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ATRIAL NATRIURETIC FACTOR IN RAT HYPOTHALAMUS, ATRIA AND PLASMA: DETERMINATION BY SPECIFIC RADIOIMMUNOASSAY

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A rapid and reproducible radioimmunoassay method was developed for rat atrial natriuretic factor (ANF)-IV. The method is also applicable to human atrial peptide. ANF was detected in rat hypothalamus (5.03 pmoles/g tissue), right (86.8 pmoles/mg tissue) and left atria (52.5 pmoles/mg tissue), and plasma (156 fmoles/ml). After high salt intake immunoreactive ANF in atria and plasma increased significantly, while a significant decrease was observed in hypothalamus. Gel chromatography revealed high and low molecular weight ANF in atria and hypothalamus while only a low molecular weight form was found in plasma.

Atrial natriuretic factors (ANF) are a group of peptides possessing a potent natriuretic activity (1,2), and the ability to inhibit agonist-induced contraction of smooth muscles (3,4). The atrial peptides have been purified from rats and human tissues and their structures have been determined (5-13). However, the lack of a rapid sensitive method for specific quantification of this peptide in physiological samples is a serious deterrent to the elucidation of its pathophysiological significance. The presence of the peptide in blood has not yet been unequivocally demonstrated. In order to establish a specific method for the quantitative identification of ANF, we have raised antibodies to rat ANF-IV and established a radioimmunoassay. Employing this method we have investigated the presence of immunoreactive ANF in rat atria, plasma, and hypothalami and its change upon sodium loading.

MATERIALS AND METHODS

Peptides - Rat ANF-IV with the amino acid sequence H_2N-RSSCFGGRIDRIGAQSGLGCNSFRY-COOH (9,13) and α-human atrial natriuretic polypeptide (α-hANP$_{4-28}$) with the amino acid sequence H_2N-RSSCFGGRMDRIGAQSGLGCNSFRY-COOH (10) were synthesized

Abbreviations used in this paper: ANF, atrial natriuretic factor; α-hANP, α-human atrial natriuretic polypeptide.

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by a solid phase method and purified by chromatography on a SP-Sephadex C-25 column and high-performance liquid chromatography (15).

Immunization - ANF-IV conjugated to bovine thyroglobulin (Sigma Chemical Co.) was used as immunogen. To a solution of 2.5 mg of ANF-IV and 10 mg of bovine thyroglobulin in 1.0 ml of 0.1 M sodium phosphate buffer, pH 7.0, were added 30 µl of 0.8 M glutaraldehyde dropwise. The mixture was stirred for 4 hr at room temperature and dialyzed against 0.01 M sodium phosphate buffer, pH 7.0, for 24 hr at 4°C. A portion of the conjugate containing 600 µg of ANF-IV was emulsified with an equal volume of Freund's complete adjuvant (Difco) and used for immunizing three Dutch-belted rabbits by multiple intradermal injections on their backs. They were boosted twice with the same amount of the conjugate emulsified with Freund's incomplete adjuvant at 4 week intervals and bled 2 weeks after the last immunization.

Iodination - ANF-IV was radioiodinated by the chloramine T method (16). The labeled ANF-IV was purified by applying the reaction mixture to a Sep-Pak C_{18} cartridge (Waters Associates) and eluting the labeled ANF-IV with 80% methanol. [^{125}I]ANF-IV was further purified with the same cartridge immediately before use. The specific activity of [^{125}I]ANF-IV was approximately 120 µCi/µg.

Radioimmunoassay - The standard diluent for radioimmunoassay was 0.1 M Tris-acetate buffer, pH 7.4, containing 0.1% bovine serum albumin (Fraction V, Sigma), aprotinin (Sigma, 500 kallikrein inhibitor units/ml), soybean trypsin inhibitor (Sigma, 50 BAEE units/ml), and 0.02% sodium azide. One hundred µl of standard ANF-IV or sample were incubated for 48 hr at 4°C with 500 µl of the standard diluent, 100 µl of antiserum (final dilution 1:1,000), and 100 µl of the labeled peptide. The separation of the antibody-bound and free peptide was performed by adding 800 µl of a suspension of dextran-coated charcoal consisting of 600 mg of Norit (Fisher Scientific Co.) and 62 mg of Dextran T-40 (Pharmacia Fine Chemicals) in 100 ml of 0.1 M Tris-acetate buffer, pH 7.4.

Experimental animals - Twelve male Sprague-Dawley rats weighing 175-200 g were housed in a temperature and humidity-controlled room with a light-dark cycle (12 hr/12 hr) for 1 week before the experiment and during experimental period. During 2 weeks of the experimental period, 6 rats were maintained with ad libitum supply of food and water (control group). Water was replaced with 1% NaCl solution as drinking water for 6 rats in the high NaCl group.

Preparation of samples - Blood samples were taken from abdominal aorta under pentobarbital anesthesia into plastic syringe containing aprotinin (500 kallikrein inactivator units/ml), soybean trypsin inhibitor (50 BAEE units/ml), and EDTA (1 mg/ml). Plasma was separated by centrifugation at 4°C and immediately processed for extraction of ANF as follows. One milliliter of each plasma sample was applied on a Sep-Pak C_{18} cartridge and the adsorbed peptide was eluted with 3 ml of 80% methanol. The eluates were evaporated under nitrogen gas stream, lyophilized, dissolved in the standard diluent and subjected to radioimmunoassay for ANF and gel chromatography.

Atria and hypothalami were removed and weighed immediately after the blood sampling. These samples were homogenized in 2 ml of 0.1 N HCl and centrifuged at 10,000 x g for 60 min at 4°C. The supernatants were lyophilized, reconstituted in 1 M acetic acid, and heated in a boiling waterbath for 10 min. These preparations were again lyophilized, reconstituted in the standard diluent, and subjected to radioimmunoassay and gel chromatography.

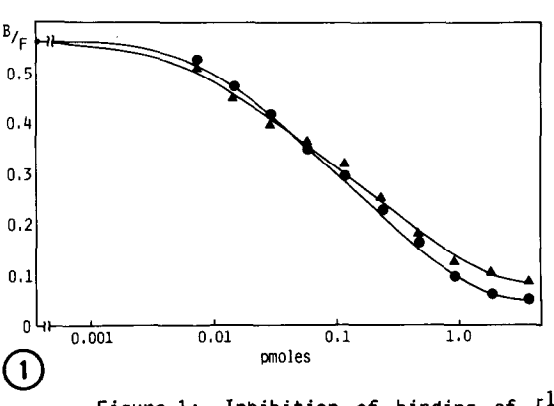
Gel exclusion chromatography - Gel filtration was performed on a Bio-Gel P-10 column (1.5 x 26 cm) eluted with the standard diluent at 4°C and a flow rate of 3.7 ml/hr. The column was calibrated with blue dextran, mouse epidermal growth factor, ANF-IV, and angiotensin I. Recovery of [^{125}I]ANF-IV in gel chromatography was 85%.

RESULTS

A typical radioimmunoassay standard curve with ANF-IV is shown in Fig. 1. Significant inhibition of the binding of [^{125}I]ANF-IV to antibodies was evident with as little as 8 fmoles/tube of cold ANF-IV. This antiserum recognized the human atrial natriuretic peptide α-hANP$_{4-28}$, the cross-reactivity of which was 81% on a molar basis. The intra- and inter-assay coefficients of variation were 5.8% and 8.9%, respectively. The serial dilution of extracts in rat atrium, plasma, and hypothalamus inhibited the binding of [^{125}I]ANF-IV to anti-ANF antiserum in parallel with the standard curve of ANF-IV (Fig. 2).

The concentrations of immunoreactive ANF in right atrium, left atrium, plasma and hypothalamus of control group and high NaCl group of rats are shown in Table 1. In high NaCl group, the concentrations in right atrium, left atrium and plasma increased significantly compared with controls (Table 1). In contrast, immunoreactive ANF in hypothalamus in high NaCl group decreased significantly.

By gel filtration at least four peaks of immunoreactive ANF were demonstrated in the extracts of rat right atrium, left atrium, and hypothalamus, while only one immunoreactive peak was seen in rat plasma as shown in Fig. 3.



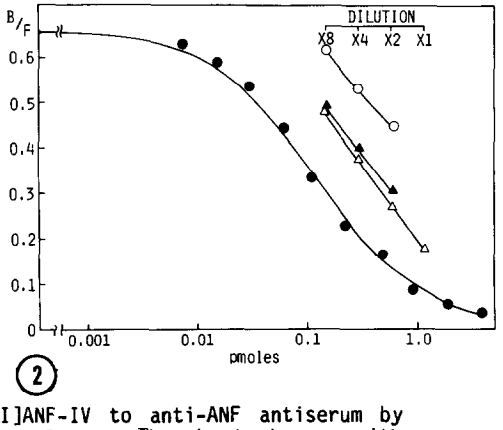


Figure 1: Inhibition of binding of [^{125}I]ANF-IV to anti-ANF antiserum by serial dilution of ANF-IV and α-hANP$_{4-28}$. The standard curve with synthetic ANF-IV shows that 0.008 to 2.0 pmol/tube of ANF-IV are measurable with this antiserum. The cross-reactivity of α-hANP$_{4-28}$ is 81%. ANF-IV (●); α-hANP$_{4-28}$ (▲).

Figure 2: Inhibition of binding of [^{125}I]ANF-IV to anti-ANF antiserum by serial dilution of extracts of rat atrium (△), plasma (○), and hypothalamus (▲), exhibiting parallel inhibition with that of the standard ANF-IV (●).

Table 1. Immunoreactive ANF in rat atrium, plasma, and hypothalamus[a]

	No.	Control group	High NaCl group
Left Atrium (pmol/mg tissue)	6	52.5 ± 2.7	94.2 ± 6.2*
Right Atrium (pmol/mg tissue)	6	86.8 ± 7.2	146.3 ± 8.1*
Plasma (fmol/ml)[b]	6	156.1 ± 13.8	354.7 ± 23.4*
Hypothalamus (pmol/g tissue)	6	5.03 ± 0.70	2.38 ± 0.29*

[a]Values are mean ± SE. [b]Corrected for 43% yield of recovery during the extraction procedure..

*$p < 0.005$ vs. control.

Data in both Table 1 and Fig. 3 are given in ANF-IV equivalent since the degree of cross-reactivity with high molecular weight ANF is not known.

DISCUSSION

Using synthetic ANF-IV and its specific antibodies, we have established a radioimmunoassay for ANF. This method permitted specific quantification of

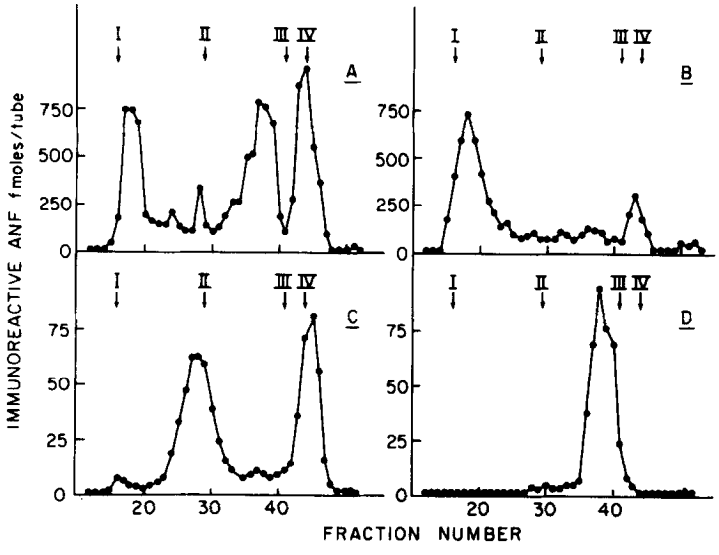


Figure 3: Gel filtration profiles on a Bio-Gel P-10 column of the extracts of rat right atrium, left atrium, hypothalamus, and plasma. A; right atrium; B, left atrium; C, hypothalamus; D, plasma. Arrows I, II, III and IV indicate elution positions of molecular weight standards, blue dextran, epidermal growth factor, angiotensin I, and ANF-IV, respectively.

ANF immunoreactive substances in tissue extract and physiological fluid without purification, providing a rapid and highly reproducible assay capability. The antiserum used also reacted equally well with the human atrial natriuretic peptide α-hANP$_{4-28}$. The parallelism of dilution curves obtained with ANF-IV and with extracts of rat atrium, plasma, and hypothalamus suggests the presence of a substance or substances immunologically indistinguishable from ANF-IV.

The present study demonstrates the presence of immunoreactive ANF in plasma and establishes ANF as a circulating hormone. Of particular interest is the finding of ANF in the hypothalamus. Possible plasma contamination is eliminated since the concentration in the hypothalamus is much higher than that of plasma and hypothalamic ANF consists of high and low molecular weight forms whereas plasma contains only a low molecular weight form. These findings suggest formation of ANF in the brain. Higher ANF concentration in right atrium than in left atrium is in agreement with an earlier report (17).

Multiple components of ANF immunoreactive substances, separated by gel filtration of atrial and hypothalamic extracts (Fig. 3), may correspond to ANF's with different molecular weight as reported recently (18-20). It is noteworthy that in plasma only one peak of immunoreactive ANF was found. The absence of a substance coinciding with ANF-IV in plasma may be due to a short half-life of the latter. However, the possibility cannot be excluded that the substance secreted to plasma from atria may have a molecular weight slightly greater than ANF-IV and the latter may be a break-down product generated during extraction of tissues.

Increase in ANF in atria and plasma caused by high salt intake suggests increased synthesis and release of ANF in rat atrium and indicates the role of ANF in the regulation of blood volume through its natriuretic activity. The inversed response of hypothalamic ANF to salt loading suggests physiological function of ANF in central nervous system independent of its peripheral action.

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