Kidney International, Vol. 51 (1997), pp. 281–287

Receptors for natriuretic peptides in a human cortical collecting duct cell line

VALERIE MILLUL, NICOLE ARDAILLOU, SANDRINE PLACIER, BÉATRICE BAUDOUIN, and PIERRE M. RONCO

INSERM U64, Hôpital Tenon, Paris, France

Receptors for natriuretic peptides in a human cortical collecting duct cell line. The aim of the present study was to analyze the expression of natriuretic peptide receptors in human collecting duct, by using a newly established SV40 cell line (HCD). ANP and C-type natriuretic peptide (CNP) induced a concentration-dependent increase in cGMP suggesting the presence of type-A (NPR-A) and type-B (NPR-B) receptors, respectively. Threshold concentrations were 1 pM and 1 nM, respectively, and stimulated over basal cGMP ratios were 500 and 160 at 0.1 μM ANP and CNP. The urodilatin concentration-response curve was similar to that of ANP. [^{125}I]-ANP bound specifically to HCD cells in a time-dependent fashion, reaching a plateau-phase between one and two hours at 4°C. Equilibrium saturation binding curves suggested a single group of receptor sites (K_d = 421 ± 55 pM, B_{max} = 49.2 ± 8.8 fmol/mg protein, Hill coefficient = 1.44 ± 0.1, N = 6). Binding of [^{125}I]-ANP was not displaced by CNP or by C-ANP (4-23), a specific ligand of clearance receptors (NPR-C), and thus occurred mainly via NPR-A. Neither Northern blot analysis nor RT-PCR could detect NPR-C mRNA, although the latter was clearly identified in control human glomerular visceral epithelial cells. In contrast, PCR products with the expected lengths were obtained for NPR-A and NPR-B. In conclusion, HCD cells express both NPR-A and NPR-B, as demonstrated by mRNA and cGMP production studies, but fail to produce NPR-C. This suggests that the human cortical collecting duct is a target for ANP, CNP and urodilatin.

The natriuretic peptide system is a family of homologous polypeptide hormones that stimulate diuresis, natriuresis, and vasorelaxation [1]. It consists of at least three distinct endogenous peptides: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). All bind to guanylate-cyclase coupled biological receptors (NPR-A and NPR-B), but the affinity of ANP and BNP is much higher for NPR-A while that of CNP is much higher for NPR-B [2]. In addition, the three peptides bind to clearance receptors devoid of guanylate-cyclase activity (NPR-C) with a function that is currently poorly defined [3].

ANP exerts multiple direct or indirect actions in the kidney that lead to increases in glomerular filtration rate and excretion of fluid and electrolytes. Its biological receptors (NPR-A) are localized mainly in glomerulus and inner medullary collecting duct by various methods in rat [4–8] and bovine kidney [9]. This suggests that in addition to its well-established effect on glomerular filtration, ANP also modulates water and electrolyte transport in the inner medullary collecting duct. Biological effects of ANP on water and sodium transport have indeed been demonstrated by Nonoguchi, Sands and Knepper [10] and Sands, Nonoguchi and Knepper [11], respectively, in rat isolated perfused terminal IMCDs. In the rat also, Terada et al [7] showed the presence of NPR-A in the cortical part of the collecting duct using the polymerase chain reaction coupled to reverse transcription (RT-PCR), and Ritter et al [8] recently demonstrated NPR-A in thin limbs of Henle's loop and in both principal and intercalated cells of cortical collecting ducts by immunocytochemistry. The renal actions of CNP are much more controversial. Sudo et al [12] and Tawaragi et al [13] found that intravenous injection of CNP increased excretion of water and electrolytes. By contrast, Stingo et al [14] reported a decrease in glomerular filtration rate, renal blood flow, and urinary sodium flow and fractional excretion of sodium, while Morita et al [15] did not find any significant effect on renal fluid or electrolyte excretion after CNP infusion. In rat cortical collecting duct, the restricted expression of NPR-B to the apical pole of alpha intercalated cells [8] suggests the possibility that CNP is involved in the regulation of bicarbonate transport in this nephron segment. On the other hand, recent studies indicate that CNP might act prominently as an autocrine/paracrine growth factor: (i) Its renal production leading to urinary excretion [16] was demonstrated by RT-PCR and/or immunohistochemistry in mouse, rat and human distal tubule segments including cortical collecting duct [16, 17]. (ii) CNP is a potent inhibitor of cell growth in bovine aortic smooth muscle cells [18] and chondrocytes [19].

In humans, the natriuretic effects of ANP at pharmacologic and pathophysiologic doses [20] and the distribution and characteristics of ANP receptors in glomerulus [21–23] are well established, but virtually nothing is known about the expression, type and function of natriuretic peptide receptors in the collecting duct. Only one study by Canaan-Kuhl et al [24] identified NPR-A, -B and -C by using RT-PCR in the human kidney, but their preparation contained glomeruli. Human studies are hampered by the scarcity of starting material for microdissection followed by RT-PCR, binding or cGMP studies, and by the lack of human tubule cell line derived from distal and collecting segments. We have recently established by immortalization with Simian Virus-40 (SV40) a human cortical cell line that exhibits major properties of

Received for publication June 20, 1996
and in revised form August 14, 1996
Accepted for publication August 16, 1996

principal cells including high responsiveness to arginine-vasopressin and lack of sensitivity to isoproterenol, parathyroid hormone and calcitonin (in terms of cAMP production), and that expresses CAM-L1, a basolateral antigen specific for principal cells [25, 26]. Using this cell model, we now show that human collecting duct principal cells express functional NPR-A and NPR-B and their mRNA transcripts in the absence of significant amounts of NPR-C. Therefore, the human cortical collecting duct may be a target for both ANP and CNP acting as an hormone and an autacoid, respectively.

Methods

Materials

The following radionuclides and reagents were obtained as indicated: rat ANP-(1–28), C ANP-(4–23), porcine CNP-(1–22) and [^{125}I]-tyr^{0}-CNP(37 TBq/mmol) from Peninsula Laboratories (Belmont, CA, USA); rat [^{125}I]-tyr^{28}-ANP-(1–28) (81.4 TBq/mmol) and [^{125}I]-cyclic GMP (81.4 TBq/mmol) from Dupont de Nemours (Germany); anti-cyclic GMP antibody from Institut Pasteur (Paris, France); oligo-dT primer and random primer from Promega (Madison, WI, USA); dNTPs (2′-deoxynucleoside 5′-triphosphate) and *Taq* DNA polymerase from Pharmacia LKB Biotechnology (Uppsala, Sweden); Moloney murine leukemia virus (MMLV) transcriptase reverse from Gibco BRL (Cergy Pontoise, France); oligonucleotide primers (20 bases for each) specific for NPR-A, -B, and -C were synthesized by Genset (Paris, France). HS-142-1, a nonpeptide natriuretic peptide antagonist of microbial origin, was a gift from Dr. Y. Matsuda (Tokyo, Japan). Cell culture media and cell culture supplies were from Gibco BRL. All other reagents were from Sigma (St. Louis, MO, USA).

Preparation of cells and characterization of natriuretic peptide receptor proteins

Cell culture. Isolation and characterization of the human collecting duct cell line (HCD) were previously reported [25]. An SV40 immortalized human podocyte cell line (HGVEC) was also studied as a positive control for expression of NPR-C receptor transcript [23]. These cells were previously characterized and shown to respond to ANP identically to the parental nontransformed cells [27].

Both cell lines were grown in a hormonally defined medium [DM; DMEM-HAM's F12, 1:1 (vol/vol); transferrin, 5 μg/ml; sodium selenate, 50 nM; glutamine, 2 mM; dexamethasone, 50 nM; insulin, 5 μg/ml; Hepes, 20 mM, pH 7.4], supplemented with only 2% of newborn calf serum (NCS), in an atmosphere of 5% CO_2–95% air, at 37°C. All experiments were performed between passages 15 and 50, and 20 and 30 for HCD and HGVEC, respectively.

Determination of cyclic GMP production. At confluence, cells grown in 12-well trays were preincubated for 20 minutes at 37°C in DM without NCS, but containing 1 mM isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor. The medium was aspirated and cells were incubated during 15 minutes at 37°C with 0.5 ml of the same medium containing ANP, urodilatin or CNP at final concentrations between 1 pM and 0.1 μM. Then the medium was collected and the stimulation was stopped by adding 1 ml of ice-cold ethanol 95%-formic acid 5% solution. After 30 minutes at 4°C, extracts were recovered, evaporated and stored at −20°C with their corresponding media. The total cyclic GMP content was measured by radioimmunoassay after resuspending the evaporated intracellular fractions in 50 mM sodium acetate buffer pH 6.2, and acetylating all the samples and the standards by a triethylamine, acetic anhydre (2/1, vol/vol) solution.

The inhibitory effect of HS-142-1, a nonpeptide ANP antagonist [28], on the cyclic GMP response was studied at concentrations between 0.1 and 100 μg/ml.

Binding studies. Human collecting duct cells grown to confluence in 12-well trays were rinsed twice with serum-free medium and were incubated in 0.5 ml of DMEM-HAM's F12, 1:1 vol/vol, containing 2 mg/ml bovine serum albumin (BSA), 1 mM phenylmethylsulfonylfluoride, 2 mg/ml bacitracin, 1 mM EDTA, 10 μM thiorphan, 1 μM aprotinin, 1 μM leupeptin, 0.1 μM pepstatin, and 20 mM Hepes, in the presence of [^{125}I]-ANP at 4°C in a room atmosphere during four hours unless otherwise stated. At the end of the incubation period, the medium was discarded and cells were washed three or four times with 1 ml of ice-cold 0.16 M NaCl. Then cells were solubilized with 1 M NaOH and cell-associated radioactivity was counted in a LKB gamma automatic counter with 65% efficiency. Specific binding was defined as the difference between total binding and nonspecific binding obtained in the presence of 1 μM unlabeled ANP. In competitive inhibition studies, unlabeled ANP, C ANP-(4–23), CNP, and urodilatin were added at increasing concentrations from 1 pM to 1 μM, and HS-142-1 was added at increasing concentrations from 1 to 100 μg/ml. Protein concentration in each well was determined by the Bradford method using BSA as standard [29].

Identification of natriuretic peptide receptor transcripts by RT-PCR and Northern blot analysis

RNA extraction and RT. Total RNA from cultured human collecting duct and glomerular epithelial cells was prepared using the guanidium thiocyanate method. RNA concentration and purity were determined from the A_{260} and A_{280} readings. Total RNA was then reverse-transcribed to first strand cDNA with oligo-dT (15 mer) and random primer (6 mer) as previously described [23].

PCR. One-fourth of the cDNA from the RT reaction was used for amplification by PCR. Three pairs of primers specific for human NPR-A, -B, and -C cDNAs were designed from the published sequences [30–32] as previously reported [23]. The sizes of the products between two primers are of 859 bp for NPR-A, 600 bp for NPR-B and 510 bp for NPR-C, respectively. PCR was performed as previously described [23]. Amplification was carried out by 30 or 40 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 2 min) using a DNA thermocycler. One-tenth of the PCR products was controlled by electrophoresis in a 0.9% agarose gel.

DNA sequencing. Each PCR product amplified from NPR-A, -B, and -C cDNAs was purified for sequencing as previously described [23].

Northern blot. The NPR-C cDNA probe was provided by Dr. J. Gordon Porter of Scios Nova (Mountain View, CA, USA) [31]. Total RNA from human collecting duct and glomerular epithelial cell line (20 or 40 μg per lane) was fractionated by electrophoresis in a 1.2% agarose gel and transferred to a nylon Gene Screen Plus membrane (New England Nuclear, Boston, MA, USA). After prehybridization, the blot was hybridized for 16 hours at 42°C with the alpha [^{32}P]-labeled cDNA probe. The filter was then washed

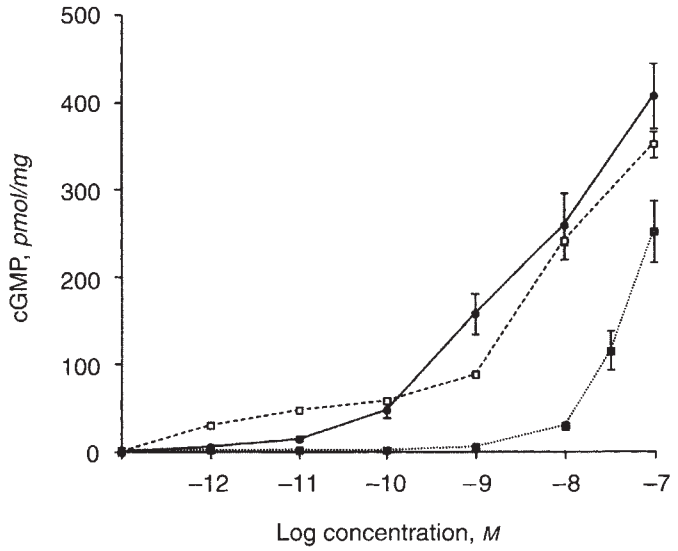

Fig. 1. *Cyclic GMP response of human collecting duct cells (HCD) to increasing concentrations of ANP (●), URO (□) and CNP (■), cGMP was measured after a 15-minutes incubation.* Means ± SE of 12 values for ANP (4 experiments) and of 6 values for CNP and URO (2 experiments).

three times at 42°C for 20 minutes in 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) containing 0.1% SDS, colored with a 0.05% methylene-blue solution, and exposed to a Fuji X-ray film at −80°C in the presence of an intensifying screen for 48 hours.

Statistics

All experiments were performed two to six times in triplicate or quadruplicate wells. Results are expressed as means ± SE. Comparisons between groups were analyzed with the use of Student's *t*-test for unpaired values.

Results

Characterization of natriuretic peptide binding sites in human collecting duct cells

Cyclic GMP production. Mean basal cyclic GMP levels were 0.823 ± 0.085 pmol/mg protein (mean ± SE; $N = 24$). ANP, urodilatin and CNP stimulated the production of cyclic GMP in a concentration-dependent manner (Fig. 1) with a threshold concentration of 1 pM for ANP and urodilatin (ANP, 5.12 ± 0.90; URO, 29.75 ± 1.10 pmol/mg protein; $P < 0.001$) and of 1 nM for CNP (2.60 ± 0.15 pmol/mg protein; $P < 0.001$). Cyclic GMP production did not reach a plateau in the range of ligand concentrations tested up to 0.1 μM. At 0.1 μM concentration, cyclic GMP was 500, 450 and 160 times higher than its basal value for ANP, urodilatin and CNP, respectively. HS-142-1, a novel competitive nonpeptide antagonist of ANP binding to NPR-A and NPR-B, reversed the stimulation by ANP and CNP of cyclic GMP production in a concentration-dependent manner (0.1 to 100 μg/ml; Table 1). Seventy-five percent of CNP-stimulated cyclic GMP was inhibited by 1 μg/ml of HS-142-1, but only 55% of the ANP-stimulated cyclic GMP was inhibited in the same cells at the same antagonist concentration. At 10 μg/ml, HS-142-1 reduced the cGMP response to both peptides by 93%.

Table 1. Effect of HS-142-1 on ANP- and CNP-stimulated cyclic GMP production in human collecting duct principal cells (HCD)

HS-142-1 concentration μg/ml	cGMP *pmol/mg protein*	
	ANP 10^{-8} M	CNP $5 \cdot 10^{-8}$ M
0	444.50 ± 57.00	55.70 ± 5.40
0.1	408.50 ± 39.00	24.62 ± 3.15[a]
1	201.00 ± 27.00[a]	14.21 ± 3.50[b]
10	32.00 ± 3.90[b]	3.00 ± 0.35[b]
50	5.10 ± 0.67[b]	1.82 ± 0.19[b]
100	2.70 ± 0.30[b]	1.91 ± 0.24[b]

Means ± SE of 8 values (2 experiments) are shown. Results obtained in the presence of HS-142-1 were compared to the stimulated values measured in the absence of HS-142-1 using Student's *t*-test for unpaired values. Basal (unstimulated) values of cGMP were 0.45 ± 0.04 pmol/mg protein.

[a] $P < 0.01$; [b] $P < 0.001$

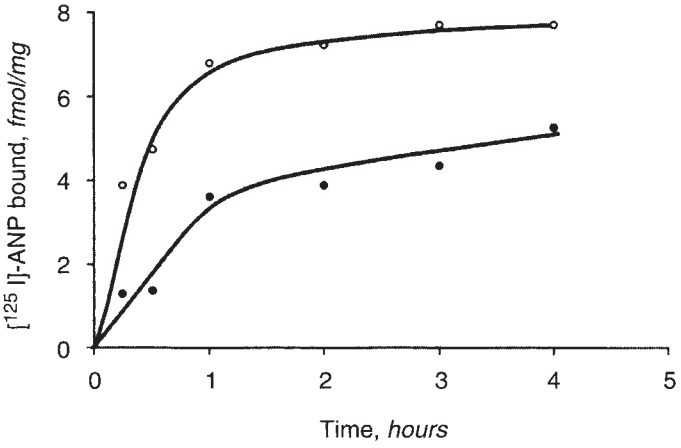

Fig. 2. *Time-course of total and specific binding of [^{125}I]-ANP to human collecting duct cells at 4°C.* Total (○) and specific binding (●) were measured during incubation with 50 pmol/liter [^{125}I]-ANP. A representative experiment ($N = 3$) performed in triplicate wells is shown.

Binding studies. [^{125}I]-ANP binding to collecting duct cells was studied at 4°C as a function of time at a concentration of 50 pM. As shown in Figure 2, binding rapidly increased within the first 30 minutes and reached a plateau between one and two hours. Nonspecific binding represented ~35% of total binding at equilibrium. When the cells were incubated for four hours with increasing concentrations of [^{125}I]-ANP, the amount of bound [^{125}I]-ANP followed a curvilinear ascending line (Fig. 3). The Scatchard plot of the data (Fig. 4) was linear ($r = 0.91$), which suggests the presence of a single group of ANP receptors. The dissociation constant (K_d) and the number of sites (B_{max}) derived from six Scatchard analyses were 421 ± 55 pmol/liter and 49.2 ± 8.8 fmol/mg protein (7,000 sites per cell), respectively. Hill equation (Fig. 4) allowed calculation of the n coefficient and of the 50% inhibitory concentration (IC_{50}). n was 1.44 ± 0.1. This value, superior to unity, suggests a positive cooperative process of [^{125}I]-ANP binding. IC_{50} was 104 ± 10 pM.

When human collecting duct cells were incubated with 100 pM [^{125}I]-ANP for four hours at 4°C in the presence of increasing concentrations of unlabeled ANP or urodilatin (1 pM to 1 μM), competitive inhibition of binding was observed (Fig. 5). The

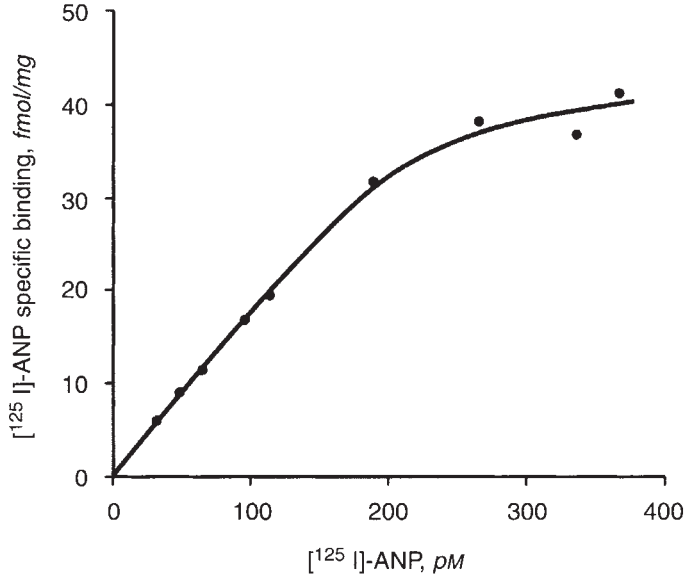

Fig. 3. *Specific binding of [^{125}I]-ANP to human collecting duct cells at equilibrium (4 hr) and at 4°C as a function of [^{125}I-tyr^{28}]-ANP-(1-28) concentration in medium.* A representative experiment (N = 6) performed in triplicate wells is shown.

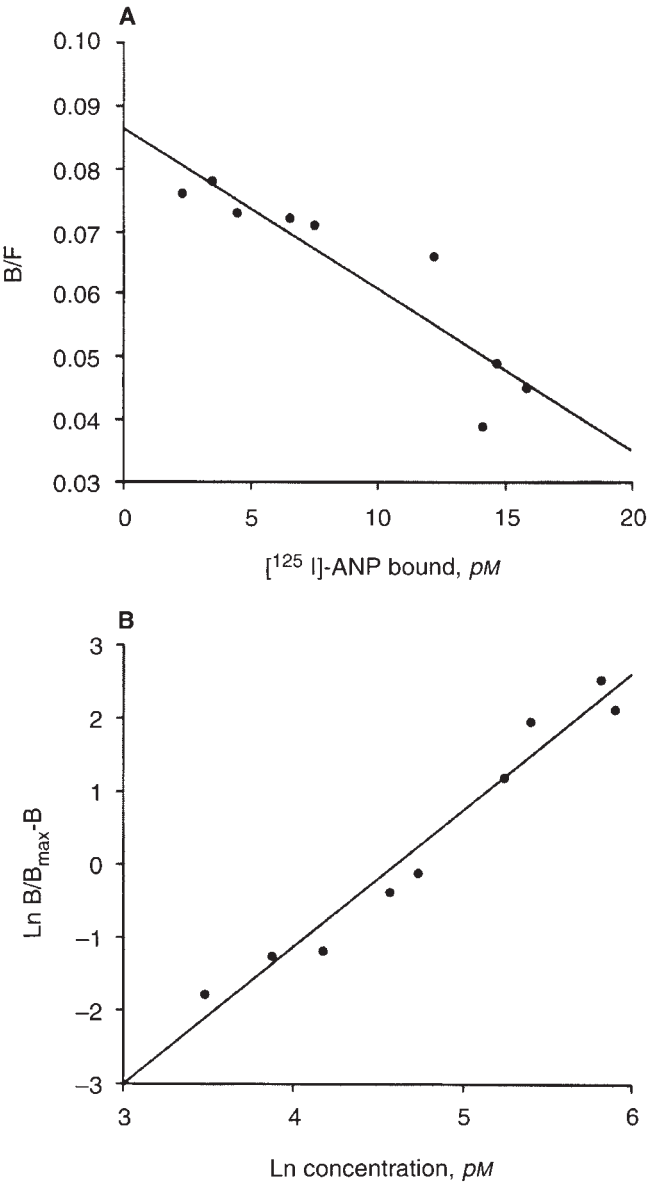

Fig. 4. *Scatchard (A; K_d = 388 pM, B_{max} = 55 fmol/mg) and Hill transformation (B; N = 1.8, IC_{50} = 102 pM) of the data.* Same representative experiment as in Figure 3.

inhibition curve rapidly decreased from 1 pM to 10 nM ANP. Concentration corresponding to 50% inhibition of total binding (ED_{50}) was ~0.7 nM. Urodilatin, another natriuretic peptide produced in the distal tubule segments of the kidney [33, 34], inhibited [^{125}I]-ANP binding with a higher affinity (ED_{50} = 0.14 nM) than unlabeled ANP.

No inhibition of [^{125}I]-ANP binding was obtained either with C ANP-(4-23) or with CNP, suggesting the absence (or the presence in very low amounts) of clearance receptors (NPR-C) in human collecting duct cells. HS-142-1 at concentrations of 1, 10 and 100 μg/ml inhibited [^{125}I]-ANP binding to the same extent as ANP did at 0.01, 0.1 and 1 μM, respectively (data not shown).

It was not possible to demonstrate specific binding of [^{125}I]-CNP to HCD cells, whereas [^{125}I]-CNP bound in a time- and concentration-dependent manner to isolated rat glomeruli incubated with [^{125}I]-CNP in parallel with HCD cells (data not shown).

Characterization of natriuretic peptide receptor transcripts in human collecting duct cells

RT-PCR identification of NPR-A, -B, and -C transcripts. cDNA samples resulting from reverse transcription of 1.5 μg total RNA from human collecting duct cells and epithelial glomerular cells as positive control were subjected to 40 and 30 PCR cycles, respectively. PCR products with the expected lengths were obtained for NPR-A (859 bp) and NPR-B (600 bp) in both cell lines, but in contrast with the glomerular cells, no signal of NPR-C amplification product (510 bp) could be detected in the collecting duct cells (Fig. 6). The signal for the NPR-B amplification transcript was weaker than that of NPR-A.

Identification of NPR-C mRNA by Northern blot analysis. The presence of specific NPR-C transcripts was also searched for in human collecting duct and glomerular epithelial cells by Northern blot analysis using a human cDNA probe. As shown in Figure 7, NPR-C mRNA could not be detected in collecting duct cells when 20 μg or 40 μg of total RNA were deposited, whereas in glomerular cells, a signal corresponding to NPR-C mRNA band (4.8 kb) was clearly detected, increasing with the amount of deposited RNA.

Discussion

Although the natriuretic effects of ANP are well-established in humans at least at pharmacologic and pathophysiologic doses [20], localization of NPR along the tubule is poorly defined. The present study demonstrates that NPR-A and NPR-B, but not NPR-C, are expressed in a human cortical collecting duct cell line whose characteristics are close to those of the parental principal cells *in vivo*. It strongly suggests, but does not prove NPR-A and

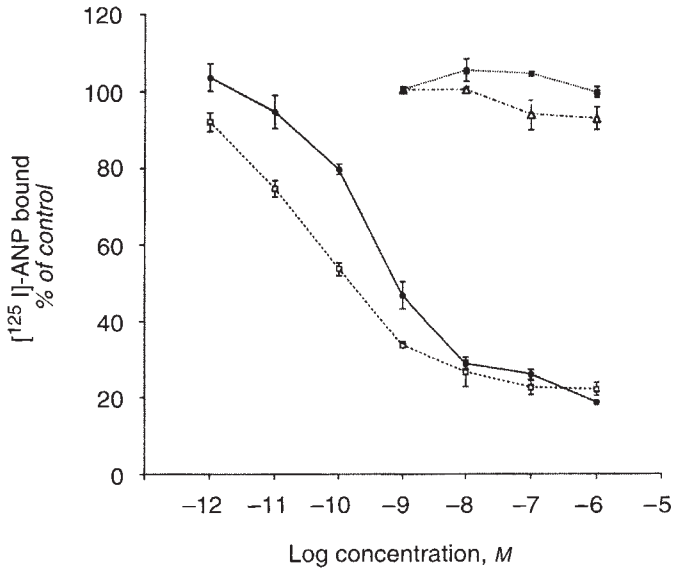

Fig. 5. *Competitive inhibition of binding of [^{125}I]-ANP to human collecting duct cells at equilibrium (4 hr) and at 4°C, in the presence of increasing concentrations of unlabeled ANP (●), urodilatin (□), CNP (■), and C-ANP-(4-23) (△).* Means ± SE of 9 values (3 experiments) are shown for ANP and C-ANP. Means ± SE of 6 values (2 experiments) are shown for CNP and urodilatin.

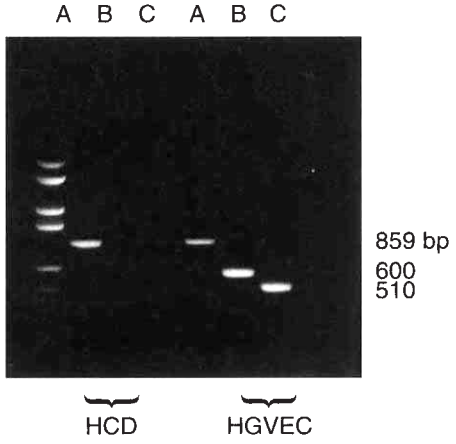

Fig. 6. *PCR products obtained after amplification of human collecting duct cells and glomerular epithelial cells (HGVEC) cDNA.* Amplification products specific for the three receptor subtypes (NPR-A, -B, and -C) were detected in glomerular epithelial cells (HGVEC, right). Although in collecting duct cells (HCD, left), identical amplification products were obtained for NPR-A and NPR-B, no signal could be detected for NPR-C. No signal was obtained when the RNA was omitted during the PCR amplification (data not shown). Specificity of PCR products was previously demonstrated by purification and sequencing [23]. A DNA molecular weight ladder is shown for size determination (left lane).

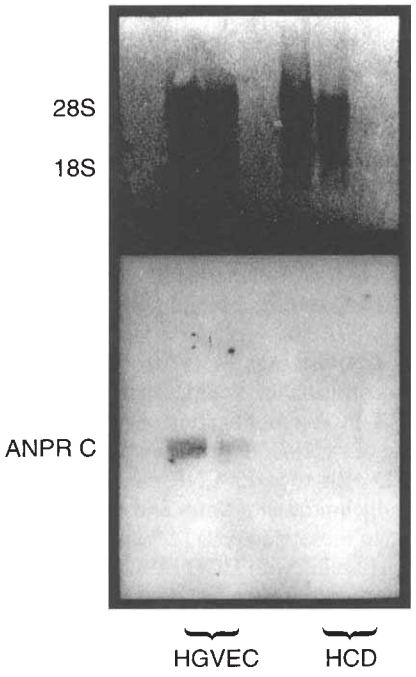

Fig. 7. *Northern blot analysis of total RNA from human glomerular epithelial cells (HGVEC, left) and collecting duct cells (HCD, right) for expression of NPR-C mRNA.* For each cell type, two concentrations of total RNA were applied: 20 μg (right lane) and 40 μg (left lane). Ethidium bromide staining of 28 S and 18 S ribosomal RNA bands is shown (top) to confirm that similar amounts of RNA were loaded for each cell type. Note the absence of hybrization signal in HCD cells (bottom).

NPR-B expression in the native human cortical collecting duct because SV40-transformed cells may acquire phenotypic differences from the cell of origin.

The assumption that human cortical collecting duct cells are targets for ANP is based on the following grounds: (1) [^{125}I]-ANP binds to these cells with a high affinity (K_d = 421 ± 55 pmol/liter); (2) ANP stimulates cyclic GMP production with a threshold concentration of 1 pmol/liter; (3) NPR-A, but not NPR-C, mRNA is expressed in HCD cells. These results indicate that cells from the cortical segment of the human collecting duct are highly responsive to ANP. In the renal tubule, NPR-A receptors have been described mainly in the inner medullary collecting duct in rat by ANP binding [4, 5] and cGMP stimulation studies [6], and by RT-PCR of microdissected segments [7], as well as in ox by *in situ* hybridization [9]. In the rat, NPR-A were also demonstrated in the cortical segment of the collecting duct by RT-PCR [7]. Nonoguchi and coworkers first reported a small rise in cGMP concentration in the rat cortical collecting duct in response to ANP [6], and then demonstrated inhibition of salt and water absorption by ANP across the perfused cortical collecting duct; they suggested that much of the natriuresis of ANP infusion may be attributable to this effect [35]. In rabbit, ANP inhibits the action of vasopressin in perfused cortical collecting ducts [36]. In humans, expression of NPR-A receptors is demonstrated in glomerular epithelial cells, but their presence in tubule segments remains to be established. The present data are in keeping with the natriuretic effects of ANP in humans, which are not entirely explained by its glomerular action [20]. HCD is the third cortical collecting duct cell line expressing functional ANP receptors. Stoos, Carretero and Garvin [37] first showed that ANP and bradykinin synergistically inhibited transport in a mouse cortical collecting duct cell line. We recently demonstrated the production and the apically polarized extrusion of ANP-stimulated cGMP in a rabbit collecting duct cell line transformed with a temperature-sensitive strain of SV40 [38, 39]. Because production of cGMP in

the rabbit cell line was maintained after SV40 large-T inactivation at the nonpermissive temperature of 39.5°C, one can exclude that ANP responsiveness in the human cell line was induced by SV40 immortalization. The results obtained with our human and rabbit cell lines as well as those of Ritter et al [8], who showed by immunocytochemistry the expression of NPR-A in principal and intercalated cells of the rat collecting duct, indicate that the cortical collecting duct is a target for ANP. Our results also suggest that it is a target for urodilatin that stimulates cGMP production concentration-dependently and that inhibits with a higher affinity than ANP, the binding of [^{125}I]-ANP to NPR-A.

In addition to NPR-A receptors, HCD cells possess NPR-B receptors: (1) CNP stimulates cyclic GMP production with a threshold concentration of 1 nmol/liter; (2) NPR-B mRNA is detected by RT-PCR amplification. We failed to demonstrate specific binding of [^{125}I]-CNP probably because of high hydrolytic activity of HCD cells towards CNP, since the same radiolabeled ligand specifically bound in a time- and concentration-dependent fashion to human mesangial cells [23] and to isolated rat glomeruli incubated in parallel with HCD cells. In the rat collecting duct, NPR-B receptors are restricted to the apical pole of alpha intercalated cells by immunocytochemistry [8]. The expression of NPR-B on HCD cells may be due to interspecies variation in the distribution of NPR or to the cellular origin of the HCD cell line that might be derived from a common ancestor to intercalated and principal cells, as suggested by the expression of band-3, a marker of alpha intercalated cells, in 100% of HCD cells [25]. Although CNP markedly stimulated cGMP production, the threshold of membrane guanylate cyclase stimulation was about 1,000 times higher with CNP than with ANP. A similar difference between ANP and CNP stimulation potencies was previously documented in rat cortical collecting duct cells [40]. The reason why CNP has less effect on cGMP accumulation than ANP in rat glomeruli, arcuate artery, and renal tubules [40], as well as in human cortical collecting duct cells is not known, and that could not be further elucidated in the present study due to the failure of binding studies. The presence of CNP in plasma is controversial [1]. In any case, its concentration is very low compared with that of ANP [41, 42] and with the threshold concentration determined in this study. Therefore, if CNP acts as a circulating hormone, then it seems to have no significant effects on human renal tubule function. On the other hand, CNP mRNA and peptide have been demonstrated by RT-PCR and immunohistochemistry in rat cortical collecting duct [17] and by immunohistochemistry in human distal tubule and medullary collecting duct as well as in human urine [16]. Urine immunoreactivity proved to be consistent by high pressure liquid chromatography with both the low molecular weight form CNP-22, as well as the high molecular weight form [16]. Hence, renal collecting duct cells may be an additional non-vascular site of synthesis of CNP that may act as an autacoid on the collecting duct. Although CNP may represent the natriuretic peptide best conserved throughout evolution, its action may not be limited to regulation of natriuresis. The restricted expression of NPR-B on rat alpha intercalated cells suggests a role of CNP in the regulation of bicarbonate transport [8]. Moreover, CNP has been shown to modulate cell growth in various cell systems including bovine aortic smooth muscle cells [18] and rat chondrocytes [19].

We failed to detect NPR-C in HCD cells: (1) C ANP-(4-23), a specific ligand of NPR-C, did not inhibit binding of [^{125}I]-ANP to HCD cells; (2) HS-142-1 (that does not bind NPR-C) inhibited [^{125}I]-ANP binding to the same extent as ANP (that does bind to NPR-A and NPR-C); (3) NPR-C mRNA transcripts could not be detected either by Northern blot or by RT-PCR of HCD cells mRNA, although in parallel experiments those transcripts were easily demonstrated in cultured human podocytes. The lack of expression of NPR-C in HCD cells was unexpected since NPR-C receptors usually increase dramatically in culture conditions [43, 44]. It was not due to SV40 tranfection since human podocytes were immortalized with the same plasmid [27]. In the kidney, NPR-C have been found thus far in bovine glomeruli [9] and in human glomerular epithelial and mesangial cells [21–23], as well as in rabbit renal cortical vascular smooth muscle cells [45], but to our knowledge, they have never been demonstrated in tubule segments by binding inhibition studies, immunohistochemistry, RNAse protection assay, or *in situ* hybridization [9]. Because NPR-C seem to act mainly as clearance receptors, one may speculate that other factors including membrane hydrolases modulate binding of natriuretic peptides to the surface of collecting duct cells. NPR-C occupancy by ANP can also inhibit cell proliferation of rat astroglial cells [46] and aortic smooth muscle cells [47]. Since the role of NPR-C remains debated, HCD cells may represent an interesting tool to investigate its function by transfecting the cells with the NPR-C cDNA.

In conclusion, we have demonstrated that human cortical collecting duct cells express both NPR-A and NPR-B as shown by mRNA and cGMP production studies, but fail to produce NPR-C. These results strongly suggest that the human cortical collecting duct is a target for ANP, CNP and urodilatin, but further studies are needed to confirm these data *in vivo*. Since ANP (or urodilatin) and CNP both increase cGMP, their physiological specificity remains to be defined.

Acknowledgments

This work was supported by grants from Institut National de la Santé et de la Recherche Médicale, Faculté de Médecine Saint-Antoine and Association Claude Bernard. We thank Ms. Christine Vial for secretarial assistance.

Reprint requests to Pierre M. Ronco, M.D., INSERM U64, Hôpital Tenon, 4 rue de la Chine, 75020 Paris, France.

References

1. Gunning ME, Ingelfinger JR, King AJ, Brenner BM: Vasoactive peptides and the kidney, in *The Kidney* (5th ed), edited by BM Brenner, New York, Saunders, 1995, pp 627–712
2. Koller KJ, Lowe DG, Bennett GL, Minamino N, Kangawa K, Matsuo H, Goeddel DV: Selective activation of the B natriuretic peptide by C-type natriuretic peptide (CNP). *Science* 252:120–123, 1991
3. Maack T, Suzuki M, Almeida FA, Nussenzweig DN, Scarborough RM, McEnroe GA, Lewicki JA: Physiological role of silent receptors of atrial natriuretic factor. *Science* 238:675–678, 1987
4. Healy DP, Fanestil DD: Localization of atrial natriuretic peptide binding sites within the rat kidney. *Am J Physiol* 250:F573–F578, 1986
5. Koseki C, Hayashi Y, Torikai S, Furuya M, Ohnuma N, Imai M: Localization of binding sites of α-rat atrial natriuretic polypeptide in rat kidney. *Am J Physiol* 250:F210–F216, 1986
6. Nonoguchi H, Knepper MA, Manganiello VC: Effects of atrial natriuretic factor on cyclic guanosine monophosphate and cyclic adenosine monophosphate accumulation in microdissected nephron segments from rats. *J Clin Invest* 79:500–507, 1987
7. Terada Y, Moriyama T, Martin BM, Knepper MA, Garcia-Perez A: RT-PCR microlocalization of mRNA for guanylyl cyclase-coupled ANF receptor in rat kidney. *Am J Physiol* 261:F1080–F1087, 1991

8. Ritter D, Dean AD, Gluck SL, Greenwald JE: Natriuretic peptide receptors A and B have different cellular distributions in rat kidney. *Kidney Int* 48:5758–5766, 1995
9. Yamamoto T, Feng L, Mizuno T, Hirose S, Kawasaki K, Yaoita E, Kihara I, Wilson CB: Expression of mRNA for natriuretic peptide receptor subtypes in bovine kidney. *Am J Physiol* 267:F318–F324, 1994
10. Nonoguchi H, Sands JM, Knepper MA: Atrial natriuretic factor inhibits vasopressin-stimulated osmotic water permeability in rat inner medullary collecting duct. *J Clin Invest* 82:1383–1390, 1988
11. Sands JM, Nonoguchi H, Knepper MA: Hormone effects on NaCl permeability of rat inner medullary collecting duct. *Am J Physiol* 255:F421–F428, 1988
12. Sudoh T, Minamino N, Kangawa K, Matsuo H: C-type natriuretic peptide (CNP): A new member of natriuretic peptide family identified in porcine brain. *Biochem Biophys Res Commun* 168:863–870, 1990
13. Tawaragi Y, Fuchimura K, Tanaka S, Minamino N, Kangawa K, Matsuo H: Gene and precursor structures of human C-type natriuretic peptide. *Biochem Biophys Res Commun* 175:645–651, 1991
14. Stingo AJ, Clavell AL, Aarthus LL, Burnett JC Jr: Cardiovascular and renal actions of C-type natriuretic peptide. *Am J Physiol* 262:H308–H312, 1992
15. Morita H, Hagiike M, Horiba T, Miyake K, Ohyama H, Yamanouchi H, Hosomi H, Kangawa K, Minamino N, Matsuo H: Effects of brain natriuretic peptide and C-type natriuretic peptide infusion on urine flow and jejunal absorption in anesthetized dogs. *Jpn J Physiol* 42:349–353, 1992
16. Mattingly MT, Brandt RR, Heublein DM, Wei C-M, Nir A, Burnett JC Jr: Presence of C-type natriuretic peptide in human kidney and urine. *Kidney Int* 46:744–747, 1994
17. Dean AD, Vehaskari VM, Greenwald JE: Synthesis and localization of C-type natriuretic peptide in mammalian kidney. *Am J Physiol* 266:F491–F496, 1994
18. Porter JG, Catalano R, McEnroe G, Lewicki JA, Protter AA: C-type natriuretic peptide inhibits growth factor-dependent DNA synthesis in smooth muscle cells. *Am J Physiol* 263:C1001–C1006, 1992
19. Hagiwara H, Sakaguchi H, Itakura M, Yoshimoto T, Furuya M, Tanaka S, Hirose S: Autocrine regulation of rat chondrocyte proliferation by natriuretic peptide C and its receptor, natriuretic peptide receptor-B. *J Biol Chem* 269:10729–10733, 1994
20. Miller JA, Tobe SW, Skorecki KL: Control of extracellular fluid volume and the pathophysiology of edema formation, in *The Kidney* (5th ed), edited by Brenner BM, New York, Saunders, 1995, pp 817–872
21. Chansel D, Pham P, Nivez MP, Ardaillou R: Characterization of atrial natriuretic factor receptors in human glomerular epithelial and mesangial cells. *Am J Physiol* 259:F619–F627, 1990
22. Suga I, Nakao K, Mukoyama M, Arai H, Hosoda K, Ogawa Y, Imura H: Characterization of natriuretic peptide receptors in cultured cells. *Hypertension* 19:762–765, 1992
23. Zhao J, Ardaillou N, Lu C-Y, Placier S, Pham P, Badre L, Cambar J, Ardaillou R: Characterization of C-type natriuretic peptide receptors in human mesangial cells. *Kidney Int* 46:717–725, 1994
24. Canaan-Kühl S, Jamison RL, Myers BD, Pratt RE: Identification of "B" receptor for natriuretic peptide in human kidney. *Endocrinology* 130:550–552, 1992
25. Prié D, Friedlander G, Coureau C, Vandewalle A, Cassinghena R, Ronco PM: Role of adenosine on glucagon-induced cAMP in a human cortical collecting duct cell line. *Kidney Int* 47:1310–1318, 1995
26. Debiec H, Christensen EI, Metz-Boutigue MH, Ronco PM: A variant of the brain L1 cell adhesive molecule (L1 CAM) is expressed in collecting duct (CD) principal cells. (abstract) *J Am Soc Nephrol* 6:374, 1995
27. Ardaillou N, Lelongt B, Turner R, Piedagnel R, Baudouin S, Estrade R, Cassingéna R, Ronco P: Characterization of simian virus 40-transformed human podocyte cell line producing type IV collagen and exhibiting polarized response to atrial natriuretic peptide. *J Cell Physiol* 152:599–616, 1992
28. Morishita Y, Sano T, Ando K, Saitoh Y, Kase H, Yamada K, Matsuda Y: Microbial polysaccharide, HS-142-1, competitively and selectively inhibits ANP binding to its guanylcyclase-containing receptor. *Biochem Biophys Res Commun* 176:947–957, 1991
29. Bradford MM: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 72:248–251, 1976
30. Chang MS, Lowe DG, Lewis M, Hellmiss R, Chen E, Goeddel DV: Differential activation by atrial and brain natriuretic peptides of two different receptor guanylate cyclases. *Nature* 341:68–72, 1989
31. Porter JG, Arfsten A, Fuller F, Miller JA, Gregory LC, Lewicki JA: Isolation and functional expression of the human atrial peptide clearance receptor cDNA. *Biochem Biophys Res Commun* 171:796–803, 1990
32. Lowe DG, Chang MS, Hellmiss R, Chen E, Singh S, Garbers DL, Goeddel DV: Human atrial natriuretic peptide receptor defines a new paradigm for second messenger signal transduction. *EMBO J* 8:1377–1384, 1989
33. Feller SM, Gagelmann M, Forssmann WG: Urodilatin: A newly described member of the ANP family. *Trends Pharmacol Sci* 10:93–94, 1989
34. Goetz KL: Renal natriuretic peptide (urodilatin?) and atriopeptin: Evolving concepts. *Am J Physiol* 261:F921–F932, 1991
35. Nonoguchi H, Sands JM, Knepper MA: ANF inhibits NaCl and fluid absorption in cortical collecting duct of rat kidney. *Am J Physiol* 256:F179–F186, 1989
36. Dillingham MA, Anderson RJ: Inhibition of vasopressin action by atrial natriuretic factor. *Science* 231:1572–1573, 1986
37. Stoos BA, Carretero OA, Garvin JL: ANF and bradykinin synergistically inhibit transport in M-1 cortical collecting duct cell line. *Am J Physiol* 263:F1–F6, 1992
38. Prié D, Dussaule JC, Lelongt B, Géniteau-Legendre M, Châtelet F, Cassingéna R, Vandewalle A, Ronco PM: Principal cell-specific antigen and hormonal regulatory network in RC.SVtsA58 cell line. *Am J Physiol* 266:C1628–C1638, 1994
39. Millul V, Prié D, Géniteau-Legendre M, Verpont MC, Baudouin B, Ronco PM: Egression of ANP-stimulated cGMP in rabbit renal collecting duct cells: Abrogation of polarity by SV40 large-T. *Am J Physiol* 270:C1051–C1060, 1996
40. Terada Y, Tomita K, Nonoguchi H, Yang T, Marumo F: PCR localization of C-type natriuretic peptide and B-type receptor mRNAs in rat nephron segments. *Am J Physiol* 267:F215–F222, 1994
41. Komatsu Y, Nakao K, Suga S, Ogawa Y, Mukoyama M, Arai H, Shirakami G, Hosoda K, Nakagawa O, Hama N, Kishimoto Imura H: C-type natriuretic peptide (CNP) in rats and humans. *Endocrinology* 129:1104–1106, 1991
42. Ogawa Y, Nakao K, Nakagawa O, Komatsu Y, Hosoda K, Suga S, Arai H, Nagata K, Yoshida N, Imura H: Human C-type natriuretic peptide. *Hypertens Dallas* 19:809–813, 1992
43. Suga S, Nakao K, Kishimoto I, Hosoda K, Mukoyama M, Arai H, Shirakami G, Ogawa Y, Komatsu Y, Nakagawa O, Hama N, Imura H: Phenotype-related alteration in expression of natriuretic peptide receptors in aortic smooth muscle cells. *Circ Res* 71:34–39, 1992
44. Ritter D, Dean AD, Guan Z-H, Greenwald JE: Polarized distribution of renal natriuretic peptide receptors in normal physiology and ischemia. *Am J Physiol* 269:F918–F925, 1995
45. Béa ML, Dussaule JC, Bens M, Ardaillou R: Characterization of ANF receptors in cultured renal cortical vascular smooth muscle cells. *Am J Physiol* 260:C424–C432, 1991
46. Levin ER, Harrison JLF: Natriuretic peptides inhibit rat astroglial proliferation: Mediation by C receptor. *Am J Physiol* 261:R453–R457, 1991
47. Cahlil PA, Hassid A: Differential antimitogenic effectiveness of atrial natriuretic peptides in primary versus subcultured rat aortic smooth muscle cells: Relationship to expression of ANF-C receptors. *J Cell Physiol* 154:28–38, 1993