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ATRIAL NATRIURETIC FACTOR IS A CIRCULATING HORMONE

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The radioimmunoassay of atrial natriuretic factor (ANF) has been applied for determination of immunoreactive ANF (IR-ANF) in rat plasma. Immunoreactive ANF has been extracted from rat plasma by immunoaffinity column on Sepharose-4B anti-ANF or by Vycor glass. The mean concentrations of IR-ANF in ether anesthetized rats were found to be 1.61 ± 0.14 ng/ml in female and 1.25 ± 0.21 ng/ml in male rats when extracted on Sepharose-4B anti-ANF, and 1.21 ± 0.10 ng/ml in females and 1.02 ± 0.11 ng/ml in males when extracted by Vycor glass. A close linear correlation has been observed between the plasma IR-ANF concentrations in aorta and jugular vein. The described results indicate that atrial cardiocytes secrete atrial natriuretic factor into plasma. The heart is, therefore, an endocrine organ.

Investigations during the last three decades have revealed that atrial cardiocytes in mammals (1-4) and atrial and ventricular cardiocytes in non mammalian vertebrates (5), apart from their contractile apparatus, harbour secretory-like granules and a large Golgi complex where protogranules are frequently found (2). These secretory-like granules were found to be made up of proteins (3-5) and the incorporation of ^{3}H-leucine (6) and ^{3}H-fucose (7) by rat atrial cardiocytes, as revealed by ultrastructural radioautography followed the pattern observed in several peptide-secreting endocrine cells. Recently, a biologically active peptide, atrial natriuretic factor (ANF) has been isolated from rat atria, sequenced and synthesized (8, 9) and localized in atrial cardiocyte secretory granules by immunocytochemistry (10). This peptide was found to be the C-terminus of a much larger molecule (pre, pro and connecting peptides) made up of 152 amino acids, first by isolation and sequencing of larger molecular weight forms (11, 12) and finally by cloning

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of the rat c-DNA (13-15). We now report that immunoreactive ANF (IR-ANF) is circulating in rat plasma, thus establishing that atrial cardiocytes are peptide-secreting endocrine cells.

MATERIALS AND METHODS

Our recently described method for direct radioimmunoassay (RIA) of ANF in rat atria has been adapted for detection of ANF in rat plasma (16).

Blood collection

Blood from male and female Sprague-Dawley rats (body weight 230-300 g) was withdrawn by aortic or jugular vein puncture under ether anesthesia. Blood was collected in tubes, which were kept in ice and which contained proteolytic enzyme inhibitors in the final concentrations: EDTA 10^{-5} M, pepstatin 5 μM and phenylmethylsulfonyl fluoride (PMSF) 10^{-5} M. The blood was centrifuged at 3,000 rpm for 10 min at 4°C.

Extraction procedures

Because the native plasma interferes with direct RIA procedures, two methods of ANF extraction were used. The first was immunoaffinity chromatography on Sepharose-4B anti-ANF columns (16). The antibodies used were produced in rabbits against a synthetic 26 amino-acid fragment (Arg 101 - Tyr 126) of ANF coupled to thyroglobulin. The antibodies were characterized by solid phase RIA (17). Two ml of a Sepharose-4B anti-ANF suspension in 0.05 M Tris NaCl, pH 7.4, were put into columns (1.0 X 15 cm); 1 or 2 ml of fresh plasma was added. The columns were then washed with Tris buffer 0.05 M, pH 7.4, with 0.5 M NaCl. ANF was eluted with 0.1 M acetic acid containing 0.5 M NaCl. Two 5-ml fractions were collected, lyophilized and assayed.

The second method consists of ANF extraction from rat plasma by Vycor glass as described in detail previously for RIA of corticotropin (ACTH) (18). One or 2 ml of fresh plasma was extracted with 0.5 or 1 ml of Vycor glass suspension (50 mg activated glass powder in 1 ml distilled water) and adsorbed ANF was eluted with 0.5 or 1 ml 60% acetone in distilled water. Acetone was evaporated under a nitrogen stream and the aqueous phase lyophilized in a Speed-Vac.

The lyophilized plasma extracts were reconstituted with 500 μl of potassium-phosphate buffer (0.05 M, pH 7.4) containing 0.1% BSA and 0.02% NaN_3 (RIA buffer) and assayed.

For further characterization, some extracts of rat plasma (10 ml) from both Sepharose-4B anti-ANF and Vycor glass were purified by reverse phase high performance liquid chromatography (HPLC) on C_{18} μBondapak columns. The Sepharose-4B anti-ANF or Vycor glass plasma extracts have been reconstituted in 0.1% trifluoroacetic acid (TFA) and injected onto a 0.39 X 30 cm C_{18} μBondapak column (Waters Inc., Milford, Mass.). The column was eluted with a gradient of acetonitrile in TFA 0.1% from 15% to 45% at 0.5%/min. The 2-ml fractions collected from HPLC were lyophilized in a Speed-Vac, then reconstituted in 500 μl of 0.1 M potassium-phosphate buffer, pH 7.4, and radioimmunoassayed for ANF as the other lyophilized samples.

Radioimmunoassay procedure

The synthetic ANF (Arg 101 - Tyr 126) was used as a standard. The stock solution of ANF (1 mg/ml) in 0.1 M ammonium acetate buffer, pH 5.5, was diluted before use in RIA buffer to a concentration of 62.5 ng/ml, the highest

concentration of the standard curve. Serial two-fold dilutions were prepared in RIA buffer to a final concentration ranging from 30 pg/ml to 62,500 pg/ml. One hundred µl of these standard solutions were used for preparation of a standard curve (16).

The RIA was performed in polystyrene tubes at 4°C by mixing 100 µl of standards or samples, 100 µl of antiserum, diluted 1:4,000 in RIA buffer, 100 µl of (^{125}I)-ANF (~20,000 cpm) and 300 µl of PBS-BSA buffer, pH 7.4. After incubation for 24 hrs at 4°C, the free (^{125}I)-ANF was separated from antibody bound (^{125}I)-ANF by dextran-coated charcoal. After centrifugation (at 4,000 rpm for 10 min, 4°C) the supernatant was promptly decanted and its radioactivity counted in a LKB 1270 Rack Gamma II counter.

Recovery, accuracy and reproducibility

Recovery of ANF was studied in each assay after addition of radiolabelled or "cold" synthetic ANF to the plasma and buffer. Different levels of radioactivity ranging from 15,000 to 46,000 cpm were added to 1 or 2 ml of plasma and extracted on Sepharose-4B anti-ANF or Vycor glass. At each step of the extraction the radioactivity was measured. Assuming that radioactive ANF behaves as endogenous ANF, the recovery of ANF during the extraction could be followed. For recovery of "cold" ANF, 1-32 ng of synthetic ANF was added to plasma, extracted on Sepharose-4B anti-ANF and/or on Vycor glass, lyophilized and assayed. The recovery of ANF was calculated by substraction of the IR-ANF concentration in plasma from the concentration of IR-ANF in the sample where ANF was added. Both recovery procedures were performed with all series of RIA determinations.

Accuracy, precision and reproducibility of the method was established by multiple measurements of ANF concentration in the plasma extracts from the same rat in the same assay (within assay variation) and by RIA of lyophilized extracts of the same plasma on separate consecutive assays (between assay variation).

Statistics

The individual results were analyzed on a computer and only mean values ± SE are given. Statistical significance of the difference between studied groups were evaluated by Student "t" test and for comparison of IR-ANF concentration in aorta and jugular vein by paired "t" test.

RESULTS

The average concentrations of IR-ANF in aortic plasma of male and female rats using both Sepharose-4B anti-ANF and Vycor glass extractions are summarized in Table 1.

The concentrations of IR-ANF in aortic and jugular vein plasma of the same rat measured simultaneously are compared in Table 2. A close linear correlation between the concentration of IR-ANF in plasma from the aorta and jugular vein was found (Fig. 1). The values from the jugular vein were found to be lower than in the aortic plasma, however, the difference did not reach statistical significance.

Table 1

CONCENTRATION OF IR-ANF IN PLASMA DETERMINED BY RIA IN INDIVIDUAL RATS

		Extraction on Sepharose-4B anti-ANF	Extraction by Vycor glass
Plasma IR-ANF concentrations (ng/ml)	Males	1.25 ± 0.21 (n = 30)	1.02 ± 0.11 (n = 44)
	Females	1.61 ± 0.14 (n = 28)	1.21 ± 0.10 * (n = 85)
Recovery of synthetic ANF added to plasma (%)		76.4 ± 2.88 (n = 8)	53.9 ± 3.37 (n = 17)
Recovery of (^{125}I)-ANF added to plasma (%)		61.2 ± 1.02 (n = 5)	60.8 ± 1.87 (n = 13)

All values are means ± SEM.

* Statistically significant differences when extraction on Sepharose and Vycor is compared.

Only fresh plasma or lyophilized extracts of fresh plasma can be used for RIA of ANF. Frozen plasma samples did not give reproducible results, mainly because of the low recovery of ANF. Lower binding capacity and recovery of added "cold" ANF from Vycor glass can be demonstrated during individual recovery steps, when extractions on Vycor glass and Sepharose-4B anti-ANF are compared, while recovery of radioactive (^{125}I)-ANF was not different. The majority of radiolabelled ANF was eluted from the Sepharose-4B anti-ANF by 0.1 M acetic acid in the first 5 ml aliquot. No corrections have been made for the recovery during the extraction.

Table 2

COMPARISON OF IMMUNOREACTIVE ANF IN AORTIC AND JUGULAR VEIN PLASMA EXTRACTED BY VYCOR GLASS

	IR-ANF ng/ml
Aorta (n = 32)	0.93 ± 0.13
Jugular vein (n = 32)	0.83 ± 0.10

Values are means ± SEM.

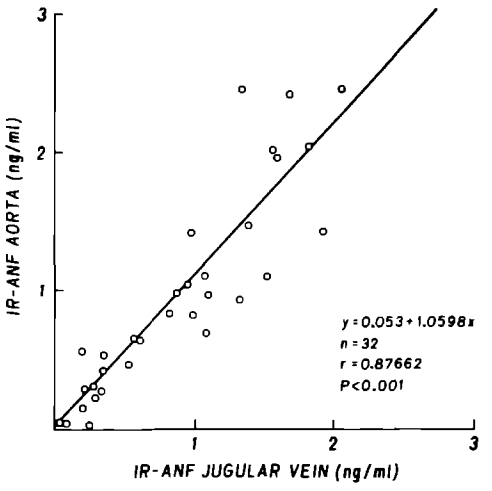

Figure 1: Correlation between IR-ANF concentrations determined in plasma taken from the same rat from aorta and jugular vein. IR-ANF has been extracted on Vycor glass.

The results of HPLC purification of both extraction procedures are given in Figure 2. IR-ANF was eluted from the HPLC columns with 30% acetonitrile. The synthetic ANF (Arg 101 - Tyr 126) was eluted from the same columns in a close vicinity with a concentration of 29% acetonitrile. The concentration of immunoreactive ANF in pools of aortic rat plasma has been estimated to be 1.7 ng/ml using the extraction by affinity column and 1.5 ng/ml when extracted by Vycor glass.

Within assay coefficient of variation calculated for two- to four-fold parallel determinations of 29 plasma samples was 7.6 ± 0.9% and the between assay coefficient of variation calculated from the consecutive RIA of ten lyophilized extracts of the same plasma was 8.1 ± 1.7%.

DISCUSSION

Detection of IR-ANF in plasma of rats give us very important evidence that ANF is a circulating hormone.

We adapted the recently developed RIA (16) for determination of IR-ANF in pool and in individual samples of rat plasma. Extraction of plasma on Sepharose-4B anti-ANF affinity columns and/or Vycor glass increased the concentration in lyophilized and reconstituted extracts and abolished the inter-

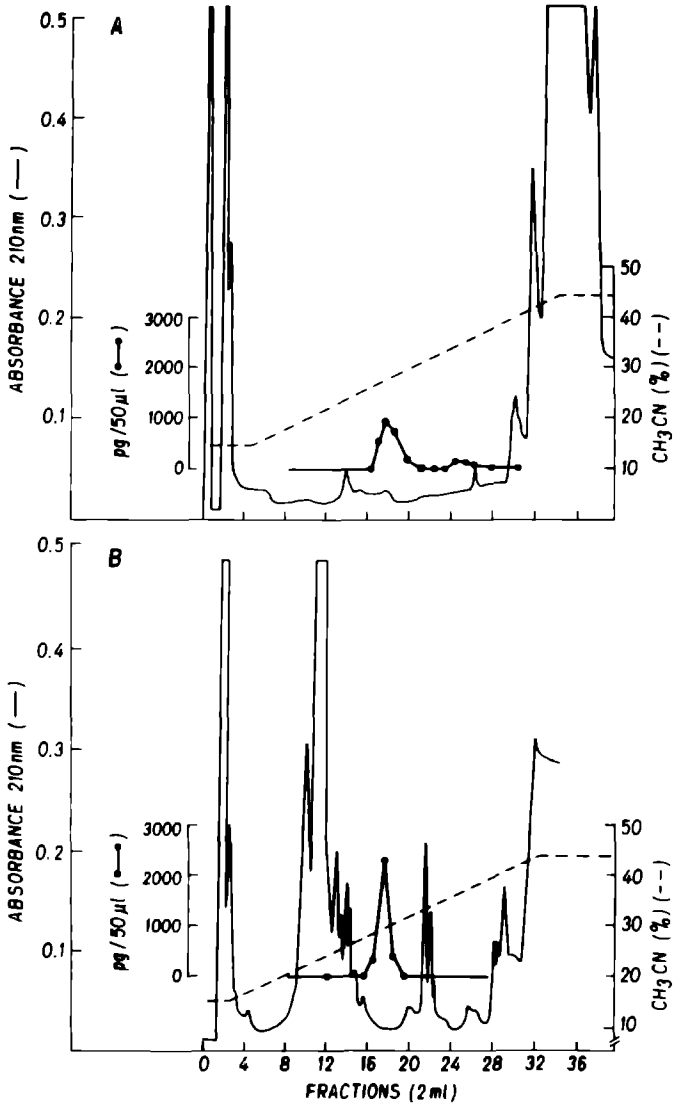

Figure 2: Reverse phase HPLC of rat plasma extracts.
Panel A: extract has been obtained from Sepharose-4B anti-ANF.
Panel B: extract from Vycor glass.

ference of different peptides present in native plasma. A good recovery of ANF was found during the extraction procedure on both Sepharose-4B anti-ANF and Vycor glass. A good parallelism has been found between different dilutions of plasma extracts with standard curves.

The presence of IR-ANF in the plasma extracts was verified by reverse phase HPLC on a C_{18} µBondapak column. IR-ANF and synthetic ANF (Arg 101 - Tyr 126) were eluted from the column in a close vicinity with the concentra-

tion of 30% and 29% acetonitrile, respectively. The presence of another small immunoreactive peak, eluted from plasma extracts with about 35% acetonitrile gradient, may be due to the presence of the propeptide (11). The presence of different fragments of ANF as well as propeptide in the circulation cannot be excluded.

The concentration of IR-ANF in aortic plasma from individual rats has been estimated to be 1.25 ± 0.21 ng/ml in males and 1.61 ± 0.14 ng/ml in females using the Sepharose extraction, and 1.02 ± 0.11 ng/ml in males and 1.21 ± 0.10 ng/ml in females when extracted on Vycor glass. Lower concentration of IR-ANF in plasma extracted on Vycor glass was probably caused by non specificity of Vycor glass extraction in comparison with Sepharose-4B anti-ANF.

The sensitivity of the method has made possible the reproducible determination of the plasma concentration of IR-ANF in ether anesthetized rats.

Only fresh rat plasma can be used for IR-ANF determination. The observed lower recovery of ANF from the frozen plasma was probably due to the destruction of IR-ANF by proteolytic enzymes during the collection and freezing of the plasma.

These results indicate that atrial cardiocytes secrete into plasma a potent peptide and are thus bona fide endocrine cells. This is confirmed by the following: 1) the ultrastructure of these cells 2) the presence of receptors for the peptide in adrenal cortex (19, 20), arterial smooth muscle cells (21), renal tubular cells (21) and pituitary (22) 3) from a variety of biological effects of the peptide: diuresis and natriuresis of rapid onset and short duration (23, 24), vasodilatation, inhibition of the arterial contraction produced by norepinephrine and angiotensin II (25) with correction of renal hypertension (26), inhibition of aldosterone and cortisol hypersecretion induced by a variety of stimulatory agents (19), and stimulation of arginine vasopressin secretion from the isolated posterior lobe of the hypophysis (22) 4) from the effects of the peptide on second messengers: inhibition of adenylate cyclase in target tissues (arterial wall, anterior and posterior hypophysis, adrenal cortex, isolated renal cortical cells) (27),

increase in cGMP in blood and urine following an intravenous injection of the peptide and similar increase in renal cortical cells in culture (28).

Although it is too early to draw definitive conclusions, the presently available evidence tends to indicate that the atria play an integrative role in cardiovascular homeostasis (blood volume and blood pressure regulation) not unlike that of the anterior lobe of the hypophysis for reproduction, growth and adaptation.

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