Collecting Duct $\text{Na}^+ / \text{K}^+$-ATPase Activity Is Correlated with Urinary Sodium Excretion in Rat Nephrotic Syndromes

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Abstract. In puromycin aminonucleoside (PAN)-treated nephrotic rats, sodium retention is associated with increased Na$^+$/K$^+$-ATPase activity in the cortical collecting ducts (CCD). This study was undertaken to determine whether stimulation of Na$^+$/K$^+$-ATPase in the CCD is a feature of other experimental nephrotic syndromes, whether it might be responsible for renal sodium retention, and whether it is mediated by increased plasma vasopressin levels or activation of calcineurin. For this purpose, the time courses of urinary excretion of sodium and protein, sodium balance, ascites, and Na$^+$/K$^+$-ATPase activities in microdissected CCD were studied in rats with PAN or adriamycin nephrosis or HgCl$_2$ nephropathy. The roles of vasopressin and calcineurin in PAN nephrosis were evaluated by measuring these parameters in Brattleboro rats and in rats treated with cyclosporin or tacrolimus. Despite different patterns of changes in urinary sodium and protein excretion in the three nephrotic syndrome models, there was a linear relationship between CCD Na$^+$/K$^+$-ATPase activities and sodium excretion in all three cases. The results also indicated that there was no correlation between proteinuria and sodium retention, but ascites was present only when proteinuria was associated with marked reduction of sodium excretion. Finally, the lack of vasopressin in Brattleboro rats or the inhibition of calcineurin by administration of either cyclosporin or tacrolimus did not prevent development of the nephrotic syndrome in PAN-treated rats or stimulation of CCD Na$^+$/K$^+$-ATPase. It is concluded that stimulation of Na$^+$/K$^+$-ATPase in the CCD of nephrotic rats might be responsible for sodium retention and that this phenomenon is independent of proteinuria and vasopressin and calcineurin activities.

Nephrotic syndromes may develop in many mammalian species as a result of primary diseases such as minimal change disease in human patients, inflammatory diseases such as membranous nephropathy or immune glomerulonephritis in human patients or laboratory animals, or exposure to toxic substances such as adriamycin or puromycin aminonucleoside (PAN) in rats (1–4). In addition to proteinuria and podocyte foot process effacement, development of edema and ascites are permanent features in the majority of models and diseases (3,4). Marked decreases in urinary sodium excretion are common clinical observations in human minimal change disease (5), and decreases in the fractional excretion of sodium are observed in animal models such as PAN nephrosis (6), adriamycin nephrosis (7), immune glomerulonephritis resulting from rabbit anti-rat serum (8), or Heymann nephritis in rats (9).

In vivo micropuncture experiments have shown that the collecting duct is the site of sodium retention in the kidney of PAN nephrotic rats (10). Na$^+$/K$^+$-ATPase activity is significantly enhanced in the cortical collecting ducts (CCD) of rats with PAN nephrosis, during a period with a positive sodium balance (6,11). Therefore, the initial aims of this study were to evaluate whether enhanced Na$^+$/K$^+$-ATPase activity in the CCD is a general feature of experimental nephrotic syndromes and whether this enhanced activity is responsible for decreased urinary sodium excretion. For this purpose, Na$^+$/K$^+$-ATPase activity was measured in CCD during periods of urinary sodium retention in PAN nephrosis and in two other experimental models of the nephrotic syndrome in rats, i.e., adriamycin nephrosis and membranous nephropathy induced by mercury chloride (12,13), and the time course of Na$^+$/K$^+$-ATPase stimulation was correlated with that of decreased urinary sodium excretion during the development of ascites in the early phases of these three experimental nephrotic syndromes. Results suggest that stimulation of Na$^+$/K$^+$-ATPase in CCD is a major molecular mechanism leading to primary sodium retention in all three experimental models of the nephrotic syndrome.

The production of edema and ascites in the nephrotic syndrome was traditionally considered to be the result of a chain of events that included successively decreased oncotic pressure, primary leakage of fluid into the interstitial compartment, hypovolemia, secondary hyperaldosteronism, and decreases in urinary sodium excretion (14). However, this hypovolemia concept, or underfill hypothesis, cannot explain all features of volume regulation and sodium reabsorption in the nephrotic syndrome (15). Hypovolemia is not a universal feature among
nephrotic patients (5), and hypervolemia has been demonstrated in some animal models of the nephrotic syndrome (16). In addition, the nephrotic syndrome occurs in adrenalectomized rats (6,8). Alternately, the overflow hypothesis now assumes that decreased urinary sodium excretion is a primary tubular disorder in nephrotic syndromes that is independent of low oncotic pressure and is responsible for primary extracellular volume expansion, which leads to secondary leakage of fluid toward the interstitium. The second aim of this study was therefore to evaluate the possible roles of factors other than aldosterone, i.e., vasopressin and calcineurin, in the physiopathologic processes of PAN nephropathy and in the stimulation of collecting duct Na\(^+\)/K\(^+\)-ATPase. Vasopressin is known to stimulate Na\(^+\)/K\(^+\)-ATPase activity in the CCD (17,18), and plasma levels of vasopressin increase during PAN nephropathy (19,20). Activation of calcineurin also stimulates Na\(^+\)/K\(^+\)-ATPase activity in rat CCD (21), and the calcineurin inhibitors cyclosporin and tacrolimus significantly decrease proteinuria in PAN nephropathy (22,23). To determine the roles of vasopressin and calcineurin in sodium retention during PAN nephropathy, we evaluated the effects of PAN in Brattleboro rats, which genetically lack vasopressin secretion, and in Sprague Dawley rats treated with either cyclosporin or tacrolimus. The results demonstrate that PAN-induced nephrosis and stimulation of CCD Na\(^+\)/K\(^+\)-ATPase are independent of vasopressin action and calcineurin stimulation.

Materials and Methods

Experimental Nephrotic Syndrome Models

Male rats (130 to 160 g body wt) of the Sprague Dawley (Iffa-Credo, L’Arbresle, France), Brown Norway (Elevage Janvier, Le Genest-St-Ise, France), Long-Evans (Harlan, Gamnat, France), or Brattleboro (Harlan France) strain were used to develop three experimental nephrotic syndrome models: PAN-induced nephrosis, mercury chloride-induced membranous nephropathy, and adriamycin-induced nephrosis. PAN nephrosis was induced in Sprague Dawley, Long-Evans, and Brattleboro rats by a single intraperitoneal injection of PAN (Sigma, St. Louis, MO) at a dose of 150 mg/kg body wt, diluted to 15 mg/ml in 0.9% NaCl. Adriamycin nephrosis was induced in Sprague Dawley rats by a single dose of 7.5 mg/kg body wt adriamycin (Sigma), diluted to 0.75 mg/ml in 0.9% NaCl and injected into the tail vein. HgCl\(_2\) nephropathy was induced in Brown Norway rats by repeated injections of HgCl\(_2\) (Sigma) at a dose of 1 mg/kg body wt, diluted to 1 mg/ml in 0.9% NaCl; subcutaneous injections into the abdominal skin were administered on days 0, 2, 4, 7, 9, and 11.

Treatment of Rats with Calcineurin Inhibitors

Cyclosporin (Novartis) and tacrolimus (Fujisawa Pharmaceutical Co., Osaka, Japan) were dissolved in DMSO and were continuously delivered for 7 d through osmotic minipumps (Azlet 2001; Charles River, St. Aubin-les-Elbeuf, France) that had been implanted subcutaneously under light ether anesthesia. Cyclosporin and tacrolimus were administered at 8 and 4 mg/kg per d, respectively. Plasma concentrations of the two drugs were analyzed by fluorescence polarization immunoassays, using a monoclonal antibody, in total blood samples obtained by retro-ocular puncture throughout the study.

Microdissection of CCD and Determination of Na\(^+\)/K\(^+\)-ATPase Hydrolytic Activity

Na\(^+\)/K\(^+\)-ATPase activity was determined by a radiochemical assay based on the measurement of phosphate released from \(\gamma\)-32PATP by the ATPase contained in pools of 2 to 5 mm of CCD. Rats were anesthetized (pentobarbital, 50 mg/kg body wt), and the left kidney of each rat was infused with 5 ml of infusion solution (see composition below), containing 12 mg of collagenase (from Clostridium histolyticum, 0.75 U/mg; Serva, Heidelberg, Germany), through an aortic catheter. The kidney was then removed and sliced into small pyra-

Metabolic Studies

Animals were housed in individual metabolic cages, starting 2 d before the beginning of the experiments. Nephrotic rats had free access to food and water throughout the study, whereas untreated, pair-fed control animals had free access to water, but each day were given the mean daily intake of the corresponding treated rats. Twenty-four-hour urine samples were collected, under mineral oil, for periods beginning 1 d before the single or first injection (day 0) and lasting for 12 d for PAN nephrosis, 14 d for HgCl\(_2\) nephropathy, and 21 d for adriamycin nephrosis. Urine volume was gravimetrically determined. Blood samples were obtained by retro-ocular puncture every day, except for adriamycin nephrosis, for which collection was restricted to days 0 to 5, 7, 10, 13, 17 to 19, and 21. Food intake was measured every day for evaluation of the net urinary sodium balance and determination of the amount of food to be given to the pair-fed rats that constituted the control group for each nephrotic syndrome model. Plasma and urinary sodium and creatinine and urinary protein concentrations were measured with an automatic analyzer (Hitachi 911; Boehringer Mannheim) by indirect potentiometry with ion-selective electrodes and colorimetric reactions, using pyrogallol red (for protein assays) and the Jaffe\’ reagent (for creatinine assays). Ascites volumes were measured after euthanasia by moistening and weighing an absorbent paper.

The daily urinary sodium balance was calculated as the difference between dietary sodium intake and urinary sodium excretion in the 24-h observation periods. To circumvent pitfalls resulting from variations in sodium concentrations attributable to dilution of the final urine samples and to artifactual errors in collection, urinary sodium excretion was calculated either as fractional excretion or as a function of urinary creatinine excretion (in mmol sodium/mmol creatinine). Because fractional sodium excretion was linearly correlated with the urinary sodium/creatinine concentrations in the three experimental models (PAN nephrosis, \( r = 0.98, 46 \) measurements from four animals, \( P < 0.0001; \) HgCl\(_2\) nephropathy, \( r = 0.91, 51 \) measurements from four animals, \( P < 0.0001; \) adriamycin nephrosis, \( r = 0.91, 55 \) measurements from five animals, \( P < 0.0001 \)), data are presented as urinary sodium/urinary creatinine concentrations. Similarly, protein excretion was expressed as a function of creatinine excretion (g/mmol creatinine).

The variations in GFR were evaluated in the three models by assessment of creatinine clearance. In PAN nephrosis, GFR decreased slightly at the beginning of the treatment and reached a minimum on day 5 (0.26 \( \pm \) 0.03 versus 0.66 \( \pm \) 0.20 ml/min on day 0, mean \( \pm \) SD, \( P < 0.05 \)) before returning to control levels. In mercury chloride nephropathy, a brief period of acute renal failure occurred on days 2 and 3 (creatinine clearance on day 0, 0.64 \( \pm \) 0.16 ml/min; day 2, 0.10 \( \pm \) 0.07 ml/min; day 3, 0.21 \( \pm \) 0.11 ml/min; mean \( \pm \) SD); thereafter, GFR returned to control levels throughout the study. GFR did not change during the 21 d of follow-up monitoring of adriamycin-treated rats.
mids, which were incubated at 30°C for 20 min. CCD were dissected in the dissection solution at 0 to 4°C, under stereoscopic observation. The CCD were identified by morphologic and topographic criteria. The infusion solution contained 120 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgSO₄, 0.2 mM Na₂HPO₄, 0.15 mM NaH₂PO₄, 5 mM glucose, 2 mM lactate, 4 mM essential amino acids, and 20 mM Hepes; the pH was 7.40, and the osmolality was adjusted at 400 mosmol/kg by mannitol addition. The dissection solution was similar except that the concentration of CaCl₂ was 0.25 mM and 0.1% bovine serum albumin (BSA) was added.

Pools of four to six CCD were individually transferred with dissection solution into the concavities of sunken bacteriologic slides coated with dried BSA and were stored on ice until the end of the dissection procedure. They were then transferred, in 0.5 μl of dissection solution, into the BSA-coated wells of a 96-well, flat-bottomed plastic microplate and were photographed for determination of the total tubular length of each pool. Two microliters of 10 mM Tris-HCl were added to each well, and the microplate was subjected to a freezing/thawing step for permeabilization of the cell membranes.

After addition of 10 μl of ATPase assay solution to each well, the microplate was incubated at 37°C for 15 min. The ATPase assay solution for measurement of total ATPase activity contained 50 mM NaCl, 5 mM KCl, 10 mM MgCl₂, 1 mM ethylenediaminetetra-acetic acid, 100 mM Tris-HCl, 10 mM Na₂ATP, and tracer amounts of [γ-32P]ATP (10 Ci/mmol; Du Pont de Nemours, Boston, MA). For measurement of basal Mg²⁺-ATPase activity, NaCl and KCl were omitted, the Tris-HCl concentration was 150 mM, and 1 mM ouabain was added. The pH of both solutions was adjusted to 7.4.

Incubation was stopped by the addition of 300 μl of an ice-cold 15% (wt/vol) suspension of activated charcoal. The microplate was centrifuged (2000 rpm for 3 min), and 100 μl of each supernatant was transferred to a 96-well sample microplate (Wallac, Turku, Finland). The radioactivity in each well was determined by Cerenkov counting (1450 Microbeta Trilux; Wallac). Total ATPase and Mg²⁺-ATPase activities were each determined in triplicate. Na⁺/K⁺-ATPase activity was calculated as the difference between the mean total ATPase activity and the mean Mg²⁺-ATPase activity and is expressed as picomoles per millimeter per hour. Data represent means and SD from at least four animals, except as otherwise indicated in the figure legends.

This semiautomated method, derived from that of Doucet et al. (24), facilitated the study of the large populations of animals required for these kinetic experiments in the three models of nephrotic syndrome. Because of the lower thermic conductance of plastic microplates compared with the aluminum plates used in the original method, the measured Na⁺/K⁺-ATPase activities were approximately one-half of those reported previously. Because ATPase activities were determined for pools of four to six CCD, the intersample biologic variability was greatly reduced. Therefore, although we used triplicate samples (instead of quintuplicate samples, as in the original method) and the activities were lower, the variability among replicates was in the range of 2 to 8% in this study, compared with 15 to 20% reported for the original technique. The linearity of Na⁺/K⁺-ATPase activity as a function of the time of incubation was verified from 5 to 40 min (r = 0.98, P < 0.001).

Statistical Analyses

Results are presented as means ± SD. Metabolic data were compared using either bilateral paired t tests for comparison of different days in the same series or unpaired t tests for comparison of different groups on a given day. Na⁺/K⁺-ATPase activities were compared using ANOVA with Fischer protected least significant difference test.
urinary sodium output then decreased markedly (1.9 to 3.7 mmol/mmol creatinine) on days 12 to 14, at a time when the sodium balance became positive (up to $1256 \pm 113 \mu$mol/d) and moderate ascites ($3.57 \pm 1.4$ g) was observed (Figure 3B). Again, these changes were not related to alterations in food intake. Moderate proteinuria (compared with that in PAN-treated rats) developed almost concomitantly with this late phase of sodium retention (Figure 3A).

Na$^+$/K$^+$-ATPase activity in CCD was significantly increased during the period of sodium retention, when ascites was present (days 12 to 14) (Figure 3C). Again, there was a
Adriamycin Nephrosis

The adriamycin model was fundamentally different from the two models described previously in that there was large inter-

linear relationship \( r = 0.978, P < 0.001 \) between \( \text{Na}^+ / \text{K}^+ \)-ATPase activity in CCD and urinary sodium excretion in this nephrotic syndrome model (days 0, 2, 9, and 11 to 14) (Figure 2).

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Early Phase of PAN Nephrosis in Long-Evans and Brattleboro Rats

This experimental series was designed for investigation of the possible role of vasopressin in the development of the nephrotic syndrome after PAN administration. For this purpose, we compared the time courses of urinary sodium and protein excretion and \( \text{Na}^+ / \text{K}^+ \)-ATPase activities in the CCD of Brattleboro rats (which genetically lack vasopressin) and their
Long-Evans strain controls, in response to PAN injections. Brattleboro rats developed PAN nephrosis, including a full-blown nephrotic syndrome and high-range proteinuria (Figure 5). On day 6, ascites (Brattleboro, 12 ± 1.1 g; Long-Evans, 10.8 ± 2.6 g; n = 5, NS) and stimulation of Na\(^+\)/K\(^+\)-ATPase in the CCD (Brattleboro, 765 ± 111 versus 479 ± 100 pmol/mm per h, P < 0.001; Long-Evans, 872 ± 77 versus 570 ± 205 pmol/mm per h, n = 5, P < 0.05) were similar in
the two strains. However, the appearance of proteinuria in the Brattleboro rats was delayed by 2 d compared with the Long-Evans rats, although proteinuria reached similar levels on day 8. In nephrotic Brattleboro rats, as in Sprague Dawley rats, there was a linear relationship between CCD Na\(^+/\)K\(^{+}\)-ATPase activities and urinary sodium excretion measured on days 0, 1, 3, and 6 (\(r = 0.98, P < 0.05\)) (Figure 5).

Effects of Calcineurin Inhibitors on the Early Phase of PAN Nephrosis in Sprague Dawley Rats

PAN-induced changes in urinary sodium excretion and proteinuria were kinetically and quantitatively similar in normal rats and rats treated with cyclosporin or tacrolimus at therapeutic levels (Figure 6). On day 6, Na\(^+/\)K\(^{+}\)-ATPase activities in CCD were significantly stimulated in cyclosporin-treated, PAN-treated rats (726 ± 75 pmol/mm per h, \(n = 3, P < 0.01\)) and tacrolimus-treated, PAN-treated rats (729 ± 197 pmol/mm per h, \(n = 3, P < 0.01\)) compared with control animals (401 ± 62 pmol/mm per h, \(n = 6\)), and were not different from those of PAN rats that had not been treated with cyclosporin or tacrolimus (805 ± 126 pmol/mm per h, \(n = 6\)).

Discussion

PAN and adriamycin are toxic agents that induce experimental nephrotic syndromes without glomerular inflammatory lesions or Ig deposits (1,2). Mercury chloride induces a systemic autoimmune disease that includes membranous nephropathy with IgG deposits. This nephropathy is responsible for the development of high-range proteinuria and a full-blown nephrotic syndrome (12). These three experimental models were used to evaluate whether activation of the sodium pump in the CCD might be directly responsible for sodium retention and correlated with ascites formation.

Because the daily time courses of urinary sodium excretion and the relationship of excretion to the appearance of proteinuria and ascites in these three models of nephrotic syndrome were not, or were only partially, described in the literature (6–8,25), we performed a systematic study of the time courses of these parameters. Our results indicated that the time courses and profiles of changes in urinary sodium excretion were different in the three models. Paradoxically, urinary sodium excretion increased on day 1 (PAN nephrosis) or day 2 (HgCl\(_2\) nephropathy) after the first exposure to the toxic agent. These increases cannot be accounted for by the volume of isotonic sodium chloride solution used as the solvent for drug administration, because: (1) the increases were quantitatively similar in the two models, although the PAN-treated rats received a 10-fold greater volume of sodium chloride; (2) the increases were observed only after the first, and not after subsequent injections of HgCl\(_2\); and (3) the increases in urinary sodium excretion were much smaller for control rats given injections of the same amount of saline solution. Instead, the increases likely result from toxic effects on renal tubules and sodium handling, because Na\(^+/\)K\(^{+}\)-ATPase activities in the CCD were reduced on those days. With respect to urinary sodium excretion, the three models differed both quantitatively and kinetically. At the peak, sodium excretion was reduced by approximately 95% in PAN nephrosis and approximately 90% in HgCl\(_2\) nephrop-
athy but only by approximately 80% in adriamycin nephrosis. These peaks lasted several days in the PAN (days 2 to 8) and HgCl₂ (days 12 to 14) models, whereas they were transient (mean duration, 1.5 d) in the adriamycin model (occurring at any time during the day 2 to 6 and/or day 13 to 21 periods). This latter observation suggests that the three drugs have different pharmacokinetic properties and that rats exhibit different susceptibilities to adriamycin, with respect to sodium reabsorption.

In the three models, proteinuria appeared after 5 to 9 d and protein levels remained elevated (although at different levels in different models) throughout the periods studied. However, the experimental models differed in the temporal relationship between decreased tubular sodium reabsorption and proteinuria. Proteinuria appeared 2 d after the onset of decreased urinary sodium excretion in PAN nephrosis and concomitantly with the marked decreases in sodium excretion in HgCl₂ nephropathy. In adriamycin nephrosis, brief peaks of sodium retention occurred randomly between days 13 and 21, whereas proteinuria was steadily present after day 7 and plateaued after day 15. In both PAN and adriamycin nephrosis, the appearance of proteinuria did not influence the course of urinary sodium excretion.

In the two strains. Such dissociation between proteinuria and renal sodium retention has also been observed in chronic Heymann nephritis and chronic serum sickness (26). These results support the hypothesis that the reduction of urinary sodium excretion may be independent of the development of proteinuria and may be attributable to a tubular disorder independent of glomerular events.

Interestingly, in all three models, the formation of ascites required the association of proteinuria and marked decreases in urinary sodium excretion. In PAN nephrosis, ascites appeared at the same time as proteinuria, i.e., at a time when the sodium balance had been markedly positive for several days. In the mercury chloride model, ascites appeared during the period associated with proteinuria and reduction of urinary sodium excretion and subsided when urinary sodium excretion returned to basal levels, despite persistent high-range proteinuria. In adriamycin nephrosis, none of the rats with low urinary sodium excretion in the period preceding proteinuria (days 2 to 6) exhibited ascites, and only those with markedly decreased sodium excretion in the period associated with proteinuria (days 13 to 21) developed ascites (or at least massive edema). The volume of ascites was different in the three models; it was moderate in the adriamycin and HgCl₂ models and was always profuse in PAN nephrosis. This likely explains why ascites resorption required several days after restoration of high urinary sodium output in the PAN model.

Figure 5. Renal excretion of sodium and protein in the early phase of PAN nephrosis in Brattleboro and Long-Evans rats. The time courses of urinary sodium excretion (filled symbols) and proteinuria (open symbols) during the 8 d after a single intraperitoneal injection of PAN (150 mg/kg) into male Long-Evans (triangles) and Brattleboro (squares) rats are shown. Twenty-four-hour urine samples were collected to measure creatinine, protein, and sodium levels. Results for day 0 correspond to the 24-h urine samples collected the day before the injection of PAN. Values are means ± SD from three to five animals. *P < 0.05, **P < 0.01, ***P < 0.001, for Brattleboro versus Long-Evans rats. (Inset) Relationship between urinary sodium excretion and Na⁺/K⁺-ATPase activity in the CCD measured on days 0, 1, 3, and 6 in Brattleboro rats (P < 0.05).
Several studies suggested that sodium retention in nephrotic syndromes is independent of alterations in GFR and originates in the distal portions of nephrons. The following findings suggest that the reduction of GFR reported in several studies (for either nephrotic patients or animal models) does not contribute to impaired sodium excretion: (1) the nephrotic syndrome has been reported for several patients with normal or high GFR (5,27,28), and (2) administration of either atrial natriuretic peptide (ANP) to PAN nephrotic rats (29) or saralasin to rats with unilateral PAN nephrosis (10) failed to increase urinary sodium excretion, despite significant increases in GFR. In vitro micropuncture studies in rats with nephrotic syndrome attributable to immune glomerulonephritis (9) or PAN-induced nephrosis (10) supported the collecting duct origin of sodium retention, because they demonstrated that: (1) the absolute reabsorption of sodium along the accessible proximal tubule is reduced in parallel with the GFR in experimental nephrotic syndromes (9,10,30,31) (clinical studies also demonstrated decreased proximal sodium reabsorption in nephrotic patients [(32,33)], so that sodium delivery to the loop of Henle remains normal, and (2) sodium reabsorption along the loop of Henle and the distal convoluted tubule accessible to micropuncture is normal (9,10,34), and the delivery of sodium to the collecting duct is normal (10). Therefore, decreased urinary sodium excretion results from increased reabsorption beyond the last accessible micropuncture site of the distal convoluted tubule, most likely in the collecting duct. In addition, treatment with bromoethylamine hydrobromide, which produces severe functional damage to inner medullary collecting ducts (IMCD) and salt wasting, does not prevent sodium retention in response to PAN, a finding that restraints the location of renal sodium retention to the CCD and/or outer medullary collecting ducts in this model (25). This hypothesis supposes that the IMCD do not compensate for the sodium imbalance originating in the CCD. Resistance of the IMCD, in particular to the natriuretic action of ANP, has been documented in the literature. As expected, the levels of ANP are increased in adriamycin and PAN nephrosis (16,29) and in nephrotic patients (35). However, both the sensitivity to ANP and the magnitude of the natriuretic and diuretic responses to ANP are reduced in rats with PAN (29) and adriamycin nephrosis (16) and in human patients with idiopathic nephrotic syndrome (35,36). This resistance, which occurs after ANP binding to its receptors in the collecting duct (16), results from the activation of a phosphodiesterase responsible for the catabolism of cGMP, the second messenger of ANP (37). Impairment of the ANP response in the IMCD could account in part for blunted volume expansion natriuresis in response to a sodium load but, in the absence of any dietary alterations, it cannot represent the only mechanism for primary sodium retention. A combination of these results and our own findings on the stimulation of the Na\(^+\)/K\(^+\)-ATPase in the CCD provides a better explanation.

\[ \text{Na}^{+}/\text{K}^{+}-\text{ATPase} \]

Figure 6. Renal excretion of sodium and protein in the early phase of PAN nephrosis in Sprague Dawley rats treated with cyclosporin or tacrolimus. The time courses of urinary sodium excretion (open symbols) and proteinuria (filled symbols) during the 7 d after a single intraperitoneal injection of PAN (150 mg/kg) in male Sprague Dawley rats treated with cyclosporin (diamonds), tacrolimus (squares), or untreated rats (circles) are shown. Plasma levels of cyclosporin (n = 9) were assayed on day 1 (384 ± 202 ng/ml), day 3 (898 ± 589 ng/ml), day 6 (306 ± 205 ng/ml), and day 7 (772 ± 310 ng/ml). Plasma levels of tacrolimus (n = 3) were assayed on day 1 (21 ± 8 ng/ml), day 3 (30 ± 0 ng/ml), and day 7 (31 ± 1 ng/ml). Twenty-four-hour urine samples were collected to measure creatinine, protein, and sodium levels. Results for day 0 correspond to the 24-h urine samples collected the day before the injection of PAN. Values are means ± SD from four animals.
but not in the cortical thick ascending limb of Henle’s loop (6) or in the outer medullary collecting duct (data not shown). The results presented here extend these observations to other nephrotic syndrome models, because they demonstrated that Na⁺/K⁺-ATPase activity in the CCD was steadily stimulated at each phase of reduction of urinary sodium excretion in the three experimental models and that there was a linear relationship between Na⁺/K⁺-ATPase activities in the CCD and urinary sodium excretion before and during the formation of ascites. In the CCD, sodium reabsorption occurs through a two-step mechanism, including primary active pumping of sodium out of the cells by basolateral Na⁺/K⁺-ATPase and secondary passive luminal entry of sodium via epithelial sodium channels. Under normal conditions, apical sodium entry is the rate-limiting step for the entire process, because it defines the intracellular sodium concentration and thus the intrinsic rate of Na⁺/K⁺-ATPase pumping (38,39). The close correlation between CCD Na⁺/K⁺-ATPase activity, as determined in vitro under Vmax conditions, and the final sodium excretion in urine suggests that Na⁺/K⁺-ATPase in the CCD becomes rate-limiting for renal sodium handling in nephrotic syndromes. This assumes a dysregulation of epithelial sodium channels. Functional and immunohistologic studies of epithelial sodium channels remain to be performed in PAN nephrosis, as in other nephrotic syndrome models.

The observation that stimulation of Na⁺/K⁺-ATPase in the CCD is responsible for sodium retention in three nephrotic syndrome models, involving different toxic agents and different histologic types of glomerular lesions, suggests that this stimulation may be involved in other nephrotic syndrome conditions, including minimal change disease and membranous nephropathy in human patients. Stimulation of Na⁺/K⁺-ATPase in the CCD of nephrotic rats could result from dysregulation of any of its known regulators, including aldosterone (40), calcineurin (21), and vasopressin (17). The role of aldosterone in sodium retention in the nephrotic syndrome has been extensively debated since the hypovolemia concept was proposed to explain this disease (14). Such a role was supported by indirect evidence, including: (1) elevated plasma renin and aldosterone levels in children with idiopathic nephrotic syndrome with edema and symptoms of hypovolemia (5) and in rats with PAN nephrosis (8,41–44); (2) a negative correlation between urinary sodium excretion and free urinary aldosterone in nephrotic patients, regardless of dietary sodium content (45–47); and (3) a partial ability of spironolactone to increase urinary sodium excretion in nephrotic patients (48) and PAN nephrotic rats (42). However, many more direct results disprove the hypovolemia concept and argue against a major role of aldosterone, namely: (1) sodium retention and edema may occur in nephrotic patients with normal blood volume and normal aldosterone levels (5,28,49); (2) ascites and edema occur in rats with nephrosis attributable to rabbit anti-rat kidney serum injection, without elevated blood aldosterone levels (8); (3) inhibition of angiotensin-converting enzyme by captopril failed to induce natriuresis in nephrotic patients (50) and in rats with PAN nephrosis (44), despite decreased aldosterone levels; (4) sodium retention was observed only in the affected proteinuric kidney in rats with unilateral models of PAN nephrosis (10) and adriamycin nephrosis (16); and (5) a full-blown nephrotic syndrome occurs in adrenalectomized rats after administration of either PAN or rabbit anti-rat kidney serum (8). Finally, in their seminal work on PAN nephrosis, Vogt and Favre (6) demonstrated that the sodium retention profiles and CCD Na⁺/K⁺-ATPase stimulation were similar in normal and adrenalectomized rats. These findings allow us to conclude that aldosterone is not involved in a determining way, in renal sodium retention in nephrotic patients and animals and cannot be the effector for the stimulation of CCD Na⁺/K⁺-ATPase in animal models of the nephrotic syndrome.

Calcineurin is a calcium/calmodulin-dependent serine-threonine phosphatase that is involved in major cell regulation, including T cell activation. Tacrolimus and cyclosporin, two inhibitors of calcineurin that are used as immunosuppressive drugs, inhibit Na⁺/K⁺-ATPase in the CCD by 90% (51) and 60% (21,51), respectively. These effects are mediated by the inhibition of calcineurin activity, because rapamycin and SDZ-220-384, which bind to calcineurin (through immunophilins) without inhibiting its activity, do not affect Na⁺/K⁺-ATPase activity. Taken together, these findings suggest that calcineurin produces basal activation of Na⁺/K⁺-ATPase and thus could be involved in the regulation of urinary sodium excretion. On the other hand, cyclosporin and tacrolimus significantly reduce proteinuria during the plateau period of PAN nephrosis (22,23). The effects of these compounds on urinary sodium excretion during the early phase of the disease had not been studied. Our results indicated that continuous delivery of cyclosporin or tacrolimus by a subcutaneous route, yielding routine therapeutic levels, slightly (but not statistically significantly) reduced proteinuria but did not modify the alterations of urinary sodium excretion induced by PAN administration. In addition, neither cyclosporin nor tacrolimus prevented the stimulation of CCD Na⁺/K⁺-ATPase activity that was observed on day 6 after PAN injection. This rules out a major contribution of calcineurin dysregulation in PAN-induced sodium retention.

Chronic administration of vasopressin increases Na⁺/K⁺-ATPase activity in the CCD of both Sprague Dawley and Brattleboro rats, whereas it has no significant effect in the outer medullary collecting ducts (17,18). In vitro exposure to vasopressin also stimulates Na⁺/K⁺-ATPase activity in the CCD (52). Because the expression of vasopressin mRNA in the hypothalamus and plasma levels of vasopressin are increased during the early phase of PAN nephrosis (19,20), we evaluated the possible role of vasopressin in urinary sodium retention and tubular Na⁺/K⁺-ATPase stimulation in PAN nephrosis. Our results with Brattleboro rats, which lack vasopressin secretion, demonstrated that vasopressin is not essential for the development of PAN nephrosis (including sodium retention, proteinuria, and ascites). Na⁺/K⁺-ATPase activity was stimulated to the same extents in the CCD of Brattleboro and Long-Evans rats 6 d after PAN administration. That increase in Na⁺/K⁺-ATPase activities in the CCD of PAN-treated rats is associated with decreased amounts of Na⁺/K⁺-ATPase at the CCD cell surface (11) and suggests posttranscriptional stimulation of the
pump. Our findings on calcineurin and vasoressin allow us to exclude contributions of phosphatase-2B and protein kinase A to this stimulation.

In summary, this study describes the temporal relationships among urinary sodium excretion, sodium balance, proteinuria, ascites, and Na\(^+\)/K\(^+\)-ATPase activities in CCD in three experimental models of the nephrotic syndrome in rats. This study demonstrates that: (1) sodium retention and proteinuria follow independent courses, (2) ascites appears only when proteinuria is associated with marked sodium retention, and (3) Na\(^+\)/K\(^+\)-ATPase activities in the CCD are closely correlated with urinary sodium excretion during the early periods of sodium retention. Our results also demonstrate that the PAN-induced nephrotic syndrome is independent of vasopressin and calcineurin activity. It is concluded that dysregulation of Na\(^+\)/K\(^+\)-ATPase in the CCD is a major factor in primary renal sodium retention in the nephrotic syndrome. Additional studies on the molecular mechanisms underlying this stimulation of Na\(^+\)/K\(^+\)-ATPase could facilitate elucidation of the pathways responsible for the tubular and glomerular disorders in the nephrotic syndrome.

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