the reduced and carboxymethylated ANF-like peptide and a partial amino acid sequence was possible. Both preliminary results confirmed the high degree of relatedness of the frog material to mammalian ANF. Indeed, the amino acid composition as revealed by the following molar ratio Asx (2.3), Ser (4.5), Glx (1.6), Gly (9.0), Ala (2.0), Met (1.4), Ile (1.7), Phe (1.8), Arg (5.7) and CM-Cys (1.9) is remarkably close to the known one of mammalian ANF (99–126); one should note the absence of Leu, Lys, His, Thr and especially Tyr residues. The amino acid sequence determination using the liquid-phase sequenator allowed the identification of 20 residues out of 30 cycles and indicated the similarity of the frog sequence in particular in the disulfide loop to its mammalian counterpart (not shown).

Due to the restricted availability of the frog atria and the low amount of material, the purification of the material obtained from 49 g of tissue was accomplished using the following three criteria, (i)

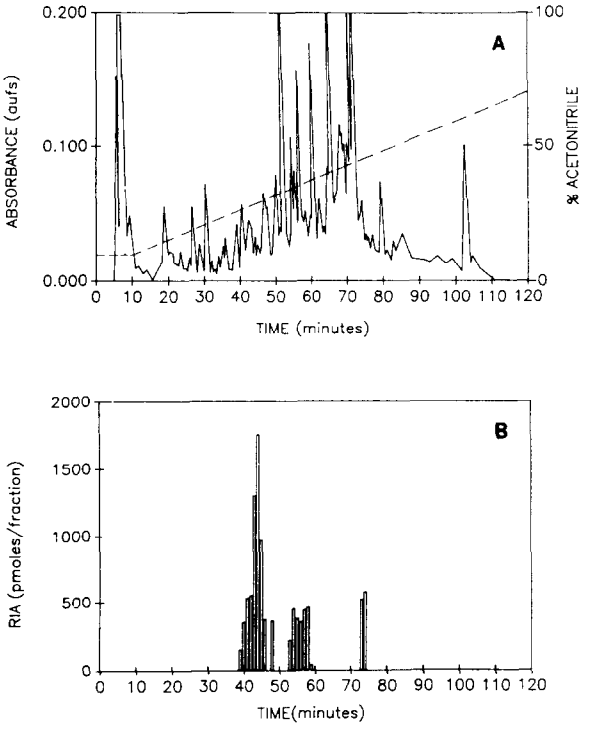

Fig.1. (A) Reverse-phase HPLC chromatogram of acid extracts of frog heart atria on semi-preparative μBondapak-C_{18} column with a linear gradient of acetonitrile as indicated by the dashed line. Monitoring of the eluate was done by measuring absorbance at 280 nm. (B) Monitoring of the eluate using RIA. The two activity zones eluting at 28.5% and 44% acetonitrile, respectively, were collected and further purified.

detection by RIA and RRA, (ii) appearance of a homogeneous peak on HPLC and (iii) an amino acid composition similar to the one obtained above. Throughout this study, these criteria were applied to all fractions eluting from the various columns used.

The initial separation, folowing the ion-exchange step, yielded three major immunoreactive fractions as shown in fig.1. The elution positions were also very close to what has been observed under the same conditions while purifying the ANF and its precursor from bovine adrenal medulla [16]. Indeed, the lower molecular mass form of ANF was found to elute at 26% acetonitrile (compared here to 28.5%) whereas the precursor form (ANF 1–126) eluted at 39% (compared here to 44%). It should be mentioned here that actually throughout the later purification steps this chromatographic correspondence was

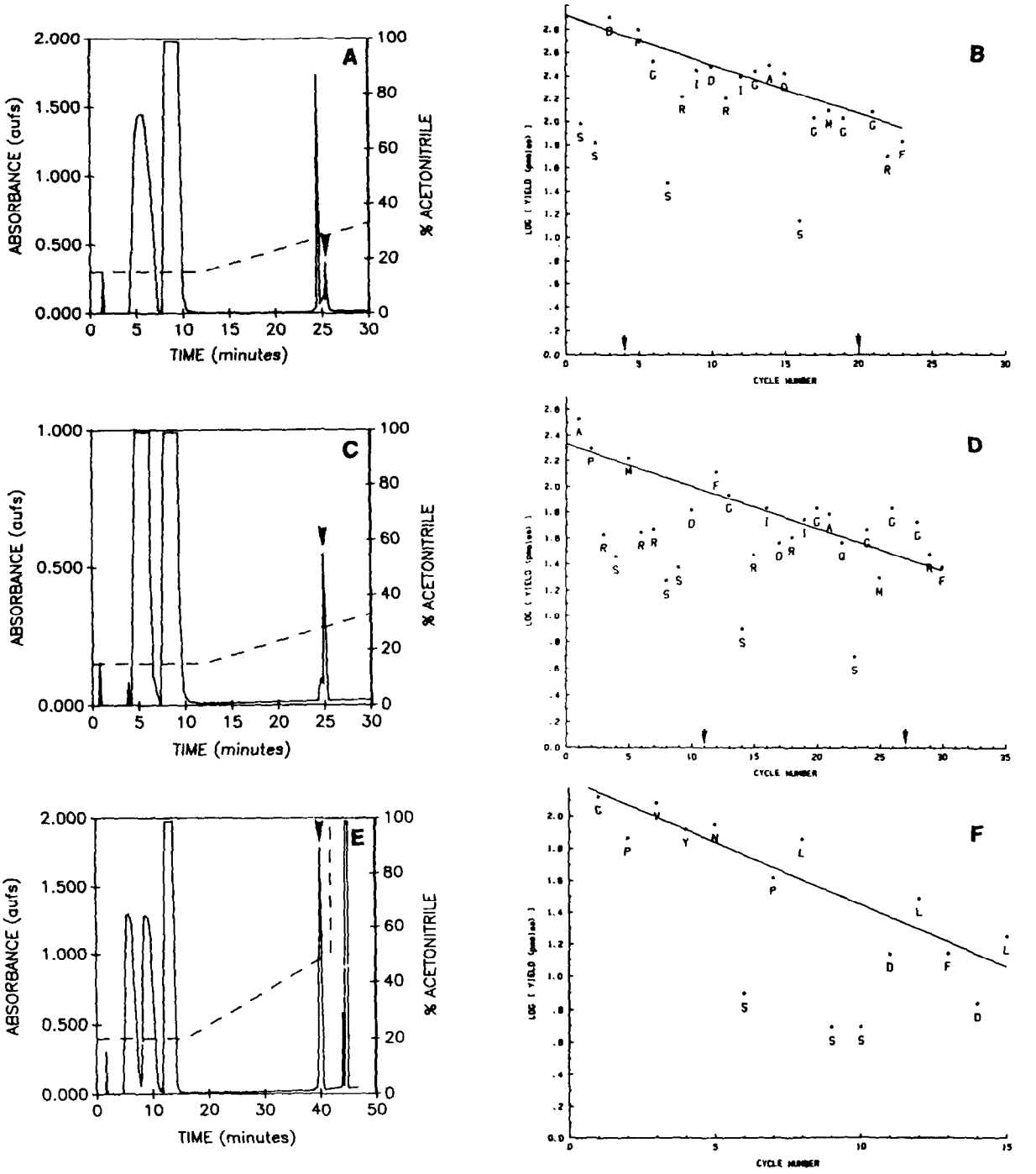

Fig.2. (A, C and E) Reverse-phase HPLC chromatograms representing the last purification step of fraction A2a (A), A2b (C) and C (E), respectively. The arrowhead indicates in each case the peak which was manually collected and submitted to amino acid sequencing. Detection of the peaks was done by measuring absorbance at 214 nm. (B, D and F) Quantitative yields of PTH-amino acid derivatives recovered during sequence analysis of fraction A2a (B), A2b (D) and C (F) are illustrated as a function of cycle numbers after normalizing to a PTH-NorLeucine internal standard. The repetitive and initial yields are determined by the slope and intercept obtained by linear regression analysis on selected stable PTH-derivatives.

observed. Two of the three fractions were processed further, the early eluting one (around 45 min, fraction A) containing the bulk of ANF immunoreactivity and the late eluting one (around 74 min, fraction C) which was recognized by RIA but minimally if at all by RRA.

Following numerous chromatographic steps as described in section 2.2, four immunoreactive fractions, albeit displaying low RIA-reactivities at the later stage of purification, were isolated and three of these, A1b, A2a and A2b, submitted to amino acid sequencing. Fraction A1b was found to contain two sequences which were both identified as (i) APRSMRRSSDX-
FGSRIDRIGAQXGMGXGR(F) for which the initial and repetitive yields were 115 pmol and 92.8%, respectively (not shown), and (ii) WIKY-QEYDEAGPSIVXXKXF for which the initial and repetitive yields were 47 pmol and 91.8%, respectively (not shown). The latter, upon searching in the Protein Data Bank (National Biomedical Research Foundation, Washington, proved to be very closely related to the COOH-terminal fragment of mammalian cardiac muscle actin starting at position 358 (11 residues out of 14 are identical). Fraction A2a, as shown in fig.2A, proved to be contaminated up to the last step with a high UV-absorbance material eluting before it which could not be identified either by composition or by sequence analysis (no PTH-derivatives were observed). However the fraction marked by an arrowhead yielded the sequence shown in fig.2B with an initial yield and repetitive yield of 829 pmol and 90.7%, respectively. Here again though, a minor sequence (not shown) was detected which corresponded to KVDAHKIG-GEALAR (initial and repetitive yields of 274 pmol and 95.1%); this sequence proved 100% identical to a fragment of frog hemoglobin β-chain. Finally, fraction A2b despite its apparent homogeneity as shown in fig.2C and its yielding a unique sequence (initial and repetitive yield of 213 pmol and 92.8%) as shown in fig.2D failed to give, as observed with the other fractions derived from pool A, an unambiguous amino acid composition. This fact complicated greatly the assignment of the COOH-terminal residues. Indeed, during the sequencing of A1b, A2a and especially A2b, the presence of Arg at cycle 29 and 30 as well as Phe at cycle 30 and 31 rendered very difficult the assignment of an Arg-Phe, an Arg-Arg-Phe or even an Arg-Arg-Phe-Phe sequence past the Arg occupying position 29. Due to the amino acid composition described previously in the preliminary results and also to the sequence similarity (as discussed in the following section) between known ANF sequences, the COOH-terminal sequence was tentatively identified as being Arg-Phe. The presence of Arg and Phe in cycle 30 and 31 could be explained by high carry-over of the preceding cycles which, however, cannot rule out entirely the other possibilities. Taking into account the identification of the contaminants and of cysteic acid upon oxidation and amino acid analysis, it can be said that the sequence results agreed with the presence of two half-cystine residues (fig.2B, cycle 11 and 27 and fig.2D, cycle 4 and 20) since these residues are not detected with the method used.

Based upon its chromatographic behavior which followed very closely the one previously observed for bovine pro-ANF [16] and its lack of reactivity in the RRA, fraction C was tentatively identified as frog pro-ANF. This identification was reinforced following final purification, shown in fig.2E, and amino acid sequence analysis, shown in fig.2F. The deduced amino acid sequence GPVYNSPLS-SDLFDL (initial yield and repetitive yield of 169 pmol and 84%) despite the need to be confirmed yet again, and the following amino acid composition, Asx (16.9), Thr (3.1), Ser (16.6), Glx (12), Pro (7.3), Gly (10.2), Ala (10.7), Val (3.2), Met (2.7), Ile (1.9), Leu (11.6), Tyr (3.2), Phe (3.0), Lys (3.2), Arg (13.2) and Cys (1.9) would be in agreement with its identification as frog pro-ANF. Indeed, the amino acid composition, where His was not detected and Trp not identified, predicts a molecule of around 121 residues and yields amino acid ratios close to those observed with mammalian pro-ANF.

4. DISCUSSION

Despite the problems encountered during purification (and probably due to protein degradation during shipment or extraction), alluded to in section 3, and despite the absence of biological data apart from its binding to the receptor in the RRA, it can be said that, indeed, an amphibian form of ANF has been isolated and sequenced.

As shown in fig.3, the deduced sequence of the

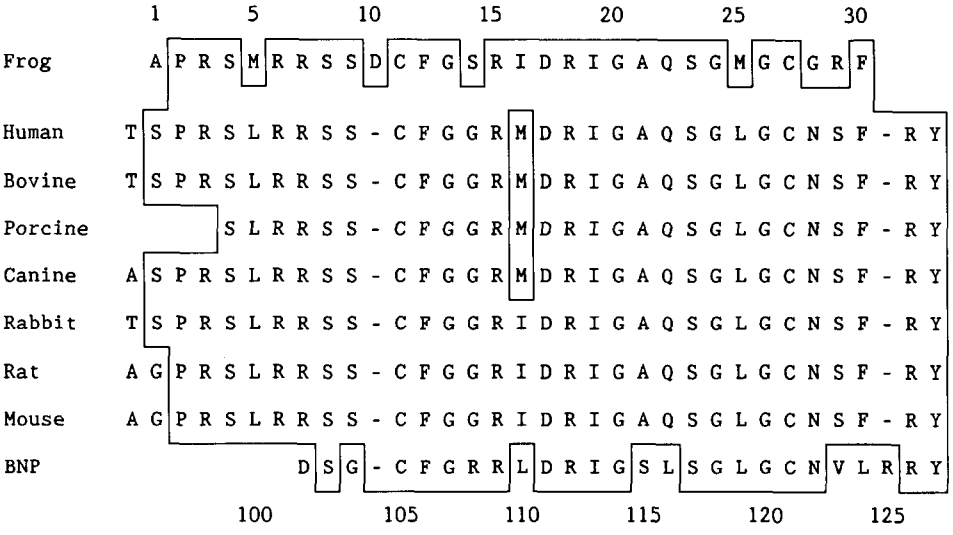

Fig.3. Amino acid sequence comparison between frog ANF and previously reported sequences of mammalian ANF taken from [2] and of BNP [11]. Identical residues are enclosed in boxes whereas the - sign indicates a gap introduced in order to maximize the identity. The numbering at the bottom of the figure refers to the residue numbering in mammalian ANF sequences. The Phe residue occupying position 30 was tentitatively assigned (see sections 3 and 4).

longer form of frog ANF isolated displays a significant homology to known mammalian sequences. The whole disulfide bridge, recognized as important for expression of biological activity in mammals [17] is entirely conserved in both systems. Within these positions (11–27), only two substitutions were observed, both of which resulted from single nucleotide substitutions and are chemically equivalent; interestingly, the only previously reported substitution, the Met/Ile at position 16, corresponds here to an Ile residue as found in rodent. Other residues thought to be important for the biological action are also conserved such as those bearing hydrophobic side chains, Phe-12, Ile-19 and Met-25 (here, replacing a Leu) and those, Gly-20, Gly-24 and Gly-26, which might be involved in conformation are retained [18]. On the COOH-terminal side, in view of the problems mentioned in section 3, the sequence has been tentatively assigned as Gly-Arg-Phe even though no unambiguous identification could be made. The COOH-terminal sequence thus needs to be reconfirmed even though no evidence of the presence of the normally encountered Tyr residue or others apart from Arg and Phe was obtained during this study. The latter observation could prove important since, in mammals, high potency is obtained as long as at least one cationic site is kept at either termini [18]. Upon maximal alignment of the region preceding the disulfide bridge, one can readily see that, in frog, a single amino acid residue was inserted at position 10 but that the other amino acids are well conserved. In particular, the cleavage site which appears to be recognized by mammalian processing enzymes, the single Arg-98, is preserved together with the region surrounding it.

Interestingly, whereas the predominant circulating ANF form which results from this cleavage is ANF 99–126 in mammals [19], here two frog ANF forms were isolated. The first one, isolated both in A1a and A2b, is longer by 7 residues than the shorter one (A2a); the former thus resembles those previously characterized by Seidah et al. [20], whereas the latter represents those denoted atriopeptin by Currie et al. [21]. Thus, whereas one can confidently predict that the frog peptides should exhibit ANF bioactivities akin to mammalian ANF, it would be hazardous to speculate on the exact secreted molecular form of the active entity in frogs.

Finally, the last fraction examined (fraction C) could possibly be related to the frog pro-ANF molecule considering that its behavior in RIA and

RRA and its amino acid composition are in agreement with what was observed in mammals. Furthermore, the partial sequence of this fraction displays a significant similarity to the mammal's sequence. Indeed, amino acids in the sequence Gly-Pro-Val-Tyr-Asn-Ser-Pro-Leu-Ser-Ser-Asp-Leu-Phe-Asp-Leu can be found within mammalian sequences; Pro-2, Tyr-4, Asp-11, Asp-14 and Leu-12 all occupy the same positions in all known sequences [2] whereas Val-3 (Met in human), Asn-5 (Gly in bovine, porcine and canine and Ser in rat and mouse), Ser-6 (Ala in human and rodents) were also observed. Thus 8 positions out of 14 are occupied by amino acid previously observed in pro-ANF sequences of mammalian origin. The extent of this correspondence will depend on further studies presently being carried out.

All in all, this report demonstrates the close similarity between mammalian and frog ANF and thus should help in future studies concerning the role and structure of ANF in lower species.

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