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Regulation of natriuretic peptide (urodilatin) release in a human kidney cell line

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Regulation of natriuretic peptide (urodilatin) release in a human kidney cell line.

Background. To identify the molecular mechanisms underlying the release of a renal natriuretic peptide (NP) we selected a human kidney cell line (HEK 293) that displays several characteristics of distal tubular cells.

Methods. Cells were exposed to different extracellular and intracellular stimuli, and the effect on NP release was measured with a specific urodilatin radioimmunoassay, as well as with an atrial NP (ANP) radioimmunoassay.

Results. In the absence of stimuli, HEK 293 cells showed a basal release of urodilatin immunoreactivity and ANP immunoreactivity. Raising the osmolality of the secretion medium with sodium chloride and various other osmolytes rapidly increased cellular NP secretion. Elevation of intracellular cAMP levels by forskolin plus 3-isobutyl-1-methylxanthine and administration of phorbol-12-myristate-13-acetate together with the calcium-ionophore A23187 also resulted in respective increases in the amount of secreted peptide. HEK 293 cells exhibit the endogenous expression of both particulate and soluble guanylyl cyclases. In the presence of 8-Br-cGMP, cell cultures showed the enhanced secretion of an ANP immunoreactive peptide only, indicating that guanylyl cyclase activation provoked the secretion of ANP immunoreactivity but not of urodilatin immunoreactivity.

Conclusions. The human embryonic kidney cell line HEK 293 represents a renal cellular model system in which we have identified a rapid and regulated release of NPs in response to the osmotic effect of increased extracellular sodium chloride and various intracellular stimuli.

Natriuretic peptides (NPs) play an important role in the regulation of body fluids, salt excretion, and blood pressure [1, 2]. They constitute a family of structurally related hormones generating a wide spectrum of vascular, renal, and endocrine actions, all of which serve to control body fluid homeostasis. Atrial NP (ANP) was the first isolated member of this peptide family after the observation of natriuretic, diuretic, and vasorelaxant activity in rat atrial extracts [3]. Brain NP (BNP) and C-type NP (CNP) represent additional NPs that were both originally isolated from porcine brain [4, 5]. On a molecular level, these peptides exert their primary effects by binding to cell surface receptors with intrinsic guanylyl cyclase (GC) activity [6]. The extracellular binding of NPs to the receptor GC leads to the intracellular synthesis of the second messenger cGMP. Clearance receptors represent another class of high-affinity cell surface receptors for NPs that lack GC activity and mediate internalization and degradation of bound ligand [7].

In the 1980s, $ANP_{99–126}$ obtained wide attention as an important regulator of natriuresis. Recently, however, we showed in many physiological studies that the renal ANP analogue urodilatin is a more likely candidate for the regulation of sodium excretion, whereas ANP exerts mainly cardiovascular effects [8–11]. Urodilatin was isolated from human urine and was identified as a 32-amino acid peptide consisting of the amino acid sequence 95–126 of the ANP prohormone [12]. The peptide has not been found in plasma [13], suggesting that urodilatin is synthesized, processed, and secreted by the kidney. Studies have provided evidence that an ANP prohormone-like peptide is produced and secreted by primary cultures of neonatal and adult rat kidney cells [14]. With immunohistochemical and immunoassay techniques, it was shown that NPs are synthesized in distal cortical tubular cells [15, 16] and that the synthesis is modulated in some pathophysiological situations in the rat [17, 18]. In human kidney, we have recently found by immunohistochemical analysis that urodilatin is present in distal tubular cells (see note added in proof). There have also been reports that demonstrated the renal expression of the gene for ANP in rat kidney, although the ANP mRNA is present only at a very low abundance in rat kidney tissue [18, 19]. All of these observations together suggest that urodilatin is probably produced by distal cortical tubular cells and is secreted luminally into the urine to induce natriuresis by interaction with receptor GCs, localized on inner medullary collecting duct cells [20].

In addition to the described NP-sensitive particulate GCs, soluble GCs (sGC) represent related cGMP-producing enzymes, which are located in the cytoplasm of cells and are activated by nitric oxide (NO). Recent

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studies indicate that the NO/sGC/cGMP system, in addition to the NP/particulate GC system, plays an important role in the regulation of salt and water transport in the kidney [21].

Currently, only little information is available concerning the extracellular and intracellular signals involved in the control of NP generation and secretion in the kidney. While the previously mentioned studies showed a close correlation between natriuresis and urodilatin secretion, the regulatory mechanisms underlying the peptide synthesis and peptide release at the molecular level remained uncertain. We therefore established and characterized the human kidney cell line HEK 293 as a kidney cell model system in order to study the regulation of renal NP secretion at the molecular level and to identify factors that are involved in this regulation.

METHODS

Materials

Cell culture media, fetal calf serum, and antibiotics were purchased from Gibco BRL (Eggenstein, Germany). Synthetic human ANP, human urodilatin, human BNP, and human CNP were obtained from Peninsula (St. Helens, UK). Human guanylin was from Bachem (Heidelberg, Germany), and human uroguanylin was from Immundiagnostik (Bensheim, Germany). [^{125}I]-iodo-human $ANP_{99–126}$ (specific activity 2000 Ci/mmol) was obtained from Amersham (Braunschweig, Germany). Sodium nitroprusside (SNP) was purchased from Merck (Darmstadt, Germany). All other chemicals were from Sigma (Deisenhofen, Germany).

Cell culture

Human embryonic kidney (HEK 293) cells were grown in Dulbecco's modified Eagle's medium/Nutrient Mix F12 (1:1) supplemented with 10% fetal calf serum, penicillin G (100 U/ml) and streptomycin sulfate (0.1 mg/ml) in a humid atmosphere consisting of 95% air and 5% CO_2 at 37°C. For experiments, cells were grown to near confluence on six-well plastic cell culture plates (approximately 10^6 cells per well). Plates had been precoated with poly-L-lysine (Mr > 300,000; 20 μg per well) to facilitate attachment of the cells to the plastic surface.

Secretion of natriuretic peptides from HEK cells

Cell culture medium from cells on six-well plastic cell culture plates (for each manipulation 3–6 wells) was aspirated. Then cells were washed twice with 2 ml prewarmed secretion-medium (140 mM NaCl, 4.3 mM KCl, 2.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.08 mM KH_2PO_4, 5.1 mM glucose, 0.3% bovine serum albumin, pH 7.4) and were subsequently incubated at 37°C in 1 ml of the same medium in the presence of 10 μM phosphoramidon, 10 μM amastatin, 2 μg/ml aprotinin and 0.2 nM rat Des-(gln^{18}, ser^{19}, gly^{20}, leu^{21}, gly^{22})-fragment 4–23 amide (C-$ANP_{4–23}$) in order to prevent a loss of the immunoreactive signal caused by proteolytic degradation or binding of secreted peptides to NP clearance receptors. Multiple additions of these substances every 10 minutes were required because NP immunoreactivity soon declined after an initial rise in single dose experiments. Cell culture supernatants were harvested by aspirating and were transferred into prechilled plastic tubes. The tubes were stored at –20°C until the determination of NP immunoreactivity in the supernatants. Cellular protein content was determined by the Lowry method [22] and averaged between 0.9 and 1.6 mg per well.

Radioimmunoassays

Urodilatin was measured by radioimmunoassay with a specific urodilatin antibody displaying no cross-reactivity with ANP ($ANP_{99–126}$), BNP, CNP, ANP prohormone, or shorter ANP analogues, as described before [13]. Because of limited availability of this specific urodilatin antibody and the fact that alternative commercial urodilatin antibodies could not measure urodilatin in cell supernatants because of matrix problems, only key experiments were performed using the urodilatin antibody. ANP immunoreactivity was determined by radioimmunoassay with a commercial ANP antibody (Amersham), which showed 100% cross-reactivity with synthetic human urodilatin.

Determination of intracellular cGMP production

Cell culture medium from cells on six-well plastic cell culture plates was aspirated, and cells were washed twice with prewarmed serum free Dulbecco's modified Eagle's medium/Nutrient Mix F12 (1:1) without Phenolred, supplemented with 15 mM N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid. Next, cells were preincubated in the same medium, supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) for 15 minutes at 37°C. After that, the respective test substances were added to the cell medium, and the plates were incubated for different times at 37°C (3–6 wells for each manipulation). Reactions were terminated by aspirating the cell culture medium followed by the addition of 5% trichloroacetic acid (TCA) to the wells. Wells were then scraped, and TCA suspensions were separated in pellets and cell extracts by centrifugation. TCA extracts were re-extracted with water-saturated diethylether and were afterward lyophilized. The cGMP contents of TCA extracts were determined after reconstitution of samples in 50 mM sodium acetate buffer (pH 5.5) by radioimmunoassay as described before [23]. Cellular protein content was determined after solution of cell pellets in 0.8 m NaOH by the Lowry method [22].

Cell characterization: Determination of intracellular cAMP accumulation

Cell cultures on six-well plastic cell culture plates were washed and preincubated in the same way as for the determination of cGMP levels. Respective test substances were then added to the cell medium, and the plates were incubated for 10 minutes at 37°C (3–6 wells for each manipulation). Determinations were proceeded including the lyophilization of cell extracts as described for the determination of cGMP (earlier here). Lyophilized samples were reconstituted in 0.2 M phosphate buffer containing 0.1% bovine serum albumin, 0.1% Tween 20, and 0.025% Thimerosal (pH 7.25) and were subsequently succinylated using 11% succinic anhydride in a 25:9 mixture of acetone/trimethylamine. According to a described nonisotopic immunoassay for cGMP [24], cAMP-contents of samples were determined in 100 μl aliquots on microtiter plates with a newly developed fluorescence immunoassay using a polyclonal goat anti-cAMP antibody, rabbit antigoat antiserum (Dako, Hamburg, Germany) biotinylated cAMP as tracer, europium-labeled streptavidin (Wallac, Freiburg, Germany) and enhancement solution for detection [25]. The EC_{50} of the assay was 3.92 ± 0.69 nM (mean ± SEM).

Cell characterization: Determination of alkaline phosphatase activity

Alkaline phosphatase activity was determined in HEK 293 membranes using p-nitrophenyl phosphate as a substrate in an automated analysis (Hitachi system 704; Boehringer Mannheim, Mannheim, Germany).

Statistical analyses

Experimental data represent means ± SEM of at least three determinations performed in triplicate. Results from time-course experiments were analyzed statistically by two-way analysis of variance (ANOVA) for repeated measures followed by the one-tailed Student's *t*-test for paired values as post hoc test. *P* values of ANOVA given in the results section indicate the significance of the combined effects of treatment on time. Other data were evaluated by the one-tailed Student's *t*-test for unpaired values. $P < 0.05$ was considered significant.

RESULTS

The epithelioid human kidney cell line HEK 293 showed a basal secretion of a renal NP-like substance into cell culture supernatants, which was measured by both a specific urodilatin and an ANP radioimmunoassay (Fig. 1). The repeated addition of protease inhibitors to the secretion medium significantly (ANOVA, $P < 0.001$) increased the immmunoreactive signal, suggesting proteolytic activities for NPs in HEK 293 cells. When tested individually, each of the inhibitors exerted a protective effect with a maximal 1.4-, 2.1-, and 2.2-fold increase of NP immunoreactivity for phosphoramidon, amastatin, and aprotinin, respectively. Additionally, incubation of cells with the NP clearance receptor ligand C-ANP_{4-23} also resulted in an increase in the immmunoreactive signal (twofold), indicating the expression of NP clearance receptors and probably binding of secreted peptide to these binding sites. In the indicated concentrations, none of the applied substances showed interference with the urodilatin radioimmunoassay or ANP radioimmunoassay, although C-ANP_{4-23} displayed cross-reactivity with the ANP radioimmunoassay when used in higher concentrations. Surprisingly, the combination-induced effect lasted only for few minutes, which may have been due to an inactivation of the protective substances themselves, suggesting the requirement for a repeated addition of the protective combination. This assumption was supported in our experiments in which substances were added every 10 minutes, and subsequent experiments were performed using the multiple addition of phosphoramidon, amastatin, aprotinin, and C-ANP_{4-23} as the control condition giving a 3.4-fold increase of signal as maximal effect.

Incubation of the cells with secretion medium containing 200 mM NaCl induced a significant increase in the amount of released urodilatin immunoreactivity in comparison to control cells incubated with medium containing 140 mM NaCl (Fig. 2; ANOVA, $P < 0.01$). To evaluate whether this effect was caused either by an osmotic mechanism of increased extracellular sodium chloride or by other actions specific for sodium and chloride ions, we tested various secretion media, each composed of 140 mM NaCl plus either 60 mM NaCl, 60 mM choline chloride, 60 mM N-methyl-D-glucamine, 60 mM sodium gluconate, or 120 mM mannitol. Figure 3 demonstrates that each of the used osmolytes was able to increase urodilatin secretion significantly when compared with control, suggesting that the effect of 200 mM NaCl was due to an increased medium osmolality. The urodilatin radioimmunoassay appeared to be slightly influenced by higher osmolyte concentrations. Presented data from cell culture supernatants were corrected for this unspecific immunoreactivity.

As shown in Figure 4, forskolin (10 μM) in combination with 100 μM IBMX as well as phorbol-12-myristate-13-acetate (PMA; 100 nM) together with the calcium-ionophore A23187 (0.5 μM) were able to stimulate the release of immunoreactive material from HEK 293 cells after five minutes of incubation. This stimulatory effect was also apparent after 15 minutes, but in contrast, no significant difference in comparison to control cells could be detected at longer incubation times (30 and 60 min; data not shown). Figures 1 and 4 also demonstrate that the used urodilatin immunoassays and ANP immunoassays measured rather comparable amounts of secreted

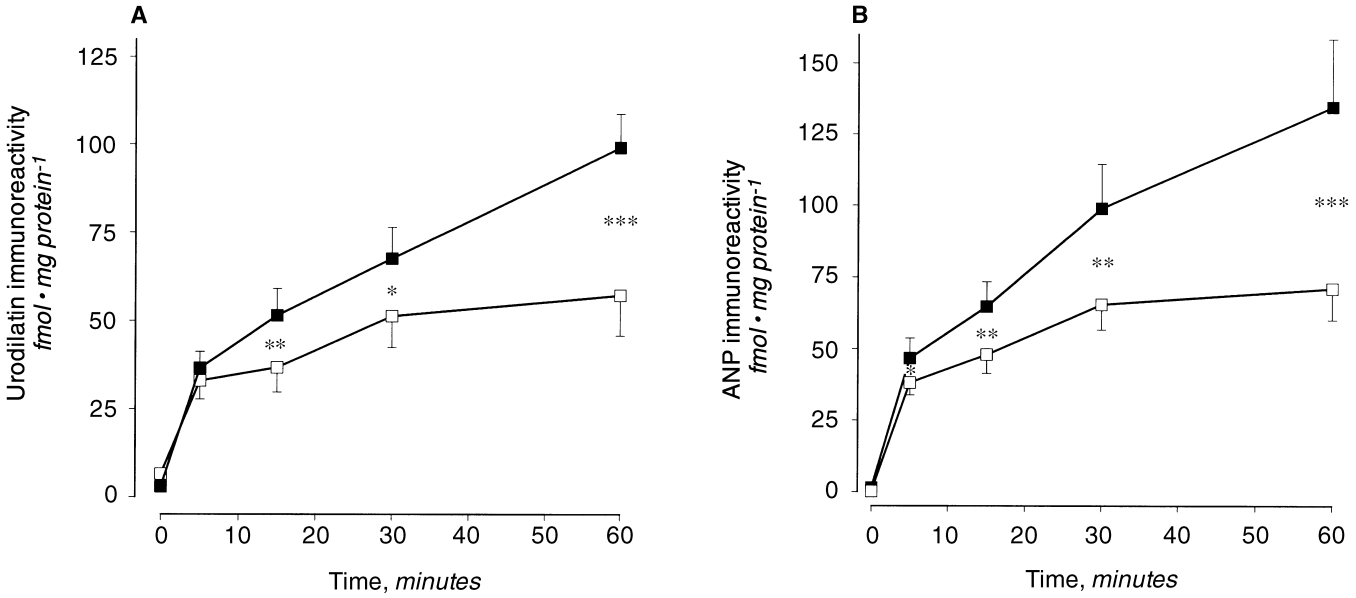

Fig. 1. Secretion of NP-immunoreactivity from HEK 293 cells. Cell cultures were incubated for the indicated times at 37°C with secretion medium in the absence (□) or presence (■) of 10 μM phosphoramidon, 10 μM amastatin, 2 μg ml^{-1} aprotinin and 0.2 nM C-ANP_{4-23}. Addition of solvent (□) or the combination of phosphoramidon, amastatin, aprotinin and C-ANP_{4-23} (■) in the above indicated final concentrations was repeated every 10 minutes. Immunoreactive peptide was determined in cell culture supernatants with (*A*) an urodilatin-specific and (*B*) with an ANP-radioimmunoassay. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

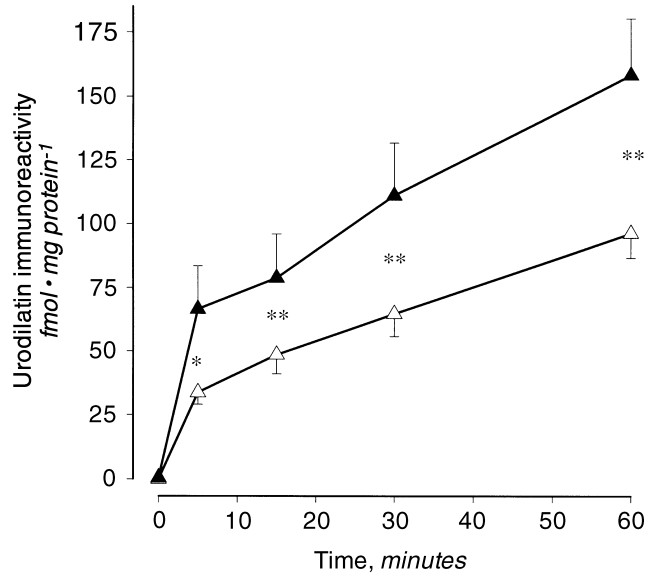

Fig. 2. Effect of sodium chloride on urodilatin secretion in HEK 293 cells. Cell cultures were incubated for the indicated times at 37°C with secretion medium containing either 140 mM NaCl (△) or 200 mM NaCl (▲). Incubations were performed in the presence of 10 μM phosphoramidon, 10 μM amastatin, 2 μg ml^{-1} aprotinin and 0.2 nM C-ANP_{4-23}. Urodilatin was determined in cell culture supernatants with a urodilatin specific radioimmunoassay. $*P < 0.05$; $**P < 0.01$.

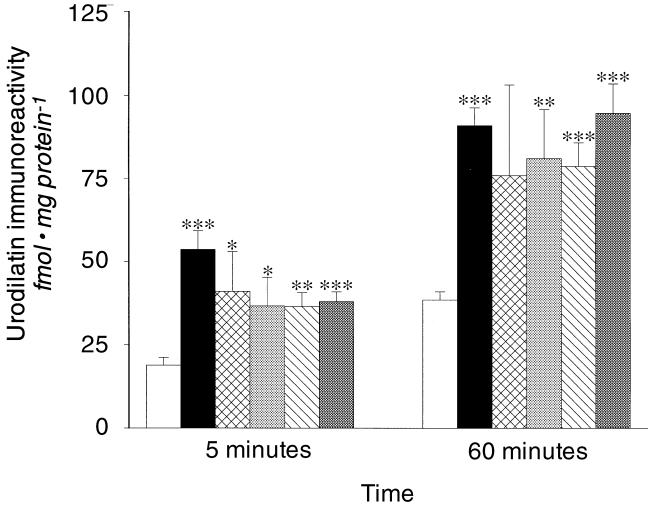

Fig. 3. Stimulation of urodilatin secretion by hyperosmolality. Cell cultures were incubated for 5 and 60 minutes at 37°C with secretion media containing 140 mM NaCl (□) plus either 60 mM NaCl (■), 60 mM choline chloride (▨), 60 mM N-methyl-D-glucamine (▩), 60 mM sodium gluconate (▧) or 120 mM mannitol (▩). Incubations were performed in the presence of 10 μM phosphoramidon, 10 μM amastatin, 2 μg ml^{-1} aprotinin and 0.2 nM C-ANP_{4-23}. Urodilatin was determined in cell culture supernatants with a urodilatin specific radioimmunoassay. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

immunoreactive substances, indicating that untreated cells and cells challenged with the applied stimuli secrete urodilatin rather than ANP_{99-126} or other ANP derivatives.

In HEK 293 cells, the expression of NP-sensitive GCs could be demonstrated by the stimulation of intracellular cGMP accumulation in response to different NP receptor ligands. Figure 5A shows the dose–response curves for

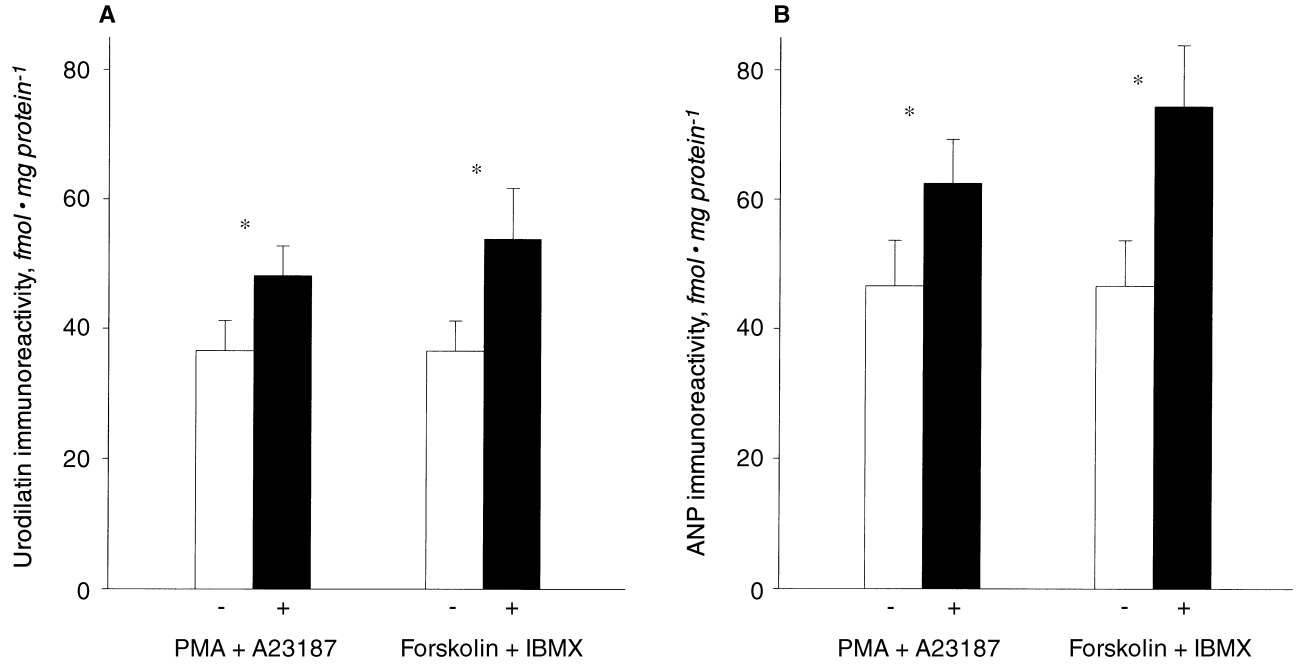

Fig. 4. Regulation of NP secretion from HEK 293 cells. Cells were incubated for 5 minutes at 37°C in the absence (□) or presence of either 100 nM PMA/500 nM A23187 or 10 μM forskolin/100 μM IBMX (■). Incubations were performed in the presence of 10 μM phosphoramidon, 10 μM amastatin, 2 μg ml^{-1} aprotinin and 0.2 nM C-ANP_{4-23}. Urodilatin (*A*) and ANP (*B*) immunoreactivities were determined in cell culture supernatants with ANP and urodilatin radioimmunoassays, respectively. $*P < 0.05$

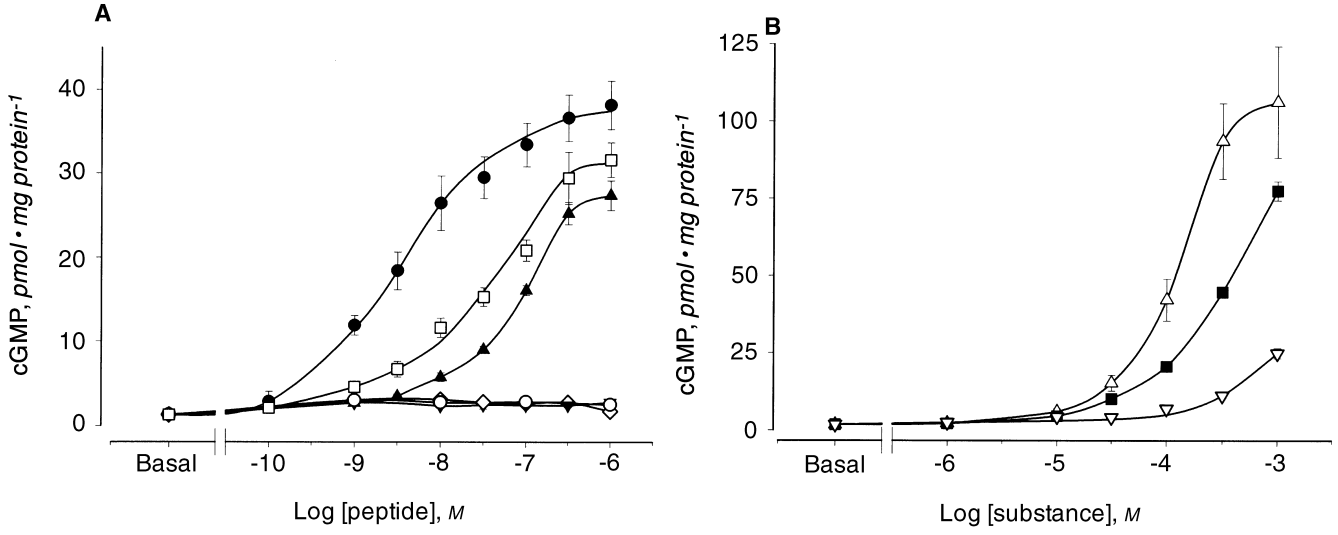

Fig. 5. Stimulation of cGMP accumulation in HEK 293 cells. (*A*) Cells were preincubated for 15 minutes with 0.5 mM IBMX and then treated for 30 minutes with solvent (Basal) or with the indicated concentrations of ANP (●), urodilatin (□), BNP (▲), CNP (◇), guanylin (○) or uroguanylin (▼). (*B*) Cells were preincubated for 15 minutes with 0.5 mM IBMX and then treated for 5 minutes with solvent (Basal) or with the indicated concentrations of YC-1 (△), SNP (■) or SIN-1 (▽). Intracellular cGMP levels were determined as described in the methods.

ANP_{99-126}, urodilatin, BNP, CNP, guanylin, and uroguanylin. Whereas ANP_{99-126}, urodilatin, and BNP elicited a dose-dependent increase of cGMP levels with EC_{50}-values of 3.4 ± 0.2 nM, 45.5 ± 1.1 nM, and 92.9 ± 23.1 nM, respectively, CNP, guanylin, and uroguanylin were without any effect. These results indicate that HEK 293 cells exhibit the endogenous expression of GC type A, which is stimulated by ANP_{99-126}, urodilatin, and BNP. The EC_{50} value for ANP_{99-126} was found to be significantly lower when compared with the EC_{50} values for urodilatin and

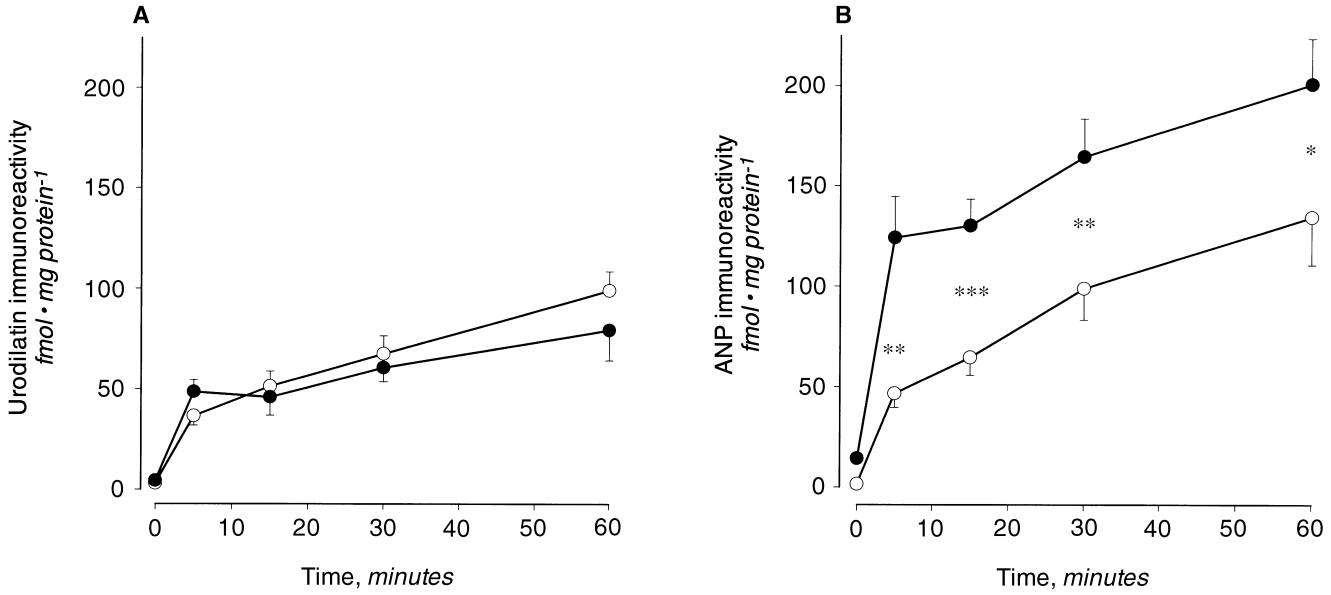

Fig. 6. Effect of 8-Br-cGMP on NP secretion in HEK 293 cells. Cells were incubated for the indicated times at 37°C with solvent (○) or with 1 mM 8-Br-cGMP (●). Incubations were performed in the presence of 10 μM phosphoramidon, 10 μM amastatin, 2 μg ml^{-1} aprotinin and 0.2 nM $C\text{-}ANP_{4\text{–}23}$. Urodilatin- (*A*) and ANP- (*B*) immunoreactivities were determined in cell culture supernatants with ANP and urodilatin radioimmunoassays, respectively. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

BNP, respectively ($P < 0.01$; *t*-test). Neither GC type B, which is specifically activated by CNP, nor the guanylin- and uroguanylin-sensitive GC type C appear to be present in HEK 293 cells. In order to test for the presence of sGC, HEK 293 cells were incubated with the NO-donors SNP, SIN-1, and YC-1, a novel NO-independent stimulator of sGC (Fig. 5B). YC-1 was shown to be most effective displaying an EC_{50} of 0.13 ± 0.013 mM and a 59-fold stimulation of intracellular cGMP accumulation over basal at a concentration of 1 mM. SNP and SIN-1 both stimulated cGMP accumulation to a lesser extent; maximal stimulations seen with concentrations of 1 mM were 43-fold for SNP and 14-fold for SIN-1.

To examine whether the activation of GCs plays a role in the regulation of renal NP secretion, HEK 293 cells were treated with a cell-permeable cGMP analogue or with the NO donor SNP. 8-Br-cGMP (1 mM) rapidly enhanced the amount of secreted ANP immunoreactivity, and this stimulatory effect could be observed over a total incubation time of 60 minutes (Fig. 6B; ANOVA, $P < 0.01$). SNP (1 mM) was likewise able to induce a comparable 1.9-fold increase of the released ANP immunoreactivity. Surprisingly, no significant increase of immunoreactive material could be detected in the same experiments with the urodilatin-specific radioimmunoassay (ANOVA, $P = 0.172$), suggesting that 8-Br-cGMP or SNP stimulated the release of ANP (Fig. 6B), but not of urodilatin (Fig. 6A). The ANP radioimmunoassay was shown to be slightly influenced by 8-Br-cGMP, giving a background signal of approximately 14 fmol/mg protein, and values were thus corrected for this unspecific immunoreactivity.

HEK 293 cells were also characterized with regard to the retention of properties specific for renal tubular cells. Cell cultures were investigated for their ability to respond to various renal-acting hormones with an elevation of intracellular cAMP levels. Compared with the basal level (12.7 ± 1.9 pmol/mg protein), marked stimulation of cellular cAMP production was observed in response to 10 μM forskolin (324.6 ± 32.2 pmol/mg protein), 1 μM vasoactive intestinal peptide (VIP; 841.0 ± 67.2 pmol/mg protein), and 10 μM isoproterenol (248.0 ± 23.8 pmol/mg protein). These results suggest the presence of both VIP- and isoproterenol-sensitive adenylyl cyclases in HEK 293 cells, which were shown previously to be restricted to distal nephron sites [26, 27]. Parathyroid hormone at 1 μM elicited a 2.4-fold increase (30.6 ± 1.1 pmol/mg protein) over basal cAMP levels, whereas 1 μM [Arg^{8}]vasopressin (AVP) had no effect (8.9 ± 0.6 pmol/mg protein), which is—in contrast to the AVP-responsive collecting duct—indicative in particular for distal convoluted tubules of the human nephron [26]. HEK 293 cells were further characterized in view of the expression of alkaline phosphatase, which represents an enzyme typically found in the brush border region of proximal tubular cells. In prepared membranes from HEK 293 cells, a low level alkaline phosphatase activity of 15 ± 3 mU/mg protein was measured, whereas isolated cultured human proximal tubular cells showed an activity of 114 ± 29 mU/mg protein [28].

DISCUSSION

In this study, we have presented a human kidney cell line (HEK 293 cells) as a cellular model system, displaying synthesis, release, and elimination of a renal NP. This suggests that the human kidney may be able to regulate the intrarenal NP processing in response to certain extracellular and intracellular stimuli such as elevation of the extracellular sodium chloride concentration, activation of adenylyl cyclase, protein kinase C (PKC), and increasing intracellular calcium concentrations in order to maintain the water and electrolyte homeostasis.

The addition of a combination consisting of the specific neutral endopeptidase inhibitor phosphoramidon, the aminopeptidase inhibitor amastatin, and the protease inhibitor aprotinin to the secretion medium resulted in an increase of NP immunoreactivity in cell culture supernatants, suggesting proteolytic activities for NPs in HEK 293 cells. The NP clearance receptor ligand C-ANP$_{4-23}$ was also able to elevate the immunoreactive signal when added to the culture medium. This finding suggests the existence of non–GC-coupled NP receptors on the surface of HEK 293 cells and probably binding of secreted peptide to these binding sites, thus reducing the amount of detectable peptide in the supernatant. Dynamic regulation of both the proteolytic activity and the number of clearance receptors may provide additional mechanisms by which bioactivity of secreted peptides is modulated. Further studies are required to identify in HEK 293 cells the exact mechanisms of NP-immunoreactivity decrease and the involved proteins.

Recent physiological studies have provided evidence that urodilatin secretion is very closely correlated with renal sodium excretion under various conditions [8–11]. This study supports the assumption of an interaction between urodilatin release and renal sodium handling, showing that an elevation of the extracellular sodium chloride concentration increased the secretion of urodilatin immunoreactive material from the cultured kidney cells. Further evidence for an influence of the extracellular sodium chloride concentration on NP release was reported in a recent study that showed that on the other hand, a reduction of the sodium chloride concentration from a normal (140 mM) to a subphysiological level (120 mM) decreased urodilatin secretion from HEK 293 cells [29]. Our results with various other osmolytes suggest that the effect of 200 mM NaCl was due to an increased osmotic pressure on the cells. Because a hyperosmotic cellular environment will induce water transport from the intracellular site to the extracellular medium, it may also represent a physiological stimulus for the transport of NP from internal to external sites of the cell.

The molecular mechanisms linking extracellular signals to NP release in the kidney are not known. For that reason, we studied the role of different second-messenger systems in the regulation of renal NP secretion. It was found that both a stimulator of PKC (PMA) in combination with the calcium-ionophore A23187, as well as the activation of adenylyl cyclase with subsequent rise of intracellular cAMP levels induced by forskolin and IBMX, were able to stimulate the rapid release of immunoreactive material from HEK 293 cells. In comparison to this, PMA and A23187 were also shown to stimulate ANP secretion in the perfused spontaneously beating rat heart [30], suggesting a common regulatory role of PKC and intracellular calcium in renal as well as in cardiac cells. Additionally, the intracellular cAMP level may likewise be involved in modulating the release of NPs from both the kidney cells and the atrial cardiocytes as has been demonstrated by the ability of forskolin to increase the ANP secretion from the perfused rat heart [31]. Furthermore, it was shown in rat adult renal cortical cell cultures that after a 24 hour treatment, PMA or forskolin had either no effect or even decreased the release of an ANP-like peptide in the culture medium [15]. Consistently, the observed stimulatory effects with PMA or forskolin in this study were apparent only after relatively short incubation times of 5 and 15 minutes, whereas longer incubation times (30 and 60 min) did not display any effect.

Because the stimulatory effects of elevated sodium chloride, as well as of PMA/A23187 and forskolin/IBMX, could already be observed after five minutes of incubation, it seems very unlikely that the increase of secreted peptide is due to enhanced gene expression and *de novo* protein synthesis. More probably, HEK 293 cells appear to be capable of storing a certain amount of NPs in an intracellular compartment prior to the release in response to an appropriate stimulus. Alternatively, it may be that certain transport processes of the constitutive secretion pathway become enhanced after administration of respective stimuli, resulting in the observed increase in peptide secretion. Other studies are in contrast to the first hypothesis, showing little or no storage capacity for a renal NP-like protein in neonatal rat kidney cells and accordingly favoring a constitutive secretion of peptides from these cells [14]. In this work, however, only secretion studies of 5 to 24 hours were performed, whereas the results from short-term incubations in the range of 5 to 60 minutes were not reported. With respect to time, our findings do not exclude the possibility of dual regulatory mechanisms underlying the secretion of renal NPs at early and late incubation times, respectively. The observed increase of secreted urodilatin immunoreactivity seen after a 60-minute treatment with 200 mM NaCl may therefore be composed of an enhanced rapid secretion from intracellular stores plus an enhanced synthesis, followed by a constitutive secretion of peptide.

Beside the obvious synthesis and secretion of a renal

NP, HEK 293 cells seem to express receptors for these ligands, therefore displaying characteristics of autocrine cells. Our results suggest that HEK 293 cells express functional GC-coupled receptors, namely GC type A but neither type B nor type C, which are considered to be activated by CNP and the guanylins, respectively. Many studies have shown that $ANP_{99–126}$ and urodilatin act through their receptor with equivalent potencies. In this work, however, urodilatin displayed an approximate 10-fold higher EC_{50} in its potency to stimulate cGMP accumulation than $ANP_{99–126}$. This may have been due to a post-translational modification of the NP receptor molecule, as has been shown previously [32]. As we consider the HEK 293 cell line to be primarily related to NP-secreting distal cortical tubular cells (see below), the comparison of HEK 293 EC_{50}-values may not reflect the conditions at the *in vivo* target cells for urodilatin, which are thought to be localized mainly in the medullary collecting duct. By incubating HEK 293 cells with the NO donors SNP and SIN-1 as well as with the novel benzyl indazole derivative YC-1, it was shown that the HEK 293 cell line expresses sGC as another cGMP-generating enzyme. To investigate the involvement of cellular GCs in the regulation of renal NP secretion, 8-Br-cGMP was applied to the cells as a membrane-permeable analogue of the second messenger cGMP. The analogue was shown to increase the amount of secreted ANP immunoreactivity from HEK 293 cells, indicating that GC activation may be involved in the regulation of renal NP release. Because SNP was found to be comparably effective to 8-Br-cGMP, it can be concluded that the NO-stimulated sGC represents a system that is able to modulate the secretion of NPs in the kidney. The extent to which the stimulation of particulate GCs will exert similar effects remains to be examined in future studies.

In contrast to the results obtained with the ANP radioimmunoassay, no increased immunoreactive material could be detected in the experiments using 8-Br-cGMP or SNP when measured with the urodilatin-specific radioimmunoassay, although both radioimmunoassays detected rather comparable amounts of immunoreactivity in all other incubations. The reason for this observation is not yet clear. It may have been due to a parallel activation of the atrial-like processing of the ANP propeptide in response to 8-Br-cGMP or SNP leading to a secretion of $ANP_{99–126}$ instead of urodilatin. Alternatively, it may be that these incubations induced a modification of the immunogenic epitope required for the recognition of the antigen by the urodilatin antibody.

The HEK 293 cell line has been shown to display several features of renal distal tubular cells, a presumed major site of renal NP localization. Characteristics are epithelial nature of cultures, formation of apical *zonae occludentes*, and absence of a brush border [33]. Additionally, the cell line is derived from renal cortical cells, which also agrees well with the reported localization of ANP or urodilatin synthesizing cells [16]. In this study, we showed that HEK 293 cells exhibit a VIP-sensitive and an isoproterenol-sensitive adenylyl cyclase, which were both shown previously to be restricted to distal nephron sites [26, 27]. Because the cell line appears to be completely unresponsive to AVP and this effect has also been reported for the human distal convoluted tubule [26], it seems very likely that HEK 293 cells may be derived from this part of the nephron and that the cells display the retention of both the unresponsiveness to vasopressin and the secretion of a renal NP. The observation that HEK 293 cells contain only low levels of alkaline phosphatase activity in comparison to cultured human proximal tubular cells additionally supports the distal character of HEK 293 cells.

In summary, we have demonstrated that HEK 293 cells secrete a renal NP-like substance that displays urodilatin immunoreactivity. The release of this urodilatin-like substance was shown to be modulated in response to various extracellular and intracellular stimuli, including the stimulation by a hyperosmotic concentration of sodium chloride. Furthermore, the cell line appears to be capable of metabolizing or eliminating secreted NPs. Therefore, HEK 293 cells may represent an *in vitro* model for a regulated intrarenal processing of NPs in the kidney, which may then exert its autocrine or paracrine effects on renal electrolyte and water excretion.

NOTE ADDED IN PROOF

Herten M, Lenz W, Gerzer R, Drummer C: The renal natriuretic peptide urodilatin is present in human kidney. *Nephrol Dial Transplant* 13:2529–2535, 1998

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APPENDIX

Abbreviations used in this article are: ANP, atrial natriuretic peptide; AVP, [Arg^8]vasopressin; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; GC, guanylyl cyclase; IBMX, 3-isobutyl-1-methylxanthine; NO, nitric oxide; NP, natriuretic peptide; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; sGC, soluble GC; SNP, sodium nitroprusside; TCA, trichloroacetic acid; VIP, vasoactive intestinal peptide.

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