The increase of cGMP by atrial natriuretic factor correlates with the distribution of particulate guanylate cyclase

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We have demonstrated previously that atrial natriuretic factor (ANF) augments urinary, plasma and kidney cGMP levels but has no significant effect upon cAMP. Using cGMP as a marker, we searched for specific target sites involved in the action of ANF in the dog kidney, and observed no change of cGMP in the proximal tubules, a 2-fold increase over basal levels in the thick loop of Henle and a 3-fold elevation in the collecting duct. The most striking action on cGMP occurred in the glomeruli with a rise of up to 50-fold being evident at 1–2 min. after the addition of ANF. The results obtained in the absence or presence of a phosphodiesterase inhibitor support the notion that the effects of ANF were exerted at the level of guanylate cyclase stimulation rather than cGMP phosphodiesterase inhibition. The action of sodium nitroprusside (SNP), a direct stimulator of soluble guanylate cyclase, differed from that of ANF. The ability of the factor to enhance cGMP levels was correlated with the distribution of particulate guanylate cyclase. This study identifies the glomeruli and the distal part of the nephron as specific targets of ANF and implicates particulate guanylate cyclase as the enzyme targetted for the expression of its action.

Atrial natriuretic factor *Cyclic GMP* *Particulate guanylate cyclase* *Glomerulus* *Tubule* *Sodium nitroprusside*

1. INTRODUCTION

Specific secretory granules of atrial myocytes contain peptides which possess potent natriuretic and diuretic properties when injected into rats [1,2]. Several of these atrial natriuretic factors (ANF) have been purified from animal and human sources and their sequences have been elucidated [3,4]. The cloning and characterization of DNA sequences encoding for rat and human ANF have recently been accomplished [5,6], suggesting that these peptides are members of a family derived from a common precursor. A fragment of one of these peptides, which was synthesized recently [7], exhibited activity similar to endogenous ANF. In addition to its diuretic and natriuretic properties, ANF appears to be a potent vasorelaxant [8,9] capable of reducing blood pressure more in spontaneously hypertensive rats (SHR) than in normotensive controls (WKY) [10]. The diminution of blood pressure is accompanied by greater ANF-induced diuresis and natriuresis in the hypertensive group [10].

While searching for the mechanism of action of ANF, we recently noted a specific elevation of cGMP levels in plasma and urine following injection of native ANF as well as of a synthetic ANF fragment [11]. The factor also heightened cGMP levels in kidney slices and primary cultures of renal tubular cells [11].

Abbreviations: MIX, 1-methyl-3-isobutylxanthine; TEA, triethanolamine

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cGMP appears to be linked to fluid and solute transport. Thus, diuretics such as furosemide increase cGMP levels, and injection of cGMP into the thoracic aorta markedly decreases tubular reabsorption of sodium in the rat [12]. A heat-stable enterotoxin from *Escherichia coli*, which changes net sodium transport by reversal of Cl^- flux and which abolishes net sodium absorption, has been shown to specifically stimulate particulate guanylate cyclase in the ileum [13,14]. It has also been suggested that cGMP acts as a negative feedback for vasocontractile agents and as a mediator of vascular smooth muscle relaxation [15,16]. Our study was undertaken to evaluate the potential involvement of cGMP in the natriuretic and vasodilating properties of ANF. We therefore looked for the specific target sites of action of ANF.

2. METHODS

2.1. *Tissue fractions*

Four different fractions (glomeruli, proximal tubules, thick ascending limbs, and collecting ducts) were obtained from dog kidneys via the following procedure: both kidneys were rapidly removed and placed in ice-cold, gassed (95% O_2/5% CO_2) Krebs-Henseleit saline (KHS). They were cut into large sagittal slices, and the outer cortex, red medulla and white medulla were carefully dissected. The tissues taken from each fraction were then sliced with a Stadie-Riggs microtome, washed with KHS and placed in siliconized Erlenmeyer flasks containing 20 ml KHS. Lactate plus pyruvate (20 and 2 mM, respectively) were added to the cortical and red medulla slices, and glucose (20 mM) to the white medulla tissue. Thirty mg of collagenase (*Clostridium histolyticum*, Boehringer Mannheim) were added. The flasks were gassed, and the tissues incubated at 37°C for 45 min with mechanical agitation, followed by tissue dissociation into tubular fragments. The cortical suspension was washed and passed through a 90 μm mesh sieve. All the glomeruli were retained and purified through successive washings. The rest of the tissues were resuspended in freshly prepared KHS/Percoll solution, as described elsewhere [17], and centrifuged at 12000 × g for 30 min. A band of virtually pure proximal tubules was collected from the Percoll gradient. Thick ascending limbs were obtained directly from digestion of red medulla tissue: the vasa-recta and thin loops of Henle were destroyed and removed by this procedure, which is similar to that described by Baverel et al. [18] except for the addition of substrates during the incubation with collagenase. The final suspension contained mostly thick ascending limbs contaminated with some collecting ducts. A 95% pure collecting duct suspension was similarly derived from the white medulla.

The following procedures verified the proper separation of these segments of nephrons: glomeruli, microscopic examination; proximal tubules, microscopic examination, positive PAS staining of brush-border membrane, rapid gluconeogenesis from lactate or glutamine, a small (10%) suppressive effect of furosemide (0.1 mM) on tissue oxygen consumption; thick ascending limbs, microscopic examination, absence of brush-border membranes (PAS), absence of gluconeogenesis, rapid oxidation of lactate, faster oxygen consumption than proximal tubules, and a major (60%) inhibitory effect of furosemide (0.1 mM); collecting ducts, microscopic examination, lack of oxygen uptake, fast glycolysis with stoichiometric release of lactate. All tissue fractions were kept on ice until they were utilized.

2.2. *Cyclic GMP levels*

Tissues or cells were incubated for varying time periods and with different concentrations of synthetic ANF. Incubation was terminated with the addition of an equal volume of 1 N perchloric acid. cGMP levels were measured by radioimmunoassay [19] after purification on Al_2O_3 and Dowex columns.

2.3. *Guanylate cyclase activity*

This parameter was essentially assessed as described earlier [20]. The reaction mixture (0.1 ml) contained 2 mM dithiothreitol, 5 mM creatinine phosphate, 50 μg creatinine phosphokinase, 1 mM MIX, 5 mM $MnCl_2$, 2 mM cGMP, 0.5 mM GTP, 0.1–0.5 μCi [α-^{32}P]GTP in 50 mM Tris buffer (pH 7.5) and 1–5 μg enzyme protein. The activities were linear for at least 60 min. Incubation was stopped after 30 min at 37°C by the addition of 500 μl of 120 mM zinc acetate and 600 μl sodium carbonate. After centrifugation, the

[^{32}P]cGMP formed was further purified on Dowex WX4 and alumina columns.

2.4. *Enzyme preparation*

After resuspension in 20 mM TEA and 1 mM EDTA (pH 7.5), the tissues were processed in a Polytron homogenizer. The homogenates were centrifuged twice at 30000 × *g* for 30 min, and the pellets were redissolved in TEA buffer. Supernatants and particulate fractions were incubated at 37°C for 30 min with GTP-Mn as substrate, as described previously. Proteins were measured by the method of Lowry et al. [21] with bovine serum albumin as standard.

3. RESULTS AND DISCUSSION

3.1. *Effect of ANF on cGMP levels in different fractions of dog nephron*

cGMP levels were assessed at varying time intervals after the addition of 0.5 μM synthetic ANF to four different fractions of the dog nephron. We observed no effect in the proximal tubules, a 2-fold increase over basal values in the thick ascending loop of Henle, and a 3-fold elevation of cGMP in the collecting ducts (fig.1). The most dramatic change was evident in the glomeruli with a maximal 50-fold augmentation over basal cGMP levels occurring at 1–2 min after ANF was added (fig.1). The threshold concentration of ANF for the cGMP rise was 5 nM (fig.2). A 4-fold enhancement of cGMP in glomeruli was recorded with 100 nM ANF and this was not significantly modified by 0.5 mM of MIX, a cyclic nucleotide phosphodiesterase inhibitor (fig.2). In contrast, in the thick loop of Henle and in the collecting duct, the heightened cGMP levels, starting with a concentration of 5–10 nM ANF, were visible only in the presence of MIX (fig.2). The relative influence of cGMP-hydrolyzing activity on resting cGMP levels appeared to be greater in the two latter fractions since the addition of MIX alone raised the cGMP values by 2-fold in these tissues. In the proximal tubules, ANF had no effect upon cGMP levels up to a concentration of 5 μM, either in the absence or presence of MIX.

These results demonstrate that ANF preferentially enhances cGMP levels in the glomeruli, followed by lower elevations in the collecting duct and thick loop of Henle, with no detectable change

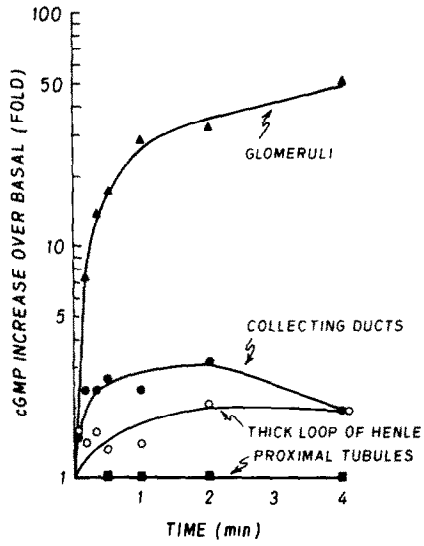

Fig.1. Time course of effect of ANF on cGMP levels. Four fractions of dog kidney, processed as described in section 2, were incubated at 37°C with constant agitation in the absence or presence of 0.5 μM ANF. The incubation was stopped at different time intervals with the addition of an equal volume of 1 N perchloric acid. cGMP levels were measured by radioimmunoassay. The basal values of cGMP were 0.5 pmol·mg protein^{-1} in the proximal tubules, 2 pmol·mg protein^{-1} in the collecting ducts, 7 pmol·mg protein^{-1} in the thick loop of Henle, and 5 pmol·mg protein^{-1} in glomeruli. Results are the mean of two different experiments.

in the proximal tubules. The differential responses of cGMP to ANF correlate well with the presence and density of ANF receptors in the same tissues (De Lean et al., in preparation). Our data on the influence of MIX support the hypothesis that the effects of ANF are exerted at the level of guanylate cyclase stimulation rather than cGMP phosphodiesterase inhibition. This, however, does not preclude a contributing inhibitory action of ANF on cGMP phosphodiesterase, as reported previously [11].

3.2. *Comparison of effects of sodium nitroprusside (SNP) and ANF on cGMP levels*

We compared the actions of ANF on cGMP levels in glomeruli and in the thick loop of Henle with those of SNP, a potent stimulator of soluble guanylate cyclase. As shown in fig.3, the increase

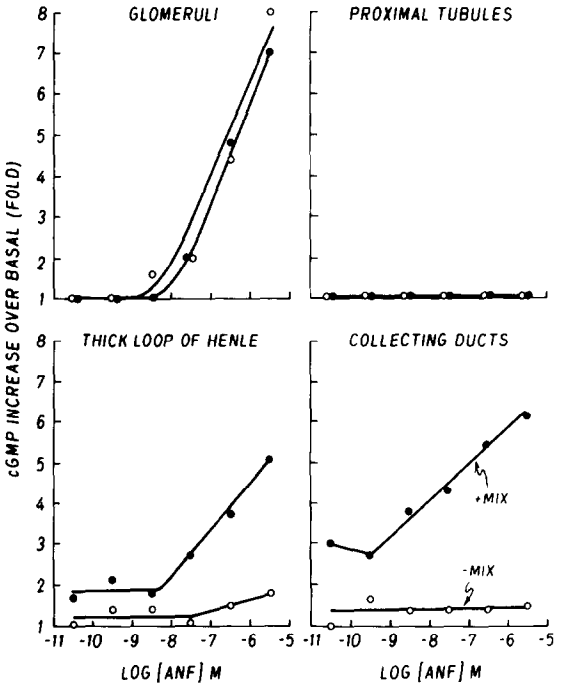

Fig.2. Dose-response curves of cGMP increase by ANF. The tissues were incubated with increasing concentrations of ANF for 2 min at 37°C in the absence (○) or presence (●) of 0.5 mM MIX. Results are the mean of 3 different experiments.

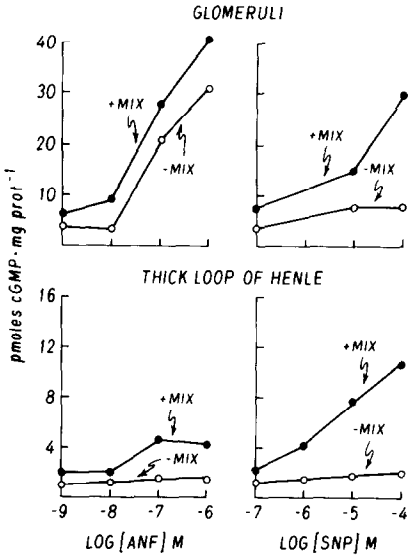

Fig.3. Effects of SNP and ANF on cGMP levels in glomeruli and the thick loop of Henle. Glomeruli and the thick loop of Henle were incubated, as described previously, with increasing concentrations of ANF (left) or SNP (right) in the absence or presence of 0.1 mM MIX.

of cGMP by SNP in the glomeruli was lower than that elicited by ANF up to a concentration of 100 μM SNP. Furthermore, the augmentation of cGMP by SNP was enhanced by the presence of MIX. Glomerulus-derived cells, cultured from outgrowths of isolated dog glomeruli revealed similar responses as seen in fresh tissues (not shown). In contrast, in the thick loop of Henle (fig.3), and in collecting ducts (not shown), the increase of cGMP levels by SNP was three times greater than that induced by ANF.

We detected no effect of ANF on cGMP in rat platelets, but a 4-fold elevation was generated by 25 μM SNP (not shown). ANF did not raise cGMP levels despite the presence of ANF receptors in this cell type (Schiffrin et al., under preparation). On the other hand, in primary cultures of rat mesenteric vascular smooth muscle cells, a similar 10-fold increase of cGMP values was induced with 100 nM ANF or 100 μM SNP (table 1). In this tissue, the threshold concentration of ANF for the cGMP increase was 0.1 nM.

Table 1

Effect of ANF and SNP on cGMP levels in rat mesenteric vascular smooth muscle cells

ANF (nM)	cGMP[a] levels	SNP (μM)	cGMP[a] levels
0	0.5	0	0.6
0.1	0.7	0.1	0.6
1.0	1.1	1.0	0.7
10	4.9	10	1.5
100	4.9	100	5.6

[a] cGMP levels are expressed in pmol·mg protein^{-1}

Mesenteric vascular smooth muscle cells were obtained from adult Wistar rats by collagenase treatment. The cells were kept in medium supplemented with 10% bovine serum and containing penicillin (100 μg/ml) and streptomycin (200 μg/ml). At the time of the experiment, the cell cultures showed about 90% confluence. These experiments were performed on cells between 3–5 passages

The results described up to now illustrate significant differences in the ability of SNP and ANF to augment cGMP levels in the tissues examined. SNP enhanced cGMP values more than ANF in the thick loop of Henle, in the collecting duct, and in platelets. In contrast, the elevation of cGMP levels by ANF was greater than that elicited by SNP in glomeruli and similar to that caused by SNP in rat mesenteric vascular smooth muscle cells.

3.3. *Distribution of guanylate cyclase activity in dog kidney*

The cellular distribution of soluble and particular guanylate cyclases varies from tissue to tissue. For example, in platelets, over 95% of guanylate cyclase recovers in platelet supernatants [20], whereas in the intestine most of it is in particulate form. In whole kidney homogenate, 90% of guanylate cyclase recovers in the soluble fraction. Craven and DeRubertis [22] have reported that guanylate cyclase is mostly soluble in the renal cortex but is particulate in the medulla. Helwig et al. [23] recorded a higher activity of particulate guanylate cyclase in rabbit glomeruli, while tubular preparations registered mainly soluble activity. The hormones and hormone-like agents tested had no effect on these enzymes [23]. cGMP levels were increased in the glomeruli by cholinergic agents and nitroso reagents in studies by Dousa et al. [24].

We determined the distribution of guanylate cyclase activity in the four fractions of dog kidney (table 2). In the glomeruli, over 80% of guanylate cyclase was found in particulate form, whereas only 20% was noted in this fraction of the collecting duct and the thick loop of Henle. In the proximal tubules, total guanylate cyclase activity was very low and not detectable in the particulate fraction. Thus, a good correlation exists between the ANF-induced elevation of cGMP levels and the distribution of particulate guanylate cyclase. The fact that ANF stimulates cGMP formation differently than SNP and is more potent in tissues rich in particulate guanylate cyclase had led us to suggest that ANF raises cGMP levels in specific target cells by stimulation of particulate guanylate cyclase.

The direct action of ANF on particulate guanylate cyclase is difficult to demonstrate in cell-free systems. However, our preliminary data indicate a two-fold stimulation of dog glomerulus particulate guanylate cyclase by ANF with no manifest effect upon the soluble fraction (Gerzer et al., in preparation).

Table 2

Distribution of guanylate cyclases in dog kidney

	Supernatant	Particulate
	(pmol cGMP · min^{-1} · mg^{-1})	
Glomeruli	83	392
Collecting duct	91	28
Loop of Henle	39	10
Proximal tubule	2	n/d[a]

[a] Not detectable

Supernatant and particulate fractions were obtained by centrifugation of whole homogenate twice at 30000 × *g*

Numerous hormonal factors, including muscarinic and α-adrenergic agonists, have been shown to stimulate cellular cGMP formation. In many tissues, the stimulation involves calcium and the release of arachidonic acid [20]. The guanylate cyclase stimulated is the soluble type, which can also be stimulated by non-hormonal compounds. Non-hormonal stimulation by agents such as nitroso compounds does not require calcium and can be demonstrated directly, in cell-free systems, on soluble or, to a lesser extent, on particulate guanylate cyclase [25].

Only a few agents have been found to stimulate particulate guanylate cyclase. A heat-stable enterotoxin from *E. coli* stimulates intestinal particulate guanylate cyclase but is ineffective in other tissues [13,14]. Speract, a decapeptide, binds to spermatozoa and increases cGMP concentrations in these cells in which particulate guanylate cyclase is predominant [26]. However, no direct stimulation of the particulate fraction has been demonstrated with this agent.

The present study identifies glomeruli and the distal part of the nephron as specific targets for ANF and indicates that particulate guanylate cyclase is the target enzyme involved in the action of ANF. In vascular smooth muscle cells, where both particulate and soluble guanylate cyclases are present, both ANF and SNP are vasodilatory. In the kidney, however, the effect of ANF appears to

correlate with the distribution of particulate guanylate cyclase, with SNP being devoid of diuretic activity [11]. It is conceivable that a specific distribution of cGMP within the cell, as synthesized by soluble or membrane guanylate cyclase, is implicated in the function of SNP and ANF in the kidney. Nevertheless, the participation of cGMP and its synthesis or hydrolysis for sodium transport warrant further study.

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