PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **182**, 358–363 (1986)

Lack of Interaction between Atrial Natriuretic Factor and Renal Organic Anion Transport System (42352)

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Abstract. The aim of the present investigation was to test the hypothesis that atrial natriuretic factor (ANF) is secreted into the proximal tubule lumen by the organic anion transport mechanism. The rationale for this hypothesis was the reported probenecid attenuation of the natriuretic effect of ANF. Probenecid is widely regarded as an inhibitor of organic acid transport in the proximal tubule. ANF was prepared in varying degrees of purity ranging from a relatively crude extract to a highly purified form. A commercially available form was also used. All forms were bioassayed using the anesthetized rat and a diuresis and natriuresis was observed in each case which was comparable to literature reports. Interaction of ANF with the organic acid transport system was evaluated using the renal cortical slice technique. Over a wide range of concentrations, there was no effect of ANF on cortical slice accumulation of either *p*-aminohippurate (PAH), the classical substrate of the organic anion transport system or tetraethylammonium (TEA), a typical organic cation. It is concluded that although ANF may indeed exert its effect at the luminal membranes of the nephron, access to the lumen is not mediated by the organic cation or anion transport system in the proximal tubule.

In 1981 de Bold *et al.* demonstrated that the intravenous injection of atrial muscle extract into anesthetized nondiuretic rats resulted in a rapid, 30-fold increase in Na excretion, a 10-fold increase in urine volume, and a doubling of K^+ excretion (1). The substance responsible for this action was named "atrial natriuretic factor" (ANF) (1, 2).

Since then, qualitatively similar effects were observed with atrial extracts obtained from many different species including dogs (2, 3), monkeys (4), and humans (2–4). Although the exact mode of action of ANF is still controversial, it was originally proposed that the ANF primarily inhibits the tubular reabsorption of filtered Na (1). The latter view was based on the observation that the ANF induces a marked natriuresis in the absence of any significant alteration of renal plasma flow and/or glomerular filtration rate (1, 4–8). Other studies, however, indicated that the atrial extract induces a significant increase in glomerular filtration rate and/or renal plasma flow and suggested that the ANF-induced natriuresis may be, at least in part, due to hemodynamic changes (9–15).

Micropuncture studies conducted to localize the tubular site of the action indicated that ANF inhibits Na reabsorption in the loop of Henle (16) or in the collecting duct (7, 8, 16). Furthermore, Sonnenberg *et al.* (17) found that the natriuretic effect of ANF is significantly attenuated in rats pretreated with probenecid, a specific inhibitor of renal organic anion secretion by the proximal tubule (18). The latter finding was interpreted to mean that the ANF is secreted into the lumen of the proximal tubule via the organic anion transport system and then acts from the luminal side of the loop of Henle or the collecting duct to inhibit the reabsorption of Na. This hypothesis was proposed before the structure of ANF was established. It is now known that ANF is a polypeptide with a minimum size in the range of 22 amino acids, and it therefore seemed unlikely to be handled like *p*-aminohippurate (PAH). Nevertheless the probenecid inhibition suggested that ANF may interact with the organic acid transport system.

The present investigation was undertaken to investigate directly the interaction between the ANF and the renal organic anion transport system. These studies utilized the renal cortical slice technique which has been extensively used to study the (secretory) transport characteristics of organic anions since its introduction to renal physiology in 1950 (19). The results indicate a lack of interaction between

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the ANF and the slice uptake of *p*-aminohippurate, a prototypical organic anion, which is at variance with the hypothesis that the ANF is first secreted by the proximal tubule via the probenecid-sensitive organic anion transport system before it reaches the site of action in the distal nephron.

Methods. The effect of various atrial extracts (crude, partially purified, and fully purified) on the uptake of PAH by the kidney cortical slice was studied in Sprague–Dawley rats of either sex. In addition, the effect of probenecid pretreatment on the natriuretic effect of various atrial extracts was also reinvestigated.

A. Preparation of atrial extracts. A crude extract of rat atria was prepared by the method of de Bold *et al.* (1). Briefly, rats were killed by cervical dislocation and the atria were immediately dissected, washed, and homogenized using a ground glass homogenizer in phosphate-buffered saline (PBS). The homogenate was centrifuged in a refrigerated centrifuge, and the supernatant was stored at −70°C until use. A crude ventricular extract was prepared in an identical manner and used as a control. In some studies, the atrial and ventricular homogenates were boiled for 10 min prior to centrifugation according to Briggs *et al.* (8).

A partially purified ANF was prepared by acetic acid extraction, desalting in a Bio-Gel P-2 column, and subsequent fractionation using gel filtration on a Sephadex G-75 column. The molecular weight of the most active fraction corresponds to less than 6000. The exact procedure for this partial purification is described elsewhere (20). The atria from 15 rats were pooled to prepare one batch of the partially purified ANF. Cardionatrin I (purified ANF) was prepared as described elsewhere (21). The atria from approximately 2000 rats were used to yield the amount of cardionatrin I used in the present work. In the last series, a synthetic atrial peptide (rat, 28 amino acids; Pennsylvania Laboratories, Inc., Belmont, Calif.) was also used for *in vitro* studies.

B. Slice uptake of organic ions. Sprague–Dawley rats of either sex were killed by cervical dislocation, and the kidneys were removed and immediately perfused through the renal artery with ice-cold isotonic saline (NaCl, 140 m*M*; KCl, 10 m*M*; $CaCl_2$, 1.5 m*M*) to remove as much blood as possible.

Thin renal cortical slices (0.4–0.5 mm) were prepared by hand using a razor blade, and were stored briefly in an ice-cold modified Cross–Taggart medium before incubation. The composition of the medium was 140 m*M* NaCl, 10 m*M* KCl, 1.5 m*M* $CaCl_2$, and 5.0 m*M* Na phosphate (pH 7.4). The renal slices (80–120 mg wet wt) were incubated in 30-ml beakers containing 4 ml of the Cross–Taggart medium with 75 μM PAH or 10 μM TEA, and a trace amount of the respective ^{14}C-labeled compound (Amersham/Searle or New England Nuclear). The incubation medium also contained variable amounts of crude cardiac extracts, partially purified ANF, cardionatrin I, or synthetic atrial peptide as specified under Results. The incubation was carried out in a Dubnoff metabolic shaker (25°C) for 30 min in a 100% oxygen atmosphere (19).

Following incubation, the slices were quickly removed, blotted, and weighed. After the tissue was solubilized in 1 *N* NaOH, the solution was neutralized with HCl. Aliquots of the medium were prepared similarly for scintillation spectrometry. These samples were assayed for ^{14}C activity using standard liquid scintillation techniques, as described elsewhere (22). Quench corrections were performed with the appropriate set of standards for each type of sample composition. The PAH and TEA uptake data are given as the slice-to-medium (S/M) ratio: the tissue concentration of organic ion (mol/g tissue) divided by that of the medium (mole/ml medium).

C. Bioassay of cardiac extracts. Rats (250–300 g) were anesthetized with Inactin (10 mg/100 g body wt, ip) and placed on a temperature-controlled table. A polyethylene cannula was placed in a jugular or femoral vein for injection. The urinary bladder was also cannulated for urine collection. One hour after completing the surgical procedures, a 20-min control urine collection was made. One of the test solutions (PBS, crude atrial or ventricular extract, partially purified ANF, or cardionatrin I) was then intravenously administered and urine collections were continued for 70 min. Since the diuretic and natriuretic responses to ANF were rapid and brief, the urine was collected every 10 min during the first 20 min,

and then over 20- and 30-min intervals thereafter. Only one test solution was administered to a rat.

In another series of experiments, the effect of pretreatment with probenecid on the renal responses to crude atrial extracts, partially purified ANF, or cardionatrin I was investigated in rats prepared as described above. At the end of the usual 20-min control urine collection period, 2.5 mg of probenecid/100 g body wt (dissolved in 0.5 ml, ~50 mM) was injected over 2 min and then the infusion of one of the above test solutions was begun 5 min later. In this series, another rat prepared in the same manner served as the paired control in which the same infusion with substitution of lactate for probenecid was made. The osmolality, Na concentration, and adjusted pH were the same for both solutions. The above dose of probenecid decreased the plasma clearance of PAH by 76% during first 10 min, which suggests marked inhibition of the organic anion transport system. All urine samples were analyzed for Na and K by flame photometry.

The data in Table I were analyzed statistically by the unpaired or paired Student t test depending on the design of experiment, and the level of probability to denote significance was chosen as $P < 0.05$. When multiple comparisons were required, such as in Fig. 1, the data were analyzed using analysis of variance. If the F value was significantly different ($P < 0.05$) the significance among individual group means was tested with the Bonferroni modification of the t test.

TABLE I. EFFECTS OF VARIOUS ATRIAL EXTRACTS ON THE SLICE UPTAKE OF PAH AND TEA

Type of extract (n)	S/M PAH	S/M TEA
A. Crude extracts		
Control (4)	4.85 ± 0.33	12.21 ± 1.59
PBS (3)	4.25 ± 0.66	13.16 ± 2.00
Atrial extract (4)[a]	5.43 ± 0.89	9.05 ± 1.12
Ventricular extract (4)[a]	6.11 ± 0.99	9.89 ± 1.60
B. Partially purified ANF		
Control (3)	3.06 ± 0.34	7.22 ± 0.80
Partially purified ANF (3)[b]	2.96 ± 0.51	6.42 ± 0.50
C. Cardionatrin I		
Control (4)	5.02 ± 0.78	10.36 ± 0.66
Cardionatrin I (4)[c]	4.68 ± 0.62	9.87 ± 1.07
D. Atrial peptide (rat, 28 amino acids)		
Control (2)	6.09	13.37
Atrial peptide (2)[d]	5.02	14.01

Note. The results obtained from experiments using lower extract concentrations are not included in this table, because S/M PAH and TEA values were the same as those for the paired control. Values are means ± SE; n = the number of rats.

[a] The original extract diluted 5- to 10-fold with the incubation medium.

[b] 0.1–0.5 ml of the suspension per 4 ml incubation medium.

[c] 10–50 μl of the suspension per 4 ml incubation medium.

[d] 10^{-11}–10^{-7} M (the data were pooled in the absence of concentration dependency).

Results. *A. Effect of ANF on organic ion transport.* The effects of various test solutions on the slice uptake of PAH (a representative organic anion) and TEA (a representative organic cation) are shown in Table I. To study the effect of crude atrial or ventricular extract, the original extract (1 g wet tissue/10 ml PBS) was serially diluted 5- to 100-fold with the incubation medium, to which PAH or TEA was added at the concentration specified under Methods. As described below, 2 ml of the original crude atrial extract (iv) gave a 5-fold increase in urine flow and Na excretion during the first 10 min after which these renal responses were rapidly dissipated. If it is assumed that the injected extract is mainly distributed within the vascular compartment (12–15 ml plasma in a 300-g rat) during the first 10 min, it is estimated that the original extract is diluted 7- to 8-fold in the blood and yet induced significant renal effects. In reality, however, the volume of distribution of the injected extract could be greater than the vascular compartment, but the highest concentration of the extract (5-fold dilution of the original) used in the slice experiment is still considerably higher than the estimated, effective plasma concentration of the atrial extract. S/M PAH values were not significantly altered by the addition of either 5-fold diluted PBS, the ventricular extract, or the atrial extract (Table I). Ventricular or atrial extracts, diluted further (up to 100-fold), also failed to alter S/M PAH (data not shown). Although S/M TEA decreased in the presence of crude ventricular or atrial extracts, there was no statistical significance. Moreover, boiling of homogenates made little difference in the results.

Partially purified ANF obtained from 26 rats was dissolved in 4 ml PBS. Intravenous administration of 0.5 ml of this solution into a rat resulted in a 5- to 6-fold increase in urine flow and Na excretion during the first 10 min (see below). Therefore, 0.02–0.5 ml of this solution was included in 4 ml incubation medium containing PAH or TEA. On the basis of the considerations given above, the highest medium concentration of partially purified ANF (i.e., 0.5 ml in 4 ml incubation medium) is considerably greater than the estimated, effective plasma concentration for the *in vivo* renal effects. However, the S/M PAH (or TEA) was little affected even in the presence of the highest concentration of this factor (Table I). Likewise, the S/M PAH (or TEA) changed little in the presence of lower concentrations of this factor (data not shown).

The effect of cardionatrin I was also studied. Cardionatrin I extracted from 2000 rats was dissolved in 1 ml PBS and 10–50 μl of this solution was added to 4 ml of the incubation medium containing PAH or TEA. Intravenous injection of 50 μl of this solution resulted in an increase in both urine flow and Na excretion 5- to 10-fold (see below). Again, the values of S/M PAH and TEA remained identical to those of the control at all concentrations of cardionatrin I. Finally, a synthetic atrial peptide at a concentration range of 10^{-11} to 10^{-7} *M* failed to affect the slice uptake of PAH and TEA.

B. Effect of probenecid on ANF-induced natriuresis. As briefly stated above, the intravenous administration of either crude atrial extract, partially purified ANF, or cardionatrin I induced marked increases in urine flow and excretion of Na and K during the first 10 min after which these renal responses dissipated quickly. Since identical patterns were observed in all experiments, the renal responses during the first 10-min period after the infusion of a test solution were summarized for comparison. As shown in Fig. 1, there were slight increases in urine flow and Na excretion when PBS alone or crude ventricular extracts were administered. However, the magnitude of these responses was significantly ($P < 0.05$) greater when the atrial extracts were administered. Since the results obtained from all three different types of ANF (crude atrial extract, partially purified ANF, and cardionatrin I) were comparable at the particular doses used in the present work, the results were pooled and expressed as "atrial extract" in Fig. 1. On the average, the effects of "atrial extract" were not significantly attenuated by the pretreatment with probenecid which, at the dose used in this study, inhibited PAH clearance by 76%.

Discussion. The exact physiological role played by ANF as a regulator of body fluid is not yet defined (9, 17, 23). Micropuncture studies indicate that it acts in the loop of Henle (16) or the collecting duct (7, 8, 16). Although the cellular mechanism of the action of the ANF is not clear, it is not mediated by the inhibition of the Na, K-ATPase localized in the basolateral membrane of the tubular cell (24–26). It has also been shown that the ANF is different from the so-called "natriuretic hormone" (25, 26) and has no effect on active Na transport across the toad urinary bladder (26) which is generally considered to be a

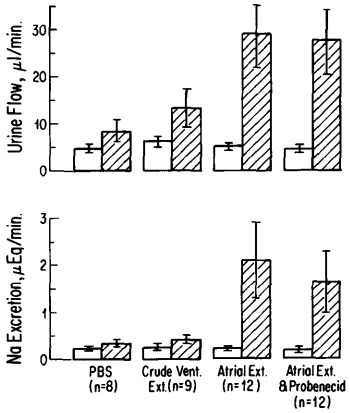

FIG. 1. Urine flow and Na excretion before (control; open bars) and during first 10 min (shaded bars) following intravenous administration of various test solutions. Data represent the means (±SE) of the number of rats indicated in parentheses. The results obtained from rats infused with crude atrial extract ($n = 6$), partially purified extract ($n = 3$), and fully purified extract ($n = 3$) are pooled under "atrial extracts." In all cases, the increments in values during first 10 min after infusion over the control period are significant ($P < 0.05$). The increments in both renal parameters after "atrial extracts" were significantly greater than those after PBS or crude ventricular extract ($P < 0.05$) but not significantly different from those after "atrial extracts + probenecid" ($P > 0.05$). See the text for the dose of each test solution infused.

functional model for the mammalian distal nephron. The fact that the ANF has no effect on the Na, K-ATPase strongly implies that it may act at the luminal membrane of the distal nephron to inhibit the passive entry of Na from the lumen into the cell. Certain diuretics such as furosemide act in this manner and, in fact, several investigators postulated that the mode of action of ANF is analogous to that of furosemide (15–17), although the latter view has been challenged by Baines *et al.* (12). Moreover, Sonnenberg *et al.* (17) showed that the diuretic effect of ANF is significantly attenuated by probenecid, a specific inhibitor of renal organic anion secretion in the proximal tubule (18). Based on this finding, Sonnenberg *et al.* (17) suggested that the ANF may reach the luminal site of the action in the distal nephron via active secretion in the proximal tubule. As shown in Fig. 1, there was a tendency toward probenecid attenuation of the ANF response but statistical significance could not be established.

In the present work, the possible interaction of ANF with the renal organic anion transport system was investigated using the renal cortical slice technique developed by Cross and Taggart (19), which has been extensively used to study the characteristics of tubular secretion. It was hypothesized that the slice uptake of PAH (an organic anion) but not TEA (an organic cation) would be inhibited by ANF. However, the uptake of neither organic ion was significantly affected by ANF (crude atrial extracts, partially purified ANF, cardionatrin I, or a synthetic atrial peptide) over a wide range of concentration in the incubation medium (Table I). Even when the concentration of ANF in the medium was higher than the estimated, effective plasma concentration needed to induce a typical natriuresis in the *in vivo* preparation, ANF had little effect on the slice uptake of organic ions. It should also be pointed out that the slice uptake of PAH, but not TEA, was indeed significantly inhibited by probenecid (S/M PAH decreased to approximately 1.0 from the control level of 5.0). These results are not consistent with the above hypothesis and cast serious doubt on the view that the ANF is secreted via a probenecid-sensitive active organic anion transport mechanism.

The authors acknowledge P. Gigliotti for her technical help, and Ms. Susan McMahon and Mrs. Elizabeth Miranda for the kind help in the preparation of this manuscript. This work was supported in part by PHS Grants AM-18918 and HL-28542, and by the Medical Research Council of Canada, the Ontario Heart and Stroke Foundation, and the American Heart Association with funds contributed in part by the Western New York Chapter.

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Received March 24, 1985. P.S.E.B.M. 1986, Vol. 182.
Accepted April 4, 1986.