Decreased amiloride-sensitive $\text{Na}^+$ absorption in collecting duct principal cells isolated from BPK ARPKD mice

Elias I. Veizis, Cathleen R. Carlin, and Calvin U. Cotton

Departments of Pediatrics and Physiology and Biophysics, Rainbow Center for Childhood PKD, Case Western Reserve University, Cleveland, Ohio 44106-4948

Submitted 1 May 2003; accepted in final form 7 October 2003

Veizis, Elias I., Cathleen R. Carlin, and Calvin U. Cotton. Decreased amiloride-sensitive $\text{Na}^+$ absorption in collecting duct principal cells isolated from BPK ARPKD mice. Am J Physiol Renal Physiol 286: F244–F254, 2004. First published October 14, 2003; 10.1152/ajprenal.00169.2003.—The main feature of polycystic kidney diseases (PKD) is formation and progressive enlargement of renal cysts. Alterations in epithelial cell proliferation, extracellular matrix, and ion transport are thought to contribute to cyst enlargement and loss of renal function. Abnormal $\text{Cl}^-$ secretion is implicated in cyst enlargement in autosomal dominant PKD (ADPKD), but little is known about transport abnormalities in autosomal recessive PKD (ARPKD). We developed a method to isolate collecting duct (CD) principal cells (site of the lesion in ARPKD) from normal and ARPKD mice. A transgenic mouse (Hoxb7/GFP) in which enhanced green fluorescent protein (GFP) is expressed in CDs was bred with an ARPKD mouse (BPK), and GFP-positive cells from normal and cystic mice were selected by fluorescence-activated cell sorting. GFP-positive CD cells ($>95 \pm 3\%$) obtained from either normal or cystic mice formed high-resistance, polarized epithelial monolayers. Expression patterns for marker proteins and the presence of a central cilium confirmed that the monolayers are composed of principal cells. Under basal conditions, the $\text{Cl}^-$ secretory responses elicited by elevation of $\text{cAMP}$ or calcium were not significantly different between normal and cystic monolayers. In contrast, the amiloride-sensitive short-circuit current was significantly reduced in monolayers of cells isolated from cystic mice (12.9 ± 1.6 $\mu\text{A/cm}^2$; $n = 10$) compared with monolayers of cells isolated from normal mice (27.3 ± 3.4 $\mu\text{A/cm}^2$; $n = 12$). The results of these studies suggest that epithelial sodium channel-mediated sodium absorption is decreased in principal cells of ARPKD CD cysts and that the reduction in sodium absorption may contribute to the accumulation of luminal fluid.

polycystic kidney disease; kidney; epithelial sodium channel; fluorescence-activated cell sorting; collecting tubule cysts

POLYCYSTIC KIDNEY DISEASES are severe genetic disorders with both autosomal dominant (ADPKD) and autosomal recessive (ARPKD) patterns of transmission. ADPKD is more common and typically presents in the third or fourth decade, and cysts can arise from all segments of the nephron (5). On the other hand, the recessive form of PKD is a pediatric disease that affects the collecting ducts (CDs) of the kidney primarily and is associated with biliary duct ectasia and portal fibrosis in the liver. ARPKD has a high mortality rate (40–60%) in the newborn period and accounts for ~35% of all end-stage renal disease in children (20). The renal cystic disease typically begins in utero and manifests as fusiform dilatation of the collecting ducts that radiate from the medulla to the cortex (7). Cyst enlargement in the kidney leads to destruction of the parenchymal architecture and functional debilitation of the kidney, which results in end-stage renal insufficiency. It has been postulated that renal cyst expansion occurs due to proliferation of cyst wall epithelial cells and fluid accumulation in the cyst lumen. Indeed, studies have shown that cystic cells have a higher index of proliferation in ADPKD (15) and ARPKD (19). Cystic cell proliferation is further increased by cAMP agonists and activation of the EGF/EGF receptor (EGFR) axis (15, 19, 40). Mislocalization of EGFRs to the apical membrane of cystic tubules is a feature common in both ARPKD and ADPKD (13, 25). Furthermore, genetic or pharmacological reduction of EGFR function significantly slows disease progression in mouse models of ARPKD (4, 35).

Detailed studies of salt and water transport in renal cysts detached from the nephron of origin and primary cultures of renal epithelial cells isolated from ADPKD patients suggest that fluid accumulation in the cysts is the result of NaCl secretion (15). Grantham and co-workers (12, 33) demonstrated that $\text{cAMP}$-stimulated, CFTR-dependent $\text{Cl}^-$ secretion contributes to fluid accumulation in renal cysts. Because the cysts are detached from the segment of origin, it is difficult to identify the precise alterations in tubule transport that accompany development of a renal cyst in ADPKD. In contrast, the predominant site of renal disease in ARPKD is the CD, and late in the disease most of the kidney is composed of dilated, fluid-filled CDs rather than isolated, detached cysts.

The CD of the mammalian kidney is a cytologically diverse segment comprised of principal cells and intercalated cells. Intercalated cells account for 10–30% of the cells in the collecting duct and are responsible for $\text{H}^+$/HCO$_3$ secretion (8, 17). Principal cells are more numerous (70–90%) and are characterized by hormonally regulated (e.g., aldosterone and vasopressin) Na$^+$, potassium, and water transport (26, 24). Principal cells play a vital role in salt and water homeostasis via regulated alterations in Na$^+$ absorption and water permeability. The expression and activity of an epithelial Na$^+$ channel (ENaC), located in the apical plasma membrane of CD principal cells (11), are the rate-limiting steps for CD Na$^+$ absorption. ENaC expression, although established early in nephrogenesis (16), is developmentally regulated and is important for postnatal Na$^+$ homeostasis (29). ARPKD is generally considered to be a disorder with developmental arrest or cellular dedifferentiation to a less mature phenotype. Therefore, ENaC-mediated Na$^+$ absorption capacity, which is considered an indication of CD maturation, represents an important ion transport pathway that may not fully develop or might be lost from less mature cystic CD cells. ARPKD cystic CDs...
are composed almost exclusively of principal cells; however, almost nothing is known about the ion transport properties of cystic CD principal cells. A limiting factor in the study of ion transport pathophysiology in ARPKD is the lack of relevant biological preparations, because cystic CDs are extremely dilated and not suitable for conventional tubule perfusion. Cell lines generated from human (28) and murine (36) ARPKD kidneys as well as freshly isolated or primary cultures of epithelial cells represent important reagents for the study of disease-related alterations in cell function.

In this study, we developed an efficient method of isolating renal CD principal cells from normal and ARPKD mice that can be grown in primary culture and are suitable for analysis of transepithelial ion transport. The results of these studies suggest that amiloride-sensitive Na\(^+\) absorption is significantly reduced in cystic CD principal cells, whereas agonist-induced Cl\(^-\) secretion is similar in normal and cystic cells. These observations highlight a fundamental difference between ion transport dysregulation in ADPKD and ARPKD.

**METHODS**

*Generation of the animal model.* The primary disease model is the BPK mouse, which arose as a spontaneous mutation on a BALB/c background and mimics the phenotype of ARPKD (22). Confirmed BPK heterozygotes (bpk\(^+/-\) determined by breeding) were crossed with a transgenic mouse (Hoxb7/GFP; B6xCBA) (32) in which green fluorescent protein (GFP) expressed under the control of Hoxb7 promoter is specifically expressed in the ureteric bud and its derivative CDs. GFP\(^+/+\) offspring (F\(_1\)) were bred with confirmed BPK heterozygotes (bpk\(^+/-\)) to determine BPK status of the F\(_1\) pups. GFP\(^+/+\)/bpk\(^+/-\) animals were identified and bred with one another. All of the resulting F\(_2\) pups were genotyped for the GFP transgene and examined at postnatal days 10–12 to identify cystic pups that have a characteristic abdominal distention. Age-matched cystic (GFP\(^+/+\)/bpk\(^+/-\)) and normal (GFP\(^+/-\)/GFP\(^+/-\)) littermates aged between days 20 and 24 were used for these studies. The offspring were genotyped by PCR analysis of DNA extracts from tail sections to identify animals that carried the GFP transgene (GFP\(^+/-\)). The primers used to screen for the Hoxb7/enhanced GFP (EGFP) transgene are *primer E* and *primer K* (see Table 1), which amplify a band of 321 bases as described by Srinivas et al. (32).

The studies described here were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of Case Western Reserve University School of Medicine.

**Primary cell isolation and cell culture.** Kidneys were dissected under sterile conditions from CO\(_2\)-anesthetized normal and cystic animals, and the renal capsule was removed. The kidneys were minced thoroughly with a razor blade, rinsed with sterile PBS, and resuspended in 10 ml of collecting tubule (CT) media supplemented with collagenase (1.5 mg/ml type IV; Worthington). The minced tissue was digested for 30–40 min at 37°C in a shaking water bath to obtain a homogenous suspension of individual cells and tubule fragments. The cellular material was centrifuged, resuspended in CT media, plated on plastic tissue culture dishes, and placed in a humidified tissue culture incubator (37°C and 5% CO\(_2\)) for 12–24 h. The unattached cells were removed, centrifuged, resuspended in fresh CT media, and plated onto additional tissue culture dishes. Media were changed every 48 h thereafter. Primary cell cultures were expanded for 4–6 days before sorting.

CT media were composed of: 1:1 mix of DMEM and Ham’s F-12 medium (Life Technologies) supplemented with 1.3 μg/ml sodium selenite, 1.3 μg/ml triiodo-l-thyronine, 5 mg/l insulin, 5 mg/l transferrin, 25 μg/l prostaglandin E1, 2.5 mM glutamine, 50 mM dexamethasone, 50,000 U/l nystatin, 50 mg/l streptomycin, and 30 mg/l penicillin G.

**Fluorescence-activated cell sorting.** After 4–6 days in culture, the cells were detached from the tissue culture dish (0.25% trypsin and 0.5 mM EDTA), resuspended in CT medium that contained fetal calf serum (10%), and passed through 40-μm mesh to remove debris. The cells were recovered by centrifugation (400 g, 5 min), and the pellet was resuspended in an appropriate volume of ice-cold HEPES-buffered salt solution (~15,000,000 cells/ml). The preparation was subjected to fluorescence-activated cell sorting (FACS) using an Elite ESP (Beckman Coulter, Miami, FL) FACS sorter equipped with an argon ion laser tuned to 488 nm. Data were processed with the Expo32 software (version 1.2b, Beckman Coulter) analysis program. GFP-positive cells were identified by their high-fluorescence intensity compared with GFP-negative cells. The dot plot analysis revealed that the population of GFP-positive cells was well separated from the GFP-negative population. The sorting gate was positioned well into the GFP-positive population to minimize contamination of the preparation by GFP-negative cells. The GFP-positive CD cells were collected under sterile conditions, resuspended in CT media supplemented with EGF (2 ng/ml) and FBS (2.5%), and plated on collagen-coated permeable supports (see below). In addition to the initial FACS, CD cells were subjected to a second analysis after electro-

---

**Table 1. Primers used for RT-PCR reactions**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Reference or Gene Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP</td>
<td><em>Primer E</em></td>
<td>5’-AGG GCC AGC AGA TGA GCC CCC GCA CT-3’</td>
</tr>
<tr>
<td></td>
<td><em>Primer K</em></td>
<td>5’-TGA GAC CAC GCA GAA GCC TCA TCT C-3’</td>
</tr>
<tr>
<td></td>
<td>Mineralocorticoid receptor</td>
<td>5’-CTA ATG ATG CTC GAC ACC ACC-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’-AAA GCG TCT GTT CCG TGA TGC-3’</td>
</tr>
<tr>
<td></td>
<td>Epithelial sodium channel α-subunit</td>
<td>5’-AGG GAG GAG TTT CCA ACC AG-3’</td>
</tr>
<tr>
<td></td>
<td>H-ATPase (kidney-specific) β1-isofom (COOH terminal)</td>
<td>5’-GCC CAC AAG AGT AGA CAG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’-TGA CAC GCA AGG GAT GCC GAC TT-3’</td>
</tr>
<tr>
<td></td>
<td>H-ATPase (kidney-specific) β1-isofom (NH2 terminal)</td>
<td>5’-CTG TCC AAA GCC ACC CAA GTA-3’</td>
</tr>
<tr>
<td></td>
<td>Anion exchanger 1, band 3</td>
<td>5’-TCA GAC AGG CAT GGG GAC TT-3’</td>
</tr>
</tbody>
</table>

EGFP, enhanced green fluorescent protein.
physiology experiments to validate the purity of the experimental preparation.

**Electrophysiological studies.** CD primary cells were seeded (1.5–2 x 10^5 cells/filter) on collagen-coated permeable supports (12-mm Millicell-CM filter). The filter surface was coated with calfskin collagen as described (37). The GFP-positive cells were grown in CT media supplemented with 2.5% FBS and 2 mg/ml EGF for 4–5 days at 37°C in a humidified 5% CO₂ atmosphere. FBS and EGF were omitted from CT media at least 24 h before electrophysiological analysis. Confluent monolayers were mounted in a thermistatically controlled Ussing chamber equipped with gas inlets and separate reservoirs for the perfusion of the apical and basolateral compartments. Both sides were bathed with an equal volume of Krebs-Ringer bicarbonate solution containing (in mM) 115 NaCl, 25 NaHCO₃, 5 KCl, 2.5 Na₂HPO₄, 1.8 CaCl₂, 1 MgSO₄, and 10 glucose. The solutions were circulated through the water-jacketed glass reservoir by gas lifts (95% O₂-5% CO₂) to maintain solution temperature at 37°C and pH at 7.4. Transepithelial voltage difference (Vₜ) was measured between two Ringer-agar bridges, each positioned 3 mm from the monolayer surface. Calomel half-cells connected the bridges to a high-impedance voltmeter. Current through an external direct-current source was passed by silver-silver chloride electrodes and Ringer-agar bridges to clamp the spontaneous Vₜ to 0 mV. The current required to clamp the voltage to a nonzero value was measured to control short-circuit current (Isc). RT-PCR analysis of gene expression from kidneys and primary cultures. RNA was obtained from cystic and normal kidneys (100 mg tissue) and primary cell cultures (1–2 x 10⁶ cells) with an RNeasy Mini Kit, which includes an on-column DNase digestion with RNase-free DNase set (Qiagen, Valencia, CA). The concentration and quality of mRNA were determined photometrically (260/280 nm). RT-PCR was performed by using Moloney murine leukemia virus, an RT system (Life Technologies, Rockville, MD) according to the manufacturer’s instructions. Appropriate primers (Table 1) were used to amplify cDNAs from whole kidney and primary cell cultures for GFR (32), mineralocorticoid receptor (MC-R) (6), the α-subunit of ENaC (α-ENaC) (31), anion exchanger 1 (AE1; band 3) (18), and COOH and NH₂ termini of the β₁-subunit of the hydrogen pump (H-ATPase β₁) (23). PCR reactions were performed on a thermocycler (94°C denaturing 1 min/55°C annealing 1 min/72°C elongation 1 min/cycle). PCR products were resolved by electrophoresis in 1% agarose gels and stained with ethidium bromide. The size of PCR products was resolved by electrophoresis in 1% agarose gels denaturing 1 min/55°C elongation 1 min/cycle).

**RESULTS**

**Characterization of GFP+/− normal and cystic mice.** The offspring of confirmed GFP+/−/bpk+/−/ parents were evaluated by PCR for the GFP transgene and by visual observation for abdominal distortion on postnatal days 10–12 to

| Table 2. Phenotype of Hoxb7/EGFP+/− cystic and normal mice and Balbc cystic mice |
|---------------------------------|--------|--------|-----------------|--------|
| Phenotype, Genotype, and Animal Background | Total Body Wt, g | Kidney Wt, g | (KW/TBW) × 100, % | Urinary Osmolality, mosmol/kgH₂O | Animal Survival, days |
|---------------------------------|--------|--------|-----------------|--------|
| Normal, GFP+/−, bpk+/− | Balbc × B6CBA | 11.3±0.8 | 0.15±0.01 | 1.4±0.2 | 1,190±95 | NM |
| Cystic, GFP+/−, bpk+/− | Balbc × B6CBA | 10.9±1.2 | 2.4±0.3* | 22±2.5* | 468±35* | 23±3 |
| Normal, bpk−/− | Balbc | 10.6±1.1 | 0.15±0.02 | 1.45±0.1 | 1,050±54 | NM |
| Cystic, bpk−/− | Balbc | 10.9±0.8 | 2.3±0.3* | 21±2.2* | 444±26* | 23±3 |

Values are means ± SE, n, no. of mice; KW, kidney wt; TBW, total body wt. *Significantly different compared with normal animals (P < 0.001, unpaired t-test). NM, not measured.

**Statistical analysis.** All results are expressed as means ± SE. Statistical significance was evaluated by either unpaired or paired Student’s t-test. P < 0.05 was considered significant.

**RESULTS**

**Characterization of GFP+/− normal and cystic mice.** The offspring of confirmed GFP+/−/bpk+/−/ parents were evaluated by PCR for the GFP transgene and by visual observation for abdominal distortion on postnatal days 10–12 to

*AJP-Renal Physiol. • VOL 286 • FEBRUARY 2004 • www.ajprenal.org*
identify cystic and normal GFP+/− pups. Cyst development and disease progression in outbred GFP+/− cystic mice are indistinguishable from inbred BALBc cystic BPK mice (Table 2). Mean survival of cystic GFP+/− mice is 23 ± 3 days, and kidney weight/total body weight is 22 ± 2.5%.

Furthermore, the GFP+/− cystic mice exhibit the urine-concentrating defect characteristic of this animal model of ARPKD (Table 2). All experiments described in this study were performed in age-matched cystic and normal littermates between 20 and 24 days old, an age that represents a
late stage in the disease (end-stage renal disease) in this murine model of ARPKD.

Histological examination of sections of GFP+/− normal mouse kidneys revealed a GFP expression pattern consistent with CDs radiating from the cortex to the medulla (Fig. 1A). On the other hand, cystic kidneys and isolated cystic nephron fragments revealed that the majority of the dilated renal tubules (cysts) were lined by a single layer of GFP-positive epithelial cells (Fig. 1B and C). Immunolocalization of principal cell AQP2 (Fig. 2A and B) and intercalated cell H-ATPase (Fig. 2C and D) demonstrated that nearly all of the cells (>95%) lining the GFP-positive cysts are principal cells, consistent with previous reports. In contrast to cystic mice, their normal littermates had the expected distribution of intercalated and principal cells in the GFP-positive collecting ducts (Fig. 2, A and B).

Isolation of GFP-positive CD cells. Individual cells and tubule fragments obtained from a collagenase digest of minced kidneys from cystic and normal mice were plated on plastic tissue culture dishes and maintained in defined, serum-free CT medium. After 4–6 days in culture, the cells were collected (15–24 × 10⁶ cells/mouse; see Fig. 4, left) and subjected to FACS. Representative sorting profiles of cystic GFP+/− and cystic GFP−/− mice and reanalysis of the GFP-positive cell population from their age-matched littermates are shown in Fig. 3. The cells derived from normal and cystic kidneys are

Fig. 3. Fluorescence-activated cell sorting (FACS) of isolated renal cells. GFP intensity profiles for cells isolated from normal and cystic mouse kidneys are indistinguishable. The dot plots show side scatter (SS) vs. forward scatter (FS) (A1, B1, and C1) and forward scatter vs. GFP intensity (A2, B2, and C2), and the histograms indicate the analyzed events vs. GFP intensity (A3, B3, and C3). A1–A3: FACS distribution of cells derived from GFP+/− cystic mouse. B1–B3: FACS distribution of cells derived from GFP−/− cystic mouse. C1–C3: FACS reanalysis of cells grown for 5–6 days in collagen-coated permeable supports and used for electrical measurements. The monolayers are composed of >97% GFP-positive cells.
similar in terms of forward scatter and side scatter, which indicate size and viability, respectively. However, based on GFP fluorescence intensity, the histogram revealed a bimodal distribution of cells derived from either GFP<sup>+/−</sup> normal or cystic mice, but not from GFP<sup>+/+</sup> animals. The mean fluorescence intensity of GFP-positive cells was ~100-fold greater than that of GFP-negative cells. The GFP-positive cells comprise 18% (n = 29) of the sorted cells in cultures from normal mice and 22% (n = 32) of the cells derived from cystic mice (Fig. 4, middle). From the total population of kidney cells, we were able to isolate 1–1.4 × 10<sup>6</sup> (n = 28–32) GFP-positive cells/animal from cystic or normal littermates (Fig. 4, right). Reanalysis of GFP-positive CD cells indicated very low contamination levels by GFP-negative cells (<3%).

Characterization of cells obtained by FACS. Primary cultures of CD cells selected by FACS were grown on collagen-coated permeable supports for 5–6 days. The confluent monolayers developed a typical “cobblestone” appearance (Fig. 5A), and normal and cystic monolayers were morphologically indistinguishable. Fluorescence microscopy revealed that 97% (n = 60 cells/monolayer in 4 different preparations) of the cells in the monolayer are GFP positive, consistent with a nearly pure population of CD cells (Fig. 5B). The cells differentiate and form junctional complexes, as demonstrated by ZO-1 staining (Fig. 5C), and develop high electrical resistance (1–1.4 kΩ·cm<sup>2</sup>; Table 3). Because both principal cells and intercalated cells are derived from the ureteric bud and are GFP positive, we sought to determine by RT-PCR the gene expression of marker proteins that are characteristic of principal cells or intercalated cells. RT-PCR analysis of RNA isolated from cystic or normal monolayers revealed the presence of mRNA for principal cell-specific proteins (mineralocorticoid receptor and α-ENaC) but not for intercalated cell-specific proteins (H-ATPase β1-subunit and anion exchanger 1, band 3) (Fig. 6). RNA isolated from normal and cystic kidneys was used as a positive control for expression. The eponymous morphological characteristic of the principal cells is the presence of a single central cilium (38) that is absent from intercalated cells.

Fig. 4. Quantitative analysis of primary collecting duct cell isolation. GFP-positive cells were sorted from mouse renal cell population grown for 4–6 days in culture. Left: total number of kidney cells obtained per mouse after expansion of the renal cell population for 4–6 days in culture (n = 28–32). Middle: FACS analysis reveals that 17.7 ± 3.5% of the cells isolated from the normal mouse are GFP<sup>+/−</sup> (n = 29) and 21.6 ± 4.3% of cells isolated from a cystic mouse are GFP positive (n = 32). Right: yield of GFP-positive cells from normal or cystic mice is 1.1–1.4 × 10<sup>6</sup> (n = 28–32). Values are means ± SE.

Fig. 5. Transmitted and fluorescent microscopy of GFP-positive cell monolayers grown on permeable supports. Collecting duct cells form confluent, polarized, well-differentiated epithelial monolayers. A: phase-contrast microscopy reveals the cobblestone appearance characteristic of epithelial cells, and almost all the cells in the monolayer are GFP positive as indicated by fluorescence microscopy (B). Collecting duct cells differentiate in culture and form confluent, polarized monolayers as indicated by tight junction formation (C; ZO-1; red). Bars: 50 μm.
ing electron microscopy revealed that nearly all of the cells
(57 of 60 cells, in 10 fields) in monolayers derived from cystic
or normal mice have a central cilium, and no morphological
differences were noted between the cystic and the normal
principal cells (Fig. 7). The average length of the cilium (1–2.5
μm, n = 30 cells) was the same in cystic and normal principal
cells. These results indicated that the primary cultures of CD
cells isolated by FACS of GFP+ /− mice and cultured for 5–6
days on permeable supports before Ussing chamber experi-
ments consisted almost exclusively of cells with features char-
acteristic of mammalian principal cells.

Bioelectric properties of normal and cystic principal cell
monolayers. The transepithelial bioelectric properties of pri-
mary cultures of CD principal cells are listed in Table 3. Cells
derived from either normal or cystic mice formed polarized,
high-resistance epithelial monolayers. Under basal conditions,
\( R_T \) was not different between normal and cystic monolayers
(Table 3). However, \( I_{sc} \) was significantly lower in cystic cells
compared with normal principal cell monolayers. In addition,
the difference in \( V_T \) of monolayers of principal cells isolated
from cystic mice (−20.0 ± 3.4 mV, n = 10) was significantly
reduced compared with monolayers composed of normal prin-
cipal cells (−32.6 ± 7.6 mV, n = 12, P < 0.005, unpaired
\( t \)-test).

As illustrated in Fig. 8, A and B, addition of the Na⁺ channel
inhibitor amiloride (100 μM; a maximally effective inhibitory
concentration) to the apical bathing solution caused a rapid
increase in \( R_T \) and a decrease in \( I_{sc} \) (Table 3). Nearly all (95%)
of the basal \( I_{sc} \) in normal monolayers was inhibited by amilo-
ride in contrast to cystic monolayers, where a significantly
smaller fraction (83%) of \( I_{sc} \) in cystic monolayers was sensitive
to amiloride. Thus the absolute and fractional inhibition of \( I_{sc} \)
(Fig. 8C) was significantly greater in monolayers derived from
normal compared with cystic mice, a response suggestive of
reduced amiloride-sensitive Na⁺ absorption in cystic collect-
ing duct principal cells. Furthermore, the amiloride-induced
current conductance decrease (\( \Delta G_T \)) in normal monolayers was nearly
twice as large as the decrease observed in cystic monolayers
(Table 3 and Fig. 8D).

Net Cl⁻ secretion, mediated by either cAMP- and/or cal-
cium-dependent apical Cl⁻ channel activation, has been impli-
cated in transepithelial fluid secretion in ADPKD (12, 30). Because CD
cells are known to express a number of Cl⁻ channels including cAMP- and calcium-activated channels, we
examined the potential role of enhanced Cl⁻ secretion in
ARPKD. We determined the effect of elevated cAMP (forsko-
lin/IBMX; 10 μM/100 μM) on \( I_{sc} \) in cystic and normal monolayers
that had been pretreated with amiloride. Addition of the
cAMP agonists to the basolateral bathing solution caused a
small, sustained increase in \( I_{sc} \). The steady-state (reached after
10-min exposure to the agonist) \( I_{sc} \) during elevation of cAMP
in normal and cystic monolayers was 4.7 ± 0.2 and 5.5 ± 0.3
µA/cm², respectively (Table 3). As expected, \( R_T \) was also
significantly decreased in response to cAMP (Table 3).

Fig. 6. RT-PCR analysis of expression of cell-specific markers.
GFP-positive cells grown for 4–6 days on permeable supports
are principal cells. RT-PCR analysis of FACS isolated cells
derived from normal or cystic mice express the α-subunit of
the epithelial sodium channel (α-ENaC) and mineralocorticoid re-
ceptor mRNAs (principal cell marker proteins) but do not
express H⁺-ATPase kidney-specific β₁-subunit and anion ex-
changer 1, band 3 mRNAs (intercalated marker proteins).
Whole kidney mRNA isolated from normal or cystic mice was
used as a positive control for each of the 6 PCR primer sets.
Control reactions without the addition of RT were negative
(data not shown).
the increase in $I_{sc}$ nor the decrease in $R_T$ was significantly different in normal compared with cystic monolayers. Calcium-activated Cl$^-$ secretion was elicited by addition of ATP (100 μM) to the apical bathing solution of epithelial monolayers pretreated with amiloride and forskolin/IBMX. Both normal and cystic monolayers responded with a large, transient increase in $I_{sc}$. The peak currents were observed at ~30 s after exposure to ATP and were not significantly different between normal and cystic monolayers (Table 3). The $I_{sc}$ of both cystic and normal monolayers returned to pre-ATP values within 3–5 min. Thus neither the magnitude (Fig. 9, A and B) nor the duration of the secretory response to extracellular ATP was abnormal in cystic cells.

DISCUSSION

ARPKD is a rapidly progressive pediatric disease that leads to renal failure due to the formation of extremely dilated CDs.
and destruction of kidney parenchyma. The dilated CDs (referred as CD cysts due to the analogy with ADPKD) are lined with a single layer of highly proliferative CD principal cells. Anatomically, the dilated nephron segments retain up- and downstream connections, but they appear to result in “functional cysts.” Hypertension frequently accompanies ARPKD, but the precise mechanisms responsible for high blood pressure in this disease remain unknown. Because ARPKD is a complex disease, hypertension might develop as a result of renin-angiotensin-aldosterone axis overactivity, local and systemic effects of substances released in response to kidney hypoxia, or aberrant renal tubule ion transport. CDs are the site of the kidney lesions in ARPKD, and ion transport phenotype changes in the disease might provide clues about fluid retention in dilated tubules and/or the etiology of hypertension.

Renal CD principal cells isolated from normal and ARPKD mice form high-resistance, polarized monolayers in primary culture. The Cl⁻ secretory responses due to elevation of cAMP or calcium are the same in normal and cystic cells, whereas amiloride-sensitive Na⁺ absorption is significantly reduced in cystic cells.

These results suggest that dysregulation of PC Na⁺ absorption may contribute to the CD dilatation and fluid retention in the kidney characteristic of ARPKD.

Aberrant ion transport in the kidney is not linked directly to the genetic defects that cause PKD but may play an important role in the rate of disease progression. In the dominant form of the disease (ADPKD), fluid accumulation driven by NaCl secretion increases cyst size, leading to kidney parenchymal destruction and, ultimately, renal failure. Little is known about ion transport in ARPKD mostly due to the lack of relevant experimental systems. The goal of these studies was to develop a method for isolating CD principal cells and to examine the ion transport phenotype of primary CD cells derived from the BPK mouse model of ARPKD. To this end, we crossed the Hoxb7/GFP mouse (32) line with the BPK murine model of ARPKD (22) to yield cystic and noncystic mice that specifically express EGFP in CD cells. The Hoxb7/EGFP⁺/+×bpk⁻/− mice had the same time course of disease progression, site of cystic lesions, and degree of renal failure due to cyst enlargement as the inbred BALBc bpk⁻/− mice, indicating that disease phenotype is independent of the mouse strain. Similar results were obtained when the BALBc bpk⁻/− mouse was bred with other mouse lines (e.g., CFTR knockout and ImmortoMouse), and there was no change in disease phenotype (21, 36).

It is clear from our immunolabeling experiments that normal and cystic animals express GFP in both principal cells and intercalated cells. However, nearly all of the GFP-positive cells in the CD cysts are principal cells, in agreement with previous studies (35). Thus GFP expression provides an easily visualizing marker for the CDs in whole cystic kidney sections in organ culture, in kidney section immunohistochemistry, and, more importantly, a nearly pure population of CD cells can be isolated by FACS. Even though we cannot ascertain the subsegment origin of isolated cells, the phenotypic characterization reveals that they are CD principal cells. GFP-positive cells were easily detected and isolated by FACS because of the uniformly high level of expression of the transgene (no evidence of intracellular GFP aggregation occurs), with an ~100-fold increase in mean fluorescence intensity compared with GFP-negative cells. A similar approach was described recently by Nelson and co-workers (41), in which PC-specific GFP transgene expression was driven by the AQP2 promoter. Their model provides a unique opportunity to study cellspecific AQP2 promoter activity; however, sustained high-level expression of GFP appears to require maneuvers such as dehydration of the animals or exposure of the cells to cAMP agonists. The relatively large number of cells obtained from each Hoxb7/EGFP⁺/+ normal or cystic mouse precludes the need for multiple passages to expand the cell number and thereby reduces the problems associated with long-term cell culture. Importantly, GFP expression status is faithfully retained in culture for at least 2 wk. As discussed above, both principal cells and intercalated cells are GFP positive in vivo, but the epithelial monolayers after 10–14 days in culture are composed exclusively of principal cells (e.g., possesses a central cilium and express GFP, α-ENaC, MC-R, and AQP2, but not H-ATPase β₁-subunit and AE1 mRNA). It is not known whether the culture conditions utilized in our studies do not support intercalated cell survival or whether there is conversion from intercalated cells to principal cells ex vivo. There is evidence of phenotypic plasticity in CD cells, in particular interconversion of α- and β-intercalated cells and principal cells in vitro (1, 14); however, the molecular mechanisms responsible for this behavior have not been elucidated.

The initial hypothesis for “reversal of Na-K-ATPase polarity” in PKD (39) is clearly not supported by our findings in primary cultures of ARPKD principal cells, because cells form high-resistance epithelial monolayers with the appropriate polarization of Na⁺ absorption and Cl⁻ secretion. The importance of CFTR-dependent Cl⁻ secretion in ADPKD is widely
accepted (12, 33), and recent observations suggest that extracellular ATP may be a paracrine mediator of calcium-dependent Cl− secretion in ADPKD cells (30). Similar studies have not been carried out in ARPKD cells. Our results indicate that the Cl− secretory responses, elicited by cAMP or calcium, were small and similar in monolayers derived from normal and cystic mice. This is in agreement with a previous study in which the BPK (ARPKD) mouse was crossed with the CFTR knockout mouse and demonstrated that cystic disease progression was not affected by loss of CFTR-dependent anion secretion (21); however, the contribution of alternative Cl− channels was not excluded. Based on these results, it appears that Cl− secretion may not play a critical role in luminal fluid accumulation in ARPKD cystic CD.

The most striking finding in this study is the lower (~50%) basal Isc recorded from cystic monolayers compared with normal monolayers. Furthermore, the absolute magnitude of amiloride-sensitive ΔIsc as well as the fractional inhibition of the current by amiloride (82 ± 2 and 95 ± 1% inhibition for cystic and normal, respectively) are lower in cystic than normal monolayers, so we conclude that electrogenic Na+ absorption is significantly reduced in cystic cell monolayers.

There are several possible explanations for the decrease in amiloride-sensitiv Isc in cystic cell monolayers, including 1) contamination of the primary cultures with non-principal cells, 2) perturbed activity or partial mislocalization of the Na-K-ATPase from the basolateral to the apical plasma membrane, 3) differences in the electrochemical driving force for Na+ entry across the apical membrane, and 4) reduced activity of apical ENaC channels. Significant contamination by non-principal cells is unlikely because in the high-resistance monolayers >98% of the cells are GFP positive (CD cells) and have a central cilium (principal cells), and expression of intercalated cell markers are undetectable by RT-PCR. It was initially proposed that reversal of polarity was an important component of altered salt and water transport in PKD, including mislocalization of the Na-K-ATPase from the basolateral to the apical plasma membrane (3, 39); however, this remains controversial (34). A more recent report (28) confirms the presence but not the activity of Na-K-ATPase in the apical side of a cell line derived from human fetal ARPKD kidney. Furthermore, they found that the immortalized cystic cells had a higher rate of apical-to-basolateral 22Na flux compared with a cell line derived from an age-matched normal human kidney. The reason for these disparate observations is unclear at present. Electrodiffusive entry of Na+ across the apical plasma membrane via the ENaC is the rate-limiting step for Na+ absorption by mammalian principal cells, and changes in apical membrane potential and/or intracellular Na+ would alter the rate of Na+ entry, independent of changes in Na+ permeability. Because amiloride-sensitive conductance was reduced by ~50% (ΔGT = 0.34 vs. ΔGT = 0.16 mS/cm² for normal and cystic monolayers, respectively) in cystic monolayers compared with normal, it is likely that reduced apical Na+ permeability (ENaC activity) in cystic cells is responsible for the decrease in Na+ transport. Postnatal maturation of CDs is associated with an increase in Na+ absorptive capacity (16, 29) which parallels ENaC expression. It is possible that in cystic disease the highly proliferative PCs do not fully differentiate (10), and as such they do not develop a mature Na+ absorptive capacity. The reduced ENaC activity might be due to lower expression or aberrant signaling processes that regulate ENaC activity such as EGFR axis overactivity (2, 27, 31) or some combination of alterations that leads to a steady-state decrease in ENaC activity in cystic PCs. Additional studies will be required to elucidate the mechanisms responsible for reduced PC Na+ absorption in ARPKD. A thorough understanding of the ion transport abnormalities associated with all forms of PKD may provide important markers of disease progression and suggest therapeutic interventions to reduce or delay the loss of renal function.

ACKNOWLEDGMENTS

The authors gratefully acknowledge helpful discussions with Bill Sweeney and Ellis Avner and thank Mike Haley, Elizabeth Carroll, and Mike Wilson for technical assistance. We thank Frank Costantini (Columbia Univ.) for providing the HoxB7/GFP mouse line, Xia-Song Xie (Univ. of Texas Southwestern) for providing antibodies, and Noel Murcia (Case Western Reserve Univ.) for scanning electron microscopy.

GRANTS

This work was supported by Polycystic Kidney Research Foundation Grant 99013 and National Institute of Diabetes and Digestive and Kidney Diseases Grants P50-DK-27651 and P50-DK-57306.

REFERENCES


