In vivo evidence that cGMP is the second messenger for atrial natriuretic factor

(glomerulus/glomerular filtration rate/cAMP/micropuncture)

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ABSTRACT cGMP generation has been associated with many of the vascular and endocrine actions of atrial natriuretic factor (ANF) in vitro. To examine the role of cGMP as a second messenger for the renal hemodynamic action of ANF in vivo, we measured glomerular filtration rate (GFR) and cGMP concentration in systemic artery, renal vein, and urine as well as in Bowman's space and end-proximal tubule (by free-flow micropuncture) after administration of ANF. ANF increased GFR by 45% and simultaneously induced a >5-fold increase of cGMP concentration in glomerular ultrafiltrate (Bowman's space) when compared to controls. There was no significant increase in either systemic artery or renal vein cGMP concentration. Thus, the source of increased Bowman's space cGMP is not from the blood via filtration but rather from either glomerular mesangial or epithelial cells, which are not in direct contact with the circulation. Although a small amount of tubular handling of cGMP occurred along the length of the nephron, the augmented cGMP production from the glomerulus accounted for most of the 10- to 12-fold higher urinary cGMP excretion observed after ANF administration. Intrarenal arterial infusion of dibutyryl cGMP, but not dibutyryl cAMP, increased GFR in a dose-dependent fashion (from 10 to 1000 μM) by a mechanism similar to that of ANF—an increase in glomerular hydraulic pressure. Thus, ANF markedly stimulated glomerular production of cGMP, which coincided with a marked increase in GFR. Since dibutyryl cGMP itself was capable of increasing GFR, cGMP is the likely second messenger for ANF in vivo.

The discovery of cGMP in mammalian urine over 20 years ago by Ashman et al. (1) initiated a search for a biological role for this cyclic nucleotide. Although cGMP has been found to mediate several cellular signal transduction processes, such as vasodilation by nitric oxide-containing drugs and acetylcholine, changes in intestinal ion transport by bacterial enterotoxin (2, 3), and phototransduction in vertebrate retinal rod cells (4), it has not yet been shown conclusively to be a second messenger for a polypeptide hormone.

The recently discovered vasoactive hormone atrial natriuretic factor (ANF) (5-7) has renewed interest in the possibility that cGMP mediates the action of a polypeptide hormone. cGMP generation has been associated with many of the actions of ANF in vitro, such as vascular smooth muscle relaxation (8, 9) and inhibition of aldosterone release from adrenal glomerulosa cell suspension (10). Since ANF has been reported to increase urinary cGMP excretion (11), it is possible that cGMP may participate in an important physiologic effect of ANF in the kidney, increase in glomerular filtration rate (GFR) (12, 13). The purpose of the present study was to investigate the potential role of cGMP as the second messenger for ANF in vivo, using functional criteria analogous to those Sutherland developed for cAMP (14): (i) ANF should increase glomerular production of cGMP, which should coincide with the important renal physiologic effect of ANF—glomerular hyperfiltration; and (ii) glomerular application of cGMP or analogs should reproduce this physiologic effect.

METHODS

Protocols. Free-flow micropuncture techniques were used to define the nephron source for urinary cGMP excretion. Five male Munich-Wistar rats were prepared for micropuncture in standard fashion after Inactin (100 mg/kg of body weight i.p.) anesthesia, as described (15, 16). [methoxy-3H]Methoxycinulin infusion [100 μCi primer injection and 100 μCi/hr in isotonic saline at 6 ml/kg per hr (1 Ci = 37 GBq)] was begun. After 45 min for equilibration, one or two timed collections (15-20 min) were made from Bowman’s space and the end-proximal tubule during the hydrogenic control period. An infusion of synthetic ANF (rat 25-amino acid Auriculin B, kindly supplied by California Biotechnology, Palo Alto, CA; 5 μg/kg primer injection and 0.5 μg/kg per min in bicarbonate Ringer’s solution at 10 μl/min) was then begun. After another 45 min for equilibration, micropuncture was repeated. Three timed urine collections with femoral arterial and renal venous blood sampling were also obtained in both control and ANF periods.

Tracer experiments were performed to evaluate cGMP handling in the kidney in the presence or absence of ANF administration. [3H]cGMP and [14C]inulin (100 and 30 μCi/hr, respectively) were given as a constant intravenous infusion (in isotonic saline at 6 ml/kg per hr). An infusion of bicarbonate Ringer’s solution (10 μl/min) with or without ANF (5 μg/kg bolus and 0.5 μg/kg per min) was begun. After 45 min for equilibration, three timed collections were made from the superficial end-proximal tubule, with simultaneous urine collections and femoral arterial and renal venous blood sampling. Since ANF administration did not affect the results obtained in the two groups, the data were combined.

To measure the effect of cGMP on GFR, we infused dibutyryl cGMP (Bt2cGMP) into the left renal artery through a 30-gauge needle in 11 Munich-Wistar rats. [3H]Inulin infusion (100 μCi primer injection and 100 μCi/hr in isotonic saline at 6 ml/kg per hr) was begun. After 45 min for equilibration, three 10-min control clearance periods were made during intravenous vehicle infusion (isotonic saline at 13 μl/min). Bt2cGMP (Sigma) was then added to the infusion at rates of 0.03, 0.3, or 3 μmol/min, sufficient to reach local glomerular plasma concentrations of =10, 100, or 1000 μM.

Abbreviations: ANF, atrial natriuretic factor; GFR, glomerular filtration rate; Bt2cAMP, dibutyryl cAMP; Bt2cGMP, dibutyryl cGMP.

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For controls, dibutyryl cAMP (Bt$_2$cAMP, Sigma) was infused in an additional six rats at 0.03 or 0.3 μmol/min; the use of 3 μmol/min was precluded because it caused hypotension and acute renal failure. After 45–60 min for equilibration, three additional clearance periods were performed.

To affirm that the mechanism by which Bt$_2$cGMP increased GFR was similar to that of ANF, either GFR or stop-flow pressure measurements were made in an additional seven male Sprague–Dawley rats. Stop-flow pressure measurements are an accurate reflection of glomerular hydraulic pressure (17). We (18) and others (19) have found that an increase in glomerular hydraulic pressure accompanies the glomerular hyperfiltration induced by ANF.

**Measurements and Calculations.** [3H]inulin, [3H]cGMP, and [3H]cGMP concentrations were determined by liquid scintillation assay in plasma, tubular fluid, and urine as described (15, 16). cGMP concentrations in aliquots of tubular fluid (200–300 nl), plasma (25 μl), and urine (0.01–0.1 μl) were determined by radioimmunoassay after acetylation with a commercially available assay kit (New England Nuclear). The detection limit was 0.0025 pmol per assay tube, equivalent to a cGMP concentration of ~10 nM in 200–300 nl of tubular fluid. Stop-flow pressure was measured by using a servo-null micropressure system (17–19). Standard methods were used for calculating GFR and fractional delivery of cGMP [ratio of the concentration in tubular fluid to the concentration in plasma (TF/P) of cGMP/inulin]. Results are presented as the mean ± SEM. Statistical significance was assessed by using the paired t test for results in the same animal.

**RESULTS**

ANF increased GFR by 45% (1.1 ± 0.1 to 1.6 ± 0.2 ml/min, P < 0.005) and simultaneously caused a marked 12-fold increase in urinary cGMP excretion (8 ± 1 to 94 ± 20 pmol/min, P < 0.01) (Table 1). To assess whether urinary cGMP was of glomerular origin, we measured cGMP concentration both in glomerular ultrafiltrate and systemic arterial plasma. Arterial plasma cGMP concentration did not change significantly after ANF, in contrast to previous reports (11). However, ANF markedly increased Bowman’s space glomerular ultrafiltrate cGMP concentration from <10 nM in the control period to 55 ± 11 nM after ANF (P < 0.005). These results suggest that glomerular cGMP production, rather than simple cGMP filtration from arterial plasma, accounted for most of the increment in urinary cGMP excretion in response to ANF.

To define more precisely the source of glomerular cGMP production during ANF administration, we also measured renal venous cGMP concentration. Renal venous and arterial cGMP concentrations were similar during ANF infusion (0.9 ± 0.1 vs. 1.1 ± 0.1 nM). Thus, little cyclic nucleotide is added to the renal circulation during ANF administration. A major component of renal metabolism of cGMP was excluded because renal extraction of [3H]cGMP (arterio–venous difference) was similar to that of inulin (35 ± 3% vs. 33 ± 4%, n = 6), a solute that is freely filtered but not metabolized or transported. Therefore, loci not contiguous with the renal circulation, such as glomerular mesangial cells or epithelial cells, were the source(s) of glomerular cGMP production.

To examine whether glomerular production of cGMP accounted for all of the urinary cGMP or whether further cGMP was added from tubular segments, we measured the relative delivery of cGMP compared to inulin (TF/P cGMP/inulin; see Fig. 1) in serial nephron segments during ANF infusion. Relative cGMP deliveries were similar in the glomerular ultrafiltrate (50 ± 13), superficial end-proximal tubular fluid (54 ± 14), and urine (54 ± 11) (Fig. 1). Therefore, the large accumulation of cGMP in the glomerular ultrafiltrate induced by ANF was not accompanied by any further net addition from either the superficial proximal tubule or from deeper or more distal tubular segments.

[3H]cGMP infused intravenously was used to examine the unidirectional fluxes of cGMP along the length of the nephron. Relative delivery of [3H]cGMP in the superficial end-proximal tubule was not significantly different from unity (0.91 ± 0.14), but that in urine was 0.69 ± 0.04, indicating slight reabsorption of [3H]cGMP in tubular segments beyond or deeper to the superficial end-proximal tubule.

To test whether cGMP can itself increase GFR, Bt$_2$cGMP was infused into the renal artery. At all doses, blood pressure and hematocrit were stable. As shown in Table 2, Bt$_2$cGMP caused a dose-dependent, significant increase in GFR over control values, by 20 ± 6% and 36 ± 10% at 100 and 1000 μM local glomerular plasma Bt$_2$cGMP concentrations, respectively. To confirm the specificity of the renal hemodynamic effect of Bt$_2$cGMP, Bt$_2$cAMP at the same doses was infused in another set of rats. GFR remained unchanged or decreased with Bt$_2$cAMP (Table 2).

ANF increases GFR by augmenting glomerular hydraulic pressure as assessed by stop-flow pressure measurement (17, 18). To confirm that Bt$_2$cGMP increases GFR by a similar mechanism as ANF, we assessed the change in glomerular hydraulic pressure when 1000 μM Bt$_2$cGMP was infused intrarenally. Bt$_2$cGMP caused a significant increase in stop-flow pressure, from 33 ± 1 to 37 ± 1 mm Hg (P < 0.05). No other vasodilator has been found previously to cause an increase in both GFR and glomerular hydraulic pressure (20).

![Fig. 1. Paired measurements of relative cGMP delivery compared to inulin delivery (ratio of the concentration in tubular fluid to the concentration in plasma (TF/P) of cGMP/inulin) in Bowman’s space, end-proximal tubule, and urine after ANF administration. The dashed line represents a relative cGMP delivery of unity, which would occur if cGMP, like inulin, were filtered but were neither produced in the glomerulus nor reabsorbed or secreted in the tubules.](image-url)
Table 2. Effect of renal arterial infusion of Bt2cGMP and Bt2cAMP on GFR

<table>
<thead>
<tr>
<th>Baseline GFR, ml/min</th>
<th>Increment of GFR over control, %</th>
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<tr>
<td>10 μM</td>
<td>100 μM</td>
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<tr>
<td>Bt2cGMP 1.1 ± 0.1 (11)</td>
<td>8 ± 7 (5)</td>
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<tr>
<td>Bt2cAMP 1.2 ± 0.2 (6)</td>
<td>-8 ± 3 (5)</td>
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Data are means ± SEM; the numbers in parentheses = n. *Significantly different from zero (P < 0.05).

DISCUSSION

The present data obtained in vivo demonstrated that ANF enhanced glomerular production of cGMP coincident with glomerular hyperfiltration (Table 1). Glomeruli in vitro have also been shown to produce cGMP in response to ANF (21, 22). The present data extends the work of Hamet et al. (11), which showed ANF administration increased urinary cGMP excretion by demonstrating that the increased urinary cGMP excretion was mostly of glomerular origin (glomerulogenous cGMP). The large glomerular ultrafiltrate delivery of cGMP without parallel addition of the cyclic nucleotide to the postglomerular renal circulation suggests that glomerular mesangial or epithelial cell functions were the source(s) of cGMP production. The lack of substantial addition of cGMP to the renal vein suggests that endothelial cells are not a major source of production, but this has not been explicitly examined. Support for the role of mesangial cells in mediating the action of ANF comes from reports that cultured mesangial cells express a cell-surface receptor for ANF and that ANF stimulates rat mesangial cell cGMP accumulation (23, 24).

The precise mechanism by which these changes in mesangial or epithelial cell function induced by ANF might increase GFR has not yet been clarified. ANF inhibits angiotensin II-induced contraction in mesangial cell culture (24), raising the possibility that ANF antagonizes mesangial effects of vasoconstrictive hormones. Alternatively, ANF may directly cause mesangial cell relaxation and/or directly or indirectly affect glomerular resistance vessels.

There was no net addition of cGMP to the tubular fluid in the superficial proximal tubule (Fig. 1), consistent with in vitro studies that ANF does not stimulate particulate guanylate cyclase activity or cause cGMP production in the proximal tubule (21, 22) and with in vivo reports that ANF does not affect proximal tubular transport (13, 25–27). The same relative cGMP delivery in the glomerular ultrafiltrate, end-proximal tubule, and urine in the rat (Fig. 1) is consistent with previous data in the human, in which neither reabsorption nor secretion of cGMP occurred (28), but contrasts with previous studies in the dog, in which tubular cGMP secretion was found (29). However, tracer cGMP reabsorption occurred beyond the rat superficial proximal tubule in the present studies. This unidirectional absorptive flux (with radiolabeled cGMP), taken in conjunction with the previous finding of no net transport (with cGMP radioimmunoassay), suggests that a relatively small amount of cGMP addition occurs beyond or deeper to the superficial proximal tubule, as has been proposed from in vitro studies (30, 31).

To test whether a permeable analogue of cGMP could reproduce the glomerular hemodynamic effect of ANF, Bt2cGMP was infused into the renal artery. Bt2cGMP increased GFR in a dose-dependent fashion (Table 2) by a similar mechanism to that of ANF—an increase in glomerular hydraulic pressure. Bt2cAMP reduced GFR as reported previously (32). Compared to other vasodilators, ANF and Bt2cGMP are unique in causing both an increase in GFR and in glomerular hydraulic pressure (20). Other vasodilators either increase GFR but not glomerular pressure (papaverine), increase glomerular pressure but not GFR (prostaglandin E2, acetylcholine, histamine), or cause neither effect (prostacyclin, bradykinin, prostacyclin) (20). The mechanistic similarity between the acute glomerular hyperfiltration induced by Bt2cGMP and ANF, not shared by any vasodilator studied to date, supports the hypothesis that cGMP mediates the effects of ANF. However, results obtained from extracellular application of this cGMP analog should be interpreted cautiously in view of the presently limited understanding of the physiological cellular concentration and mechanism of action of cGMP. cGMP may have other effects on renal function in addition to those that are involved in the action of ANF. In addition, since the cGMP concentration in renal tissue homogenate has been reported to be ~40 nmol/kg wet weight (33), the 0.1–1 mM Bt2cGMP concentrations used in the present study may be unphysiological. However, a homogenate of renal tissue includes many nonglomerular cell types in the cortex and medulla without ANF receptors or guanylate cyclase (21, 22). Cytosolic cGMP concentration may be much higher in cells in which cGMP is a second messenger. For example, cytosolic cGMP concentration in the outer segment of retinal rod cells has been found to be as high as 0.1 mM (4). Finally, compartmental cGMP concentrations within the cell may vary, perhaps correlating with the distribution of the different guanylate cyclases and/or different cGMP-dependent protein kinases (8, 34), as has also been observed with cAMP (35).

While a number of hormones and other agents modify cellular cGMP concentration, none has been shown to consistently increase renal cGMP production and urinary cGMP excretion under physiological conditions (36). Therefore, urinary cGMP excretion may prove to be a useful marker of the biological activity of ANF in the kidney, analogous to the use of urinary cAMP as a reflection of renal parathyroid hormone activity (36, 37). Further studies will be required to confirm the sensitivity and specificity of urinary cGMP as a marker of ANF’s glomerular activity.

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