Stimulation of cyclic GMP synthesis in human cultured glomerular cells by atrial natriuretic peptide

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Recently a stimulatory effect of atrial natriuretic peptide (ANP) on the particulate guanylate cyclase system has been reported in the glomeruli from different species. Using cultures of homogeneous human glomerular cell lines, we found that rat and human ANP stimulated markedly cGMP formation in epithelial cells with a threshold dose of 1 nM. A 20-fold increase was obtained at 5 μM. Stimulation was also present but less substantial (2-fold at 5 μM) in mesangial cells. cGMP was formed rapidly and released in the medium. ANP and sodium nitroprusside, an activator of soluble guanylate cyclase, had additive effects on cGMP formation. ANP did not inhibit cAMP formation in both cell lines. These results demonstrate that, at least in the human species, epithelial cells represent the main target of ANP in the glomerulus. Synthesis of cGMP in the glomerular epithelial cells in response to ANP also suggests that the excess of urinary cGMP produced by the kidney which is observed after ANP administration is of glomerular rather than of tubular origin.

Atrial natriuretic factor *cyclic GMP* *(Glomerulus, Epithelial cell)*

1. INTRODUCTION

Atrial natriuretic peptides (ANP) stimulate renal excretion of water and sodium. Increase in glomerular filtration rate (GFR) appears to be the major mechanism responsible for natriuresis. In many studies, GFR rose by 30–50% [1–4]. The mechanisms for the change in GFR are still unknown. Increased renal plasma flow can be ruled out since it decreased or remained unchanged after ANP administration [1,2]. Thus, ANP enhances GFR by other mechanisms such as increases of glomerular filtration surface area, glomerular capillary permeability or capillary hydraulic pressure. The latter effect has been recently demonstrated in the rat using micropuncture techniques [5]. In vitro studies have also contributed to analysis of the mode of action of ANP in glomeruli. ANP binds specifically to rat glomeruli [6,7] and stimulates cGMP formation in rat [6] and dog [8,9] glomeruli. Activation of the particulate but not the soluble guanylate cyclase is responsible for this effect [8]. The greatest accumulation of cGMP in the kidney has been observed in the glomeruli whereas there was no change in the proximal tubule [8,9] and only slight increases in the thick segment of the ascending limb of the loop of Henle and in the collecting tubule [8]. In contrast, with renal tubular epithelial cells, vascular smooth muscle cells respond to ANP by marked cGMP generation [10,11]. These results suggested that glomerular mesangial cells which possess many of the properties of smooth muscle cells should be the target of ANP within the glomeruli. In agreement with this hypothesis, Ballerman et al. [6] demonstrated recently that ANP bound to rat mesangial cells and stimulated markedly cGMP formation in these cells. A functional response was also observed in glomerular epithelial cells but was considerably smaller. We have previously reported that ANP stimulated particulate guanylate cyclase in human glomeruli [12].

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The aim of this study was to localize the effect of ANP within the glomerulus in the human species. Unexpectedly, we observed that ANP stimulated cGMP essentially in the epithelial cells.

2. MATERIALS AND METHODS

Synthetic rat ANP (Ser 128–Arg 150; atriopeptin II) was donated by Ciba-Geigy (Basel, Switzerland) and synthetic human ANP (Ser 124–Tyr 151) was purchased from Peninsula Laboratories (Belmont, CA). The following radionuclides and reagents were obtained as indicated: ^{125}I-tyrosine methyl ester cGMP and ^{125}I-tyrosine methyl ester cAMP, the Radiochemical Centre (Amersham); cGMP and cAMP, Boehringer (Mannheim); isobutylmethylxanthine (MIX), Sigma (St. Louis, MO); collagenase, Cooper Biomedical (Malvern, PA).

Human glomerular epithelial and mesangial cells were cultured as described [12]. Briefly, glomeruli were prepared from the cortex of human cadaver kidneys judged to be unsuitable for transplantation. Isolated glomeruli were treated with collagenase (1 mg/ml) for 30 min at 37°C. Digested glomeruli were then passed on a 25 μm sieve. Retained glomeruli and filtered dissociated cells were resuspended separately in Waymouth's medium supplemented with 20% human serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were plated on culture dishes and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. When outgrown, the cells were cloned in order to obtain pure preparations of mesangial and epithelial cells. Epithelial and mesangial cells were studied after 2–3 and 3–7 passages, respectively. They were plated on 6-well dishes (Nunclon; Nunc, Roskilde, Denmark) and used 48 h later. At this time each well contained approx. 200000 cells (200 μg protein).

Epithelial and mesangial cells were identified from their morphological aspect and their synthetic functions. Further identification of each batch was provided using the immunofluorescence technique with an antikeratin antiserum (Miles, Paris) raised in guinea pig. Cells were grown on 12-well multitest slides (Flow Labs, Puteaux, France) and fixed with acetone for 3 min. After several washings with phosphate-buffered saline, the cells were incubated with antikeratin antiserum, washed again, and incubated with fluoresceinated anti-guinea pig IgG.

Cells were washed 3 times with Waymouth's medium without human serum and incubated in situ at 37°C with 500 μl of Waymouth's medium supplemented with 5 mM $MgCl_2$ and 0.1 mM MIX in the presence of 0.1 nM–5 μM ANP. The reaction was stopped at 3 min, unless otherwise stated, by addition of 0.5 ml of 0.1 M HCl. cGMP and cAMP were measured in the HCl extract by radioimmunoassays. In some experiments the medium was collected and HCl was added separately to the medium and to the cells in order to measure intra- and extracellular cGMP. The samples were acetylated and aliquots of 25, 50 and 100 μl were collected for radioimmunoassay. Acetylated samples and standards were incubated for 16 h at 4°C with either ^{125}I-cGMP and the anti-cGMP antibody at 1:200000 or ^{125}I-cAMP and the anti-cAMP antibody at 1:40000 (final dilutions). Both antibodies had been raised in the laboratory. It was verified they did not crossreact with ANP or MIX. The protein content of each well was measured according to Lowry et al. [13]. Results were expressed as pmol cGMP or cAMP per mg protein.

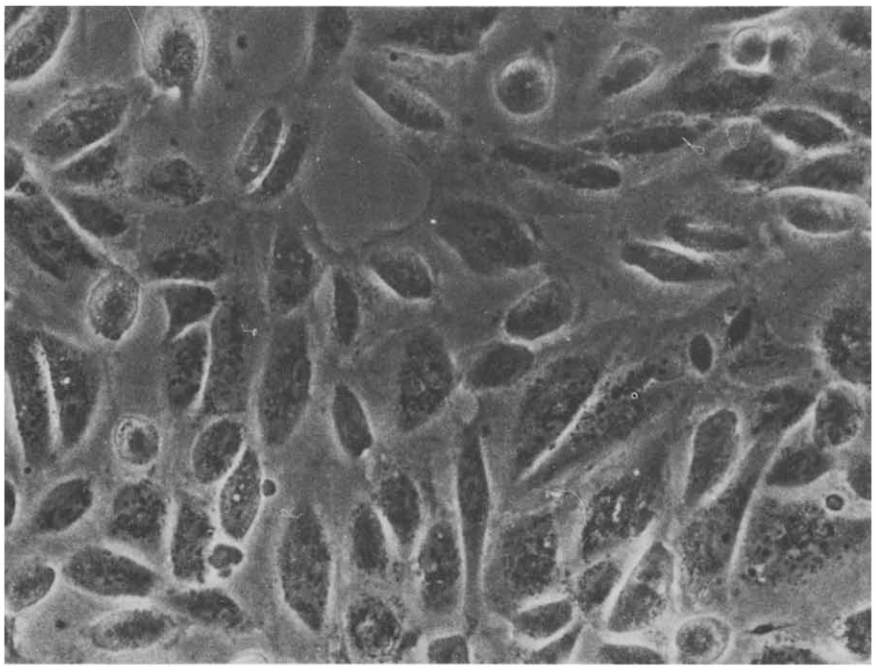
The results shown in figs 2 and 4 were analysed using two factor analysis of variance with repetition. The additive effect of ANP and sodium nitroprusside on cGMP production shown in fig.3 was investigated using Student's *t*-test. We compared the sum of the effects obtained separately with ANP and sodium nitroprusside with the experimental value obtained when both agents were tested in combination. It was considered that the variance of the predicted sum was equal to the sum of the individual variances of its two components.

3. RESULTS

3.1. *Cell identification*

The morphological characteristics of the two cell types in culture are shown in fig.1. Epithelial cells are polyhedral with a cobblestone-like appearance at confluency whereas mesangial cells have a typical stellate appearance, pile up, and exhibit thin cytoplasmic extensions. In addition, the two human cell types were identified by their cytoskeleton proteins, specific response to several agonists and synthetic functions. Using the im-

A



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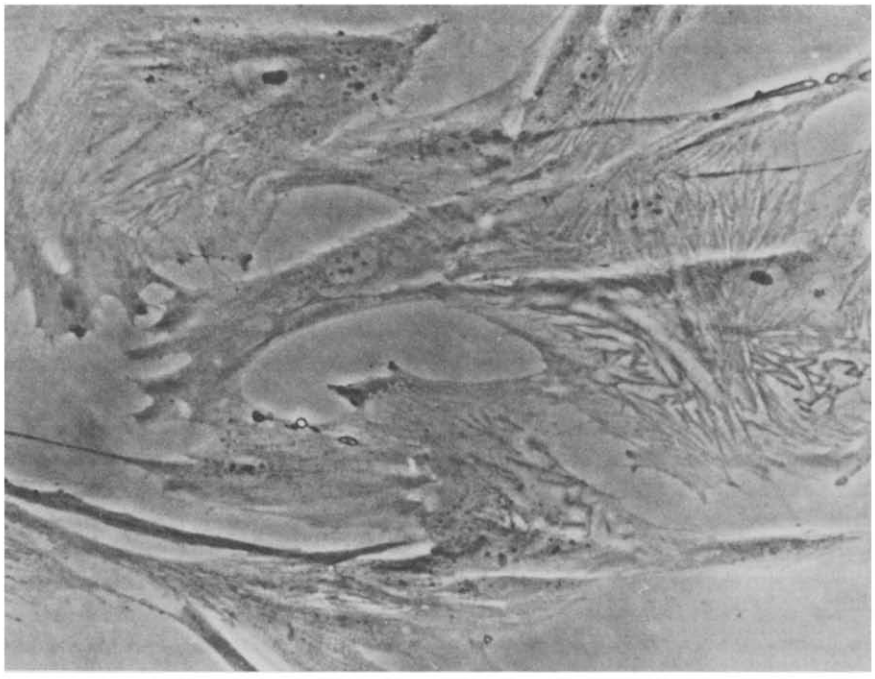

Fig. 1. Morphological characteristics of cultured glomerular epithelial (A) and mesangial (B) cells. Magnification, × 450.

munofluorescence technique, epithelial cells stained markedly after incubation with a specific antikeratin antiserum whereas there was no staining with mesangial cells. Mesangial cells contracted in the presence of 1 nM angiotensin II or 10 nM AVP whereas epithelial cells did not [14]. Epithelial cells synthesized predominantly type IV collagen whereas mesangial cells synthesized several types of collagen [15]. Finally, epithelial cells synthesized and released in the incubation medium urokinase, an activator of plasminogen well characterized by its fibrin-independent activity and inhibition of this activity in the presence of specific antiurokinase antibody [16]. In contrast, human mesangial cells did not produce urokinase.

3.2. *Effect of ANP on cyclic GMP synthesis*

Mean basal cGMP levels were 2.91 ± 0.68 and 3.81 ± 1.17 (mean ± SE; n = 16) pmol/mg in epithelial and mesangial cells respectively. In epithelial cells, rANP markedly stimulated accumulation of cGMP with a threshold between 1 and 10 nM, and increasing stimulation up to 5 μM as shown in fig.2. At this concentration cGMP content was 20-times higher than its basal value.

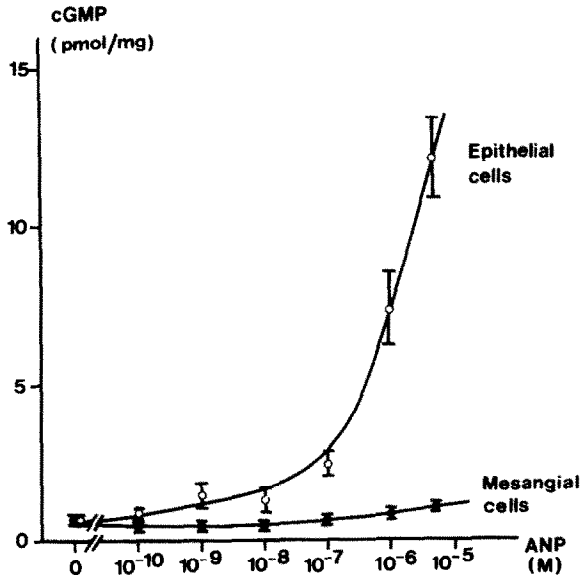

Fig.2. Effect of rat atriopeptide on cGMP concentration in glomerular epithelial (○) and mesangial (●) cells. Means ± SE of the results of 4 experiments (2 values per experiment) are shown. cGMP was measured after 3 min incubation.

There was also an increase in cGMP in mesangial cells exposed to rANP but the response was considerably lower than in epithelial cells. A 2-fold stimulation was only observed with 5 μM rANP. It was verified that the response of mesangial cells did not change between the 3rd and 7th passage. Since we utilized glomerular cells from human origin we also studied the effects of hANP on cGMP accumulation in epithelial cells. hANP stimulated cGMP generation with a threshold dose of 10 nM, but stimulation observed with 5 μM was 10-times the basal value, thus less than that observed using rANP.

Sodium nitroprusside, a well-known activator of soluble guanylate cyclase without tissue specificity, produced at 1 mM a marked increase in cGMP formation in both glomerular cell types. However, stimulation was more substantial with epithelial (× 10–50) than with mesangial cells (× 6–20). When studied in combination, rANP (1 μM) and sodium nitroprusside (1 mM) produced an additive effect. Indeed, there was no significant difference between the value observed and the calculated sum of the separate effects of both agents (fig.3).

To study the time course of cGMP production and release, cGMP accumulation with time was measured in parallel in the incubation medium and the epithelial cell extract under basal conditions

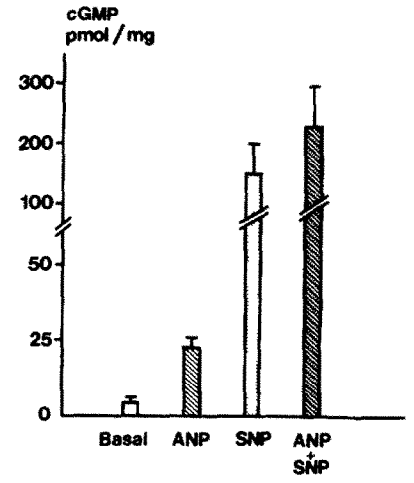

Fig.3. Separate and combined effects of rat atriopeptide (1 μM) and sodium nitroprusside (1 mM) on cGMP concentration in glomerular epithelial cells. Means ± SE of the results of 3 experiments (2 values per experiment) are shown.

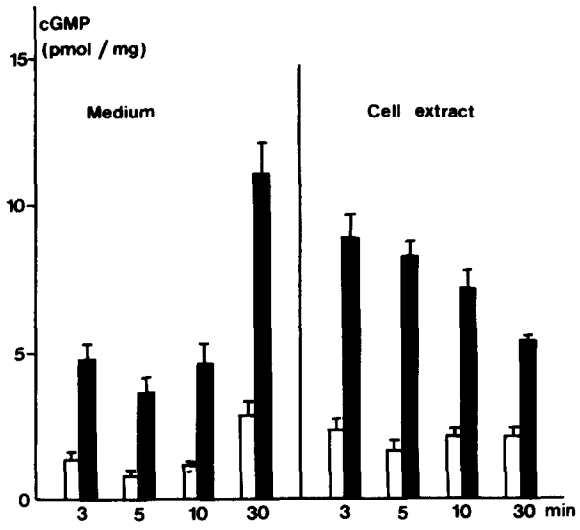

Fig.4. Accumulation of cGMP with time in the medium and the cell extract of glomerular epithelial cells incubated with (shaded bars) or without (open bars) 1 μM rat atriopeptide. Means ± SE of the results of 2 experiments (2 values per experiment) are shown.

and in the presence of 1 μM rANP. The effect of this hormone was apparent after 3 min incubation. Intracellular cGMP decreased progressively with time and extracellular cGMP represented approx. 70% of the total at 30 min (fig.4).

Mean basal cAMP levels were 5.8 ± 0.5 and 8.9 ± 2.3 (mean ± SE; n = 12) pmol/mg in epithelial and mesangial cells, respectively. There was no change in the cAMP content of both cell types after 3 min incubation with 5 μM rANP.

4. DISCUSSION

Our findings indicate that ANP stimulates cGMP in human glomerular epithelial and mesangial cells. However, the response is considerably greater in epithelial than in mesangial cells. These results are thus different from those of Ballerman et al. [6] who reported that ANP-stimulated cGMP accumulation was predominant in rat cultured mesangial cells. These authors also observed that cell surface receptors for ANP were expressed on mesangial but not epithelial cells. However, they concluded that cGMP formation in mesangial cells was not directly coupled to the ANP receptors. The discordance between their study and ours may be due either to a species difference or to the conditions of culture. It has been already shown that murine and human glomerular cells do not possess exactly the same synthetic functions. For example, rat mesangial and epithelial cells synthesize high levels of PGE_2 and, to a lesser extent, $PGF_{2\alpha}$ whereas PG production is very low in both human glomerular cell lines due to the lack of cyclooxygenase [17]. We also studied cultured cells earlier (epithelial cells between the 2nd and 3rd passage and mesangial cells between the 3rd and 7th passage) than did Ballerman et al. (both cell lines between the 12th and 20th passage). Furthermore, recent findings demonstrate that mesangial cells are not the only intra-glomerular target of ANP even in the rat. Bianchi et al. [18], using autoradiography techniques, reported that ^{125}I-ANP was present essentially in the endothelial cells. More recently the same group concluded from more precise ultrastructural studies that the majority of ANP receptors were localized in the visceral epithelial cells [7]. In agreement with these findings, Huang et al. [5] found a high concentration of cGMP in the urinary Bowman's space after administration of ANP in the rat. A mesangial source for this nucleotide would be unlikely since the products synthesized by the mesangial cells are excreted via the renal circulation. On the contrary, these experiments suggest that cGMP was generated by the epithelial cells which are directly in contact with the glomerular ultrafiltrate. It has also been shown [19] that administration of ANP in the rat produced a greater increase in the urinary excretion (10-fold) than in the plasma concentration (3.8-fold) of cGMP. The resulting increase (2.6-fold) in the renal clearance suggests a direct addition of the nucleotide to the tubular fluid. The tubular cells represent a possible origin since atrial extracts elevated cGMP in primary cultures of renal tubular cells [19]. However, the effect was less marked than that which we have observed with cultured glomerular epithelial cells in the present study. Furthermore, the study of fresh preparations showed that the most striking action of ANP on cGMP occurred in the glomeruli (50-fold) and not in the tubular segments. Indeed, there was no effect in the proximal tubule [8,9] and only minor effects in the thick loop of Henle and the collecting duct [8]. It is thus possible that the excess of cGMP observed in the final urine may originate from the

glomerular epithelial cells. This hypothesis is strengthened by the recent results of Huang et al. [5], showing that the ratio cGMP concentration to inulin concentration did not change along the rat nephron including the final urine. The fact that cGMP formed in the cultured epithelial cells is rapidly released in the incubation medium is also in favor of this hypothesis.

It is likely that ANP acts on the particulate guanylate cyclase in human glomerular epithelial cells similarly to what we reported with whole human glomeruli. Since it was not possible to study the increase in endogenous cGMP using subcellular fractions, we studied the separate and combined effects of rANP and nitroprusside on cGMP formation. We observed that addition of rANP, although at a dose which could not be considered as providing the maximum response, further increased the effect of a maximally active dose (1 mM) of sodium nitroprusside. This result suggests that ANP and nitroprusside, a well-known stimulus of soluble guanylate cyclase, act on different pools of cGMP. In accordance with the results of Umemura et al. [20] with rat isolated glomeruli, we found that ANP did not inhibit endogenous cAMP levels in glomerular epithelial cells. These results confirm that ANP operates in glomeruli only via activation of the particulate guanylate cyclase and not via inhibition of the cell membrane adenylate cyclase.

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