Atrial Natriuretic Factor
Characterization and Partial Purification

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SUMMARY  One of the main differences between atrial and ventricular cardiocytes is the presence in former of specific granules with morphological characteristics very similar to secretory granules found in peptide-secreting endocrine cells. It has been suggested that these granules are the storage place for the atrial natriuretic factor. In the rat, water deprivation produces an increase in atrial granularity but a significant decrease in acid-extractable diuretic and natriuretic activity, suggesting that the number of atrial specific granules does not necessarily represent natriuretic activity. The atrial natriuretic factor activity is destroyed by incubation with several proteases and does not inhibit the sodium-potassium ATPase, suggesting that the active substance is a small peptide that is probably different from the so-called natriuretic hormone. After a series of chromatographic steps in Sep-Pak cartridges, Bio-Gel P-10, CM Bio-Gel, and Mono S columns, the specific activity of the atrial natriuretic factor was increased from 193, corresponding to atrial homogenates, to 242,000, which corresponds to the last chromatographic step representing a 1250-fold purification. This material showed a potent natriuretic activity, as 10 picomoles increased natriuresis by 100%.

(Hypertension 5 (supp I): I-75-I-80, 1983)

KEY WORDS • natriuretic activity • atrial natriuretic factor • atrial specific granules

DURING the last 20 years, several natriuretic substances have been described in the plasma and urine of volume-expanded experimental animals and human subjects.1-3 A low molecular weight natriuretic factor that inhibits the sodium-potassium (Na-K)-dependent ATPase and whose origin is still unclear has been related to several forms of hypertension.4,5 It has been demonstrated that stimulation of atrial baroreceptors results in an increase in urinary flow and sodium excretion,6,7 but the mechanisms by which these changes are accomplished are still uncertain. It has been suggested that the release of a diuretic agent could be one of the factors involved.8

Mammalian atrial cardiocytes contain specific granules, rich in protein, and with morphological characteristics similar to secretory granules found in endocrine cells.9,10 Changes in atrial granularity occur in experiments that modify salt and water balance,11,12 they suggest that atrial specific granules may play a role in the mechanisms by which atrial distension modifies diuresis and natriuresis in response to volume changes.

It has been reported13-15 that the injection of crude atrial homogenates produces a rapid and short increase in diuresis and natriuresis in the rat. The atrial specific granules have been suggested as the storage place for this natriuretic substance.16 Our purpose has been to purify this factor and to study some of its physiochemical characteristics and possible involvement in water balance.

Material and Methods
Female Sprague-Dawley rats (180–200 g) were used as bioassay animals, as previously described.15 They were anesthetized with pentobarbital (60 mg/kg i.p.), and a bladder catheter and intrajugular vein catheter were installed. The animals received an infusion of 5% dextrose (3 ml/hr) 45 minutes before the assay and during the evaluation period. Urine was collected in preweighed vials for 20-minute intervals. After a basal collection period, samples from animal experiments or from the different chromatographic steps were injected to test their natriuretic activity. Sodium concentration was measured in a flame photometer.

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Supported by Grant MA-7665 from the Medical Research Council of Canada and by a group grant given by the Medical Research Council of Canada to the Multidisciplinary Research Group on Hypertension of the Clinical Research Institute of Montreal.

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Protocol
Female Sprague-Dawley rats (180–200 g) were fed a normal sodium rat chow. The water-deprived group was left without water for 5 days. The control group had free access to tap water. At the end of the experimental period the rats were decapitated, the heart rapidly excised, and both atria dissected and processed together for each individual animal. The tissue was homogenized in 2 ml of 1 M acetic acid, centrifuged at 15,000 g for 10 minutes, and the supernatant lyophilized. This material was redissolved in 0.5 ml of 0.1 M acetic acid, centrifuged again for 5 minutes and 200 μl injected into a bioassay rat. The results were expressed as Δ μl urine and Δ μEq of sodium excreted in the 20-minute period following the injection per milligram of protein.

Some of the animals in each group were subjected to electron microscopy studies.

Electron Microscopy and Specific Granule Count
The right atria was fixed by perfusion with 2% glutaraldehyde, as previously described.17 The specific granules were counted in fine sections of longitudinally cut cardiocytes at a magnification of × 4773. Only longitudinal sections framed on two sides by myofilaments and containing in their center part of a nucleus and at least one Golgi complex were photographed. For each of five controls and five experimental rats, five photographs from five different blocks were taken and magnified at × 13,380. The granules were then counted and averaged as already described.17

Na-K-ATPase Activity
The effect of a partially purified natriuretic factor was verified on a Na-K-ATPase prepared from rat renal medulla according to Lo et al.18 The Na-K-ATPase activity was determined by the method of Gruber et al.,19 and the liberated inorganic phosphate (Pi) was measured by a colorimetric assay.20

ATPase activity was measured in the presence and absence of atrial natriuretic factor and compared with the inhibitory effect produced by increasing concentrations of ouabain (10−7 to 5 × 10−2 M). Samples were processed in duplicate.

Purification of the Atrial Natriuretic Factor
Atria (5.3 g) from female Sprague-Dawley rats (200–250 g) were homogenized in 1.0 M acetic acid (7 ml/g) and centrifuged at 40,000 g for 20 minutes. The pellet was reextracted with 1.0 M acetic acid, centrifuged again for 5 minutes and 200 μl injected into a bioassay rat. The results were expressed as Δ μl urine and Δ μEq of sodium excreted in the 20-minute period following the injection per milligram of protein.

The supernatant was next passed through two sets of Bio-Gel P-10 column (2.5 × 90 cm) and eluted with 0.1 M acetic acid. The fractions corresponding to protein peaks were pooled, lyophilized, and dissolved in 2 ml of 0.02 M ammonium acetate pH 5.0.

Fractions containing natriuretic activity were then added to a CM Bio-Gel A column (1.0 × 20 cm) equilibrated with 0.02 M ammonium acetate, pH 5.0. The material was eluted with a linear gradient of 100 ml of 0.02 to 1.0 M ammonium acetate, pH 5.0. Fractions were pooled, lyophilized, and those with natriuretic activity were further purified in a cationic exchange Mono S column (Pharmacia Fine Chemicals, Uppsala, Sweden) adapted to be used with a Waters HPLC system. The material was eluted with a 30-minute linear gradient of 0.1 to 1.1 M ammonium acetate pH 5.0 at a flow rate of 1.5 ml/min.

The biological activity of the different fractions during the purification was measured by bioassay as described above and represents the mean of duplicate assays. Protein concentration was measured by the method of Bradford as modified by Spector21 with bovine serum albumin as a standard.

Incubation in the Presence of Proteases
In two separate experiments, atrial natriuretic activity equivalent to 297 and 133.8 μmol of Na excreted in 20 minutes was incubated in duplicate with 100 μg of trypsin, chymotrypsin, aminopeptidase A, and carboxypeptidase A, B, and C, for 3 hours at room temperature in 0.1 M Tris-HCl buffer, pH 7.0. After the incubation, the residual activity was measured by bioassay. Comparisons of the results, when appropriate, were made by Student’s unpaired t test.

Results
Water deprivation induced a significant (p < 0.01) increase in the number of atrial specific granules from 90 ± 7 (mean ± SEM) in the control group, to 150 ± 9 in the water-deprived group (fig. 1 and 2). Atrial extracts from water-deprived rats produced a significantly lower diuresis and natriuresis, expressed as specific activity, when they were compared with the atrial extracts prepared from rats receiving tap water ad libitum (table 1).

<table>
<thead>
<tr>
<th>Rat group</th>
<th>Δ μl urine/20 min/mg protein</th>
<th>Δ μEq Na+/20 min/mg protein</th>
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<tbody>
<tr>
<td>Control (n = 7)</td>
<td>6365 ± 1072</td>
<td>1218 ± 240</td>
</tr>
<tr>
<td>Water deprivation (n = 8)</td>
<td>2860 ± 565*</td>
<td>522 ± 1061*</td>
</tr>
</tbody>
</table>

Values are means ± SEM.
* p < 0.01 and f p < 0.02 vs control.
As can be seen in table 2, whereas ouabain at a concentration of $10^{-3}$ M induced a maximal inhibition on the Na-K-ATPase, the atrial natriuretic factor at a concentration able to increase natriuresis 100-fold (equivalent to 436 $\mu$Eq Na/20 min/ml) had no inhibitory effect. The ATPase activity from rat renal medulla was $9.5 \pm 2.2 \mu$mol of inorganic phosphate (Pi) liberated/hr/mg protein. It has been demonstrated that the Na-K-ATPase of rat kidney is not very sensitive to ouabain inhibition, and the $K_i$ for ouabain for our preparation was $10^{-4}$ M. The maximum inhibition obtained on the ATPase activity was 60%.

The effects of the same concentrations of atrial natriuretic factor were also studied in commercially available preparations of Na-K dependent pork cerebral cortex and dog kidney ATPases (not shown). No inhibition was found.

The natriuretic activity (table 3) was completely abolished by incubation in the presence of trypsin, chymotrypsin, aminopeptidase A, carboxypeptidase B and C, and partially abolished by carboxypeptidase A.

Chromatography with a Bio-Gel P-10 column (fig. 3) showed that 80% of the natriuretic activity was found between fractions 75 and 110. The fractions with higher activity were pooled and further purified with a cationic exchange column, a CM Bio-Gel A (fig. 4). As in figure 3, the elution pattern read at 280 nm. Most of the activity was eluted in fractions 51 to 62, corresponding to the last eluted peak.

In figure 5, the results from chromatography with a Mono S column are shown. Two well-defined activity peaks were seen (upper panel), but because of the low protein concentration (4.5 $\mu$g/ml), no absorbance was seen (lower panel).

In table 4, the different steps of purification can be followed. There is an increase in specific activity ($\mu$Eq Na/20 min/mg of protein) from 193 in atrial homogenates to 45,000 after chromatography on a Mono S
FIGURE 3. Results of chromatography with a Bio-Gel P-10 column. Upper panel: Natriuretic activity, expressed as μEq of sodium excreted during the first 20 minutes following injection of the fractions. Lower panel: Elution pattern, at 280 nm.

FIGURE 4. Results of chromatography with a CM Bio-Gel A column. Upper panel: Natriuretic activity (μEq sodium excreted during first 20 minutes after injection of the fractions). Lower panel: Elution pattern, at 280 nm.

FIGURE 5. Results of chromatography with a Mono S column. Upper panel: Natriuretic activity (μEq sodium excreted during first 20 minutes after injection of the fractions). Lower panel: Elution pattern, at 280 nm.
column, which means a purification of 236-fold; these values represent the mean of two purifications. The last line in table 4, between parenthesis, corresponds to the main peak of activity of figure 4, corresponding to a specific activity of 242,000, which represents a purification of 1250-fold. About 10 picomoles of this material increased natriuresis by 100%.

**Discussion**

Both high and a low molecular weight natriuretic substances have been found in plasma and urine after volume expansion.1-3 The latter has a molecular weight probably lower than 1000 and the ability to inhibit active sodium transport by inhibition of the Na-K-ATPase.23 The origin of this natriuretic factor remains uncertain, but a digitalis-like activity has been found in the guinea pig brain24 and bovine hypothalamus.25 The presence of natriuretic activity in rat atria has been recently demonstrated,13-15 localized in the atrial specific granules.16 An increase in the number of these granules has been previously demonstrated with light microscopy in sodium-deficient or water-deprived animals,11,12 suggesting a direct role in salt and water balance. Previous studies have not shown whether an increase in atrial granularity represented a simultaneous increase in atrial natriuretic activity; if so, it seemed incongruous to have an increase in natriuretic activity in the presence of antinatriuretic and antidiuretic states. Our results clearly indicate that, at least in water-deprived rats, an increase in granularity, as shown by electron microscopy and by granule counts, is not an indication of increased natriuretic activity.

The natriuretic ability is completely abolished after incubation with several proteases, suggesting that it may be a polypeptide. A molecular weight of about 4000 has been previously suggested.26 These characteristics together with the fact that the atrial natriuretic factor does not inhibit the Na-K-ATPase, as demonstrated in table 2, clearly differentiate this factor from the so-called natriuretic hormone.27

After several chromatographic steps, we have succeeded in purifying the atrial natriuretic factor 1255-fold, with a very potent natriuretic activity. Ten picomoles of this partially purified material are enough to increase natriuresis by 100%.

Our data suggest that an increase in atrial granularity does not necessarily reflect parallel changes in atrial natriuretic activity. This activity is probably due to a potent peptide whose effect is not, at least in vitro, linked to an inhibition of the Na-K-ATPase. As there is not, as yet, a method to determine whether this atrial natriuretic factor is released into the circulation, its actual physiological role remains to be elucidated.

**Acknowledgments**

The authors thank Suzanne Diebold, Suzanne Olivieri, and Micheline Pelletier for their excellent technical assistance.

**References**

Atrial natriuretic factor. Characterization and partial purification.
G Thibault, R Garcia, M Cantin and J Genest

Hypertension. 1983;5:175
doi: 10.1161/01.HYP.5.2_Pt_2.175

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1983 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

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