Increased Synthesis and AVP Unresponsiveness of Na,K-ATPase in Collecting Duct from Nephrotic Rats

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Abstract. Renal sodium retention is responsible for ascites and edema in nephrotic syndrome. In puromycin aminonucleoside (PAN)–induced nephrosis, sodium retention originates in part from the collecting duct, and it is associated with increased Na,K-ATPase activity in the cortical collecting duct (CCD). The aims of this study were to evaluate whether the outer medullary collecting duct (OMCD) also participates to sodium retention and to determine the mechanisms responsible for stimulation of Na,K-ATPase in CCD. PAN nephrosis increased Na,K-ATPase activity in the CCD but not in OMCD. The two-fold increase of Na,K-ATPase activity in CCD was associated with two-fold increases in the number of α and β Na,K-ATPase subunits mRNA determined by quantitative RT-PCR and of the total amount of Na,K-ATPase α subunits estimated by Western blotting. PAN nephrosis also increased two-fold the amount of Na,K-ATPase α subunit at the basolateral membrane of CCD principal cells, as determined by Western blotting after biotinylation and streptavidin precipitation and by immunofluorescence. The intracellular pool of latent Na,K-ATPase units also increased in size and was no longer recruitable by vasopressin and cAMP. This unresponsiveness of the intracellular pool of Na,K-ATPase to vasopressin was not the result of any alteration of the molecular and functional expression of the vasopressin V2 receptor/adenylyl cyclase (AC) complex. It is concluded that PAN nephrosis (1) does not alter sodium reabsorption in OMCD, (2) is associated with increased synthesis and membrane expression of Na,K-ATPase in the CCD, and (3) alters the normal trafficking of intracellular Na,K-ATPase units to the basolateral membrane.

Nephrotic syndrome is defined by a marked proteinuria that leads to hypoalbuminemia (1). In humans, nephrotic syndrome is either a primary disease without glomerular morphologic alterations visible at light microscopy (minimal change disease) or a complication of inflammatory or deposit glomerular diseases. The primary forms of the disease correspond to either functional alterations of specific genes, such as nephrin, α-actinin-4, podocin, or WT1 (2–5), or to the idiopathic nephrotic syndrome, which is a functional disease that is likely caused by a circulating factor that alters the filtration properties of the glomerulus (1). Experimental nephrotic syndromes can be induced in laboratory mammals by injection of toxic substances, such as Adriamycin (Pharmacia & Upjohn, Kalama-zoo, MI) or puromycin aminonucleoside (PAN) (6), by induction of experimental glomerulonephritis (6), or by trans-

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whether the outer medullary collecting duct (OMCD) also participates to sodium retention. Sodium retention in collecting duct of PAN nephrotic rats is independent of vasopressin and aldosterone (14, 15), the two main factors that control sodium reabsorption in the collecting duct. Thus, elucidating the mechanism of sodium retention in nephrosis is also important from a fundamental point of view: it will reveal unknown pathways of regulation of sodium transport that might also be important in other diseases, particularly in essential hypertension.

Decreased urinary sodium retention in several models of nephrotic syndrome is closely correlated with enhancement of Na,K-ATPase activity in the CCD (14,15), which provides the driving force for tubular sodium reabsorption (19). Féraillé et al. (20) reported that increased Na,K-ATPase activity in CCD from rats with PAN-induced nephrotic syndrome relies on increased maximal activity (V\text{max}) of a subclass of Na,K-ATPase with low ouabain sensitivity. The aims of this study were to determine whether Na,K-ATPase is also enhanced in OMCD of PAN nephrotic rats and to elucidate the molecular mechanism of Na,K-ATPase activation in CCD.

Increased V\text{max} of Na,K-ATPase may be accounted for by stimulation of Na,K-ATPase units that preexist in the basolateral membrane—for example, through phosphorylation of the catalytic subunit of the pump (19)—and/or by increased number of Na,K-ATPase units in the membrane. In rat CCD, these two mechanisms have been described: insulin increases the activity of pre-existing Na,K-ATPase units (21), whereas aldosterone and vasopressin increase the number of units at the basolateral membrane through de novo synthesis and recruitment of an intracellular pool of latent pumps, respectively (22–25). Although neither aldosterone nor vasopressin are involved in sodium retention and stimulation of Na,K-ATPase activity in CCD of PAN nephrotic rats (14,15), all these possible mechanisms of stimulation of Na,K-ATPase were investigated. For this purpose, (1) we evaluated the amount of Na,K-ATPase units present in the membrane of CCD cells not only by its enzymatic activity, but also by Western blotting after cell-surface biotinylation and streptavidin precipitation and by immunofluorescence; (2) we quantitated the mRNA encoding the α and β subunits of Na,K-ATPase; and (3) we determined the size of the intracellular pool of Na,K-ATPase and its recruitment by cAMP and vasopressin. Furthermore, given the unresponsiveness of the intracellular pool of Na,K-ATPase to cAMP and to vasopressin in CCD from PAN nephrotic rats, we evaluated the molecular and functional expression of the vasopressin V\text{2} receptor/AC complex in PAN nephrosis.

Materials and Methods

Animals and Isolation of Tubules

Experiments were carried out in control and nephrotic male Sprague-Dawley rats weighing 130 to 160 g and fed the usual laboratory chow ad libitum. Nephrotic syndrome was induced by a single injection of PAN (150 mg/kg body wt, intraperitoneally). Rats were studied 6 d after PAN injection, when renal sodium retention was minimal and proteinuria fully developed (15).

After pentobarbital anesthesia (50 mg/kg body wt), the left kidney was infused with incubation solution (120 mM NaCl, 5 mM RbCl, 4 mM NaHCO\text{3}, 1 mM CaCl\text{2}, 1 mM MgSO\text{4}, 0.2 mM Na\text{2}H\text{2}PO\text{4}, 0.15 mM Na\text{2}HPO\text{4}, 5 mM glucose, 10 mM lactate, 1 mM pyruvate, 4 mM essential and nonessential amino acids, 0.03 mM vitamins, 20 mM HEPE, 0.1% BSA, pH 7.45) that contained 0.44% (wt/vol) collagenase (CL251, 0.75 to 0.87 U/mg; Serva, Heidelberg, Germany). The kidney was sliced into small pieces, which were incubated 20 min at 30°C in oxygenated (95% O\text{2} and 5% CO\text{2}) incubation solution that contained 0.08% (wt/vol) collagenase. Tubules were dissected at 0 to 4°C under stereomicroscopic observation and photographed to determine their length, which served as reference for data expression. For RNA extraction, tubule isolation was run under the “RNase-free conditions” (26). For Western blotting analysis, antiproteases were added to the dissection solution.

Measurement of Na,K-ATPase Activity

The hydrolytic activity of Na,K-ATPase was determined on pools of 4 to 6 permeabilized segments of nephron under V\text{max} conditions by a radiochemical microassay (15). When necessary, CCD were preincubated at 37°C with or without dibutyryl-cAMP (db-cAMP, 10\text{–}3 M, 20 min) or a protein kinase A inhibitor (H89, 10\text{–}6 M, 30 min). Tubules were permeabilized either by the classical procedure (27) that consists of the addition of 2 μl of 10 mM Tris-HCl (pH 7.4) and then a freezing-thawing step or by addition of 2 μl of 10 mM Tris-HCl (pH 7.4) that contains 0.5 mg/ml saponin and then incubation for 10 min at room temperature and freezing-thawing (25). These two permeabilization procedures allow for quantitation of Na,K-ATPase activity that originates from the cell surface (basolateral membrane) only and from the whole cell (basolateral membrane plus intracellular pool), respectively (25). Total ATPase activity was measured after the addition of 10 μl of a solution that contained 100 mM NaCI, 5 mM KCl, 10 mM MgCl\text{2}, 1 mM ethylenediaminetetraacetic acid (EDTA), 100 mM Tris-HCl, 10 mM Na\text{2}ATP, and ~5 nCi/μl [γ\text{32P}]-ATP (DuPont, Boston, MA) (2 to 10 Ci/mmol) at pH 7.4. For Na\text{2}++K\text{+}-independent ATPase activity measurements, NaCl and KCl were omitted, Tris HCl was 150 mM, and 2 mM ouabain was added. Na,K-ATPase activity (in picomoles of ATP per millimeter of tubule per hour) was taken as the difference between total and Na\text{2}++K\text{+}-independent ATPase activities.

Measurement of Ouabain-Sensitive 86Rb Uptake

The transport activity of Na,K-ATPase was measured by the ouabain-sensitive 86Rb uptake under conditions of initial rate (28). Pools of 8 to 10 CCD were preincubated at 37°C for 5 min to allow the restoration of transmembrane ion gradients as well as the action of ouabain (2.5 mM), when necessary. Thereafter, pools of CCD were incubated for 5 min at 37°C with or without db-cAMP (10\text{–}3 M) or vasopressin (10\text{–}6 M). The following incubation with 86RbCl solution (100 nCi/ml Amersham Pharmacia Biotech Europe, Saclay, France) lasted 1 min at 37°C and was stopped by addition of ice-cold rinsing solution. After rinsing three times, the intracellular radioactivity was measured by liquid scintillation counting. Ouabain-sensitive 86Rb uptake was calculated as the difference between the mean values measured in samples incubated with and without ouabain, respectively.

AC Activity and cAMP Accumulation in CCD

cAMP accumulation under basal condition (pool of 10 CCD) and vasopressin (AVP) stimulation (single CCD) were measured (29) in the presence of indomethacin (5 mM), adenosine deaminase (0.5
U/ml), and isobutyl methyl xanthine (1 mM). Amounts of cAMP (fmol/mm per 6 min) were measured by RIA (NEN Life Science Products, Boston, MA).

AC activity was determined on single CCD by the rate of conversion of [α-32P]-ATP into [32P]-cAMP under basal and AVP-stimulated conditions (30). Briefly, CCD permeabilized by hypotonic shock and freezing-thawing were incubated at 30°C for 30 min with [α-32P]-ATP in the presence of an ATP-regenerating system (phosphocreatine and creatine kinase). After incubation, the [32P]-cAMP was separated from the other 32P nucleotides by double column (Dowex [Sigma-Aldrich Chimie, Saint Quentin-Fallavier, France] and alumina) filtration procedure. The yield of the whole separation procedure was evaluated in each sample by the recovery of [3H]-cAMP added to each sample at the end of the incubation.

**RNA Extraction and Quantitative RT-PCR in CCD**

RNA were extracted from pools of 20 to 50 microdissected CCD (26) and stored at −80°C until use. Expression of mRNA that encoded the α1 and β1 subunits of Na,K-ATPase, the vasopressin V2 receptors, and the type 6 AC were quantitated by RT-PCR with known amounts of target-specific mutant cRNA as internal standards (31–33). Reverse transcription and PCR (carried out in the presence of [α-32P]-dCTP) were performed in the same tube. DNA fragments were separated by electrophoresis on 2% agarose slab gels and quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

For Na,K-ATPase α1 and β1 subunits, deletion mutant cRNA were used, and quantification was performed by comparison with a standard curve run in the same experimental series (31). For vasopressin V2 receptors (32) and type 6 AC (33), a restriction site for HindIII was introduced in the mutant cRNA and quantitation was performed after HindIII digestion of RT-PCR products by comparing the radioactivity of the DNA fragments generated from known amounts of mutant cRNA and mRNA from CCD co-processed in the same tubes.

**Western Blot Quantification of Total, Cell-Surface, and Intracellular Na,K-ATPase in CCD**

For measurement of total tubular content of Na,K-ATPase, pools of 50 CCD were pelleted and lysed in 100 μl of homogenization buffer (20 mM Tris-HCl [pH 7.4], 2 mM EDTA, 2 mM ethyleneglycoltetraacetic acid, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 1 mM phenylmethyl sulfonyl fluoride, 1 mM AEBSF, 30 mM NaF, 30 mM Na pyrophosphate along with 0.1% (wt/vol) sodium dodecyl sulfate (SDS), and 1% (vol/vol) Triton X100) and freeze-dried until SDS-PAGE was performed. Samples were resuspended in 100 μl of Laemmli 2X buffer and analyzed by SDS-PAGE. The amount of material loaded into each lane corresponded to the same initial length of CCD (±5%). After electrophoresis on 7% polyacrylamide gels, proteins were electrotransferred on polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA) and incubated overnight at 4°C with a polyclonal antibody (dilution, 1:10,000) raised against the 56 kD subunit of H-ATPase (dilution, 1:200) (36), two antibodies directed against aquaporin 3 (dilution, 1:300) (35) or specific markers of principal cells and intercalated cells in CCD, respectively (35,36). Glass slides were washed 3 times for 10 min in PBS and incubated for 45 min with a 1:100 dilution in PBS/BSA of a rabbit polyclonal antibody directed against the ETYY peptide corresponding to the C-terminus of Na,K-ATPase α subunits (antibody kindly provided by Dr J. Kyte, University of California, San Diego). Control experiments were carried out with rabbit polyclonal antibodies directed against aquaporin 3 (dilution, 1:300) (35) or against the 56 kD subunit of H-ATPase (dilution, 1:200) (36), two specific markers of principal cells and intercalated cells in CCD, respectively (35,36). Glass slides were washed 3 times for 10 min in PBS and incubated for 45 min with a 1:100 dilution in PBS/BSA of goat anti-rabbit antibodies coupled to FITC (Jackson Immunoresearch, West Grove, PA). Tissue sections were washed 3 times for 10 min in PBS, counter-colored with Evans blue, mounted in 50% glycerol in 0.2 M Tris-HCl (pH 8.0) and examined with a fluorescence microscope.

**Electron Microscopy**

Kidneys were fixed by perfusion with 2% glutaraldehyde in PBS followed by 1 h incubation in the same solution and washed in 0.1 M...
cortex, anti–H9251
1037/H11006
France).

U Whitney
SE: control, 361/H11006
(35), and negative for H-ATPase (Figure 2d), a marker of
positive for aquaporin 3 (Figure 2c), a marker of principal cells
duct cells that displayed a low staining by Evans blue were
stains mitochondria-rich intercalated cells. Indeed, collecting
was localized to the basolateral pole of the cells, which were
phrotic rats (Figure 2b, *), staining for Na,K-ATPase antibody
that of CTAL/DCT (Figure 2b, T). In CCD from PAN ne-
much stronger than in PCT (Figure 2b, P) and comparable to
staining for CCD in PAN nephrotic rats (Figure 2b, *) was
PCT (Figure 2a, P) and CCD (Figure 2a, *), whereas the
–Na,K-ATPase antibody were observed in both
iments for anti
(Figure 2b, T). In normal animals, weak and comparable stain-
strong signal in the distal nephron (CTAL and/or DCT) from
ATPase activity, adenylyl cyclase activity, cAMP accumulation, and
mRNA expression were done by unpaired t test. Statistical analysis of
Na,K-ATPase α subunit immunoreactivity was done using the Mann-
Whitney U test; P < 0.05 was considered significant. Results are
expressed as mean ± SEM from several independent experiments,
each one being performed with tubules from one animal.

Results
Expression of Na,K-ATPase along the Nephron of
Nephrotic Rats
To assess the specificity of CCD for increased tubular Na,K-
ATPase activity in nephrotic rats, cell-surface Na,K-ATPase activity
was measured along the nephron of control and PAN nephrotic rats by using the freezing/thawing permeabilization procedure (Figure 1A). Na,K-ATPase activity was increased in the CCD of PAN nephrotic rats, whereas it was not altered in proximal convoluted tubule (PCT), medullary and cortical thick ascending limb of Henle’s loop (MTAL and CTAL), and OMCD. Similarly, the whole-cell expression level of Na,K-
ATPase α subunit was specifically increased in the CCD of
PAN nephrotic rats but not in PCT, MTAL, CTAL, and OMCD (Figure 1B).

In CCD, PAN treatment also increased the number of
mRNA encoding Na,K-ATPase α1 subunit (10^3 copies/mm ±
SE: control, 361 ± 46; PAN, 804 ± 94; n = 4; P < 0.005) and
β1 subunit (10^3 copies/mm ± SE: control, 554 ± 80; PAN,
1037 ± 64; n = 4; P < 0.005).

By indirect immunofluorescence on cryosections of kidney
cortex, anti–Na,K-ATPase α subunit antibody produced a
strong signal in the distal nephron (CTAL and/or DCT) from
both normal (Figure 2a, T) and PAN nephrotic rat kidneys
(Figure 2b, T). In normal animals, weak and comparable stain-
ings for anti–Na,K-ATPase antibody were observed in both
PCT (Figure 2a, P) and CCD (Figure 2a, *), whereas the
staining for CCD in PAN nephrotic rats (Figure 2b, *) was
much stronger than in PCT (Figure 2b, P) and comparable to
that of CTAL/DCT (Figure 2b, T). In CCD from PAN ne-
phrotic rats (Figure 2b, *), staining for Na,K-ATPase antibody
was localized to the basolateral pole of the cells, which were
weakly stained in red by Evans blue, a dye that preferentially
stains mitochondria-rich intercalated cells. Indeed, collecting
duct cells that displayed a low staining by Evans blue were
positive for aquaporin 3 (Figure 2c), a marker of principal cells
(35), and negative for H-ATPase (Figure 2d), a marker of α
(apical pole) and β (basolateral pole) intercalated cells (36).

These results indicate that increased Na,K-ATPase activity
relies on an increase in expression level of Na,K-ATPase at the
basolateral pole of CCD principal cells secondary to an
increased pump subunits synthesis.

Subcellular Distribution of Na,K-ATPase in CCD of
Nephrotic Rats
The distribution of Na,K-ATPase units between the cell
surface and the intracellular pool was estimated by measuring
the whole-cell, the cell-surface, and the intracellular Na,K-
ATPase activities and expression levels in CCD from control
and PAN nephrotic rats.

Figure 3 shows that PAN nephrotic syndrome increased both
the whole-cell Na,K-ATPase activity (pmol/mm per h ± SE:
control, 869 ± 91, n = 4; PAN, 1720 ± 93, n = 5; P < 0.001)
and the cell-surface Na,K-ATPase activity (pmol/mm per h ±

Figure 1. Na,K-ATPase activity and expression of α subunit along the
nephron of normal and PAN nephrotic rats. (A) Cell-surface Na,K-
ATPase activity was measured by using the freeze/thawing permeabi-
ligation procedure in proximal convoluted tubules (PCT), medullary
and cortical thick ascending limbs of Henle’s loop (MTAL and
CTAL), and OMCD. Values are mean ± SE from different animals
(number in bars); **, P < 0.01. (B) Total amount of Na,K-ATPase
α subunit was detected by Western blotting with a specific polyclonal
antibody on same amounts of microdissected tubules of control (C,
open bars) and nephrotic rats (PAN, dark bars). Values are mean ±
SE from different animals (number in bars); **, P < 0.01.
SE: control, 477 ± 63, n = 4; PAN, 1147 ± 50, n = 5; P < 0.001). Na,K-ATPase activity originating from the intracellular pool of Na,K-ATPase, as estimated by the difference between the activities measured after saponin and freeze/thawing permeabilization respectively, was slightly (+46%) but not significantly increased in CCD from PAN nephrotic rats (pmol/mm per h ± SE: control, 392 ± 46, n = 4; PAN, 573 ± 85; n = 5; NS).

Western blot analysis indicated that PAN treatment also increased both the whole-cell (Figure 4A) and the cell-surface expression levels (Figure 4B) of the Na,K-ATPase α subunit (percent of normal rat ± SE: whole cell, 159 ± 16%, n = 9, P < 0.05; cell surface, 177 ± 7%, n = 20, P < 0.05). The fraction of intracellular Na,K-ATPase α subunit, estimated as the ratio of nonbiotinylated (supernatant taken after streptavidin precipitation of biotinylated proteins) over whole-cell α.

Figure 2. Indirect immunofluorescence staining of rat kidney cortex for Na,K-ATPase α subunit. In the kidney cortex, the anti-Na,K-ATPase α subunit antibody (a and b) mostly stained the basolateral membrane of the distal nephron segments CTAL and DCT (T). In normal animals (a), a weak basolateral staining of PCT (P) and CCD (*) was also observed. In PAN nephrotic rats (b), a strong staining of basolateral plasma membranes was observed in most but not all CCD cells. Cells stained by the anti-Na,K-ATPase antibody in CCD were weakly stained by Evans blue; they were also stained by an anti-aquaporin 3 antibody (c) but not by an antibody directed against the 56-kD subunit of vacuolar H-ATPase (d). G, glomerulus; bar = 25 μm.
subunit, was not significantly increased in CCD from PAN-treated rats (percent of the whole-cell α subunit ± SE: control, 35 ± 4, n = 4; PAN, 41 ± 3, n = 6; NS) (Figure 4C). However, the whole-cell content of Na,K-ATPase α subunit was increased by 59% in CCD from PAN nephrotic rats; therefore, the actual size of the intracellular pool of Na,K-ATPase was increased by 85% as compared with the pool in CCD from normal rats.

Thus the increase in whole-cell Na,K-ATPase content in CCD from PAN nephrotic rats is associated with increased plasma membrane expression of active Na,K-ATPase units. In addition, the two methods for evaluating the intracellular pool of Na,K-ATPase (saponin permeabilization and Western blotting) suggest that its size increased in CCD from PAN nephrotic rats.

Recruitment by cAMP of the Intracellular Pool of Na,K-ATPase is Blunted in PAN Nephrosis

In normal rats, the intracellular pool of Na,K-ATPase is recruited at the basolateral membrane by AVP via cAMP (24,25). The slight increase in size of the intracellular pool of Na,K-ATPase may indicate that its mobilization by AVP is altered in nephrotic rat CCD. Thus, effects of the cAMP analogue, dibutyryl cAMP (db-cAMP), on activity and cell-surface expression of Na,K-ATPase were compared in CCD from normal and PAN nephrotic rats.
Figure 5. Effect of db-cAMP on the subcellular distribution of Na,K-
ATPase in CCD from normal and PAN nephrotic rats. (A) Ouabain-
sensitive $^{86}$Rb uptake was measured after preincubation of CCD from
normal (Control) and PAN nephrotic rats (PAN) in the absence
(Basal) or presence of $10^{-3}$ M db-cAMP (cAMP) for 15 min at 37°C.
Results are percents of the basal value, and data are mean ± SE from
different animals (numbers in bars); **, $P < 0.01$. (B) Cell-surface
Na,K-ATPase activity was determined in CCD preincubated with or
without db-cAMP as above. Upper and lower panels show a representative Western blot and the densitometric quantification
(mean ± SE) from different animals (numbers in bars). Results are
percents of the optical density values from untreated samples; *, $P < 0.05$.

Figure 6. Expression of the vasopressin V$_2$ receptor/adenylyl cyclase
system in CCD from control and PAN nephrotic rats. (A and B) Adenylyl cyclase activity (A) and cAMP accumulation (B) were
determined in CCD from normal (open bars) and nephrotic rats (dark bars) in the absence (Basal) or presence of $10^{-6}$ M vasopressin
(AVP). Results are mean ± SE; number of animals in bars. (C) mRNA encoding type 6 adenylyl cyclase (AC 6) and vasopressin V$_2$
receptors (V$_2$-R) were quantified by RT-PCR in CCD from normal
(open bars) and PAN nephrotic rats (dark bars). Results are mean ±
SE from different animals (numbers in bars).
In control rat CCD, db-cAMP (10^{-3} M, 20 min) increased by similar factors ouabain-sensitive 86Rb uptake (Figure 5A) (pmol/mm per min ± SE: basal, 4.7 ± 0.7; db-cAMP, 7.9 ± 1.2; n = 7; P < 0.01), cell-surface Na,K-ATPase activity (Figure 5B) (pmol/mm per h ± SE: basal, 336 ± 54; db-cAMP, 606 ± 67; n = 6; P < 0.01), and cell-surface expression of the Na,K-ATPase α subunit (percent of basal condition ± SE: db-cAMP, 216 ± 50; n = 6, P < 0.05) (Figure 5, C and D). Similarly, AVP (10^{-6} M) increased ouabain-sensitive 86Rb uptake (pmol/mm per min ± SE: basal, 4.1 ± 0.8; vasopressin, 11.0 ± 1.4; n = 6; P < 0.01).

In contrast, in CCD from PAN nephrotic rats, db-cAMP did not alter ouabain-sensitive 86Rb uptake (Figure 5A) (pmol/mm per min ± SE: basal, 7.3 ± 0.3; db-cAMP, 7.4 ± 1.2; n = 7; NS), cell-surface Na,K-ATPase activity (Figure 5B) (pmol/mm per h ± SE: basal, 623 ± 99; db-cAMP, 590 ± 109; n = 6; NS), or cell-surface expression of Na,K-ATPase α subunit (percent of basal condition ± SE: db-cAMP: 96 ± 17; n = 6; NS) (Figure 5C). Consistently, AVP stimulation of ouabain-sensitive 86Rb uptake was blunted in nephrotic rat CCD (pmol/mm per min ± SE: control, 7.1 ± 1.4; vasopressin, 10.2 ± 2.4; n = 7; NS).

The stimulatory effect of cAMP on ouabain-sensitive Rb uptake in CCD from normal rats was abolished by 10^{-6} M protein kinase A inhibitor H89 (pmol/mm per min ± SE: H89, 5.1 ± 2.2; db-cAMP + H89, 5.6 ± 1.3; n = 6; NS). On the other hand, H89 had no effect in CCD from PAN nephrotic rats (pmol/mm per min ± SE: control, 10.4 ± 1.1; H89, 11.6 ± 1.0; n = 4; NS), which suggests that the protein kinase A pathway was not stimulated beforehand.

**Molecular and Functional Expression of the Vasopressin V2 Receptor/AC Complex**

The marked increase in cell-surface expression of Na,K-ATPase in the CCD of PAN nephrotic rats raises the question of its selectivity with regard to other basolateral membrane proteins. In addition, the resistance of Na,K-ATPase to the stimulatory effect of vasopressin may reflect a defect in the initial steps of AVP signaling. Thus we evaluated the mRNA abundance and the functional expression of two well-defined components of the basolateral membrane of principal cells: the vasopressin V2 receptor and the type 6 AC. Figure 6 indicates that PAN nephrosis altered neither the vasopressin V2 receptor nor the type 6 AC expression. Basal and vasopressin-stimu-
lated AC activities, measured either under $V_{\text{max}}$ conditions in permeabilized CCD (Figure 6A) or under rate-limiting conditions in intact CCD (Figure 6B), were similar in normal and PAN nephrotic rats. Consistently, the expression level of mRNA encoding type 6 AC and vasopressin V$_2$ receptor were similar in CCD from normal and nephrotic animals (Figure 6C).

**Alterations of Tubular Morphology in Nephrotic Rats**

Electron microscopic observation revealed that CCD principal cells from PAN nephrotic rats displayed marked dilations of the extracellular spaces between the basal membrane digitations and of the lateral intercellular spaces (Figure 7b, *) as compared with normal CCD (Figure 7a). In contrast, the topology of the apical cell membrane and the tight junctions (Figure 7, arrowheads) were not modified apparently. The whole volume of principal cells was apparently reduced in PAN nephrotic animals. The interdigitations of the basolateral membrane of intercalated cells were also dilated in CCD from nephrotic rats (Figure 8a, I). However, these digitations are much shorter than in principal cells; therefore, the whole-cell morphology was not as profoundly altered. Morphologic alterations of the basolateral membrane and intercellular spaces were also found in cells from the CTAL (Figure 8b) and the S2 segment of PCT (Figure 8d), i.e., in cells from all the nephron segments located in the medullary rays. In contrast, cells of the S1 segment of PCT showed no alteration (Figure 8c). These findings are consistent with accumulation of extracellular water in microenvironment of the medullary rays, which might be secondary to increased salt reabsorption by the CCD.

**Discussion**

These results clearly demonstrate that stimulation of tubular Na,K-ATPase in PAN nephrosis is specifically observed in the

![Figure 8. Morphology of cells in cortical nephron segments of PAN nephrotic rats. (a) In CCD, both principal (P) and intercalated (I) cells display dilated interdigitations of their basolateral membrane and intercellular spaces. However, the whole morphology of intercalated cells is much less altered than that of principal cells because the digitations of their basolateral membrane are much less developed. (b) In CTAL, cells also display dilated basolateral membrane digitations. (c) Cells from the S1 segment of the proximal tubule show normal morphology. (d) In contrast, cells from the S2 segment of the proximal tubule have dilated intercellular spaces. bar = 4 μm.](image-url)
CCD (Figure 1); in particular, no change in Na,K-ATPase was observed in OMCD. Along with the stimulation of Na,K-ATPase activity, we observed that the interdigitations of the basal membrane and/or the intercellular spaces of CCD cells but also of the cells from all the nephron segments that constitute the medullary rays were markedly dilated in nephrotic rats (Figures 7 and 8). Similar morphologic changes were previously attributed to a fixation artifact because they were no longer observed when kidney tissue was fixed by immersion instead of perfusion (37). In fact, it is probably not an artifact because morphologic changes were observed repeatedly in all sections from nephrotic rats but in none from control animals. Rather, these morphologic changes may be accounted for by the fact that sodium accumulated in the interstitium that surrounds the CCD, i.e., the interstitium of the medullary rays, is not washed rapidly toward the capillaries and therefore drains water. Because of their osmotic origin, the dilations of basolateral interdigitations and intercellular spaces are preserved by rapid fixation of kidney tissue, whereas they disappear when kidneys are fixed by immersion in an isoosmotic medium. This suggests that morphologic alterations are not the result of intrinsic modifications of the membrane structure but are reversible changes in topology induced by osmotic forces.

In CCD, PAN-induced stimulation of cell-surface Na,K-ATPase activity (Figure 3, left portion) is associated with a parallel increase in the amount of Na,K-ATPase α subunit determined by Western blotting (Figure 4B), and immunofluorescence (Figure 2). These observations contrast with our previous finding that the number of [3H]-ouabain binding sites was decreased in CCD of PAN nephrotic rats as compared with normal rats (20). This paradoxical decrease in [3H]-ouabain binding might be the result of (1) an experimental artifact due to the very low affinity for ouabain of rat kidney Na,K-ATPase, which limits the sensitivity of the ouabain binding method, and/or (2) the presence of ouabain binding proteins other than Na,K-ATPase, such as type III H,K-ATPase (38), which may be down-regulated in CCD of PAN nephrotic rats

The increased amount of Na,K-ATPase in the basolateral membrane appears to be specific to the principal cells in CCD of PAN nephrotic rats. This fits with the role of these cells in sodium reabsorption and with the sensitivity to amiloride of sodium reabsorption in CCD of PAN nephrotic rats (18). Increased cell membrane expression of Na,K-ATPase is paralleled by an increase in the total amount of Na,K-ATPase—determined either by Western blotting (Figure 4A) or by the enzymatic activity under saponin permeabilization (Figure 3, right portion)—and an increased expression of the mRNA encoding the α and β subunits of the enzyme. These findings demonstrate a transcriptional induction of Na,K-ATPase expression. Although plasma aldosterone level is high in PAN nephrotic rats (39) and aldosterone induces the synthesis of Na,K-ATPase in CCD (40), aldosterone is not responsible for this transcriptional induction of Na,K-ATPase. Indeed, (1) sodium retention and stimulation of Na,K-ATPase in CCD are observed in adrenalectomized rats (14), (2) aldosterone controls Na,K-ATPase in both CCD and OMCD (22), whereas nephrotic syndrome alters the Na,K-pump in the CCD only (Figure 1), and (3) aldosterone controls the expression of mRNA encoding the α but not the β subunit of Na,K-ATPase (23), whereas expression of both mRNA was altered in nephrotic syndrome. The lack of induction of Na,K-ATPase in OMCD by PAN-induced hyperaldosteronemia suggests that Na,K-ATPase is resistant to aldosterone in PAN nephrotic rats. Since independent from aldosterone, induction of the Na,K-pump in the CCD of PAN nephrotic rats might result alternately from a transient elevation in intracellular sodium concentration secondary to increased apical sodium entry. This hypothesis is supported by the following: (1) activity of the apical sodium channels is increased in CCD from PAN nephrotic rats (18); (2) the V_max of Na,K-ATPase activity increases in response to the elevation of intracellular sodium concentration in many cells including renal cells (41,42); and (3) conversely to aldosterone but like PAN nephrosis, increased intracellular sodium concentration induces the mRNA transcription of both α and β subunits of Na,K-ATPase in kidney cells (43).

Rat CCD contain an intracellular pool of latent Na,K-ATPase units which is recruited in response to protein kinase A activation (24,25); therefore, the question arises whether recruitment of this pool participates to the increased expression of Na,K-ATPase to the basolateral membrane. Results indicate that not only the size of the intracellular pool of Na,K-ATPase increased in nephrotic rats, but its recruitment by cAMP and AVP was blunted (Figure 5). This unresponsiveness of the intracellular pool of Na,K-ATPase to AVP is not accounted for by a defect of vasopressin V2 receptor/AC system, the functional expression of which is not altered in nephrotic rats (Figure 6). Nor can it be attributed to a pre-stimulation of the protein kinase A pathway, because enzyme inhibition by H89 did not alter cell-membrane Na,K-ATPase activity. Blockade of the intracellular pool of Na,K-ATPase indicates that (1) newly synthetized Na,K-ATPase units are targeted to the plasma membrane without transiting through the cAMP-re- cruitable intracellular pool and (2) PAN nephrosis alters intracellular vesicle traffic in CCD principal cells. These results also indicate that despite increased synthesis and plasma levels of vasopressin (37,44), a strong stimulus for Na,K-ATPase activity and sodium reabsorption in the rat CCD (24,45,46), vasopressin does not participate to Na,K-ATPase induction in PAN nephrotic rats. This conclusion confirms the previous report that administration of PAN to Brattleboro rats that genetically lack vasopressin secretion induces full-blown nephrotic syndrome with induction of Na,K-ATPase and sodium retention (15).

The unresponsiveness of Na,K-ATPase to aldosterone and to vasopressin in OMCD and in CCD, respectively, in PAN nephrotic rats might be seen as feedback adaptations that prevent sodium reabsorption to increase even more.

Finally, the lack of alteration in the molecular and functional expression of the vasopressin V2 receptor/AC complex in CCD from PAN nephrotic rats indicates that the observed induction of Na,K-ATPase is not just due to increase in the surface area of basolateral membrane of CCD principal cells.

In conclusion, renal sodium retention in PAN nephrotic
syndrome results in part from an increased amount of Na,K-ATPase α and β subunits mRNA leading to increased synthesis and cell-surface overexpression of the Na,K-pump in the CCD. This CCD-specific up-regulation of Na,K-ATPase is independent of aldosterone and vasopressin. Finally, the intracellular pool of Na,K-ATPase becomes unresponsive to vasopressin during PAN nephrosis.

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