Enzymatic and binding effects of atrial natriuretic factor in glomeruli and nephrons

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Enzymatic and binding effects of atrial natriuretic factor in glomeruli and nephrons. Atrial natriuretic factor (ANF) has been suggested to exert a tubular effect on the mammalian nephron, perhaps in part by interacting with other hormones. In the present study, the effect of ANF was examined on glomeruli (Gm) and different renal tubule segments including medullary (MAL) and cortical thick ascending limb (CAL) and cortical (CCT), outer medullary (OMCT) and inner medullary collecting tubules (IMCT). This effect of ANF was assessed by alteration in adenylate cyclase and cGMP in the various nephron segments in the presence and absence of arginine vasopressin (AVP), parathyroid hormone (PTH) and calcitonin (SCT). An effect of ANF (10^{-8} M) was not demonstrated on adenylate cyclase (fmol cAMP formed/30 min/μg protein) in Gm, CAL, MAL, CCT, OMCT or IMCT. Nor did ANF (10^{-8} M) interfere with the effect of PTH (5 IU/ml) on the Gm (PTH 35.1 ± 3.7 vs. PTH + ANF 32.5 ± 1.8, NS), CAL (PTH 50.5 ± 10.9 vs. PTH + ANF 46.2 ± 1.4, NS) or AVP (10^{-8} M) on the CCT (AVP 40.8 ± 6.6 vs. AVP + ANF 33.0 ± 3.1, NS), OMCT (AVP 56.0 ± 11.8 vs. AVP + ANF 42.1 ± 6.7, NS), IMCT (AVP 66.5 ± 4.6 vs. AVP + ANF 53.5 ± 7.0, NS) or MAL (AVP 15.5 ± 1.6 vs. AVP + ANF 14.0 ± 2.6, NS). ANF also did not affect SCT (1.5×10^{-8} M)-induced adenylate cyclase on CCT (SCT 69.8 ± 11.3 vs. SCT + ANF 79.9 ± 7.2, NS). ANF (10^{-8} M), however, significantly increased cGMP in the Gm (6.4 ± 1.7 to 121.3 ± 32.4 fmol/μg protein, $P < 0.001$) and IMCT (0.63 ± 0.16 to 1.46 ± 0.29 fmol/μg protein, $P < 0.05$). However, no effect of ANF on cGMP was observed in the CAL, CCT, OMCT, and MAL even at 10^{-7} M ANF. PTH (5 IU/ml) did not alter either basal or ANF-stimulated cGMP in the Gm. Also, specific ANF binding was studied in the microdissected IMCT. K_d was 6.08×10^{-9} M and B_{max} was 8.07×10^{-11} M. These results suggest that ANF does not exert a direct effect on the cellular action of AVP, PTH or SCT, but may exert a significant physiological role in the Gm and IMCT by increasing cGMP.

Atrial natriuretic factor (ANF) has been demonstrated to be released from myocardiocytes [1], and has been reported to exert potent natriuretic and diuretic effects in several species [1–6]. Although it is generally accepted that ANF increases the renal excretion of sodium and water, there have been conflicting reports about the mechanism of action and target sites of ANF action in the kidney. In studies using renal tissue plasma membranes, specific binding of ANF was demonstrated in rabbit and rat renal cortical plasma membranes [7, 8] and in LLC-PK$_1$ cells [7]. The major ANF binding in the renal cortex appears to be in the glomeruli [9]. Moreover, specific ANF binding has been shown in the isolated rat and human glomeruli and in cultured rat glomerular mesangial cells [9]. Autoradiography studies have indicated that ANF receptors were distributed mainly in glomeruli in cortex [10, 11] with low amounts of receptors occurring in proximal tubules [11] and in inner medullary collecting tubules (IMCT) [10, 11].

Although it has been suggested that the natriuretic effect of ANF is due primarily to an increase in glomerular filtration and filtered sodium load, a specific inhibition of sodium reabsorption in the collecting tubule has been proposed [4]. In this regard, an effect of ANF to decrease sodium permeability of the IMCT has been demonstrated in studies by Zeidel et al [12].

Recently it has also been reported that ANF inhibited the hydraulic conductivity response to arginine vasopressin (AVP) but not to either cAMP or forskolin in the microperfused rabbit cortical collecting tubules (CCT) [13]. This finding thus suggests a potential effect of ANF on the action of AVP in the mammalian distal nephron. ANF also has been shown to inhibit AVP-stimulated cAMP production as well as stimulate cGMP in the cultured rat inner medullary cells [14]. A physiological role for cGMP in the action of ANF in the kidney has been suggested since ANF was shown to increase cGMP levels in cultured or dissected rat inner medullary cells [14–16], rat, rabbit, and human glomeruli [8, 17, 18], and in cultured rat glomerular mesangial cells [9]. Taken together, these studies suggest that the natriuretic and diuretic effect of ANF might be due to a direct action of ANF on renal tubules as well as a potential interaction with other hormones, perhaps involving cGMP generation. The present study was therefore undertaken to examine the effect of ANF and its interaction with AVP, PTH and calcitonin on the stimulation of adenylate cyclase and cGMP production along the rat nephron, including the glomeruli, proximal tubule, cortical (CAL) and medullary thick ascending limb of Henle's Loop (MAL), collecting tubules of cortex (CCT), outer (OMCT) and inner medullary (IMCT), and ANF binding to IMCT.

Methods

Synthetic ANF (Ile-ANF-26) was from Dr. Edward Blaine (Merck Sharp and Dohme, West Point, Pennsylvania, USA). This peptide has been shown to have properties identical to native ANF of a low molecular weight [19].

Microdissection of tubules and glomeruli

The rats (200 to 250 g body wt) were lightly anesthetized with pentobarbital (3 mg/100 g body wt) and the left kidney was

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perfused [20–22] with 10 ml of collagenase medium [137 mM NaCl, 5 mM KCl, 0.8 mM MgSO$_4$, 0.8 mM Na$_2$HOP$_4$, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 10 mM Tris, pH 7.4, 120 to 200 U/ml collagenase (Millipore Corp., Greenhold, New Jersey, USA), 1 mg/ml hyaluronidase (Sigma Chemical Co., St. Louis, Missouri, USA), and 1 mg/ml bovine serum albumin with 20 U/ml heparin] at an infusion rate of 1 ml/min. The cortical, outer medullary and inner medullary (papillary) tissue was dissected and sliced into thin strips with direction from cortex to papillary tip. Then the pieces were incubated in an aerated collagenase medium for 60 minutes for papillary tissue and 30 minutes for cortical and medullary tissues at 35°C. After incubation the tissues were washed with cold microdissection medium (same as the collagenase medium except 0.25 mM CaCl$_2$ and no collagenase, hyaluronidase, and bovine serum albumin) and kept on ice while microdissection was performed. CCT, OMCT and IMCT, and CAL and MAL and glomeruli were dissected under a stereomicroscope using thin needles. Dissected glomeruli (10 glomeruli for each assay) and tubules (1.5 to 2.0 mm for each assay) were transferred to a concave bacteriological slide by aspiration of tubules in a small droplet of microdissection medium. For assays of adenylate cyclase, lengths of the tubules were measured using a microscope with computerized planimeter (Carl Zeiss, Oberkochen, FRG). Tubules then were disrupted by hypoosmotic shock with freezing and thawing. After the microdissection, the medium was aspirated and 0.5 μl of hypoosmotic medium (1 mM MgCl$_2$, 0.25 mM EDTA, 0.1% bovine serum albumin, and 1 mM Tris, pH 7.4) was added under the microscope; the samples were then immediately frozen by placing them on a block of dry ice. The frozen tubules were thawed once and refrozen, then kept at −80°C until assayed. The basal and hormone stimulated activities of adenylate cyclase were stable at least for five days at −80°C.

Assays of adenylate cyclase activity

The activity of adenylate cyclase was measured according to a modification [20–22] of the method of Morel, Chabardes and Imbert-Teboul [23]. Briefly, slides with tubules (1.5 to 2 mm total tubule length per sample) were incubated at 37°C for 30 minutes in a final volume of 5.5 μl containing 0.25 mM alpha-^{32}P-ATP (3 to 4 × 10^6 cpm/sample), 1 mM cAMP, 3.8 mM MgCL$_2$, 0.25 mM EDTA, 20 mM creatine phosphate (Calbiochem, San Diego, California), 200 U/ml creatine phosphokinase (Calbiochem), and 100 mM Tris, pH 7.4, with or without addition of ANF or hormone, or ANF + hormone [AVP, Bachem, Torrance, California, USA; calcitonin, Calbiochem; parathyroid (PTH), Sigma]. The reaction was stopped by addition of 150 μl stopping solution (3.3 mM ATP, 5 mM cAMP, 50 mM Tris HCl, pH 7.4, and ^{3}H-cAMP containing 1 × 10^4 cpm/ sample to determine recovery). Produced ^{32}P-cAMP was separated according to the method of Salomon [24] using Dowex 50-x4, 200 to 400 mesh (Biorad, Richmond, California, USA), and albumin oxide columns (ICN, Cleveland, Ohio, USA). Most of the enzyme activity was expressed as fmol cAMP produced/min/μg protein for tubules and fmol cAMP produced/ min/μg of protein for glomeruli.

Determination of in situ cGMP

The cGMP content was determined by radioimmunoassay and the cells were treated as in the cAMP measurement [20–22].

The microdissection medium was aspirated off and 2.5 μl of modified Krebs-Ringer buffer (140 mM NaCl, 5 mM KCl, 1.2 mM MgSO$_4$, 10 mM glucose, 0.8 mM CaCl$_2$, 10 mM sodium acetate, 2 mM NaH$_2$PO$_4$), 0.2 mM MIX (1-methyl-3-isobutylxanthine), and 20 mM Tris-HCl, pH 7.4, was added without (basal) or with ANF (Ile-ANF-26) or other hormones. The samples were covered with vaseline-coated concave slides and were incubated at 30°C for 10 minutes. The incubation was stopped by freezing on dry ice, then the frozen samples were transferred to 12 × 75 glass tubes. One hundred microliters of buffer of 50 mM sodium acetate, pH 6.2, was added and boiled for three minutes. The samples were stored at −20°C until assayed. Following acetylation of the experimental samples and standard, cGMP was determined by radioimmunoassay using cGMP [^{125}I] tracer (RIA kit from Dupont Co., New England Nuclear Prod., Billerica, Massachusetts, USA). In all experiments microdissection and assay of basal activities or with hormone was performed on the same tissue. The assays were performed in four replications. The standard curves ranged from 1 to 100 fmol/tube, the coefficient of determination was 0.988 to 0.997 and the cGMP concentration of samples was not lower than 1 fmol/assay. The cGMP content was expressed as fmol/μg protein of glomeruli or tubules. With this procedure, recovery of cGMP applied on blank slides was 96 to 99%. Therefore, ^{3}H-cGMP was not added to each sample for recovery.
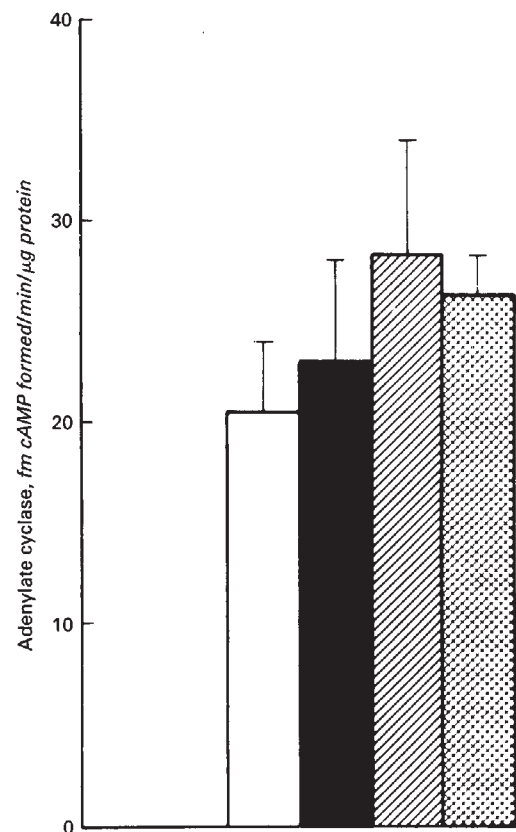

Fig. 1. *Effect of ANF on adenylate cyclase of rat glomeruli.* Each bar represents mean ± SEM. Symbols are: (□) basal, (■) ANF 10^{-9} M, (▨) ANF 10^{-8} M, (▩) ANF 10^{-7} M. N = 5, number of rats.
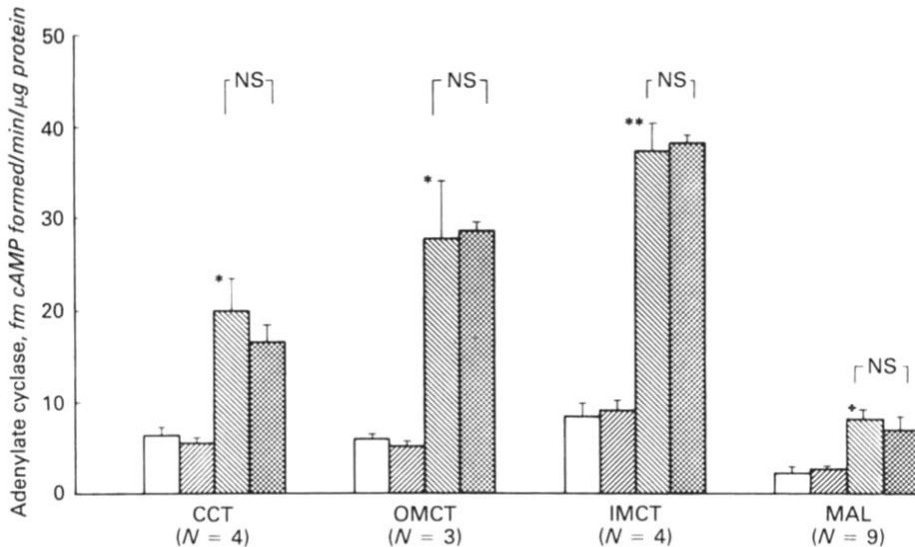
Fig. 2. *Effect of ANF on AVP-stimulated adenylate cyclase of rat cortical collecting tubule (CCT), outer (OMCT) and inner medullary collecting tubule (IMCT), and medullary thick ascending limb (MAL).* Each bar represents mean ± SEM. Symbols are: (□) basal, (▨) ANF 10^{-8} M, (▨) AVP 10^{-8} M, (▨) ANF + AVP 10^{-8} M; *$P < 0.02$ vs. basal, **$P < 0.05$ vs. basal, + $P < 0.001$ vs. basal (basal vs. AVP). N = number of rats.

ANF binding to isolated IMCT

^{125}I ANF binding to IMCT was performed by a modified method of Napier et al [9]. 3-[^{125}I] iodotyrosyl^{28}ANF (specific activity 1950 Ci/mmol) was purchased from Amersham (Arlington Heights, Illinois, USA). The binding assay was performed in total volume of 50 μl in a microcentrifuge tube containing dissected IMCT (total length 20 mm), and Tris-BSA buffer containing 10 mM $MgCl_2$, 0.1% bacitracin, 0.2% BSA, 50 mM Tris-HCL, pH 7.4, 1 × 10^{-10} M ^{125}I-ANF and various concentrations of unlabeled ANF (5 × 10^{-10} M, 1 × 10^{-9} M, 5 × 10^{-9} M, 1 × 10^{-8} M). The reaction mixture was incubated at 4°C for 30 minutes, and the reaction was stopped by adding 950 μl cold Tris-BSA buffer. The tubes were immediately centrifuged for 10 minutes at 15,000 × g. After the centrifugation, the supernatant was removed and the pellet was carefully washed twice with 1 ml cold Tris-BSA buffer. The time course study showed that the binding equilibration was reached at 15 minutes at 30°C and maintained up to 40 minutes. The nonspecific binding was determined in the presence of 1 × 10^{-6} M unlabeled ANF. K_d and B_{max} were calculated using an equilibrium binding data analysis of the Radioligand Binding Analysis Programs by G.A. McPherson (Elsevier-BIOSOFT). This computer program was designed to analyze the radioligand binding studies by Scatchard plot. The equation is $[B] = B_{max} - K_d \cdot [B]/[L]$, where [B] is bound; [L], free; B_{max}, maximal number of receptor; and K_d, dissociation constant. Protein was determined by a modified method from Lowry et al [25]. With this micromethod, proteins ranged in 0.2 to 1.0 μg can be measured [20–22].

Results

Adenylate cyclase determinations

ANF alone at concentrations of 10^{-9} to 10^{-7} M did not show any significant effect on adenylate cyclase activities in the isolated glomeruli (Fig. 1), although there were slight but insignificant increases in adenylate cyclase activities by 10^{-8} and 10^{-7} M ANF. To determine whether ANF exerted any effect on the AVP-stimulated adenylate cyclase, the effect of 10^{-8} M ANF on a submaximal stimulatory concentration of
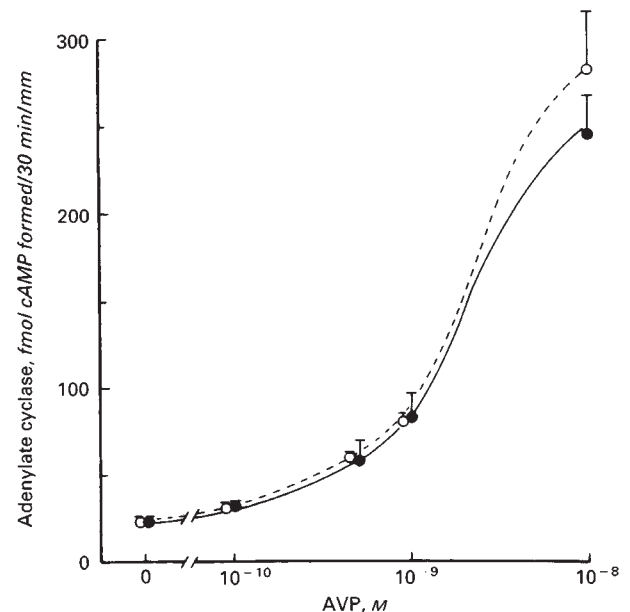

Fig. 3. *Effect of ANF on AVP dose-responsive adenylate cyclase of rat IMCT.* Each bar represents mean ± SEM. Symbols are: (●——●) control, (○--○) + ANF 10^{-8} M. N = 4, number of rats.

10^{-8} M AVP was examined on the adenylate cyclase of AVP-sensitive renal nephron segments. As shown in Figure 2, ANF alone at 10^{-8} M did not affect the basal activities of adenylate cyclase in the CCT, OMCT, IMCT, and MAL, and also showed no effect on the AVP-stimulated adenylate cyclase in these renal tubules. In Figure 3 is shown the effect of 10^{-8} M ANF on the AVP dose-responsive stimulation of adenylate cyclase in IMCT. There was also no significant effect of ANF on the AVP stimulation of adenylate cyclase in these studies.

In glomeruli (Fig. 4), 5 IU/ml of PTH significantly increased adenylate cyclase (basal 20.6 ± 3.5 vs. 5 IU/ml PTH 35.1 ± 3.7 fmol cAMP formed/min/μg, $P < 0.02$) and 10^{-8} M ANF did not affect this PTH-stimulated adenylate cyclase. The same pattern of results was shown in the CAL (Fig. 4); 10^{-8} M ANF did not

A

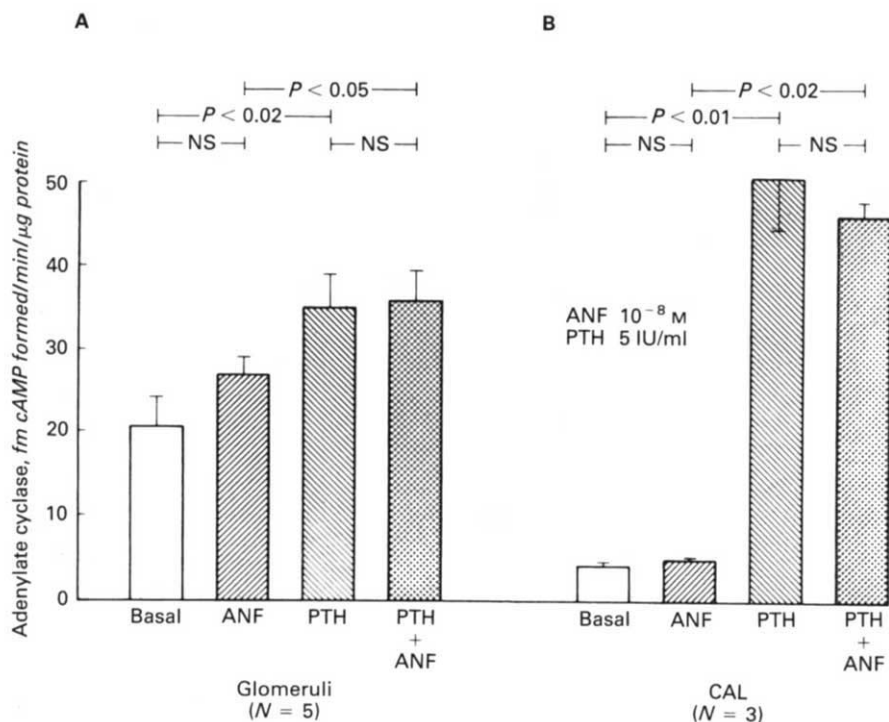

B

ANF 10^{-8} M
PTH 5 IU/ml

Fig. 4. *Effect of ANF on PTH-stimulated adenylate cyclase in rat glomeruli and cortical thick ascending limb (CAL).* Each bar represents mean ± SEM. N = number of rats.
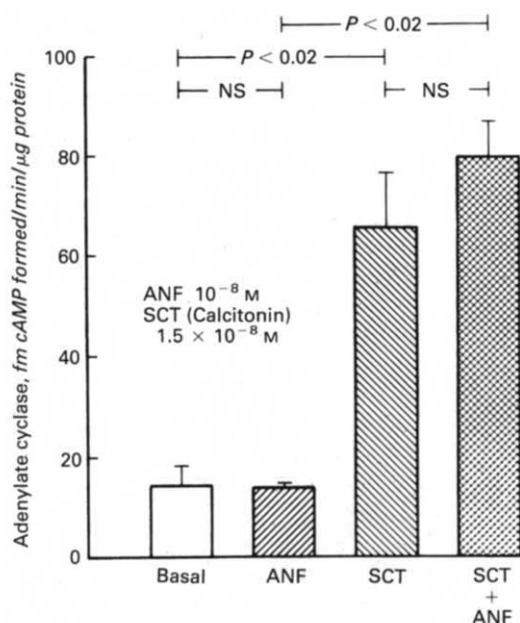
Effect of ANF on cGMP

ANF significantly increased cGMP levels in the isolated rat glomeruli (basal 6.1 ± 3.1 vs. 10^{-8} M ANF 119.4 ± 38.9, $P <$ 0.01, 10^{-7} M ANF 121.3 ± 37.4, $P < 0.01$; Fig. 6). As shown in Figure 7, 5 IU/ml PTH showed no effect on either basal cGMP levels or ANF-stimulated cGMP concentrations in the isolated glomeruli. The effect of ANF on the cGMP concentrations was studied in several nephron segments, including the proximal tubule (P × T), CAL, MAL, CCT, OMCT, and IMCT. These results are shown in Figure 8. ANF (10^{-7} M) significantly increased cGMP levels only in the IMCT (0.63 ± 0.16 to 1.46 ± 0.29 fmol/µg protein, $P < 0.05$) and this increase was consistent in every rat. There were no significant increases in cGMP induced by ANF in any of the other renal tubules. The basal concentrations of cGMP were higher in the CCT as compared to other renal tubules.

^{125}I ANF binding to IMCT

^{125}I ANF binding was studied only in the microdissected IMCT, since IMCT was the renal tubule in which ANF stimulated cGMP. In Figure 9 is shown the Scatchard plot of ANF binding to IMCT. It suggests one homogeneous receptor population of ANF in rat IMCT. K_d was 6.08 × 10^{-9} M and B_{max} was 8.08 × 10^{-11} M. Nonspecific binding was 23 to 28%.

Fig. 5. *Effect of ANF on calcitonin-stimulated adenylate cyclase of rat CCT.* Each bar represents mean ± SEM. N = 4, number of rats.

Discussion

There is little doubt that the atrial natriuretic peptides increase urine flow and sodium excretion in a number of different species [1–6]. The exact mechanism for these effects, however, remains a matter of some debate. The glomerular binding and stimulation of cGMP production is the most profound effect of ANF in the kidney, and ANF has been shown to increase glomerular filtration rate (GFR) significantly in several studies
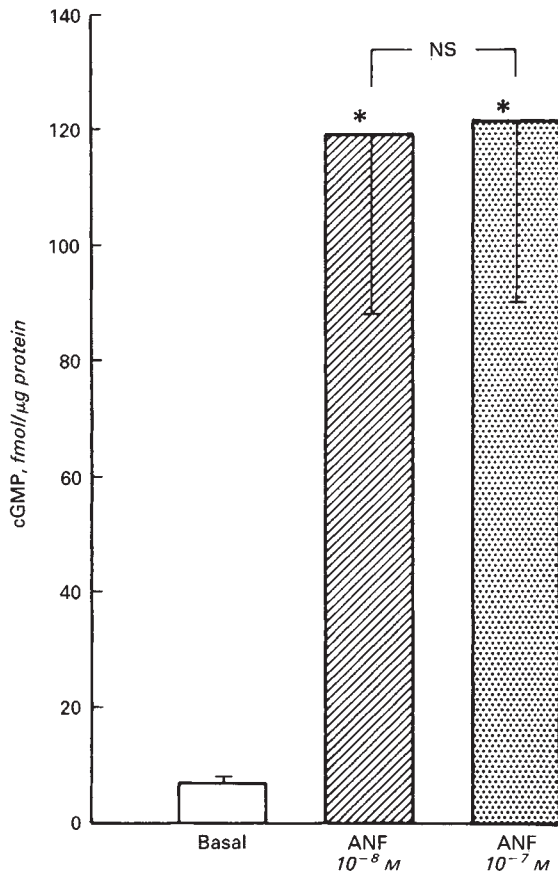
show any effect on either basal activities or PTH-stimulated adenylate cyclase.

Calcitonin is known to stimulate adenylate cyclase in CCT. As shown in Figure 5, 10^{-8} M ANF slightly but insignificantly increased the calcitonin-stimulated adenylate cyclase (1.5 × 10^{-8} M SCT 69.8 ± 11.3, SCT + 10^{-8} M ANF 79.9 ± 7.2 fmol cAMP formed/min/µg protein, NS).

Fig. 6. *Effect of ANF on cGMP of rat glomeruli.* Each bar represents mean ± SEM. $N = 5$, number of rats; *$P < 0.01$.

Fig. 7. *Effect of PTH and ANF on glomerular cGMP.* Each bar represents mean ± SEM. $N = 4$, number of rats.

[3, 26, 27]. However, against the role of GFR as the primary modulator of the natriuretic and diuretic effect of ANF is the finding of several investigators that ANF causes a diuresis and natriuresis in spite of any detectable increases in GFR [6, 28].

Recent attention has therefore been focused on potential effects of ANF on the distal nephron. The potential role of the distal nephron in the natriuretic and diuretic response to ANF has been proposed for several reasons. First of all, in addition to the renal cortical binding of ANF, which occurs primarily in the glomerulus, ANF binding has been observed by autoradiography in the inner medullary or papillary region of the kidney [10, 11]. Such membrane binding, however, could occur on interstitial cells, vasculature or tubular epithelium. In fact, Ballerman et al [9] initially suggested that a vascular effect of ANF in the papilla increased the hydrostatic driving force in the vasa recti, a consequence that could result in the passive movement of sodium from the interstitium into the collecting duct. The same group has, however, now shown a direct effect of ANF to decrease ouabain-sensitive oxygen consumption of rabbit inner medullary collecting duct cells in suspension [12]. On the basis of these studies, an effect of ANF to decrease sodium entry across the IMCT has been proposed. Such a direct effect of ANF on sodium transport in the rat inner medullary collecting duct is quite compatible with the present findings that ANF both binds to IMCT and also significantly stimulates cGMP production in the same segment. Koseki et al
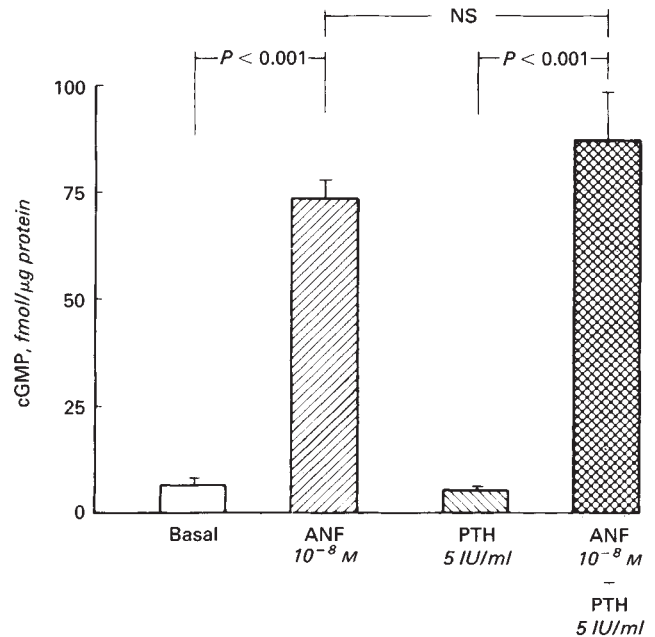
[10] have also recently shown that ANF binds to rat inner medullary rich cells separated by gradient centrifugation, showing a K_d of 2.1×10^{-8} M. The ANF binding results in the present study, however, revealed an ANF affinity with a K_d of 6.08×10^{-9} M. Differences in the K_d for ANF binding might be due to the two different preparations of tubular cells studied. The receptor number for ANF on IMCT in our study as assessed by B_{max} was 8.07×10^{-11} M or 1900 fmol/mg protein.

Another possible mechanism of action of ANF in the mammalian nephron has been suggested by results which indicated an effect of ANF to interfere with other hormones, particularly AVP, which stimulate the adenylate cyclase-cAMP system. In a recent study in the dog, ANF inhibited adenylate cyclase in glomeruli, loops of Henle and collecting tubules [29]. ANF has also been shown to inhibit AVP-induced water flux in the rabbit cortical collecting tubules [13]. On the other hand, in other studies, ANF did not inhibit AVP-stimulated cAMP in cultured rabbit CCT [30], or dissected CCT and OMCT from the rat or rabbit [18, 31]. ANF has also not been found to alter transmural voltage or the lumen to bath chloride fluxes in perfused loops of Henle [32].

A comprehensive investigation was therefore performed in the present study in search for an interaction between ANF and hormones whose action involves the adenylate cyclase-cAMP system. Several hormones were used and different segments of the rat nephron were studied; the results were rather clear. There was no evidence of ANF inhibition of the effect of calcitonin, PTH or AVP to stimulate adenylate cyclase in isolated rat nephron segments. It should be emphasized, however, that these results on adenylate cyclase activity were obtained in permeablized tubular cells, and may not be comparable to studies performed in membrane preparations as well-preserved living tubules which measured cAMP generation from endogenous ATP rather than adenylate cyclase activity. In
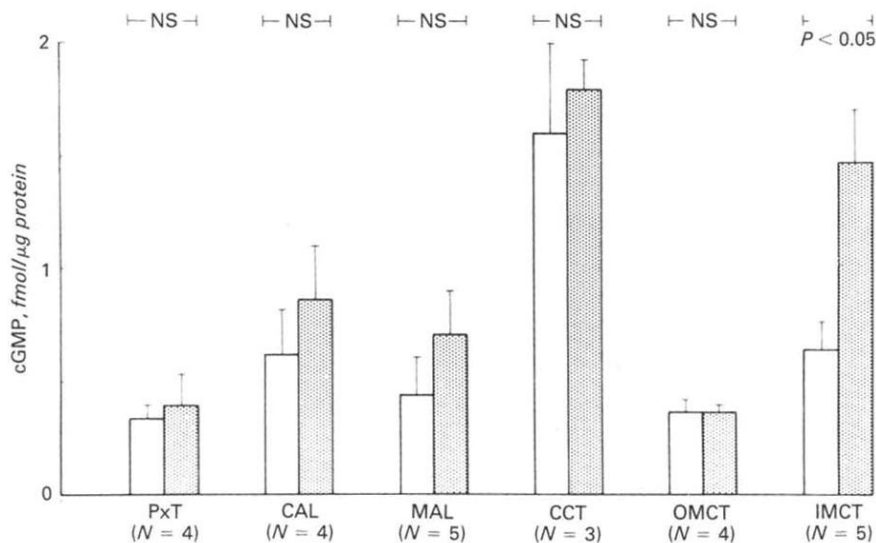
Fig. 8. *Effect of ANF on cGMP in the rat proximal tubules (PxT), CAL, MAL, CCT, OMCT, and IMCT.* Each bar represents mean ± SEM. N = number of rats. Symbols are (□) basal, (▨) ANF 10^{-7} M.
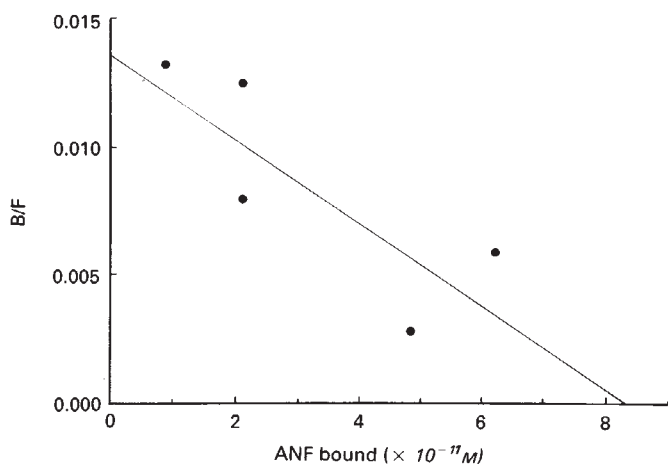

Fig. 9. *Scatchard plot of ^{125}I ANF binding to rat IMCT.* Each point is the mean of three experiments, and each point of each experiment is a mean of duplicate. Nonspecific binding was 23 to 28%. K_d 6.08 × 10^{-9} M; B_{max} 8.07 × 10^{-11} M.

this regard, a previous report using an alpha-2-adrenergic agonist showed no effects on adenylate cyclase response to AVP, but inhibition of cAMP stimulation by AVP was demonstrated in rat OMCT [33]. However, other studies [16, 18, 31] with cAMP measurements in intact nephron segments reported no effect of ANF on AVP-stimulated cAMP accumulation.

In the present investigation, ANF was found to bind to the IMCT of the rat, the only site in which ANF stimulated cGMP production in the nephron. These findings are consonant with the results of a recent preliminary micropuncture study in the rat in which an effect of ANF to inhibit tubular sodium reabsorption in the collecting tubule was demonstrated [34]. Whether cGMP is the secondary messenger of such an effect of ANF in the collecting tubule or a pari passu event, however, must await further study. In this regard, it is interesting that ANF analogues which do not stimulate cGMP production in collecting duct cells are still modestly natriuretic in vivo (J. Lewicki, personal communication).

In summary, ANF was shown not to interfere with hormones which stimulate the adenylate cyclase-cAMP system including AVP, PTH and calcitonin. Stimulation of cGMP production by ANF, however, was observed not only to occur in the glomerulus but also in the IMCT of the rat, both sites of ANF binding. The IMCT may therefore be the major site of ANF action in the rat distal nephron.

Acknowledgments

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