PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 170, 133–138 (1982)

Atrial Natriuretic Factor of the Rat Heart. Studies on Isolation and Properties[1] (41408)

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Abstract. Studies on a chemical extraction and purification procedures are described for rat atrial natriuretic factor. This factor—first detected by injection of crude rat atrial heart muscle homogenates—induces a powerful diuretic and natriuretic response when injected into nondiuretic assay rats. Studies on the properties of purified natriuretic factor suggest that it is of a proteinaceous nature. It is efficiently extracted by dilute acetic acid, readily degraded by protease, and recoverable after gel filtration at a fractionation range corresponding to less than 6000 daltons. Dose–response studies show that at maximal doses the response is a 10- to 15-fold increase in urine output and a 30- to 40-fold increase in urinary sodium excretion. These responses are characteristically of rapid onset (1–2 min) and decay (10–15 min).

Studies on secretory-like morphological features of mammalian atrial cardiocytes (3) has led to the finding that injection of crude rat atrial myocardial extracts induces a very powerful diuretic and natriuretic response in assay rats (4). This response occurs without an increase in glomerular filtration rate (4) and appears to be due to inhibition of reabsorption in the loop of Henle and the medullary collecting duct resulting in equivalent increases of Na^+ and Cl^- excretion in the urine (1).

The substance responsible for this action, referred to here as atrial natriuretic factor, is of unknown chemical nature. In the present work, our studies on methodology for isolation and purification of rat atrial natriuretic factor is reported. In addition, some of the properties of the partially purified factor were studied. The data gathered indicates that rat atrial natriuretic factor is a distinct substance of proteinaceous nature.

Materials and Methods. Atria were obtained from male Sprague–Dawley rats (300–350 g). Up to 100 atria (10 g wet weight) were used in each experiment. The animals had free access to food and water until sacrificed by decapitation. The hearts were rapidly removed and placed in ice-cold phosphate-buffered saline (PBS: 0.9% NaCl in 5 m*M* sodium phosphate buffer, pH 7.2). The atria were dissected, thoroughly rinsed in PBS, blotted dry, and weighed. Five volumes of cold 1.0 *M* acetic acid, containing 1.0 mg/L of the protease inhibitors pepstatin A and phenylmethylsulfonyl fluoride, were added per gram of tissue wet weight. The tissue was then homogenized in a Polytron, let to stand for 1 hr on ice, and centrifuged at 4° for 30 min at 10,000*g*. The supernatant from this centrifugation was saved and the pellet rehomogenized in 2.5 vol of acetic acid and treated as above. The combined supernatant from the two acetic acid extractions was then adjusted to pH 7.6 with concentrated NH_4OH and centrifuged once again. The supernatant from this centrifugation was freeze-dried, resuspended in 10 ml of 1.0 *M* acetic acid, and desalted at 4° in a Bio-Gel P-2 column (2.6 × 60 cm) equilibrated with 1.0 *M* acetic acid. The void volume from this column was freeze-dried, resuspended in 10 ml of 1.0 *M* acetic acid, and applied to a Sephadex G-75 column (2.6 × 90 cm) equilibrated with 1.0 *M* acetic acid. Pooled active fractions obtained after this chromatography were freeze-dried. The product

[1] Supported by the Ontario Heart Foundation, The J. P. Bickell Foundation, and The Banting Research Foundation. A preliminary report on this work was presented to a meeting of the Federation of American Societies for Experimental Biology, Atlanta, Ga., April 1981.

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0037-9727/82/060133-06$01.00/0

obtained is referred to as "partially purified natriuretic factor." Dilutions of partially purified natriuretic factor in PBS were used to determine dose–response relationships.

To study the effect of protease (Pronase) on partially purified natriuretic factor, dilutions were made in 0.05 *M* Tris buffer, pH 7.6. Digestions were carried out by incubating 50 μg of partially purified natriuretic factor with 0.1 U of Pronase for 4 hr at 37° in a total volume of 1.1 ml of 0.05 *M* Tris buffer, pH 7.6. The reaction was terminated by addition of 0.2 ml of glacial acetic acid and cooling on ice. After freeze-drying, the samples were resuspended in 1.0 ml of PBS, centrifuged, and 0.2-ml aliquots tested for natriuretic activity.

Further purification of natriuretic factor was accomplished by ion-exchange chromatography in CM Bio-Gel equilibrated with 0.01 *M* ammonium acetate buffer, pH 4.7. Column size was 0.9 × 12 cm. Elution was carried out with 13 column volumes under starting conditions followed by a 13-column volume gradient to 0.1 *M* ammonium acetate, pH 7.0, and, finally, with 13 column volumes from 0.1 *M* ammonium acetate, pH 7.0, to 0.5 *M* ammonium acetate, pH 7.0. Natriuretic factor assay was carried out in nondiuretic rats essentially as previously described (4). The total assay period was, however, considerably shortened as follows: after a priming 20-min period in which a total of 1.2 ml of Ringer's solution was administered, an infusion of Ringer's solution at a rate of 1.2 ml/hr was started and maintained for the duration of the assay. The animals were let to stabilize for a further 20 min. During the following 20 min urine was quantitatively collected (control period) after which injection of 0.2 ml of the test sample was made over approximately 5 sec. Urine was then collected for the next 20 min (test period). A photoelectric drop counter was positioned between the bladder catheter and the collection tube to qualitatively assess the diuretic response. Urine sodium and potassium concentrations were measured by flame photometry, chloride by electrometric titration, and urine volumes by weighing. Protein estimation was made by measuring absorbance at 280 nm (10-mm path) assuming 1 AU = 1 mg protein/ml. Statistical comparisons were carried out using unpaired Student's *t* test.

Results. Pooled active freeze-dried fractions, obtained after chromatographic desalting in Bio-Gel P-2 (Fig. 1) or after fractionation in Sephadex G-75 (Fig. 2), and injected dissolved in PBS, induced a typical response in assay rats (Fig. 3). In terms of urine output, this response was characterized by rapid onset (1–2 min) and decay (10–15 min). By the end of the test period—i.e., 20 min after injection—urine output was essentially the same as that observed during the control period. Doses approaching maximal response (see below) had the effect of gradually decreasing arterial blood pressure so that the blood pressure values observed at the end of the assay were 10–30 mm Hg lower than values observed prior to the injection of the extracts.

Both diuretic and natriuretic responses were dose dependent (Fig. 4). Maximal response obtained after injection of 18 μg of partially purified factor corresponded to a 30- to 40-fold and 10- to 15-fold increase in total sodium excretion and urine volume, respectively.

Incubation of partially purified natriuretic factor with protease (Table I) completely abolished activity while samples incubated with inactivated enzyme had activities comparable to that of samples incubated with buffer alone.

Natriuretic factor distribution during chromatography in Sephadex G-75 (Fig. 2) was consistently multimodal although most of the activity was recovered in peaks corresponding to molecular weights of less than 6000 daltons. Further purification of this fraction could be accomplished by ion-exchange chromatography in CM Bio-Gel (Fig. 5) after which natriuretic activity was recovered in a single, though not symmetrical, peak.

Discussion. The presence of secretory-like "specific atrial granules" in cardiac atrial muscle fibers (5), together with the finding that crude rat atrial muscle extracts are able to induce a very potent diuretic and natriuretic response in assay rats (4) is both

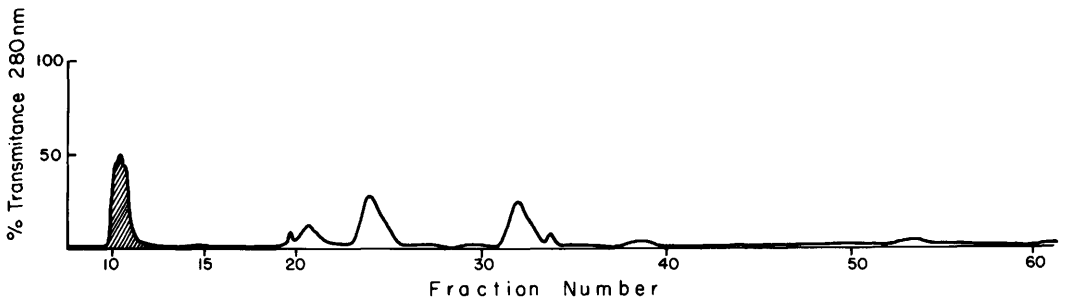

FIG. 1. Chromatographic desalting of rat atrial extract in Bio-Gel P-2. Hatched area indicates fractions where natriuretic activity is recovered.

intriguing and of great potential in reaching a better understanding of the control of water and electrolyte balance. These findings appear more than merely coincidental. Recent studies (2) using ultracentrifugation techniques and the acetic acid extraction methodology described here have shown that subcellular fractions which microscopically contained purified specific granules had the highest specific natriuretic activity. In these studies, ventricular muscle, which lacks specific granules, was used as control. Neither ventricular homogenates, nor any of the derived fractions, displayed natriuretic activity. This lack of activity of ventricular extracts is consistent with earlier studies (4) using crude homogenates.

The investigations reported here represent our first attempts to determine the feasibility of chemically extracting rat atrial natriuretic factor and to infer from some of its apparent biochemical properties the possible nature of the substance itself. This

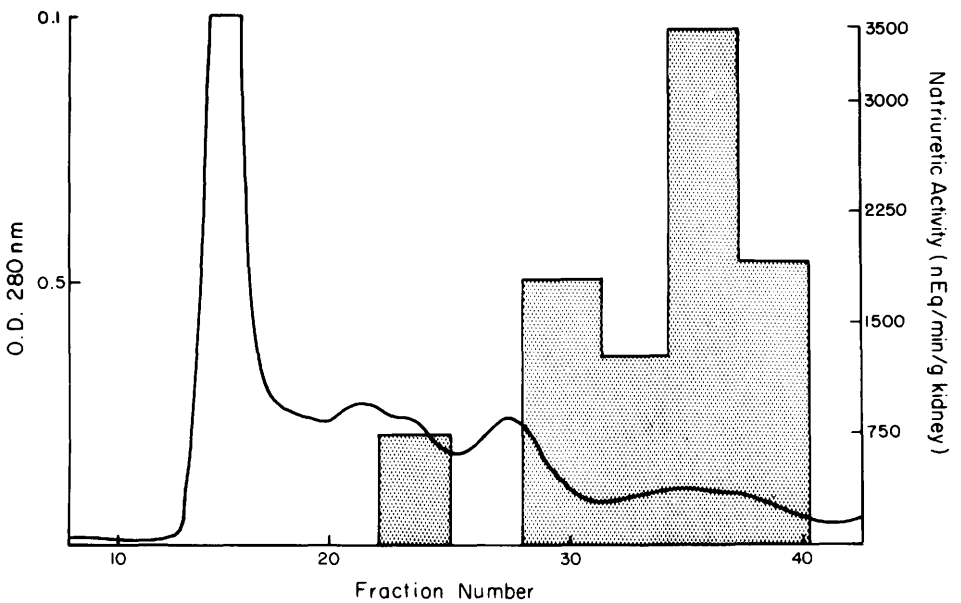

FIG. 2. Chromatography of rat atrial extract (after desalting in Bio-Gel P-2) in Sephadex G-75. Natriuretic activity (bars) was determined by injecting freeze-dried aliquots of pooled fractions resuspended in PBS.

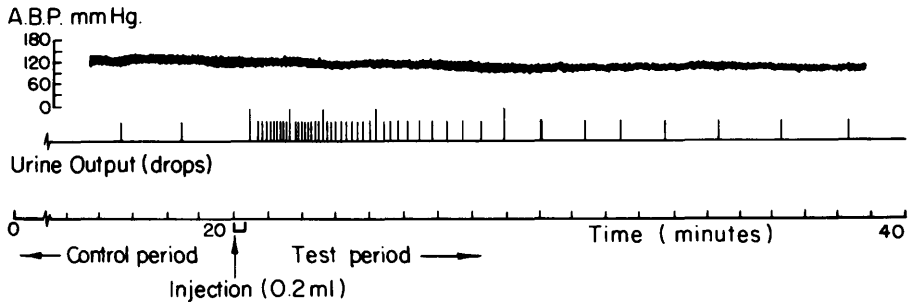

FIG. 3. Typical effect of an injection of partially purified rat atrial natriuretic factor on arterial blood pressure and urine output of bioassay rats.

information was deemed essential before more elaborate physiological and biochemical studies could be planned.

The acetic acid extraction developed for rat atrial natriuretic factor was based on two considerations. First, our working hypothesis is that atrial specific granules are a site of storage for atrial natriuretic factor. Second, histologically, it has been found (6) that the staining intensity of these granules

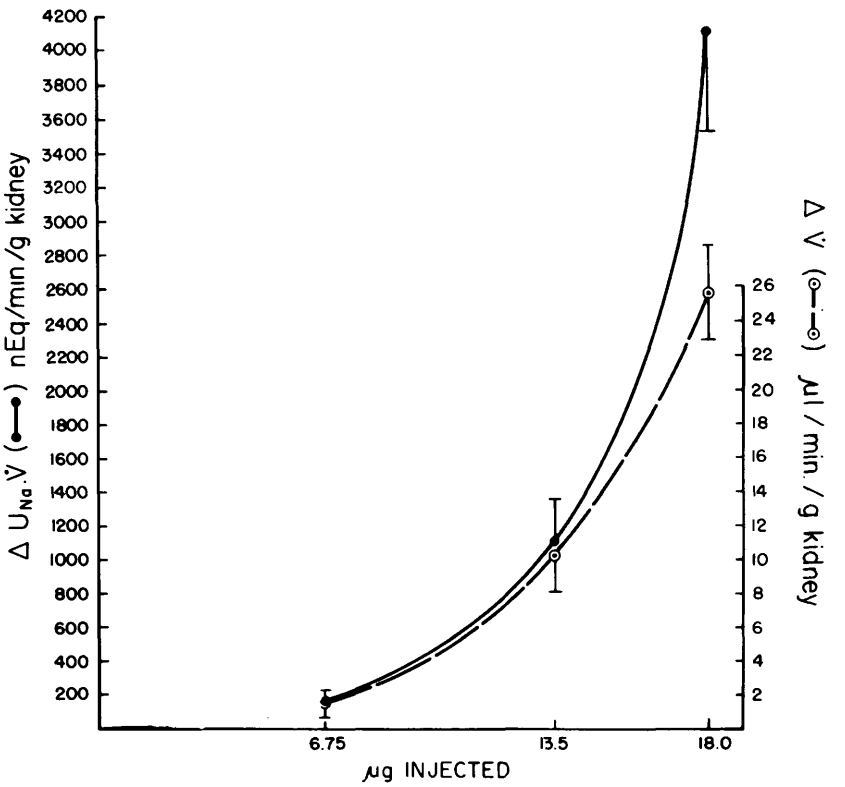

FIG. 4. Net natriuretic and diuretic effect of partially purified natriuretic factor injected in a total volume of 0.2 ml of PBS. Net values were obtained by subtracting total sodium excretions and urine output of animals injected with PBS alone from corresponding values of animals injected with natriuretic factor. (Values ± SEM)

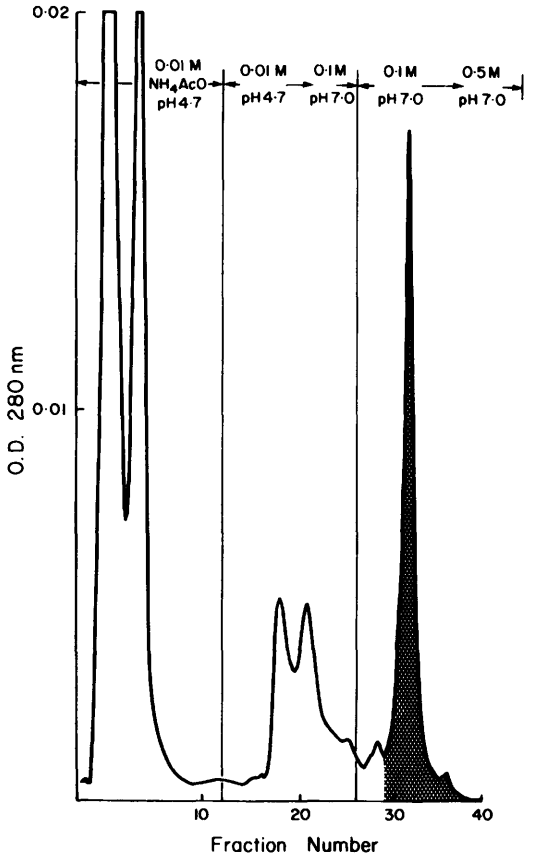

FIG. 5. Ion-exchange chromatography purification of partially purified natriuretic factor. Hatched area indicates fractions where natriuretic activity is recovered.

is diminished after fixation in fixatives containing acetic acid. This is consistent with, on the one hand, histochemical data (6) which suggest that atrial granules contain a polypeptide and, on the other hand, the fact that acetic acid is a good peptide solvent. During pilot investigations it soon became apparent that acetic acid, indeed, was the most efficient of several extraction procedures tested. The view that the ability of this acid to extract atrial granule material is the reason why it is an efficient natriuretic factor extractant appears to find support in the tissue fractionation studies (2) mentioned above.

The finding that natriuretic activity is recovered after desalting in a Bio-Gel P-2 column (fractionation range: 100–1800 daltons) is of considerable interest because it shows that natriuretic factor is distinct from inorganic salts and low-molecular-weight organic compounds which are likely extracted by acetic acid and which may be expected to be cardioactive and/or affect kidney function.

It was observed that desalting of the acetic acid extract eliminates the acute fall in blood pressure observed after injection of nondesalted extracts. However, the gradual fall in blood pressure previously observed (4) after injection of crude extract may also be observed after injection of purified extracts. It is not clear if this is a consequence

TABLE I. EFFECT OF PROTEASE (PRONASE) ON PARTIALLY PURIFIED RAT ATRIAL NATRIURETIC FACTOR

	Group	Urine volume (μl/min/g kidney)	Total excretions (neq/min/g kidney)		
			Na^+	K^+	Cl^-
Bioassay Control Period	A (N = 6)	2.39 ± 0.49	117 ± 27	643 ± 137	573 ± 158
	B (N = 4)	2.03 ± 0.84	68 ± 6	316 ± 52	151 ± 46
	C (N = 4)	2.14 ± 0.73	102 ± 39	578 ± 221	819 ± 250
Bioassay Test Period	A (NF)	13.20 ± 3.24*	1074 ± 208*	1902 ± 513*	2850 ± 779*
	B (NF + enzyme)	2.86 ± 1.09†	71 ± 12†	463 ± 181†	383 ± 235†
	C (NF + boiled enzyme)	14.68 ± 2.36**	1434 ± 190**	1460 ± 96*	2549 ± 343*

Note. Protease digestion was carried out by incubating approximately 50 μg of partially purified natriuretic factor with 0.1 U of protease for 4 hr at 37° in a total volume of 1.1 ml of 0.05 *M* Tris buffer, pH 7.6 (Group B). Controls were incubated with either boiled protease (Group C) or with buffer alone (Group A). All values ± SEM.

* Significant difference from control, $P < 0.05$.

** Significant difference from control, $P < 0.01$.

† No significant difference from control values.

of fluid loss (diuresis) or a direct effect on peripheral resistance.

The significance of multimodal distribution of natriuretic factor after chromatography in Sephadex G-75 is not clear. Protein interaction, possibly including polymerization appears as the most likely explanation.

The general behavior of natriuretic factor during isolation and partial purification suggest that it is a polypeptide. This is supported by the finding that natriuretic activity is readily abolished by incubation with protease. However, the protease preparation used may not be assumed to be entirely specific for peptides.

Natriuretic factor appears to be but a very minor component of the active UV-absorbing material isolated after ion-exchange chromatography. In investigations preliminary to the present work, it was observed that ventricular muscle extracts subjected to the same chromatographic techniques as atrial extracts, give virtually identical UV profiles even though the ventricular fractions so obtained display no natriuretic activity. The use of large hearts (e.g., beef) as a source of natriuretic factor is not as economical as it may appear at first because, although the atria of these hearts contain natriuretic activity, the total extractable activity is much lower than that obtained from an equivalent amount of rat tissue. Nevertheless, isolation and purification of natriuretic factor on a preparative scale appears to be one of the most important endeavors to be undertaken in the understanding of the findings described above.

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Received October 26, 1981. P.S.E.B.M. 1982, Vol. 170.