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Atrial natriuretic peptide inhibits renin release from juxtaglomerular cells by a cGMP-mediated process

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ABSTRACT **We have examined the effect of a synthetic analogue of human α-atrial natriuretic peptide (ANP), APII, on renin release in cultured renal juxtaglomerular cells (JGA cells). Using cell cultures containing 80–90% renal juxtaglomerular cells, we found that ANP (10^{-13}–10^{-9} M) strongly inhibited renin release from the cells in a dose-dependent fashion (k_i, 10 pM) to about 10% of control. Inhibition of renin release by ANP was paralleled by an increase in cellular cGMP levels; while in the presence of the cGMP-phosphodiesterase inhibitor M&B 22948 (1 mM), concentrations of ANP lower by a factor of 100 were required to obtain the same effects on renin release and cGMP levels. The guanylate cyclase inhibitor methylene blue (10 μM), on the other hand, shifted the dose–response curves for renin release and cGMP levels to 100-fold higher concentrations of ANP. Neither the influx of ^{45}Ca into the cells nor the intracellular quin-2 signal, which is a measure for changes of intracellular Ca concentration, was in any way altered by ANP. Our results suggest that ANP inhibits renin release from juxtaglomerular cells by a cGMP-dependent process that does not involve changes in intracellular calcium.**

Two major effects of atrial natriuretic peptide (ANP) on renal function have been described, namely the enhancement of sodium and water excretion (cf. refs. 1 and 2) and the suppression of renin release (3–5). Inhibition of renin release by ANP leading to a diminished formation of angiotensin II (AII) would result in decreased vascular tone and impaired aldosterone secretion. Both effects are of physiological importance during states of expanded extracellular volume, in which enhanced release of ANP from the atrium is observed (6, 7).

The mechanism by which ANP inhibits renal renin release is as yet a matter of debate (2). In view of the increased filtration rate of NaCl caused by the ANP, which leads to an increased NaCl load at the macula densa, it has been speculated that ANP inhibits renin release from juxtaglomerular cells via the macula densa receptor (3, 4). However, a direct effect of ANP on juxtaglomerular cells could not be excluded on the basis of experimental evidence. Therefore, we wanted to investigate whether or not ANP directly inhibits renin release by renal juxtaglomerular cells.

We have developed a cell culture system that contains around 50% juxtaglomerular cells (8, 9). We now have improved this method by including a Percoll-density gradient in the cell preparation procedure. As a result, cell cultures can be obtained that contain regularly between 80 and 90% juxtaglomerular cells.

We found that ANP strongly inhibits renin release from these cultured cells and, furthermore, obtained strong evidence that the inhibitory effect of ANP is mediated by cGMP and does not involve an increase in intracellular calcium.

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MATERIALS AND METHODS

Cell Culture. Isolation of juxtaglomerular cells was essentially as described (8, 9). In brief, rat kidneys were perfused *in situ* with citrate buffer. After exstirpation of the kidneys, renal cortex was minced and subsequently incubated with a collagenase–trypsin solution. The suspension was poured over a 22-μm screen, and single cells passing the sieve were washed with culture medium. As described (9), cell cultures containing about 50% juxtaglomerular cells can be obtained by culturing these single cells. To improve the enrichment of juxtaglomerular cells, we centrifuged the cells in a Percoll-density gradient. About 15×10^6 cells were mixed with 30 ml of an isotonic 25% (vol/vol) Percoll solution and centrifuged at $15,000 \times g$ in a vertical rotor (SV-288, Sorvall) for 30 min. Four different bands with densities of 1.02 g/ml, 1.05 g/ml, 1.06 g/ml, and 1.13 g/ml were regularly obtained. Table 1 gives typical values for the distribution of protein and specific renin activity within the gradient. Band III cells (1.06 g/ml) were incubated with culture medium [RPMI 1640, 25 mM Hepes, streptomycin at 100 μg/ml, penicillin at 100 units/ml, 2% (vol/vol) fetal bovine serum] at 37°C in 5% CO_2/95% air. All experiments as described below were performed on the second day of culture.

Renin Demonstration by Immunofluorescence. Immunofluorescence staining for renin was done exactly as described (9). In brief, cell cultures were fixed in 4% (wt/vol) paraformaldehyde/Dulbecco's phosphate-buffered saline (PBS). After 5 min in 10% (vol/vol) normal swine serum/0.1% bovine serum albumin/PBS, the cultures were incubated with rabbit antiserum against rat renin diluted 1:200 in 10% (vol/vol) normal swine serum/PBS. After rinsing in PBS, a 30-min incubation followed with tetramethylrhodamine B isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (1:500, Sigma). The specificity of the immunoreaction was tested with the usual control procedures (9). Immunofluorescence was examined using a Polyvar fluorescence microscope (Reichert–Jung, Vienna, Austria). Rabbit antiserum against rat renin, used in the studies, was a generous gift of M. Celio (Zürich) who obtained it from T. Inagami (Nashville, TN). For immunocytochemical demonstration and localization with the protein A/gold technique, pellets of 10^6 cells were fixed in 1% glutaraldehyde and embedded in London White resin (London Resin, Basingstoke, England). Ultrathin sections were immunostained for renin as described by Taugner *et al.* (10). Rabbit antiserum against rat renin used in these studies was a generous gift from E. Hackenthal.

Renin Release. Renin release from the cultured cells was determined exactly as described (8). In brief, the culture medium was replaced with prewarmed, Hepes-buffered saline [132 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 2 mM $CaCl_2$,

Abbreviations: ANP, atrial natriuretic peptide; AI, angiotensin I; AII, angiotension II; $[Ca]_i$, intracellular Ca^{2+} concentration.

Table 1. Protein distribution and specific renin activity of cells separated by a 25% (vol/vol) Percoll gradient

Band	Density, g/ml	Protein, mg	Specific renin activity, ng of AI per hr per mg of protein
I	1.02	2.01 ± 0.35	30 ± 11
II	1.05	3.36 ± 0.75	143 ± 60
III	1.06	0.51 ± 0.19	1170 ± 280
IV	1.13	3.27 ± 0.62	7 ± 5

Data are mean ± SEM of three experiments.

10 mM NaOAc, 2 mM NaH_2PO_4, 10 mM glucose, 20 mM Hepes (pH 7.2)]; and the culture dishes were placed on a heating block at 37°C. The renin secretion rate was calculated from the linear increase of the renin activity of the cell-conditioned buffer before and 10, 20, and 30 min after addition of the agents.

Renin Activity Assay. Renin activity was determined by its ability to generate angiotensin I (AI) from the plasma of bilaterally nephrectomized rats exactly as described (11). AI was determined by radioimmunoassay (Isotopen Dienst West, Teufen, Switzerland).

Intracellular cGMP Levels. Intracellular cGMP concentrations were examined under the same experimental conditions as the renin release. Five minutes after the addition of the agents, the buffer was removed from the cultures, and the dishes were placed on an ice block. After the addition of 0.4 ml of ice-cold 5 mM potassium phosphate, 0.2 mM EDTA, 0.5 mM 3-isobutyl-methylxanthine, and 150 mM KCl (pH 6.8), the cells were scraped off with a Teflon policeman. The cell suspension so obtained was sonicated, boiled for 5 min, and centrifuged. An aliquot was removed from the sonicated cell suspension for protein determination. The supernatants were assayed for cGMP using a cGMP radioimmunoassay (New England Nuclear).

^{45}Ca-Uptake. ^{45}Ca-uptake into the cultured cells was determined exactly as described (8).

Intracellular Ca^{2+} Measurement. Intracellular Ca^{2+}, $[Ca]_i$, was measured using quin-2. About 2×10^7 cells were incubated with 25 μM quin-2 AM [tetrakis(acetoxymethyl) ester of quin-2] in RPMI 1640 for 20 min followed by another 40-min incubation with 4 vol of medium. After the incubation period, aliquots of 1×10^6 cells were washed twice and resuspended in Hepes-buffered saline. Fluorescence of quin-2-loaded cells was measured at 37°C in a Perkin–Elmer fluorescence spectrophotometer L93 using excitation wavelength of 340 nm and emission wavelength of 490 nm. The fluorescence signal was calibrated at the end of each individual trace essentially as described by Tsien *et al.* (12). Cell numbers were determined using a Coulter counter. Protein was determined according to the method of Lowry (13) with bovine serum albumin as a standard.

Reagents. All reagents for the culture medium were obtained from Boehringer Mannheim. Synthetic atrial peptide (Arg-101 to Tyr-126) was a generous gift from F. Spinelli (CIBA–Geigy, Basel, Switzerland). The cGMP-specific phosphodiesterase inhibitor M&B 22948 was kindly provided by the May & Baker Company (Dagenham, Essex, U.K.). Sodium nitroprusside, methylene blue, and AII were purchased from Sigma. ^{45}Ca was from Amersham (U.K.). Quin-2 AM was obtained from Calbiochem.

RESULTS

Cell Culture. Fig. 1 (*Upper*) Phase-contrast photomicrograph of Band III cells cultured for 2 days. The cultures consisted of 80–90% round, single cells. These cells exhibited specific immunofluorescence for renin (Fig. 1, *Lower*). As we have also shown elsewhere (9), the round cells that stain for renin contain prominent renin granules (Fig. 2). We, therefore, conclude that these cells are juxtaglomerular cells. The cellular renin activity of the cultured cells used in this study was between 100–200 ng of AI per hr per mg of protein on the second day of culture. The renin activity in the culture medium was between 1 and 2.6 μg of AI per hr per mg of cellular protein.

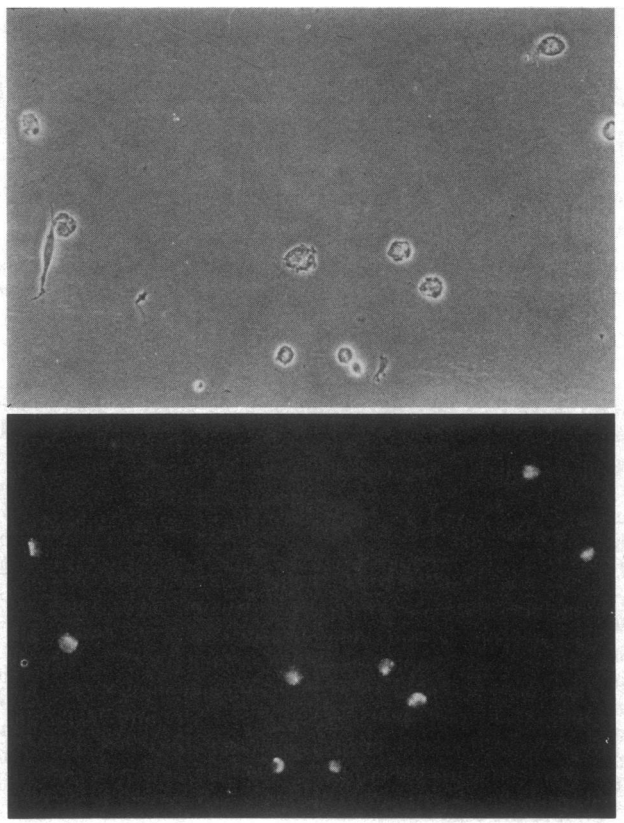

FIG. 1. Microphotograph of cultured cells on second day of culture. (*Upper*) Phase-contrast and (*Lower*) immunofluorescence micrographs of cells treated with rabbit anti-rat renin and TRITC-labeled goat anti-rabbit γ-globulin. (×2902.)

Effect of ANP on the Cultured Cells. Fig. 3 shows the effect of ANP on the spontaneous renin release from the cultured cells. It can be seen that ANP strongly inhibited renin release in a dose-dependent fashion between 10^{-13} and 10^{-9} M. In parallel with the inhibition of renin release, ANP led to an increase in intracellular cGMP levels (Fig. 4). To find out whether or not the inhibitory effect of ANP on renin release is causally linked to the increase in intracellular cGMP, we examined the effects of ANP on renin release and cGMP levels in presence of the cGMP phosphodiesterase inhibitor M&B 22948 (14) and the guanylate cyclase inhibitor methylene blue (15). Fig. 4 shows that M&B 22948 (1 mM) shifted the dose–response curves for renin release and cGMP levels to concentrations of ANP lower by a factor of 100. Methylene blue (10 μM) on the other hand decreased the ANP sensitivity of renin release and cGMP levels of juxtaglomerular cells by a factor of 100. Nitroprusside also elevates intracellular cGMP levels by stimulating soluble guanylate cyclase (14, 16). We, therefore, tested the effect of sodium nitroprusside on renin release and cGMP levels of the cultured juxtaglomerular cells. We found that 1 μM sodium nitroprusside decreased renin release to 54 ± 10% of control (n = 5) and elevated cGMP levels to 154 ± 9% of control (n = 5).

Because there is strong evidence that an increase in the intracellular calcium concentration is an inhibitory signal for renin release from juxtaglomerular cells (8, 17, 18), we

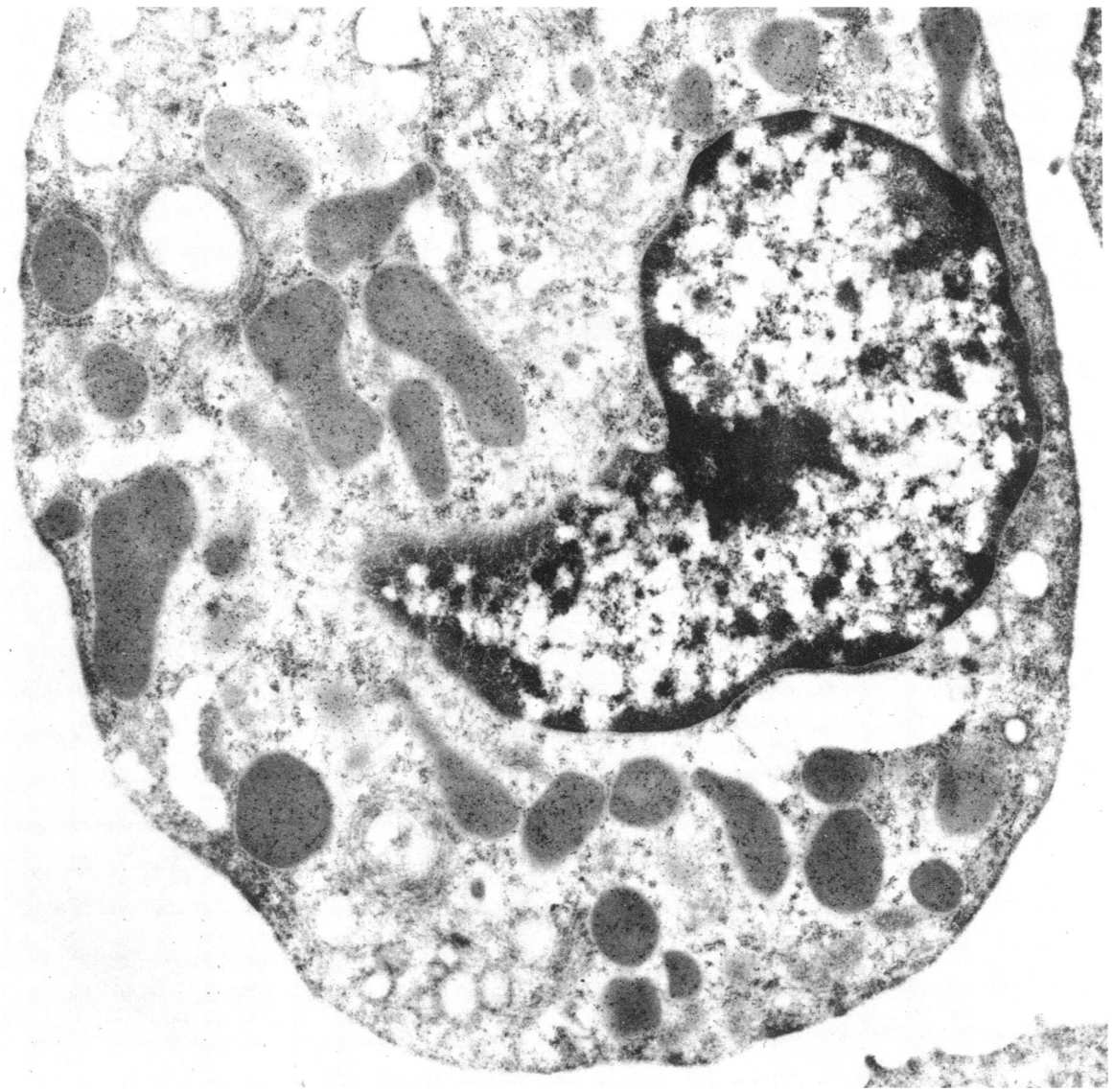

FIG. 2. Electron micrograph of isolated epitheloid cells with mature secretory granules labeled with antiserum against rat renin. All granules are intensely labeled. (×15,800.)

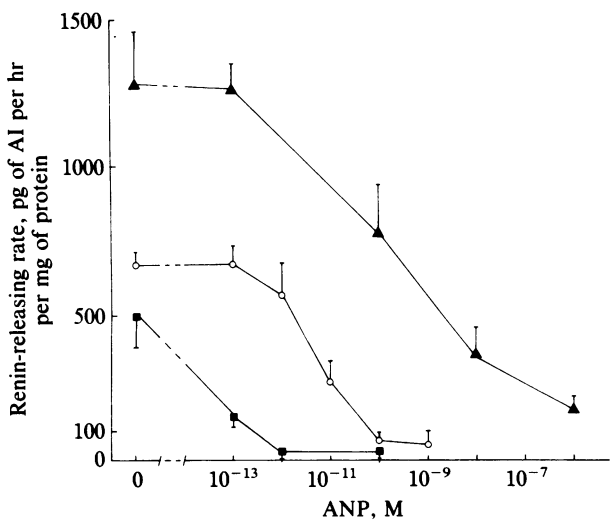

FIG. 3. ANP concentration dependence of renin-releasing rate from the cultured cells. ANP alone (○) or ANP in the presence of 1 mM M&B 22948 (■) or in the presence of 10 μM methylene blue (▲). Data are mean ± SEM of 10 experiments.

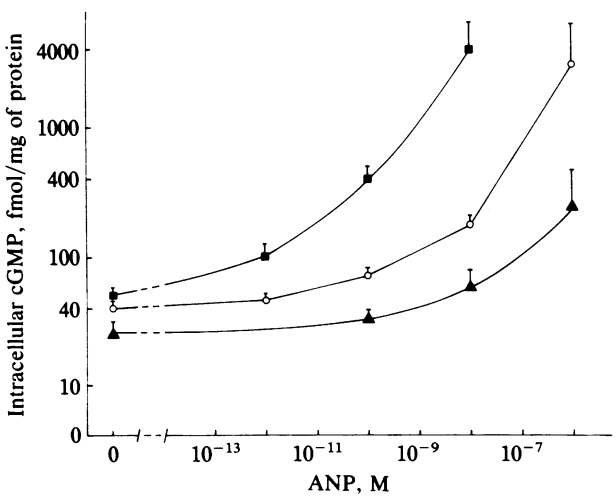

FIG. 4. ANP concentration dependence of cellular cGMP content of the cultured cells. ANP alone (○) or ANP in the presence of 1 mM M&B 22948 (■) or in the presence of 10 μM methylene blue (▲). Data are mean ± SEM of six experiments.

wanted to find out whether or not ANP influences calcium metabolism in cultured juxtaglomerular cells. We, therefore, studied the effect of ANP on the influx of $^{45}Ca^{2+}$ into cells. The data shown in Table 2 provide strong evidence that ANP (0.1 nM) does not stimulate calcium influx into the cells. AII (0.1 μM) on the other hand, which was used as control, significantly increased calcium influx. We, furthermore, examined the effect of ANP on $[Ca]_i$ as monitored by the quin-2 signal. Fig. 5 shows one typical recording out of five. In no instance did ANP (0.1 nM) alter the quin-2 signal, while AII (0.1 μM) regularly increased $[Ca]_i$ to 126 ± 3% of control (n = 5).

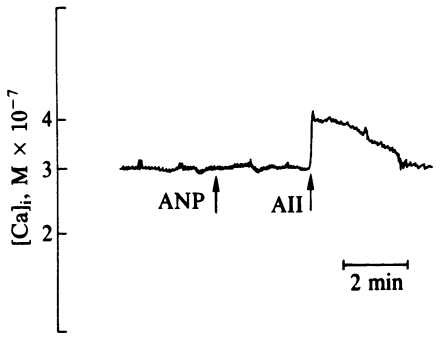

FIG. 5. Quin-2 fluorescence of the cultured cells after addition of synthetic 100 pM ANP and 100 nM AII. $[Ca^{2+}]_i$ is on a logarithmic scale. Arrows indicate addition of agents.

DISCUSSION

There is evidence to indicate that ANP inhibits renal renin release (3–5). Since the mechanism of this inhibitory effect is unclear, the present study examined whether or not ANP acted directly on juxtaglomerular cells and if so which intracellular events mediated the inhibition of renin release. For our study we used cell cultures containing 80–90% cells that stained positive for renin (Fig. 1) and that contained prominent renin granules (Fig. 2). Since juxtaglomerular cells are the only renal cells that have these two characteristics, we infer from our results that the cells we used in the present culture originate from juxtaglomerular cells.

We found that synthetic ANP, identical to human α-ANP (19), strongly inhibited renin release from the juxtaglomerular cells (Fig. 3). Half-maximal inhibition could be observed at 10 pM ANP. It is noteworthy in this context that the normal plasma concentration of ANP is around 10 pM in humans and rats (6, 7, 20, 21).

During volume expansion ANP levels were found to increase up to 0.1 nM (6, 7). It appears, therefore, that the concentration range in which the ANP can be physiologically varied is well within the concentrations at which we observed inhibition of renin release (Fig. 3). Therefore, ANP is likely to have a physiologically regulatory function on renin release from the kidney. Moreover, it has been shown that half-maximal inhibition of aldosterone secretion from adrenal cells by ANP is observed at 20 pM ANP (22). This figure is very close to the results obtained in this study.

We have obtained three pieces of evidence for a causal link between cGMP and inhibition of renin release by ANP. (*i*) ANP elevates cGMP levels, and this effect parallels the inhibition of renin release (Fig. 4). (*ii*) Inhibition of the cGMP-specific phosphodiesterase (by M&B 22948) leads to an apparent increase of the ANP sensitivity of juxtaglomerular cells for the inhibition of renin release and increase of cGMP levels. Inhibition of guanylate cyclase by methylene blue on the other hand lowers the ANP sensitivity of juxtaglomerular cells for renin release and increase in cGMP levels (Fig. 3). (*iii*) Sodium nitroprusside, which is known to increase cGMP levels in muscle tissues by activating guanylate cyclase (23), also elevated cGMP levels in the juxtaglomerular cells and simultaneously inhibited renin release.

Fig. 6 shows a plot of renin releasing rate vs. intracellular cGMP levels as a function of ANP in the absence and presence of M&B 22948, methylene blue, and sodium nitroprusside. It is evident that the data for cGMP levels and renin-releasing rate fall on the same curve under all conditions tested. This may be taken as a further indication of a causal role for cGMP in the inhibition of renin release from juxtaglomerular cells. It is noteworthy in this connection that a stimulation of particulate guanylate cyclase by atrial natriuretic factor has been demonstrated for a number of different tissues (23–27).

Since an increase in cytosolic Ca is accepted as an inhibitory signal for renin release from juxtaglomerular cells (17, 18), we wanted to find out whether or not the effect of ANP on renin release is initiated by cGMP, which leads eventually to an increase in $[Ca]_i$. However, we failed to obtain any evidence for a stimulation of Ca influx into juxtaglomerular cells by ANP or for an increase in $[Ca]_i$ as measured by the quin-2 method. As a control, AII, that inhibits renin release by increasing $[Ca^{2+}]_i$ (8, 17, 18) increased both the Ca influx (Table 2) and $[Ca^{2+}]_i$ (Fig. 5).

From the entirety of our results we conclude that ANP inhibits renin release from juxtaglomerular cells by a mechanism that, at least in isolated juxtaglomerular cells, does not involve an increase in intracellular cGMP and that does not

Table 2. Effects of 0.1 nM ANP and 0.1 μM AII on ^{45}Ca uptake into cultured juxtaglomerular cells

	Rate of ^{45}Ca uptake, cpm per mg of protein		
	30 sec	60 sec	120 sec
Control	1748 ± 372	5778 ± 702	10,110 ± 1500
ANP	2385 ± 468	4539 ± 435	8,172 ± 675
AII	4014 ± 252	8280 ± 300	14,463 ± 750

Data are mean ± SEM of five experiments.

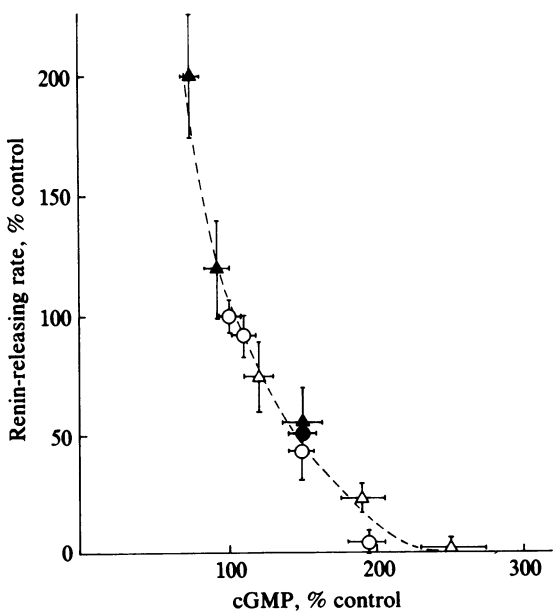

FIG. 6. Renin-releasing rate vs. cellular cGMP content. Renin-releasing rate values are taken from Fig. 2, and cGMP levels are taken from Fig. 3. Data are given as the percentage of the control value, which was obtained in the absence of ANP and agents. ○, ANP without M&B 22948 and methylene blue; △, ANP with 1 mM M&B 22948; ▲, ANP with 10 μM methylene blue; ●, 1 μM sodium nitroprusside.

alter the cellular Ca metabolism. Our results confirm the idea proposed by Freeman *et al.* (28) that ANP has an important physiological role in the control of renin release.

We thank Dr. Spinelli for the kind gift of synthetic ANP and the May & Baker Company for the generous gift of M&B 22948. We are indebted to Dr. Celio for his help with the immunofluorescence procedure. We, furthermore, thank Dr. Inagami and Dr. Hackenthal for anti-rat renin serum. The skillfull technical assistance of Ingrid Weissbrodt and Hans Niederberger is gratefully acknowledged. Moreover, we thank Olga Stoupa for her secretarial help and Werner Gehret for doing the artwork.

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