Abnormal sodium pump distribution during renal tubulogenesis in congenital murine polycystic kidney disease

ELLIS D. AVNER*†, WILLIAM E. SWEENEY, JR.*, AND W. JAMES NELSON‡

*Department of Pediatrics, University of Washington and Children's Hospital and Medical Center, CH-46, 4800 Sand Point Way, N.E., Seattle, WA 98105; and ‡Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Beckman Center for Molecular and Genetic Medicine, Stanford, CA 94305-5426

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ABSTRACT Congenital polycystic kidney disease is characterized by the formation of large fluid-filled cysts in kidney tubules. It has been postulated that increased epithelial cell proliferation and altered transtubular fluid transport are necessary for cyst formation. To address the latter problem, we have studied the plasma membrane distribution of Na+/K+ ATPase during progressive stages of proximal and collecting tubular cyst formation in the CPK model of autosomal recessive polycystic kidney disease. In both control and cystic proximal tubules, Na+/K+ ATPase distribution was restricted to the basal-lateral membrane of cells. However, in newborn through day 5 kidney tissue, 16% of control vs. 47% of cystic outer cortical, 6% of control vs. 46% of cystic inner cortical, and 2% of control vs. 63% of cystic medullary collecting tubules demonstrated apical and lateral membrane distribution of Na+/K+ ATPase. In all nephrogenic zones, the percentage of control or cystic collecting tubules demonstrating apical membrane distribution of Na+/K+ ATPase decreased over time, but the percentage of cystic collecting tubules with apical membrane Na+/K+ ATPase remained significantly greater than in developmentally matched controls. No alterations in the normal distributions of other apical or basal-lateral membrane marker proteins were noted at any stage of control or cystic proximal or collecting tubule development. We conclude that apical-lateral membrane Na+/K+ ATPase expression is a normal transient feature of early collecting tubule development. However, apical membrane Na+/K+ ATPase persists in cystic kidneys, suggesting that such expression may be a manifestation of the relatively undifferentiated phenotype of epithelial cells lining collecting tubule cysts. The persistence of apical membrane Na+/K+ ATPase, if the enzyme is functional, may have pathogenic import in abnormal transtubular fluid transport in polycystic kidney disease.

Autosomal dominant polycystic kidney disease (ADPKD) and autosomal recessive polycystic kidney disease (ARPKD) affect over 500,000 individuals in the United States and are responsible for significant morbidity and mortality (1). Both diseases are characterized by progressive accumulation of fluid in dilated renal tubules, which leads to distortion of renal architecture, and ultimately, to renal failure. The altered gene(s) responsible for ADPKD and ARPKD has not been identified.

The pathophysiology of renal tubular cyst formation and progressive enlargement has been studied in a variety of in vivo and in vitro experimental models (2). Such studies, as well as mathematical analysis of cyst growth kinetics and meticulous anatomical study of ADPKD, demonstrate that two basic criteria necessary for cyst formation are increased epithelial cell proliferation and altered transtubular fluid transport (3–6).

Altered fluid transport in renal cystic tubular epithelium leading to net tubular secretion and intratubular fluid accumulation could result from alterations in ion pump activity or more global abnormalities of polarized cell structure and function (2, 5, 6). Studies in the C57BL/6jcpk/cpk (CPK) mouse, a murine model of ARPKD (2, 7, 8), and cultured ADPKD cystic epithelium (6, 9) suggest a significant role for altered Na+/K+ adenosine triphosphatase (Na+/K+ ATPase) activity in cystic tubular epithelial fluid secretion. In normal renal tubular epithelial cells, Na+/K+ ATPase is restricted to the basal-lateral membrane (10). Through coupled Na+/K+ countertransport, Na+/K+ ATPase is the major driving force for tubular Na+ reabsorption and the "secondary active" transport of a number of other solutes (11). Significantly, increased Na+/K+ ATPase activity and consequent tubular epithelial hyperplasia are early markers of cyst formation in CPK mice (2, 7, 8).

We can consider two hypotheses for the role of abnormal Na+/K+ ATPase activity in intratubular fluid accumulation and tubule cyst formation. If present in its normal basal-lateral membrane location, increased Na+/K+ ATPase activity in cystic tubular epithelium might lead to increased secondary active transport and net tubular secretion of solutes, thereby osmotically obligating intratubular fluid accumulation (2, 5, 12). Alternatively, if Na+/K+ ATPase is mislocated to apical membranes of cystic tubular cells it could stimulate net basal-to-apical vectorial transport of Na+ and fluid, resulting in tubular fluid secretion and cyst formation (6, 9). In this latter regard, it is of particular interest that Na+/K+ ATPase has been reported to be transiently expressed in the apical membrane of collecting tubule epithelium during normal renal development (13, 14), and it is sorted to both apical and basolateral domains during establishment of polarity in MDCK cells, a canine renal epithelial cell line (15). Furthermore, Na+/K+ ATPase is normally expressed in the apical membrane of choroid plexus epithelium (16) and retinal pigmented epithelial cells (17).

In the current study, we have sought to test these hypotheses by comparing the subcellular patterns of Na+/K+ ATPase distribution during normal mammalian renal tubule development with different stages in the ontogeny of congenital cystic tubular maldevelopment in CPK mice. The results clearly show that during normal renal tubule development Na+/K+ ATPase is transiently localized to both the apical and lateral membranes of cells and that polarized basolateral membrane distribution is acquired later in fully differentiated cells. In contrast, in cystic tubules, apical membrane Na+/K+ ATPase persists at developmental stages when Na+/K+ ATPase has become restricted to the cell line (15). Furthermore, Na+/K+ ATPase is normally expressed in the apical membrane of choroid plexus epithelium (16) and retinal pigmented epithelial cells (17).

Abbreviations: ADPKD and ARPKD, autosomal dominant and recessive polycystic kidney disease, respectively; LTA, Lotus tetragonolobus agglutinin; DBA, Dolichos biflorus agglutinin.

†To whom reprint requests should be addressed.
basal–lateral membrane in normal tubules. These results show that abnormalities associated with cystic tubule formation arise during early tubular development. The results also support the hypothesis that abnormal Na+/K+-ATPase distribution to the apical membrane may play an important role in abnormal transtubular fluid transport in this disease.

METHODS

Kidney tissue was obtained from control C57BL/6J and cystic CPK mice at seven postnatal stages: days 0 (newborn), 3, 5, 8, 10, 12, and 21. In CPK mice, days 0–5 represent a stage of proximal and early collecting tubule cyst development, days 8–12 represent a stage of predominant collecting tubule development, and day 21 represents the terminal phase of cystic disease, as we have previously described (7, 18). Tissue was rapidly fixed by immersion in 3.5% paraformaldehyde (pH 7.4) for 1 hr at 4°C. Fixed tissue was then washed, dehydrated through a graded acetone series, and infiltrated and embedded with Immunobid plastic embedding medium (Polysciences). Serial sections (3 μm each) were cut parallel to the long axis of the kidney. Sequential sections were stained with antibodies specific for either the α or the β subunit of Na+/K+-ATPase, biotinylated lectins, and a series of antibodies to apical and basal–lateral membrane marker proteins (see below). Apical membrane markers included GP-135, γ-glutamyltranspeptidase, Lotus tetragonolobus agglutinin (LTA), and Dolichos biflorus agglutinin (DBA). Basal–lateral markers included ZO-1, type IV collagen, laminin, entactin, band 3 anion-exchanger, and carbonic anhydrase II. For immunohistologic analysis, staining patterns were evaluated separately in outer cortical, inner cortical, and medullary zones. Since active nephron formation continues for 2–3 weeks postnatally in murine kidneys, this permitted direct comparison of nephron segments at similar development stages. Distributions of Na+/K+-ATPase subunits were compared in proximal tubules, proximal tubule cysts, collecting tubules, and collecting tubule cysts. Segments were clearly identified through systematic evaluation of lectin–profiled serial sections. As we have previously described (19–21), lectin staining patterns allow clear discrimination of proximal tubules and proximal tubular cysts (LTA+, DBA−) from collecting tubules and collecting tubule cysts (LTA−, DBA+) in developing murine renal tissue. Over 200 lectin-identified cystic or control proximal and collecting tubule segments derived from five to seven kidneys were analyzed in each nephrogenic zone, at each of the seven postnatal developmental stages (see above). The percentage of tubules exhibiting apical–lateral or basal–lateral membrane Na+/K+-ATPase was expressed as the mean ± standard deviation, and significance of differences of means (control vs. CPK at any developmental stage) was determined by the Student t test. For graphical presentation, means ± standard deviations were expressed as the percentage of total apical membranes demonstrating apical–lateral or basal–lateral membrane Na+/K+-ATPase in relation to the total number of tubules analyzed.

Specific methods for protein immunolocalization were established during our previous studies of matrix and membrane antigen expression during normal and abnormal murine renal development (7, 8, 19, 20, 22) and have been recently described in detail (21). We have previously characterized the antibody used to detect the α1 subunit of Na+/K+-ATPase, a rabbit polyclonal antibody to the purified dog kidney α1 subunit (23). The antibody utilized to localize Na+/K+-ATPase β1 subunit distribution was a rabbit polyclonal antibody raised against a β1 isoform-specific fusion protein (24), which was obtained commercially (Upstate Biotechnical, Lake Placid, NY). Antibodies to ZO-1 were prepared as previously reported (25). Antibodies to γ-glutamyltranspeptidase, the band 3 anion-exchanger GP-135, and carbonic anhydrase II were generously provided by N. P. Curthoys (Colorado State University), P. S. Low (Purdue University), G. K. Ojakian (State University of New York at Brooklyn), and P. J. Linser (University of Florida), respectively, and prepared as previously described (26–29). Antibodies to type IV collagen were obtained from DMI (Westbrook, ME), antibodies to laminin and entactin were obtained from Upstate Biotechnical, and biotinylated lectins were obtained from Sigma.

RESULTS

Analysis of control proximal tubules, identified by their lectin-staining pattern (LTA+, DBA−), showed that the distribution of both the α1 and β1 subunits of Na+/K+-ATPase was restricted to the basal–lateral membrane domain at all developmental stages (Fig. 1A). Similarly, localization of Na+/K+-ATPase subunits in unaffected or cystic CPK proximal tubules demonstrated exclusive distribution to the basal–lateral cell membrane domain (Fig. 1B). We did not detect expression of Na+/K+-ATPase subunits on the apical membrane of cells in control proximal tubules or unaffected or cystic CPK proximal tubules. Analysis of other marker proteins of the apical or basal–lateral membrane domain revealed normal distributions in those tubule cells (data not shown).

In the majority of control collecting tubules (LTA−, DBA−), both α1 and β1 subunits of Na+/K+-ATPase were localized to the basal–lateral membrane of cells in all nephrogenic zones. However, a subpopulation of control collecting tubules in the outer and inner cortical zones of active tubulogenesis clearly demonstrated apical, as well as lateral, membrane staining of both Na+/K+-ATPase subunits (Figs. 1C and 2A and C). The basal membrane staining was low compared with the intensity of the apical and lateral staining in the same cells and with the basal staining in other collecting tubules elsewhere in the tissue or in normal proximal tubules (see Fig. 1A and C). Na+/K+-ATPase distribution at the apical membrane was observed in a maximum of 16% of outer cortical, 6% of inner cortical, and 2% of medullary collecting tubules at days 0–5. However, apical membrane Na+/K+-ATPase staining declined progressively in all zones at subsequent developmental stages, as the normal basal–lateral membrane staining pattern became prominent (Fig. 2A and B).

In cystic CPK collecting tubules, apical and lateral membrane staining of Na+/K+-ATPase α1 and β1 subunits was significantly increased compared with Na+/K+-ATPase staining patterns in control tubules (Fig. 1D and 2). This was particularly true in the developing medulla, where a maximum of 63% of cystic tubules demonstrated apical membrane Na+/K+-ATPase distribution in newborn kidneys. In addition, significant differences were present between Na+/K+-ATPase distributions in cystic and control collecting tubules at all developmental stages studied. In the inner cortical zone, apical and lateral membrane Na+/K+-ATPase expression was observed in a maximum of 46% of cystic collecting tubules at day 3 [the first developmental stage at which collecting tubular cysts are seen in the inner cortex (7, 18)], with significant differences persisting between cystic and control collecting tubules at all developmental stages studied (Fig. 2B).

In the outer cortical zone, a maximum of 47% of collecting tubule cysts expressed apical and lateral membrane Na+/K+-ATPase distribution at day 5. Significant differences in the percentage of cells exhibiting apical and lateral membrane Na+/K+-ATPase between cystic and control collecting tubules were observed from day 5 to day 21 (Fig. 2A).

In all nephrogenic zones, the percentage of cystic collecting tubules with apical membrane Na+/K+-ATPase distribution decreased over time, paralleling the pattern seen in

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control collecting tubules. However, as noted, the numbers of cystic tubule cells exhibiting apical membrane distribution of Na+/K+-ATPase were greater than the numbers of control tubule cells with the apical enzyme at all stages analyzed (see Fig. 2).

We did not detect any abnormalities in immunolocalization of other apical or basal-lateral cell surface marker proteins at any stage of development in control or cystic collecting tubules (data not shown). These results indicate that the abnormal distribution of Na+/K+-ATPase to the apical membrane domain of cystic collecting tubule cells does not reflect a general defect in the polarized distribution of membrane proteins in these cells.

**DISCUSSION**

Regulation of Na+/K+-ATPase expression, activity, and distribution is critical for renal function. Regulation of Na+/K+-ATPase expression can occur at the transcriptional level or post-translationally by a variety of hormonal and ionic modulators including triiodothyronine, steroid hormones, and potassium (30, 31). In addition, regulation of Na+/K+-ATPase distribution in the plasma membrane of polarized epithelial cells has a direct effect on the direction of ion and fluid transport. For example, basal-lateral membrane distribution of Na+/K+-ATPase in renal tubule epithelia results in ion and fluid reabsorption, whereas Na+/K+-ATPase distribution to the apical membrane in choroid plexus results in ion and fluid secretion (16).

Our results show that during normal and cystic CPK proximal tubular development, both subunits of Na+/K+-ATPase are localized to only the basal-lateral membrane domain (Fig. 1 A and B). This suggests that intracellular sorting of Na+/K+-ATPase was normal during cystic transformation of renal proximal tubular epithelium. Proximal tubular cyst formation in the CPK model has previously been correlated with increased Na+/K+-ATPase activity (7, 8, 32). The current data demonstrate that intratubular cyst fluid accumulation in this nephron segment is not secondary to simple reversal of vectorial Na+ and fluid transport as has been proposed for ADPKD epithelium (6, 9). Instead, proximal tubular cyst formation in this murine model of ARPKD may involve increased organic anion secretion driven by a basal-lateral membrane Na+/K+-ATPase (8, 12, 33). The intratubular sequestration of osmotically active organic anions would then oblige net intratubular fluid accumulation, resulting in cyst formation and enlargement (2, 5).
The current study demonstrates that Na\(^+\)/K\(^+-\)ATPase is transiently localized to both the apical and lateral membranes of cells as a normal feature of renal collecting tubule development (Fig. 1C). This greatly extends previous observations made at a single developmental time point in rabbit and human kidneys (13, 14). At each developmental stage, apical membrane distribution of Na\(^+\)/K\(^+-\)ATPase was frequently found in the most immature collecting tubules of the outer cortical zone of active nephrogenesis, and it was only rarely demonstrated in the more mature collecting tubules of the medulla (Fig. 2 A and C). As might be predicted from the centrifugal gradient of tubular differentiation, which extends from medulla (most mature) to outer cortex (least mature) at any given developmental time point studied, the inner cortical zone demonstrated intermediate percentages of collecting tubules with apical membrane Na\(^+\)/K\(^+-\)ATPase (Fig. 2B). In all nephrogenic zones, the percentage of tubules demonstrating apical membrane Na\(^+\)/K\(^+-\)ATPase expression decreased at progressive developmental stages. This suggests that apical membrane Na\(^+\)/K\(^+-\)ATPase expression is a normal, transient phenotypic feature of early collecting tubule differentiation which is lost with subsequent cell maturation. If the apically expressed Na\(^+\)/K\(^+-\)ATPase is functional, it may partially explain the well-documented reduction in Na\(^+\) reabsorptive ability of immature distal nephrons (34).

Like immature control collecting tubules, cystic collecting tubules demonstrated apical and lateral membrane Na\(^+\)/K\(^+-\)ATPase expression (Fig. 1D). Cystic collecting tubules expressing apical membrane Na\(^+\)/K\(^+-\)ATPase were found in all nephrogenic zones paralleling the medullary to cortical pattern of cystic ontogeny that we have previously described in the CPK model (18). In parallel with control collecting tubules, the percentage of cystic collecting tubules with apical and lateral membrane Na\(^+\)/K\(^+-\)ATPase expression decreased in relation to the total cystic nephron population at successive developmental stages. However, with the exception of the outer cortical zone at day 3, the percentage of cystic collecting tubules in each nephrogenic zone with apical membrane Na\(^+\)/K\(^+-\)ATPase expression was significantly greater than matched controls. From these data, we conclude that a large population of cystic collecting tubules exhibit a relatively undifferentiated phenotype in terms of the membrane distribution of Na\(^+\)/K\(^+-\)ATPase. This conclusion is consistent with recent reports that cystic CPK collecting tubule epithelium exhibits elevated steady-state mRNA levels of a variety of protooncogenes and the SGP2 gene that are normally expressed during an early stage of collecting tubule differentiation (35, 36). Further, preliminary ultrastructural studies of cystic CPK collecting tubules have revealed characteristic anatomical features of a relatively undifferentiated epithelium (37). The normal distribution of a series of marker proteins of either the apical or basal–lateral membrane, as well as the presence of normal cell–cell junctions in cystic CPK epithelium (37), rules out ischemic injury as a factor in the observed pattern of apical membrane Na\(^+\)/K\(^+-\)ATPase expression (38).

In the current study, the distributions of other apical or basal–lateral membrane marker proteins were unaffected in immature control and cystic collecting tubule epithelial cells. This rules out the possibility that the abnormal distribution of Na\(^+\)/K\(^+-\)ATPase to the apical membranes of these cells is the result of a general deregulation of mechanisms involved in establishing and maintaining cell surface polarity. Furthermore, the fact that Na\(^+\)/K\(^+-\)ATPase distribution is restricted to the basal–lateral membrane of both normal and cystic proximal tubule epithelial cells indicates that CPK epithelial cells are capable of normal sorting of Na\(^+\)/K\(^+-\)ATPase. It remains possible that distinct, as-yet-undetermined, abnormalities in Na\(^+\)/K\(^+-\)ATPase sorting that are unique to cystic

**Fig. 2.** Localization of Na\(^+\)/K\(^+-\)ATPase α1 subunit to apical, as well as lateral, cell surfaces in cystic CPK (○) and control (▲) collecting tubules. Data are expressed as percentages (mean ± SD) of total control or cystic DBA\(^+\) collecting tubules (19) in outer cortex (A), inner cortex (B), and medulla (C). *P < 0.001, cystic vs. controls; **P < 0.02, cystic vs. controls.
CPK epithelium are responsible for the apical membrane location of Na+/K+-ATPase in those cells. However, since Na+/K+-ATPase distribution to the apical membrane is a normal, albeit transient, characteristic of the development of normal collecting tubule epithelial cell surface polarity, we suggest an alternative explanation. We hypothesize that the sorting pathway of Na+/K+-ATPase is the same in normal and cystic epithelial cells, but that Na+/K+-ATPase retention in the apical membrane is transient in normal development but persists in cystic epithelial cells.

Mechanisms of membrane protein sorting in polarized renal epithelial cells have been analyzed extensively (39–41). Recent studies indicate that the polarized cell surface distribution of Na+/K+-ATPase in an in vitro model of normal renal transporting epithelia, MDCK cells, is regulated by differences in the relative stability and activity of newly synthesized Na+/K+-ATPase that is normally delivered to both the apical and basal–lateral membranes (15). Na+/K+-ATPase delivered to the apical membrane is inactive and rapidly removed (t½ < 2 hr). In contrast, Na+/K+-ATPase delivered to the basal–lateral membrane is active and retained in the membrane (t½ > 36 hr), probably as a result of direct binding to the membrane-associated cytoskeleton (23, 31, 42). At present, we do not know if retention of Na+/K+-ATPase on the apical membrane during normal collecting tubule development or in cystic tubule epithelial cells is due to the assembly of the membrane-cytoskeleton at that membrane. However, it is noteworthy in this context that in retinal pigmented epithelium (17) and choroid plexus (J. A. Marts, R. W. Mays, and W.J.N., unpublished data) the membrane-cytoskeleton is codistributed and complexed with Na+/K+-ATPase on the apical membrane. The distribution of membrane-cytoskeletal proteins in the cystic epithelial cells is not yet known.

A further regulatory mechanism that can be considered in this context is the retention of apical membrane Na+/K+-ATPase in cystic epithelial cells. In normal epithelial cells, Na+/K+-ATPase delivered to the apical membrane does not bind ouabain and is inactive (15). The mechanism(s) of inactivation of Na+/K+-ATPase at the apical membrane is unknown. However, previous studies indicate that Na+/K+-ATPase activity is downregulated by decreased fluidity of the lipid bilayer (43). In normal polarized epithelial cells, the apical membrane contains a high concentration of glycosphingolipids compared with the basal–lateral membrane (44). Glycosphingolipids form extensive intermembranous hydrogen bonds (45), resulting in a decrease of membrane fluidity that could decrease Na+/K+-ATPase activity (46); a reversal of this process could lead to Na+/K+-ATPase activation. At present, though, it is not known whether the distribution of glycosphingolipids is altered in the apical membrane of cystic tubule cells.

In conclusion, we have shown that control collecting tubules, but not proximal tubules, have a developmental profile of decreasing apical membrane Na+/K+-ATPase expression. Though exhibiting a similar development profile, cystic CPK collecting tubules, but not proximal tubules, demonstrate significant levels of apical membrane Na+/K+-ATPase expression relative to developmentally staged controls. Thus, increased apical membrane Na+/K+-ATPase expression in cystic kidney epithelia may be a marker of the relatively undifferentiated phenotype of cyst lining cells, and it may have pathogenic import in cyst formation and progressive enlargement.

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