Effects of Atrial Natriuretic Factor on Cyclic Nucleotides in Rabbit Proximal Tubule

Eveline Eitle, Peter J. Harris, Trefor O. Morgan

Abstract Atrial natriuretic factor induces renal sodium excretion by several mechanisms, including inhibition of angiotensin II-stimulated sodium reabsorption in the proximal tubule. In most tissues, the action of atrial natriuretic factor involves generation of the intracellular second messenger, cyclic GMP, but in the proximal tubule the presence of this signal transduction pathway has remained controversial. We used intrarenal arterial infusion of iron oxide followed by enzymatic dispersion and magnetic separation to obtain suspensions of rabbit kidney cortex enriched with either glomeruli or proximal tubules. When suspensions enriched with proximal tubules or preparations of microdissected proximal tubules were incubated with atrial natriuretic factor (1 μmol/L), cyclic GMP concentrations increased significantly. Addition of angiotensin II (1 μmol/L) together with atrial natriuretic factor had no significant effect on the stimulation of cyclic GMP accumulation observed with atrial natriuretic factor alone. Neither atrial natriuretic factor nor angiotensin II altered intracellular concentrations of cyclic AMP in tubule-enriched suspensions or microdissected tubules. We conclude that cyclic GMP acts as a second messenger for atrial natriuretic factor in rabbit proximal tubule. However, we found no evidence to support the view that alterations in intracellular cyclic AMP levels are involved in the proximal tubular actions of angiotensin II and have not been able to demonstrate that interactions between cyclic AMP and cyclic GMP underlie the antagonistic effect of atrial natriuretic factor on angiotensin II-stimulated proximal sodium transport. (Hypertension. 1994; 23:358-363.)

Key Words • atrial natriuretic factor • angiotensin II • cyclic GMP • kidney • kidney tubules, proximal

The remarkable potency of low concentrations of atrial natriuretic factor (ANF) in promoting renal sodium excretion can be ascribed to its ability to inhibit sodium reabsorption at several sites along the nephron. At higher doses these actions are combined with direct effects on the glomerular mesangium that result in hyperfiltration and further loss of salt and water. The cellular mechanism of action of ANF in most cell types within the kidney involves interaction with a specific, high-affinity receptor shown to be identical to membrane-bound guanylate cyclase, and binding of ANF thus results in elevation of intracellular cyclic GMP (cGMP) levels. ANF inhibits angiotensin II (Ang II)-induced contraction in mesangial cells through lowering cytosolic free calcium, suppresses renin release from purified rat juxtaglomerular cells, and inhibits apical sodium uptake in the inner medullary collecting duct.

In the proximal tubule ANF has no direct action on transepithelial transport but inhibits sodium reabsorption that has been stimulated by low concentrations of Ang II. The cellular mechanism of ANF action has been controversial because early studies showed no change in cGMP levels. However, ANF has been found to inhibit sodium-coupled bicarbonate and phosphate transport in proximal brush border membrane vesicles and to increase Ca²⁺-Mg²⁺ ATPase activity in isolated basolateral membranes from rat kidney cortex. We have used an enriched cortical tubule preparation and microdissected tubules to examine the possibility that cGMP mediates suppression of Ang II-induced proximal sodium reabsorption by ANF.

Some reports have suggested that stimulation of sodium reabsorption by Ang II is mediated by changes in cytosolic cyclic AMP (cAMP) acting as a second messenger. However, several other signal transduction pathways have been implicated in this response to Ang II, and the mechanisms involved in the interaction between ANF and Ang II remain unclear. Total cellular cAMP content was measured in enzyme-dispersed tubule suspensions and microdissected proximal tubules after addition of low concentrations of Ang II alone and in combination with ANF.

Methods

Preparation of Enriched Suspensions of Proximal Tubules and Glomeruli

Suspensions of renal cortical tissue enriched with proximal tubules or glomeruli were prepared using a modification of the procedure described by Wrenn et al. Thirteen New Zealand White or New Zealand crossbred rabbits (1.2 to 2 kg body weight; obtained from Commonwealth Serum Laboratories) were anesthetized with Saffan (0.8 to 2.0 mL/kg IV). An intrarenal arterial cannula was inserted, and both kidneys were perfused with 20 to 40 mL of an ice-cold isotonic buffer (pH 7.5) containing (mmol/L) NaCl 136, NaHCO₃ 5, Na₂HPO₄ 1.6, NaH₂PO₄ 0.4, CaCl₂ 1.3, KCl 5, MgSO₄ 1, sodium acetate 10, HEPES 5, and glucose 10. The kidneys were then perfused with a further 20 mL of this solution containing 0.5% (wt/vol) magnetic iron oxide (Ajax Chemicals) and finally with 15 mL of the solution containing 0.4 mg/mL collagenase (type 2, Worthington Biochemical Co) and 1 mg/mL hyaluronidase (Sigma...
Chemical Co). The kidneys were then removed and decapsulated, and the cortex was carefully separated and chopped into small pieces with scissors. The tissue was incubated in a horizontal shaking water bath (50 rpm) with 20 to 40 mL of the collagenase and hyaluronidase solution described above at 37°C for 10 to 20 minutes. The suspension was filtered once through a 200-μm mesh sieve with the buffer solution and washed three times by sedimentation in a 200-ml beaker. The suspension of tubules and glomeruli was then passed four to six times through a magnetic field to remove small vessels and glomeruli containing trapped iron oxide. Suspensions derived from the tissue bound to the magnet were enriched with glomeruli, and suspensions of the remaining tissue were enriched with proximal tubules.

### Quantification of Glomeruli in Tissue Suspensions

The total number of glomeruli in 10 μL of each type of suspension (before magnetic separation, tubule- and glomeruli-enriched suspensions) was counted by light microscopic examination. Protein concentration was determined according to the method of Lowry et al. Percentage glomerular protein in a suspension was estimated from the mean protein content of microdissected glomeruli. Suspensions were prepared from four animals, and from each suspension 40 to 110 glomeruli were separated by microdissection, and their protein content was determined. From this value the mean protein content of a single glomerulus was calculated.

### Microdissection of Proximal Tubules

Kidneys were perfused as described, and the cortex was cut into small pieces. After enzymatic digestion (as above) and washing, proximal tubules (both convoluted and straight segments) were bathed in cold (4°C) saline buffer and selected by observation under a stereomicroscope, with care taken to avoid contamination by vascular elements. Twelve samples of microdissected tubule segments were included per prepara-

### Hormone Stimulation of Tubule- and Glomeruli-Enriched Suspensions

Suspensions were stored on ice until used. Samples (100 μL) in polypropylene tubes were incubated for 3 minutes at 37°C in a horizontal shaking water bath. An aliquot (11 μL) of solution containing the required hormone (Auspep) was then added to experimental tubes or a similar volume of buffer to control tubes. The suspension was mixed by manual agitation and reincubated at 37°C in the water bath for a timed period.

### Measurement of Cyclic Nucleotides (cGMP and cAMP)

The freeze-dried, deproteinized samples were redissolved in 180 μL of the appropriate buffer (Amersham) and assayed for cGMP or cAMP according to standard Amersham protocols. Assay of cGMP was carried out using the acetylation method for all samples. For assay of cAMP the acetylation protocol was followed for microdissected tubules, and the nonacetylation method was used for tubule suspensions. Standards were prepared in a manner identical to that used for the samples with the same buffer—addition of trichloroacetic acid, ether extraction, and freeze-drying.

Some tissue extracts (those used for control incubations and ANF stimulation) and standard solutions of cGMP were treated with phosphodiesterase (bovine heart, Sigma) to estimate the presence of cross-reacting compounds. Ten micromoles of phosphodiesterase (1 μM/mL) was added to 500 μL of tissue extract (containing 150 to 400 fmol cGMP), adjusted to pH 6 to 7, and incubated for 30 minutes at 37°C. The samples were then boiled for 3 minutes to inactivate the phosphodiesterase and after freeze-drying were assayed for cyclic nucleotides as described above. In ANF-stimulated and control tubule suspensions, some unidentified material (10 to 30 fmol cGMP/mg protein) that cross-reacted with the cGMP antibody was not destroyed by the phosphodiesterase. Similarly, in microdissected tubules, treatment with phosphodiesterase reduced cGMP concentration from 101 ± 52 to 32 ± 4 fmol/mg protein in samples of control tubules (n = 3) and from 376 ± 140 to 17 ± 3 fmol/mg protein in ANF-stimulated tubules (n = 3).

In standard solutions all cGMP was destroyed by the addition of phosphodiesterase. A known amount of cGMP (100 fmol) was added to some tissue extracts and was almost completely recovered (98 ± 11%, SD, n = 3) by assay.

### Results

#### Composition of Suspensions After Magnetic Separation

The number of glomeruli (expressed per milligram of protein) in the proximal tubule- and glomeruli-enriched suspensions is shown in Table 1. Magnetic separation resulted in removal of approximately 90% of the glomeruli from the suspension. Light and electron

<table>
<thead>
<tr>
<th>Suspension</th>
<th>No. of Glomeruli/ mg Protein</th>
<th>Glomerular Protein, % of total protein</th>
<th>fmol cGMP/ mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspension before magnetic separation</td>
<td>606 ± 287</td>
<td>4.1 ± 2.0</td>
<td>...</td>
</tr>
<tr>
<td>Proximal tubule-enriched suspension</td>
<td>51 ± 21</td>
<td>0.34 ± 0.14</td>
<td>58 ± 27</td>
</tr>
<tr>
<td>Glomeruli-enriched suspension</td>
<td>2809 ± 1200</td>
<td>19.0 ± 8.0</td>
<td>1778 ± 731</td>
</tr>
</tbody>
</table>

cGMP indicates cyclic GMP. Data represent the mean value of four different preparations (±SD). The total number of glomeruli in 10 μL of suspension was counted under a microscope as described in "Methods."
microscopic examinations confirmed that the tubule-enriched suspension contained predominantly proximal tubules. Counts of the number of glomeruli in these suspensions together with measurement of the mean protein content of a single glomerulus (67.5 ± 12.5 ng) indicated that glomeruli contributed only 0.34 ± 0.14% of the total protein. Electron microscopic examination indicated a healthy appearance of the tubules, with no visible swelling of mitochondria, open lumens, and intact brush border membranes. Basal values for cGMP concentration (Table 1) were approximately 30-fold higher in glomeruli-enriched suspensions than in proximal tubule-enriched suspensions.

Effect of Atrial Natriuretic Factor on cGMP Levels in Proximal Tubule Suspensions

The time course of changes in cGMP concentration after addition of 1 μmol/L ANF to proximal tubule-enriched suspensions is shown in Fig 1. ANF treatment resulted in consistent elevation of cGMP concentrations, with maximum levels being reached between 1 and 2 minutes after addition of the peptide and remaining elevated at 5 minutes. There was considerable variation in the extent of stimulation observed with tubules from different preparations. The time courses of changes in cGMP in glomeruli- and tubule-enriched suspensions were similar (not shown). Concentration dependency of the effect of ANF on cGMP accumulation is shown in Fig 2. The incubation was stopped after 3 minutes, and the extent of maximal stimulation (achieved with 1 μmol/L ANF) was consequently less than the optimum observed after 1 to 2 minutes in the time course experiments (see Fig 1). The phosphodiesterase inhibitor IBMX was used to reduce the breakdown of cGMP so that changes in cyclic nucleotide concentration would more accurately reflect the rate of cGMP production. However, optimal inhibition of phosphodiesterase was probably not achieved in these experiments, because IBMX was added only 3 minutes before the hormone. Longer periods of exposure to IBMX were expected to be more effective but were avoided to minimize deterioration of these highly metabolically active cells during incubation.

Effects of Angiotensin II and Atrial Natriuretic Factor on cGMP and cAMP Levels in Dissected Proximal Tubules

We also examined the effects of ANF in microdissected proximal tubule segments to confirm that elevation of cGMP concentrations in proximal tubule suspensions was not due to stimulation of contaminating glomeruli or other vascular cells. In tubules isolated from four animals, mean control values for cGMP were 171 + 42 (SD) fmol/mg protein. In the absence of IBMX, addition of 1 μmol/L ANF caused marked increases in intracellular cGMP concentration ranging from 3.9- up to 6-fold (Table 2), thus confirming the direct effect of ANF on proximal tubule cells. The results from microdissected proximal tubules also show that Ang II (1 μmol/L) did not affect either the increase in cGMP induced by ANF (1 μmol/L) or the intracellular cAMP levels (Table 2).

Effects of Angiotensin II and Atrial Natriuretic Factor on cGMP and cAMP Levels in Enriched Tubule Suspensions

Addition of Ang II (1 μmol/L) together with ANF (1 μmol/L) to tubule-enriched suspensions did not consistently alter the extent of stimulation of cGMP compared with that observed with the same concentration of ANF alone (Table 3). Neither 1 μmol/L ANF, 1 μmol/L Ang II, nor 50 pmol/L Ang II altered cAMP levels in these experiments. Data are presented in Table 3 to allow comparison of the stimulatory effect of ANF on cGMP accumulation in individual experiments using proximal tubule–enriched and glomeruli-enriched suspensions. In
Cyclic Nucleotides in Proximal Tubule

TABLE 2. Effects of ANF, Ang II, and ANF+Ang II on cGMP and cAMP in Microdissected Proximal Tubules

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>1 μmol/L ANF</th>
<th>1 μmol/L Ang II</th>
<th>1 μmol/L ANF+1 μmol/L Ang II</th>
</tr>
</thead>
<tbody>
<tr>
<td>[cGMP]</td>
<td>501±89</td>
<td>89±15</td>
<td>424±103</td>
</tr>
<tr>
<td>[cAMP]</td>
<td>112±16</td>
<td>112±13</td>
<td>109±10</td>
</tr>
</tbody>
</table>

ANF indicates atrial natriuretic factor; Ang II, angiotensin II; cGMP, cyclic GMP; and cAMP, cyclic AMP. Data are expressed as percent of control and represent mean values (±SD) from tubules taken from preparations from each of four animals. Proximal tubules were microdissected as described in “Methods.” Samples were incubated for 3 minutes at 37°C followed by 3 minutes of incubation with hormone and without addition of isobutylmethylxanthine.

the glomerular fraction (containing 3112 glomeruli per milligram of protein), it can be calculated that on average each glomerulus contributed 8.12 fmol cGMP/mg protein to the observed rise in cGMP concentration, assuming no cGMP production from the tubular component of the suspension. Analysis of the data from the enriched proximal tubule preparation (containing only 62 glomeruli per milligram of protein) indicated that the small amount of glomerular contamination (0.34% of total protein) could not account for the observed rise in cGMP concentration, because each glomerulus would have to contribute an average of 12.8 fmol cGMP/mg protein, 1.6 times that predicted from the glomerular fraction. Thus, although a substantial contribution of cGMP derived from glomeruli is likely in this preparation, these results provide supporting evidence that ANF stimulates cGMP production in proximal tubules.

Discussion

In this study we examined the effects of ANF and Ang II on cGMP and cAMP levels in enriched suspensions of rabbit proximal tubules and in tubules microdissected from rabbit kidneys. We observed a very substantial rise in intracellular cGMP concentration after treatment with ANF but found no significant changes in cAMP levels with either ANF or Ang II.

Previous in vivo micropuncture studies from this laboratory showed that although ANF alone had no effect on sodium reabsorption in the rat proximal tubule, ANF inhibited the stimulatory action of low doses of Ang II on transepithelial sodium and water transport. This finding was subsequently confirmed in vitro by Garvin using perfused isolated rat tubules. Garvin also found that the inhibitory effect of ANF on Ang II-stimulated sodium reabsorption was mimicked by dibutyryl-cGMP and concluded that cGMP acts as a second messenger for ANF in the proximal tubule, as in other tissues.

Attempts to measure changes in intracellular cGMP in response to ANF in the proximal tubule have provided apparently contradictory data. Nonoguchi et al measured cGMP concentrations in microdissected tubule segments from rat and found substantial stimulation by ANF in glomeruli and inner medullary collecting ducts and much smaller (but still significant) increases in midproximal convoluted and late proximal straight tubules. In contrast, Chabardes et al could not detect cGMP generation in any ANF-treated nephron segment from rat kidney. The disparities between these two sets of data were discussed by Knepper et al, who considered that the sensitivity of the nucleotide assay used by Chabardes and coworkers was insufficient to detect the comparatively small changes occurring in the proximal convoluted tubule. Similar arguments can be applied to other studies that used microdissected proxi-

TABLE 3. Effects of ANF and Ang II on cGMP Concentrations In Proximal Tubule-Enriched and Glomerull-Enriched Suspensions

<table>
<thead>
<tr>
<th>Suspension</th>
<th>Experiment</th>
<th>Glomerull/mg Protein</th>
<th>Control</th>
<th>1 μmol/L ANF</th>
<th>1 μmol/L ANF+1 μmol/L Ang II</th>
<th>1 μmol/L Ang II</th>
<th>50 pmol/L Ang II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal tubule-enriched</td>
<td>1</td>
<td>57</td>
<td>81</td>
<td>782</td>
<td>794</td>
<td>87</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>80</td>
<td>934</td>
<td>1 061</td>
<td>1074</td>
<td>106</td>
<td>112</td>
</tr>
<tr>
<td>3</td>
<td>76</td>
<td>31</td>
<td>754</td>
<td>571</td>
<td>571</td>
<td>57</td>
<td>57</td>
</tr>
<tr>
<td>4</td>
<td>34</td>
<td>40</td>
<td>938</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>62±21</td>
<td>58±26</td>
<td>852±97</td>
<td>707±286</td>
<td>54±27</td>
<td>50±35</td>
<td></td>
</tr>
<tr>
<td>Glomerull-enriched</td>
<td>1</td>
<td>3567</td>
<td>2405</td>
<td>30 639</td>
<td>37 166</td>
<td>27 046±975</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2416</td>
<td>1071</td>
<td>14 660</td>
<td>9 936</td>
<td>1188</td>
<td>1124</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3112</td>
<td>1681</td>
<td>25 718</td>
<td>22 407</td>
<td>1302</td>
<td>1526</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3354</td>
<td>1956</td>
<td>37 166</td>
<td>43 354</td>
<td>1422</td>
<td>1212</td>
<td></td>
</tr>
<tr>
<td>Mean±SD</td>
<td>3112±500</td>
<td>1778±558</td>
<td>27 046±9496</td>
<td>23 307±14 315</td>
<td>1402±218</td>
<td>2628±703</td>
<td></td>
</tr>
</tbody>
</table>

ANF indicates atrial natriuretic factor; Ang II, angiotensin II; and cGMP, cyclic GMP. Values for each experiment represent an average from triplicate samples.
imal tubules23,24 and in which ANF apparently did not affect cGMP levels.

In the studies reported here we used enriched suspensions of proximal tubules and microdissected tubules from rabbits. Both preparations showed much stronger stimulation of cGMP than reported by Nonoguchi et al using microdissected tubules from rats. This may be a species difference or reflect differences in the procedure for enzymatic digestion of the cortex. The combination of a short digestion time followed by magnetic removal of glomeruli and vascular tissue allowed us to avoid centrifugation. The enriched tubule suspensions had a healthy appearance characterized by open lumens and intact brush border membranes and were largely free from contaminations when viewed by electron microscopy.

The procedures reported here for tubule preparation provided enriched suspensions of proximal tubules that responded to ANF with enhanced generation of cGMP. The response to ANF strongly supports a role for cGMP as a second messenger in the proximal action of ANF and this inference is confirmed by the observation of similar responses in microdissected proximal tubules.

Modulation of the activity of adenyl cyclase has been proposed to mediate the stimulatory actions of Ang II on sodium, bicarbonate, and water reabsorption in the proximal tubule.17 Ang II has been shown to inhibit adenyl cyclase in purified membranes from renal cortex,25,26 but the concentrations required were several orders of magnitude higher than those associated with stimulation of transport. A role for this cyclase in the transduction of the Ang II response is also challenged by experiments on rat renal proximal tubulalateral membranes,27 in which no effect of Ang II on basal or parathyroid hormone-stimulated transport could be detected. In contrast, studies using in vivo microperfusion28 and cultured rabbit proximal tubule cells29 have suggested that stimulation of sodium transport by picomolar to nanomolar concentrations of Ang II is accompanied by inhibition of adenyl cyclase and reduced intracellular cAMP levels.

The data reported in this article do not support the notion that suppression of intracellular cAMP is a necessary step in the Ang II signal transduction pathway in the proximal tubule. In our preparation of proximal tubule suspensions, Ang II at either 50 pmol/L or 1 μmol/L had no significant effect on cAMP levels. cAMP concentration did not increase when cGMP generation was stimulated by ANF, and addition of Ang II had little effect on the ability of ANF to stimulate cGMP accumulation. Our results do not reveal any interaction between cAMP and cGMP during the responses to either ANF or Ang II, and it may be inferred that such mechanisms therefore are unlikely to underlie the suppression by ANF of Ang II–stimulated sodium reabsorption. However, it should be noted that compartmentation of intracellular cyclic nucleotides cannot be detected by the techniques used in this study, which measure total intracellular content. Local changes in the cytosolic free concentration of either cAMP or cGMP might occur in response to peptide hormone stimulation and therefore could participate in signal transduction or its modulation.

In renal inner medullary collecting ducts cGMP has been shown to suppress sodium reabsorption by directly inhibiting an amiloride-sensitive cation channel and activating a cGMP-dependent protein kinase. This mechanism does not appear to be relevant for proximal tubule cells, because luminal sodium uptake is achieved mainly through secondarily coupled co-transporters such as the "amiloride-insensitive" Na⁺-H⁺ exchanger rather than cation channels.

We conclude that the modulatory effect of ANF on Ang II–stimulated proximal tubular sodium transport is likely to be mediated by cGMP acting as an intracellular second messenger. The mechanism does not appear to involve interaction with cAMP. The data do not indicate a role for suppression of adenylyl cyclase in the stimulatory action of Ang II on proximal tubular transport.

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