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Regulated ion transport in mouse liver cyst epithelial cells

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Abstract

Derived from bile duct epithelia (BDE), secretion by liver cyst-lining epithelia is positioned to drive cyst expansion but the responsible ion flux pathways have not been characterized. Cyst-lining epithelia were isolated and cultured into high resistance monolayers to assess the ion secretory pathways. Electrophysiologic studies showed a marked rate of constitutive transepithelial ion transport, including Cl^- secretion and Na^+ absorption. Na^+ absorption was amiloride-sensitive, suggesting the activation of epithelial sodium channels (ENaC). Further, both $cAMP_i$ and extracellular ATP induced robust secretory responses. Western blotting and immunohistologic analysis of liver cyst epithelia demonstrated expression of P2X4, a potent purinergic receptor in normal BDE. Luminometry and bioassaying measured physiologically relevant levels of ATP in a subset of liver cyst fluid samples. Liver cyst epithelia also displayed a significant capacity to degrade extracellular ATP. In conclusion, regulated ion transport pathways are present in liver cyst epithelia and are positioned to direct fluid secretion into the lumen of liver cysts and promote increases in liver cyst expansion and growth.

Keywords: Autosomal dominant polycystic kidney disease (ADPKD); Ussing analysis; Purinergic signaling; cAMP; Epithelial sodium channel

1. Introduction

Cystic liver diseases are a source of significant morbidity and mortality. Three genetic forms of liver cyst disease have been identified, including autosomal dominant polycystic kidney disease (ADPKD), polycystic liver disease (PCLD) and autosomal recessive polycystic kidney disease (ARPKD) [1,2]. Occurring in 1 in 800 individuals, ADPKD is the most common and best understood of the liver cyst diseases. Liver cysts begin as focal out pockets of cells from the intrahepatic bile duct, detach from the parent duct to form an enclosed, autonomous cysts and continue to enlarge. Grossly affected livers have innumerable cysts that are readily visualized by CT and MRI imaging, can individually exceed 5 cm in diameter and, as a group, more than double the volume of the liver [3]. The overt clinical manifestations of ADPKD arise around the fourth decade of life and stem from the compression of neighboring organs, tissues and vasculature. Surgical procedures, ranging from cyst fluid aspiration to liver transplantation, represent the mainstay of therapy since there are currently no alternative medical therapies available. Understanding the molecular and cellular events that promote cyst expansion may direct the development of mechanism-based therapies to treat individuals with liver cyst disease.

ADPKD is caused by mutations in PKD1 or PKD2, the genes that encode for polycystin-1 and polycystin-2. While loss of function of the ADPKD gene products leads to the initiation of liver cyst formation, the disease progression is markedly heterogeneous. Genetic considerations, such as the site or type of gene mutation, may influence the variability in the course of the disease but epigenetic factors not directly associated with PKD1 or PKD2 gene mutations contribute substantially to the pathogenic heterogeneity [4]. Since ADPKD liver cysts are fully enclosed, it has been postulated that fluid secretion into the cyst lumen might increase intralumenal pressures, mechanically stretch the lining epithelium, induce proliferation of the epithelial cells and, consequently, promote cyst expansion. Such a

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paradigm is supported by prior observations. Human liver cysts have a positive lumenal pressure (16 to 40 cm H_2O) sufficient to result in epithelial cell stretching [5]. Stretching of alveolar epithelial cells induces the release of interleukin-8 [6], a pro-proliferative cytokine that is found at physiologic levels in human ADPKD liver cyst fluid [7]. Finally, stretching of Madin–Darby canine kidney cells increases the proliferation index of the epithelial cells [8]. Transepithelial fluid secretion is driven by the vectorial movement of ions and solutes across an epithelium and, consequently, the osmotic movement of water. Given the potential for fluid secretion to drive the expansion of liver cysts, the present study sought to directly measure the ion flux pathways present in the liver cyst epithelium.

Normal intrahepatic bile ducts are lined by a simple epithelial cell type termed cholangiocytes. Cholangiocytes, when appropriately mutated, give rise to liver cysts. Normal cholangiocytes have two distinct Cl^- secretory pathways to initiate the secretion of a bicarbonate-rich fluid into bile. Under physiologic conditions, the hormone secretin interacts with specific receptors on the basolateral membrane of cholangiocytes to increase $cAMP_i$ levels. Subsequently, increased $cAMP_i$ leads to PKA-dependent activation of cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels in the apical membrane. In human ADPKD studies, the administration of secretin increases fluid secretion into liver cysts [5], suggesting cAMP-mediated secretory pathways are intact in 'cystic' cholangiocytes. In 'normal' cholangiocytes, autocrine/paracrine signaling by extracellular ATP initiates a distinct Cl^- secretory response. Binding of ATP to purinergic receptors activates Ca^{2+}-dependent Cl^- channels and initiates a robust and prolonged Cl^- secretory response. To date, the lack of available cellular models of liver cyst epithelia has hampered the direct determination if $cAMP_i$- or ATP-mediated Cl^- secretory pathways are present in 'cystic' cholangiocytes. To address this important point, 'cystic' cholangiocytes were isolated from two genetic mouse liver cyst models and the ion transport characteristics in these epithelial cells were measured. Molecular and biophysical observations support the concept that cystic epithelial cells have a high basal rate of Cl^- secretion and Na^+ absorption and retain a robust Cl^- secretory response to both $cAMP_i$ and extracellular ATP. Given the potential contribution of secretion into the enclosed lumen of liver cysts to drive expansion of these cysts, pharmacologic targeting of the identified secretory pathways may represent an attractive target for amelioration of pathologic liver cyst expansion.

2. Materials and methods

2.1. Cpk(+/−) and pkd2(WS25/−) mice

Both Balb/c cpk(+/−) and C57/Bl6 pkd2(WS25/−) mice were utilized in the present studies. The cpk gene encodes for the protein 'cystin', a protein that has not been directly linked to a specific human disease. The pkd2 gene is a direct homolog of PKD2 in humans, one of the two genes directly linked to ADPKD in humans. Cpk(+/−) mice were bred and reared at the Indiana University School of Medicine Animal Resource Center. Female cpk(+/−) mice (~1 years of age) were transferred to the University of Colorado Health Sciences Center (UCHSC) Animal Care Facility prior to studies. These animals reproducibly form large, fluid-filled liver cysts that appear detached from the bile duct system (Fig. 1).

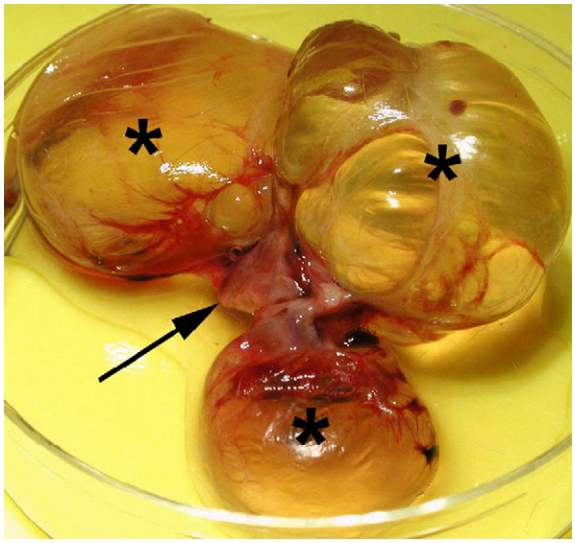

Fig. 1. Superficial cysts in liver from a cpk(+/−) mouse. The image is of a cystic liver from a 12-month-old Balb/c cpk(+/−) mouse in a 100 mm plate. During the initial year of life, cpk(+/−) mice develop large fluid-filled liver cysts (asterisks) that initiate from the intrahepatic bile ducts and expand out of the body of the liver (arrow). Pkd2(WS25/−) mice develop similar liver cysts in a similar time course (not shown). The cells lining these superficial liver cysts were isolated, cultured and studied for their secretory characteristics.

Breeding pairs of pkd2(+/−) and pkd2(WS25/+) mice were graciously provided by Stefan Somlo (Yale University). The WS25 allele is sensitive to recombination and either reverts back to the wild type allele or forms an allelic knockout [9]. The temporal somatic loss of the second pkd2 allele in pkd2(WS25/−) mice mirrors the 'second-hit' somatic mutation that is thought to occur in humans with ADPKD. Pkd2(WS25/−) consistently form histologically discernible liver cysts by 4 months of age [9] and large, fluid-filled liver cysts at 8–12 months of age. Wild type C57/Bl6 mice were used to harvest control tissue for Western and Northern blotting. Animals were euthanized (150 mg pentobarbital/kg body weight) and their livers removed under aseptic conditions as approved by the UCHSC Institutional Care and Use Committee. All animals received humane care as outlined by the National Institutes of Health in 'Guide for the care and use of laboratory animals'.

2.2. Development of cpk(+/−) and pkd2(WS25/−) liver cyst epithelial cultures

Unless otherwise noted, media and reagents for epithelial cell cultures were obtained from Gibco-Invitrogen (Carlsbad, CA). Cpk(+/−) and pkd2(WS25/−) liver cyst wall epithelial cells were isolated and cultured from superficial cyst wall explants. Blocks (~5 mm × 5 mm) of cyst wall tissue were removed from superficial liver cysts and finely minced under sterile conditions. The minced tissues were seeded atop a ~3 mm slab of rat tail collagen in cholangiocyte growth medium (Dulbecco's modified Eagles medium/F12 media, 5% fetal bovine serum (Hyclone; Logan, UT), 2 mM glutamine, 1% non-essential amino acid solution, 1% lipid concentrate, 1% vitamin solution, 393 ng/ml dexamethasone (Sigma-Aldrich; St. Louis, MO), 25 ng/ml epidermal growth factor (Upstate Biologicals; Lake Placid, NY), 30 μg/ml bovine pituitary extract (Upstate Biologicals; Lake Placid, NY), 3.4 μg/ml triiodothyrodine, 1% insulin–transferrin–Se, 0.4 μg/ml forskolin (Sigma-Aldrich; St. Louis, MO), 50 μg/ml soybean trypsin inhibitor). Epithelial cells were allowed to grow out from minced tissue and were passaged as small epithelial islands by digesting away the underlying collagen bed (1 mg/ml collagenase, 2 mg/ml dispase; 60 min at 37 °C). The complete enrichment of epithelial cells from cpk(+/−) (termed HL-cpk cells) and pkd2(WS25/−) (termed pkd2 cells) was evaluated by light microscopy, electron microscopy, Western blotting and electronic voltage-ohm meter/Ussing analysis of transepithelial resistance.

Normal bile duct cells were enriched from wild type mice to comparatively assay the expression of proteins in normal cholangiocytes. This was done by collagenase digestion. Briefly, the portal vein was cannulated and the liver perfused with collagenase (M.M. mg/ml in DMEM; 37 °C; gassed with 95% O_2/5% CO_2; 45 min). After livers became translucent, hepatocytes were shaken free from the biliary tree; the biliary tree was then further digested while shaking in collagenase solution. Additional hepatocytes were then teased from the biliary tree and the biliary tree was solubilized in 5×PAGE buffer (5% sodium dodecyl sulfate, 25% sucrose, 50 mM Tris–HCl, 5 mM ethylenediamine tetraacetic acid (EDTA), 0.2 M dithiothreitol; pH 8.0; Complete Protease Inhibitor II (Roche; Mannheim, Germany)). Western blotting of ezrin and bile salt export protein (BSEP) were used as marker proteins for cholangiocytes and hepatocytes, respectively and used to assess bile duct enrichment.

Normal rat cholangiocytes (NRC) cells were used to bioassay human liver cyst fluid. NRC cells were initially isolated by the LaRusso laboratory [10] and were cultured on collagen slabs and passaged in cholangiocyte growth medium, as previously described [11].

2.3. Electron microscopy of liver cysts and cultured liver cyst epithelial cells

For electron microscopy (EM), tissues were fixed, processed and examined as previously described [12]. For native liver cysts, superficial cyst walls were trimmed from the cpk(+/−) and pkd2(WS25/−) livers and immediately immersed in EM fixative (2% glutaraldehyde, 2% sucrose, 100 mM cacodylate; pH 7.4). Cultured cpk(+/−) and pkd2(WS25/−) liver cyst epithelial cell monolayers were prepared similarly. Following >60 min of fixation, these tissues were post-fixed in 1% osmium, dehydrated in ethanol, embedded in Epon812, stained with uranyl acetate and lead citrate and viewed by transmission electron microscopy.

2.4. Western blot analysis

Western blot analysis was performed as previously described [13]. Briefly, HL-cpk cells, pkd2 cells or mouse liver tissues were solubilized in 5×PAGE buffer, separated on a 4%–14% gradient polyacrylamide gel, transferred onto nitrocellulose and probed with primary antibodies, labeled with peroxidase-tagged secondary antibodies and detected by enhanced chemiluminescence. Primary antibodies were directed against actin (1:5,000 dilution; Calbiochem; San Diego, CA), ezrin (1:100 dilution; NeoMarkers; Temecula, CA), pan-cytokeratin (1:100 dilution; Sigma-Aldrich; St. Louis, MO), P2Y2 (1:300 dilution; Sigma Chemicals; St. Louis, MO), P2Y4 (1:300 dilution, Sigma Chemicals; St. Louis, MO) or P2X4 (1:100 dilution; Alamone Labs; Jerusalem, Israel).

2.5. Northern blotting

Northern blotting was performed as previously described [14]. Briefly, 20 μg of total RNA from HL-cpk cells or mouse kidney (positive control) was separated on a 1% agarose-formaldehyde gel and transferred onto a nylon membrane (MSI). Probes against ENaC-α were generated by random priming of cDNA generated with ENaC-α primers ((Sense: CAAGACCCTTGTCCTCCT-CAG; Antisense: GATGTTGAGGCTCACTGGCTAG), DecaPrimeII, Ambion; Austin, TX) and spiked with ^{32}P-dCTP. Membranes were blocked, probed and washed prior to detection by film exposure.

2.6. Immunofluorescence staining

Human and pkd2(WS25/−) mouse liver cyst wall were subjected to immunostaining and fluorescence analysis as previously described [13]. Briefly, portions of the cyst wall were excised, fixed in 3% paraformaldehyde for 30+ min, embedded in OCT compound (Torrence, CA), frozen in liquid nitrogen, cryosectioned (6 μm), blocked, stained with antibodies against P2X4 (1:100 dilution; Alamone Labs; Jerusalem, Israel), counterstained with FITC-labeled secondary antibody and imaged for immunofluorescence (Nikon DiaPhot with Diagnostics Instruments CCD camera). Control sections were incubated with non-immune serum prior to counter staining with FITC-labeled secondary antibody. The black level and sensitivity of detection were set such that these control sections appeared black. Consequently, all fluorescence detected above the black level in the paired sections that received P2X4 Ab is specific to that antibody.

2.7. Ussing chamber analysis of epithelial monolayers

Transepithelial ion currents were measured across HL-cpk and pkd2 monolayers by Ussing chamber analysis, as previously reported [11]. Briefly, monolayers were grown to confluence on semi-permeable, collagen-coated PTFE membrane supports (Costar-Corning; Corning, NY) and allowed to develop between 10 and 14 days following seeding. Monolayers were mounted in the Ussing chamber in an extracellular buffer (E-buffer; 140 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 1 mM KH_2PO_4, 10 mM HEPES, 10 mM glucose; pH 7.35; 295–300 mosM). Following a 10 min equilibration period, monolayers with transepithelial resistances >1,000 Ω cm^2 and basal short-circuit currents (Isc) between 10 and 40 μAmps were used in the studies. When exchanging solutions, the chamber solutions were exchanged with 10 times the chamber volume of the desired solution. Colorimetric analysis showed this had a >99.5% exchange efficiency. Low Cl^- solutions were prepared by substituting Na–gluconate for NaCl. Presented studies retained 5 mM chloride to stabilize the Ag–AgCl electrode signal. Sodium was removed from the apical chamber solution by substituting Tris–chloride for NaCl. cAMP-dependent Cl^- secretion was activated by the addition of 500 μM 8-cpt-cAMP and 450 μM 3-isobutyl-1-methylxanthine (IBMX). Purinergic receptor-dependent ion secretion was activated by the addition of 100 μM ATP to the apical chamber. Amiloride was added to the apical chamber to evaluate epithelial Na^+ channel (ENaC) activity. An amiloride dose–response included 0.1 μM, 1 μM, 10 μM and 100 μM added to the apical chamber. Amiloride addition to the basal chamber had no effect on Isc levels from HL-cpk cells (data not shown).

2.8. Measurements of ATP concentrations

The concentration of ATP was measured in liver cyst fluid samples and in culture media by a luciferin-luciferase (L-Lase) assay as described by the manufacturer (Promega; Madison, WI). Briefly, liver cyst fluid samples and epithelial cell culture media were collected, immediately frozen in liquid N2 and stored at −80 °C until assayed. For ATP degradation studies, the cell culture media were initially spiked with 1 μM ATP and the rate of ATP loss was measured. In the ATP assay, 10 μl of sample or ATP standards and 200 μl L-Lase buffer were added to a clear 0.5 ml PCR tube. These tubes were placed inside a 7 ml scintillation vial and counted in a scintillation counter on single photon mode (Beckman LS6000; Fullerton, CA).

2.9. Statistical analysis

The quantitative results are presented as mean±SEM. As noted in the text, statistical analyses included *t*-tests and analyses of variance. Statistical significances between groups were denoted when $p<0.05$.

3. Results

3.1. Development of the mouse liver cyst cell lines

Fig. 1 shows a notable example of the liver cysts that develop in livers of cpk(+/−) and pkd2(WS25/−) mice. In pkd2(WS25/−) mice, all animals develop discernible liver cysts after 4 months [9]. At 12 months, the liver weight as a percentage of body weight in male pkd2(WS25/−) mice were markedly greater than wild type animals (pkd2(+/+): 4.8±0.2% ($n=12$); pkd2(WS25/−): 21.0±4.8% ($n=12$); $p<0.01$). In extreme examples, the livers from grossly affected animals 12 months of age or older exceeded 50% of the total body weight. The wall tissue from superficial cysts was isolated, minced and seeded on

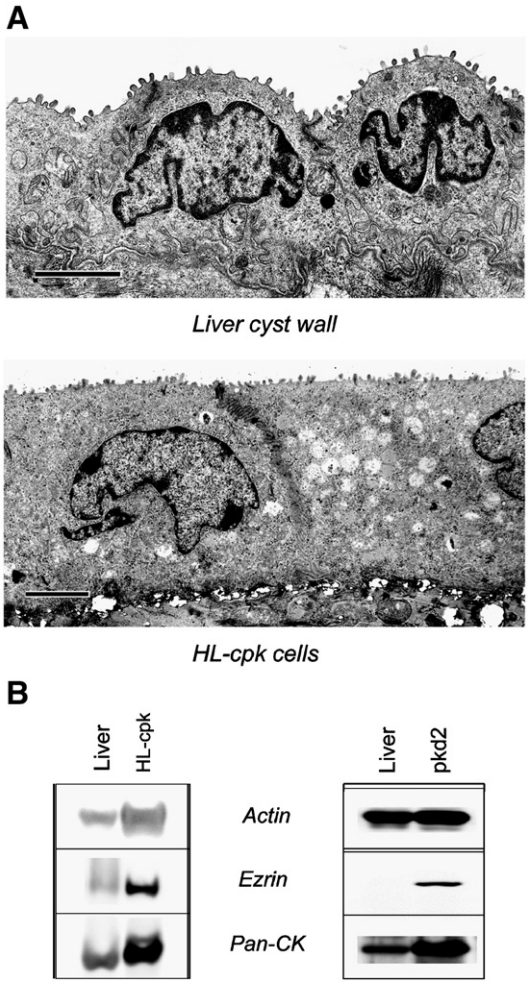

Fig. 2. HL-cpk and pkd2 cells in culture retain characteristics of cyst wall epithelia. (A) Transmission electron microscopy shows cpk(+/−) liver cyst lining epithelia retain the morphologic features of normal cholangiocytes. Upper panel shows native liver cyst epithelial cells; lower panel shows cultured liver cyst epithelial cells (bars = 1 μm). Morphologic features include a cuboidal epithelium with short apical microvilli and electron dense junctional complexes. Pkd2(WS25/−) liver cyst epithelia had similar histologic features (data not shown). (B) Western blotting shows HL-cpk cells, from cpk(+/−) liver cysts, and pkd2 cells, from pkd2(WS25/−) liver cysts, express epithelial cell and cholangiocyte-specific marker proteins. A pan-cytokeratin Ab (pan-CK, an epithelial cell marker) readily detected cytokeratins in the lysates. Ezrin, a cholangiocyte-specific protein in liver, was readily detected in the HL-cpk and pkd2 lysates. Ezrin is weakly detected in whole liver lysates since cholangiocytes constitute <4% of the liver cell mass. Actin served to approximate relative protein loading.

a bed of collagen to allow the lining epithelium to grow into a monolayer. Transmission electron microscopy of HL-cpk liver cyst epithelial cells showed they retained ultrastructural features of native cpk(+/−) liver cyst epithelia (Fig. 2A). These features include a short (<10 μm), cuboidal epithelium with small abundant apical microvilli and well-developed junctional complexes at the apical pole. Western blot analyses for cytokeratins and ezrin in HL-cpk and pkd2 cells were both positive (Fig. 2B). Cytokeratins are distinctly expressed in epithelial cells; ezrin is expressed in cholangiocytes (intrahepatic bile duct epithelium) but not hepatocytes, the second epithelial cell type in liver. Therefore, as anticipated, cholangiocytes are the likely progenitor cell of the liver cyst lining epithelium in these animals. Western blotting for vimentin, a marker protein for mesenchymal cells, was positive in some samples (data not shown), suggesting there may be some fibroblast contamination in these primary cultures. However, cultured HL-cpk and pkd2 epithelial cells formed an electrically 'tight' monolayer with transepithelial resistances >1000 Ω cm^2 and the presence of any fibroblasts did not appear to affect epithelial cell functions (see below). Finally, HL-cpk monolayers secrete a similar profile of cytokines as the native cpk(+/−) liver cyst epithelium (data not shown). Collectively, these findings indicate the non-transformed HL-cpk and pkd2 cells in culture originate from the bile duct epithelium (i.e. cholangiocytes) and retain general characteristics of the parent cell type.

3.2. HL-cpk epithelia exhibit basal levels of Cl^- secretion and Na^+ absorption

Fluid within enclosed liver cysts has a positive hydrostatic pressure [5], consistent with active secretion into the cyst lumen by the cyst-lining epithelium. Transepithelial fluid secretion is generally driven by the vectorial movement of ions across the epithelium and a consequent osmotic flux of water. Accordingly, ion substitution studies were performed to determine if appreciable levels of Cl^- and Na^+ ion transport were present in liver cyst epithelia. In Fig. 3A, Cl^- in the basal chamber of HL-cpk cells was reduced from 140 mM to 5 mM by substituting Na–gluconate for NaCl. This substitution resulted in a marked drop in the short-circuit current; values were restored following reintroduction of Cl^-. The Cl^- substitution studies required a

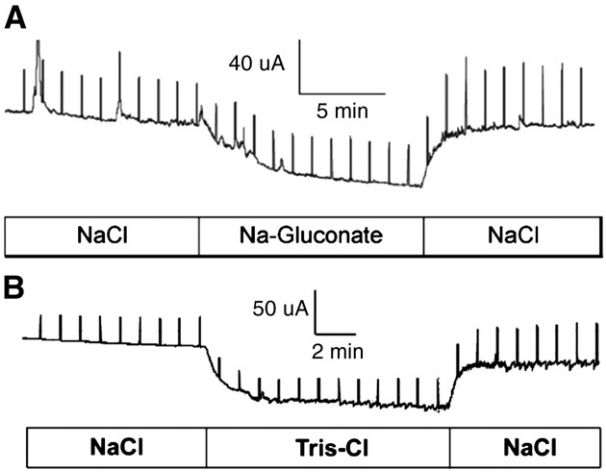

Fig. 3. HL-cpk monolayers secrete Cl^- and absorb Na^+ under basal conditions. Cells in monolayer culture were mounted in an Ussing chamber and Isc was measured under voltage clamp conditions (0 mV). (A) Substitution of Na^+-gluconate for NaCl in the basal chamber resulted in a marked decrease in the Isc under basal conditions. A residual amount of chloride (5 mM) was required for signal stabilization and precluded accurate quantitation of the basal Cl^- efflux. (B) Complete exchange of Tris for Na^+ in the apical chamber resulted in a pronounced drop in the Isc across HL-cpk cells (−55 ± 10 μA; n = 4). Both currents were restored upon reintroduction of Cl^- and Na^+, respectively.

residual amount of Cl^- to stabilize the electrode signal so the precise basal Cl^- current was not quantitated. However, the observations indicate HL-cpk cells have marked vectorial Cl^- transport (basal to apical) that contributes importantly to the observed high Isc under basal conditions.

Similarly, apical Na^+ substitution in HL-cpk cells resulted in a pronounced drop in the Isc (Fig. 3B), indicating that electrogenic Na^+ absorption (55±10 μAmps; $n=4$) contributes significantly to the basal Isc. In many epithelial cell types, the epithelial Na^+ channel (ENaC) contributes to apical Na^+ absorption. In normal cholangiocyte cells and monolayers, however, ENaC activity has not been described and amiloride has no effect on basal Isc values in normal rat cholangiocytes (data not shown). Lack of an effective ENaC antibody precluded the direct assessment of ENaC expression in HL-cpk cells but RT-PCR (data not shown), Northern blotting and Ussing analysis indicate ENaC is expressed and active in liver cyst epithelial cells (Fig. 4). Northern blotting of HL-cpk mRNA readily detected ENaC-α message at levels similar to those found in mouse kidney tissue. Amiloride added to the apical chamber induced a marked decrease in the basal Isc of HL-cpk cells. At 0.1 μM amiloride, the inhibition was relatively weak (−7±3 μA; $n=3$) but was more robust and significantly inhibited at 1 μM (−13±2 μA; $n=8$; $p<0.01$) and 10 μM amiloride (−14±2 μA; $n=10$; $p<0.01$). Application of amiloride (10 μM) to the basal chamber of HL-cpk monolayers failed to alter the basal Isc level (data not shown). These findings indicate ENaC is expressed in cyst epithelia, is constitutively active and accounts for a significant portion of the basal Isc in HL-cpk cells.

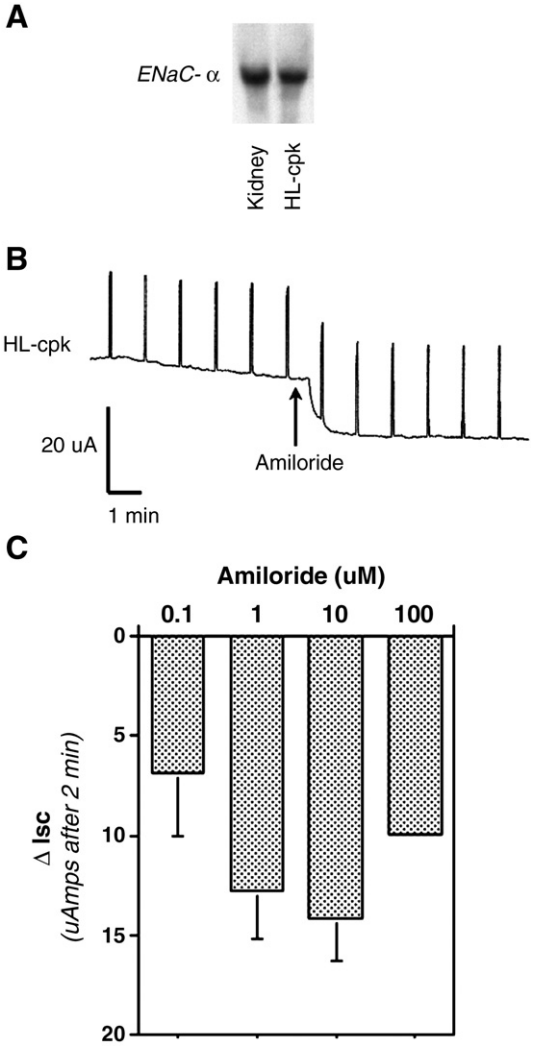

Fig. 4. HL-cpk monolayers have an amiloride-sensitive current under basal conditions. The potential contribution of the epithelial Na^+ channel (ENaC) to the basal Na^+ absorption current was evaluated. (A) Northern blotting found ENaC-α mRNA was present in HL-cpk cells at similar levels as found in mouse kidney. Equal amounts of mRNA (20 μg) were added in both lanes. (B) The addition of 10 μM amiloride to the apical chamber resulted in a significant decrease in the basal Isc (−14±2 μA; $n=10$) (C) A dose–response analysis showed that 0.1 μM amiloride induced a modest decrease in the basal Isc (−7±3 μA; $n=3$) while 1 μM amiloride (−13±2 μA; $n=8$) and 10 μM (−14±2 μA; $n=10$) amiloride induced a more robust inhibition. High dose amiloride (100 μM; −10 μA; $n=2$) also inhibited the basal current.

3.3. HL-cpk cells retain $cAMP_i$- and ATP-mediated secretory responses

In normal cholangiocytes, elevated $cAMP_i$ and extracellular ATP stimulates Cl^- secretion [15–19]. To assess if 'cystic' cholangiocytes retain these regulated secretory pathways, monolayers were exposed to agonists during measurement of Isc (Fig. 5). In HL-cpk monolayers, increased $cAMP_i$ (500 μM 8-cpt-cAMP; 450 μM IBMX) induced an increase in the Isc (15±5 μA; $n=4$; $p<0.05$). Likewise, extracellular ATP stimulated an increase in the Isc (40±9 μA; $n=4$; $p<0.05$). Thus, in parallel with native cholangiocytes, regulated secretory pathways are present in 'cystic' cholangiocytes and could drive fluid secretion into the liver cyst lumen.

3.4. Pkd2(WS25/−) liver cyst epithelia also retain regulated secretion characteristics

To determine if the functional properties in HL-cpk(+/−) liver cyst epithelia are present in other liver cyst models, liver cyst epithelial cells were isolated from pkd2(WS25/−) mice and tested for their responses to amiloride, 8-cpt-cAMP and extracellular ATP. Ussing chamber analysis of pkd2 monolayers had a sustained lumen-negative electrical potential and a positive Isc. Application of amiloride to the apical membrane decreased the basal Isc (−5.0±1.4 μA; $n=3$) and incubation with either 8-cpt-cAMP/IBMX or extracellular ATP induced marked secretory responses (Fig. 6). Thus, liver cyst epithelia from pkd2-mutated mice also perform robust basal and regulated transepithelial ion transport.

3.5. Cystic epithelia express P2X4 purinergic receptors

These above findings indicate that liver cyst epithelial cells exhibit a robust secretory response to extracellular ATP. Normal cholangiocytes have a complex purinergic receptor profile [15,20,21]. In normal cholangiocytes, P2Y2, P2Y4 and P2X4 are the most prominent ATP receptor types and P2X4 receptors

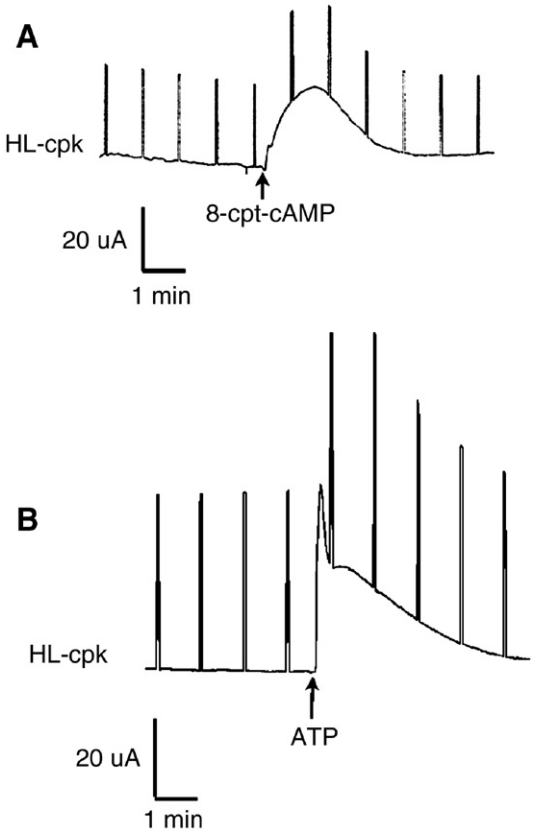

Fig. 5. HL-cpk monolayers have cAMP-induced and ATP-induced currents. In normal cholangiocytes, $cAMP_i$ and extracellular ATP induced Cl^- secretory responses [11,15,19]. (A) Likewise, HL-cpk cells displayed an increased Isc (peak: 15±5 μA; $n=4$) when $cAMP_i$ was elevated (500 μM 8-cpt-cAMP; 450 μM IBMX). (B) HL-cpk cells also displayed an increased Isc (peak: 40±9 μA; $n=4$) when 100 μM ATP was added to the apical media. The pattern of the HL-cpk response to ATP had two components, an immediate, short-lived increase in Isc followed by a slower, prolonged increase.

appear to account for a significant portion of the cellular response [15]. The relative expression of these three purinergic receptors in liver cyst epithelial cells was evaluated by Western blotting. The expression level was compared to levels in whole liver, enriched 'normal' bile ducts, liver cyst wall tissue and cultured liver cyst epithelial cells. The enrichment of the 'normal' bile ducts was demonstrated by blotting for ezrin (cholangiocyte marker protein) and BSEP (hepatocyte marker protein). Whole liver (~80% hepatocyte; ~3% cholangiocyte), enriched hepatocytes had marked levels of BSEP and little ezrin signal. Conversely, the enriched bile ducts (i.e. cholangiocytes) had comparatively little BSEP signal by marked levels of ezrin (Fig. 7A). Consistent with previous reports [15,20,21], 'normal' bile ducts expressed P2Y2, P2Y4 and P2X4 protein (Fig. 7B). Isolated liver cyst wall tissue had more modest but detectable expression of all three purinergic receptor types. The relative distribution of these three proteins in the epithelial, endothelial and mesechymal cells of the cyst wall was not specifically determined. Cultured pkd2 cells, however, had minimally detected

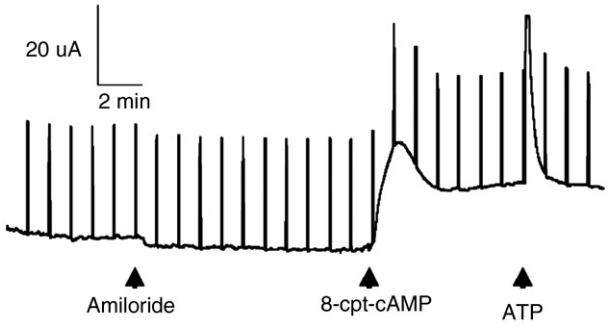

Fig. 6. Pkd2(WS25/−) cells also have secretory responses. Similar to HL-cpk liver cyst epithelial cells, liver cyst epithelial cells from pkd2(WS25/−) mice (pkd2) showed a similar short-circuit current responses in to apical amiloride, increased $cAMP_i$ and extracellular ATP.

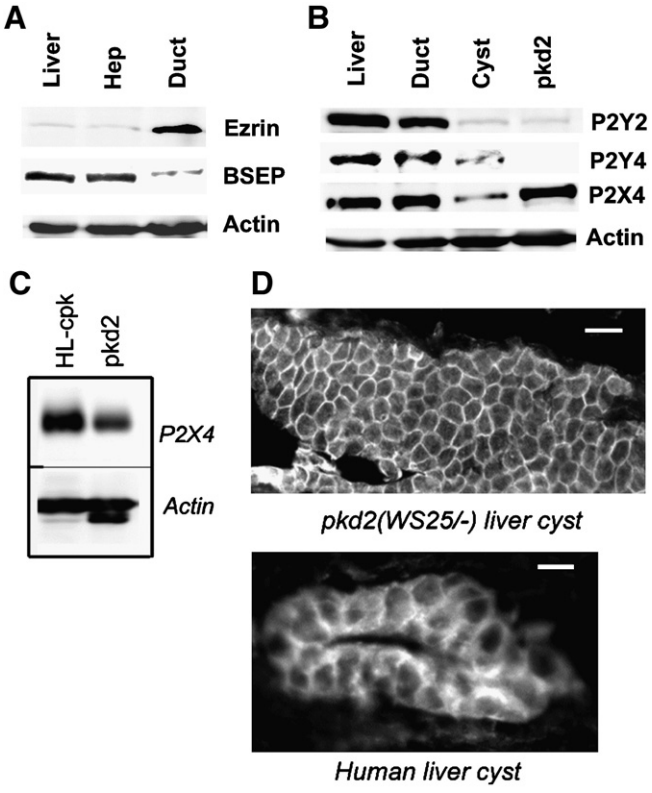

Fig. 7. Liver cyst epithelia express purinergic receptors. Normal cholangiocytes express P2Y2, P2Y4 and P2X4 purinergic receptors [15,19,21]. The expression of these receptors was evaluated in liver cyst epithelial cells. (A) Normal bile ducts were enriched from mouse liver to serve as a positive control. Using BSEP and ezrin for markers of hepatocytes and cholangiocytes (i.e. bile ducts), respectively. Compared to liver and hepatocytes (Hep), isolated bile ducts (Duct) had significant enrichment of ezrin and de-enrichment of BSEP (B) Normal bile ducts (Duct) expressed P2Y2, P2Y4 and P2X4. Relative expression of each of these was diminished in liver cyst wall tissue (Cyst), comprised of epithelial cells, endothelial cells and mesenchymal cells. Isolated liver cyst epithelial cells (pkd2) also showed weak expression of P2Y2 and P2Y4 but had robust expression of P2X4. Actin served as a loading control. (C) This robust expression in pkd2 cells was mirrored in HL-cpk cells. (D) Immunofluorescence staining for P2X4 demonstrated expression both in native mouse pkd2(WS25/−) liver cyst epithelia (upper panel; bar=15 μm) and in human ADPKD liver cyst epithelial cells (lower panel; bar=10 μm). As in normal cholangiocytes, P2X4 is expressed broadly across the cell membrane domains. The sectioning across the cyst lumen in the human liver cyst (lower panel) shows P2X4 is present at the apical membrane domain.

levels or P2Y2 and P2Y4 but had robust expression of P2X4. In parallel with pkd2 cells, P2X4 is also readily detected in HL-cpk cells (Fig. 7C). The expression of P2X4 protein in mouse liver cyst cultures was confirmed by immunofluorescence with P2X4 observed in native liver cyst-lining epithelial cells from both mouse pkd2(WS25/−) and human ADPKD (Fig. 7D). The cross section through human liver cysts shows that P2X4 is broadly expressed in the cyst lining epithelial cells including expression at the apical domain. These observations demonstrate, at a minimum, that P2X4 purinergic receptors are expressed and positioned in the cystic epithelial cells and to mediate the secretory response to ATP. To begin to evaluate whether these pathways are operative *in vivo*, ATP concentrations in liver cyst fluids were measured.

3.6. Physiologic ATP concentrations in liver cysts

Secretion of ATP into enclosed cyst lumens could permit the accumulation of ATP to levels that activate purinergic receptors. The Km of ATP for the P2Y and P2X families of receptors ranges from 100 to 500 nM. Accordingly, ATP concentrations were assayed in liver cyst fluids aspirated from cpk(+/−) and pkd2 (WS25/−) mice and found to be markedly variable. In three cpk (+/−) samples, ATP levels ranged from 49 to 197 nM (Fig. 8A). In pkd2(WS25/−) liver cyst fluid, the majority of samples also had ATP concentrations below 200 nM. Three samples, however, had ATP levels well above the Km for purinergic receptors (710 nM; 1,616 nM; 2,660 nM). Thus, in a minority of liver cyst fluid samples, ATP was present at physiologic/supraphysiologic levels.

Bioassaying of five human liver cyst fluid samples for ATP-mediated secretory responses was consistent with this observation. In these studies, normal rat cholangiocyte (NRC) monolayers were mounted in an Ussing chamber and the buffer in the apical chamber (18 ml) received 2 ml human liver cyst fluid. Four of the five samples tested failed to elicit a change in the short circuit current. One sample elicited an Isc response that was similar in magnitude and duration as an ATP-induced response (Fig. 8B). Pre-treatment of the 'positive' cyst fluid with apyrase, an enzyme that degrades ATP, ablated the secretory response. Taken together, these observations suggest that physiologically important concentrations of ATP are present in a subset of liver cyst fluids.

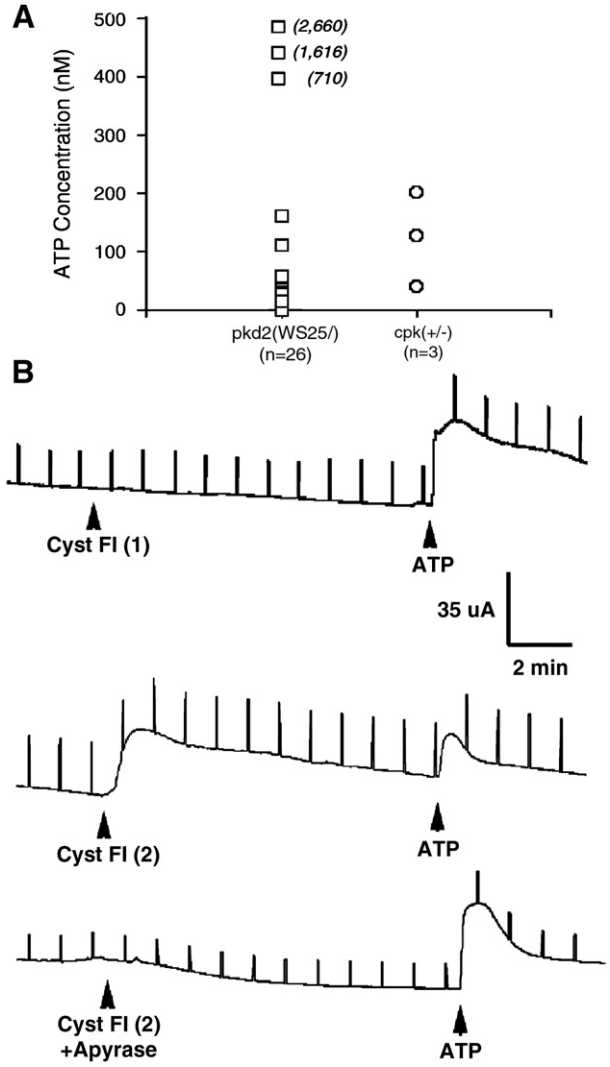

Fig. 8. ATP levels are markedly variable in disparate liver cysts. Direct measurement and bioassay of liver cyst fluid reveals ATP is present in liver cyst fluids but their concentrations are markedly variable. (A) In pkd2(WS25/−) liver cyst fluid samples, ATP ranged from 2,660 nM to 0 nM. The majority of samples (21 of 26) were under 100 nM and below the Km for purinergic receptors. Of the 26 samples measured, two samples were near the Km for purinergic receptors and three samples were significantly greater than the Km for purinergic receptors. Three samples from cpk(+/−) mice had concentrations ranging from 49 nM to 197 nM. (B) Bioassay of human liver cyst fluid did not elicit any change in the Isc of NRC cells in 4 of 5 samples tested (upper tracing). One of 5 samples, however, elicited an increased Isc that was similar in magnitude and duration to ATP responses (middle tracing). Pre-treatment of the positive cyst fluid sample with apyrase, an ATP degrading enzyme, abolished the fluid-evoked increase in Isc (lower tracing). ATP was added to the apical chamber at the end of each run to ensure monolayer responsiveness.

3.7. HL-cpk cells have significant extracellular nucleotidase activity

ATP concentrations in cyst fluids reflect the balance of release and degradation of ATP at the time of sampling. To determine if the observed variability in cyst fluid ATP levels was related to the capacity of cyst epithelia to degrade extracellular ATP, liver cyst epithelial cells were grown to confluence, the media (5 ml) was spiked with exogenous ATP (final concentration of ~1 μM) and the rate of degradation was followed over time (Fig. 9). In the presence of serum and absence of cells, there was a small but measurable loss of ATP over time. Thus, studies were performed using ATP-spiked serum-supplemented media or PBS, each with or without cells. The inclusion of pkd2(WS25/−) cell monolayers resulted in a rapid and robust loss of ATP (Fig. 9A). Quantitative analysis showed only 7±2% of the initial ATP remained after 15 min ($n=5$; $p<0.01$) and only 1±1% of the initial ATP remained after 30 min ($n=5$; $p<0.01$; Fig. 9B). Similarly, <1% of the initial ATP was detectable after 30 min in HL-cpk ($n=1$) and NRC ($n=1$) monolayers. Thus, liver cyst epithelial cells retain

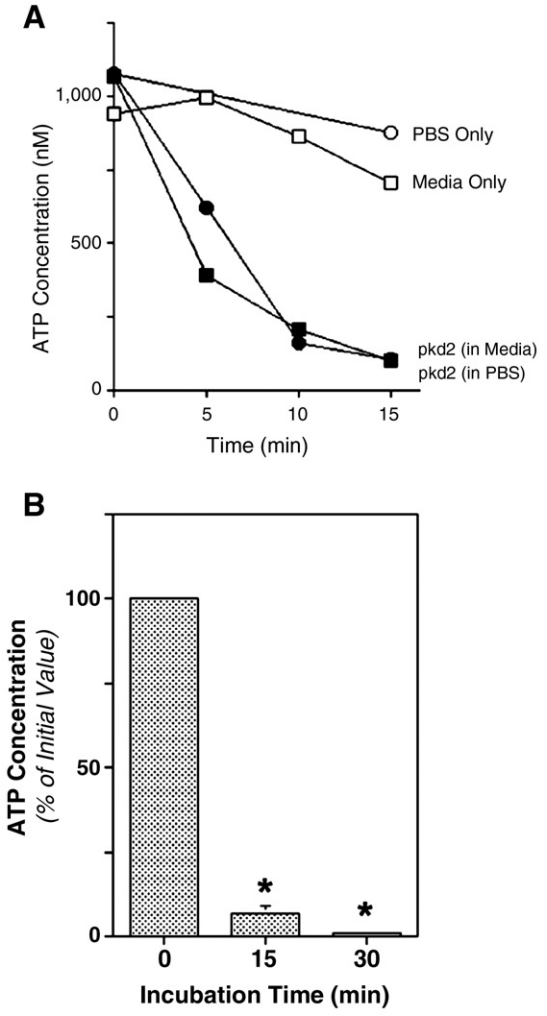

Fig. 9. Extracellular ATP is rapidly degraded by liver cyst epithelia. The capacity of pkd2 liver cyst epithelia to degrade extracellular ATP was measured. (A) In the absence of cells, incubation of ATP in either PBS (open circles) or supplemented media (open squares) showed little decrease in ATP over 15 min. In contrast, the rate of ATP degradation was markedly increased in the presence of pkd2 monolayers. Values were similar with PBS covering pkd2 monolayers (closed circles) or supplemented media covering pkd2 monolayers (closed squares). Shown is a representative experiment. (B) Quantitative analysis ($n=5$) found extracellular ATP levels were degraded to 7±2% ($p<0.01$ vs. 0 min) of initial concentrations after 15 min and to 1±1% ($p<0.01$ vs. 0 min) of initial concentrations after 30 min of incubation. In both HL-cpk ($n=1$) and NRC ($n=1$) monolayers, ATP was similarly degraded to ≤5% and 1% of the initial concentration after 15 min and 30 min of incubation, respectively (data not shown).

the ability to degrade ATP in the lumenal cyst fluid and temporally moderate purinergic signaling of the cyst lining epithelium.

4. Discussion

4.1. Extragenetic factors impact liver cyst disease severity

ADPKD, the leading cause of human kidney and liver cyst formation, is a 'molecular recessive' disease that is linked to mutations in PKD1 and PKD2. ADPKD associated with mutations in PKD2 has a later onset and milder course than that associated with mutations in PKD1 [22]. Further, the position of the mutation within the gene and susceptibility to the second somatic mutation may influence disease expression [23]. Beyond this, however, other modifier genes and environmental factors largely dictate disease severity [24]. The pathology in rodent renal cyst models is impacted by epigenetic factors and studies of human sibships and monozygotic twins strongly corroborate the contribution of modifier genes [25–28]. Many of the concepts and hypotheses in the present liver cyst study stem from the study of ADPKD renal cyst disease. Identifying the pivotal epigenetic factors that specifically promote ADPKD liver cyst growth is particularly poignant since the clinical sequelae of cystic liver disease are largely determined by the increases in liver volumes.

Liver cysts originate from mutated cholangiocytes that proliferate and emerge from the intrahepatic bile duct. In humans, the intrahepatic bile duct epithelium normally constitutes 2–4% of the liver cell mass but contributes up to 40% of the total bile volume in a regulated manner. If 'cystic' cholangiocytes retained this robust secretory capacity, the fluid secretion into the lumens of enclosed liver cysts would likely increase the lumenal pressure and result in epithelial cell stretching. In culture models, epithelial cell stretching initiates cytokine release and induces cell proliferation [8,29]. Interestingly, interleukin-8 is a potent proliferative cytokine that is specifically released during epithelial cell stretching and is also concentrated to physiologic levels in human ADPKD liver cyst fluid [6,7]. Consequently it was hypothesized that fluid secretion into liver cysts promotes stretching of the cyst lining epithelium, causing the release of cytokines into the cyst fluid that then signal an acceleration of cyst growth. The present studies begin to test this hypothesis by evaluating if basal and regulated secretory pathways are present in liver cyst epithelial cells.

4.2. Epithelial sodium channel activity is elevated in liver cystic epithelial cells

In pulmonary epithelial monolayers, ENaC activity mediates net fluid absorption and controls the volume of the apical fluid layer [30]. In renal distal tubules, aldosterone moderates ENaC distribution and activity at the apical membrane of renal principal cells to control Na^+ reabsorption and urinary Na^+ loss [31]. In autosomal recessive polycystic kidney disease (ARPKD), ENaC is dysregulated with higher levels of amiloride-sensitive Na^+ absorption; this activity is inhibited by apical EGF receptor activation [32–34]. ENaC expression and function in cholangiocytes has not been reported and amiloride-sensitive Isc was not observed in normal rat cholangiocytes (data not shown). In liver cyst epithelial cells, however, ENaC mRNA and amiloride-sensitive Isc was readily detected (Figs. 4 and 6). Theoretically, this might represent an adaptive response since increased apical ENaC activity would increase Na^+ absorption from the cyst lumen and thereby reduce fluid pressure within liver cysts. In other epithelia, ENaC activity is regulated by a variety of mechanisms, including

moderating its expression, distribution and open channel probability. The complete characterization of these ENaC regulatory pathways may elucidate a role for regulated ENaC activity in normal bile duct function, provide insight into the molecular mechanisms of cyst expansion and unveil new treatment strategies to blunt fluid secretion into liver cysts.

4.3. cAMP-mediated secretory pathway persists in liver cyst epithelia

Treatment of ADPKD renal monolayers with cAMP agonists induces a secretory response [35] and the CFTR Cl^- channel appears to contribute significantly to the cAMP-mediated secretory response [36,37]. This includes cell proliferation assays in three dimensional renal cell cultures where CFTR-mediated Cl^- secretion is directly associated with renal cyst growth [38,39]. In humans, an ADPKD family with concurrent CFTR gene mutations had a milder ADPKD disease phenotype, suggesting control of $cAMP_i$-mediated secretion can influence ADPKD disease severity [40]. $cAMP_i$-mediated, CFTR-dependent Cl^- secretion plays a central role in regulated secretion in bile ducts [16,17]. A number of gut hormones positively (e.g. secretin) and negatively (e.g. somatostatin) influence ductular secretion by moderating $cAMP_i$ levels. In an *in vivo* human ADPKD liver cyst study, administration of secretin resulted in an increase in fluid secretion [5]. The present study corroborates these findings at the cellular level by directly demonstrating that liver cyst epithelial cells retain potent $cAMP_i$-mediated secretory responses (Figs. 5 and 6). In bile ducts, the pro-secretory effects of secretin are inhibited by somatostatin. ADPKD patients treated with the somatostatin analogue octreotide exhibited slower rates of growth in renal volume [41]. While the study did not evaluate hepatic cyst growth, the known physiologic effects of somatostatin on ductular secretion predict octreotide should be effective in inhibiting hepatic cyst enlargement.

4.4. ATP-mediated secretory pathways persist in liver cyst epithelia

Extracellular ATP is a potent stimulus for Cl^- secretion in a variety of epithelia including the intrahepatic bile duct [15,20]. The potential for ATP to drive lumenal fluid secretion and cyst growth has been studied in ADPKD renal cysts. In a subset of ADPKD renal cysts, ATP accumulates to physiologic levels [42,43] and demonstrable ATP release from cultured ADPKD renal cyst epithelial cells suggests the lining epithelium is the source of ATP [43,44]. The presence of purinergic receptors on renal cyst epithelial cells permits the secreted and accumulated ATP to participate in autocrine/paracrine signaling [43,45]. The potential significance of this autocrine/paracrine signaling axis is demonstrated in 3D MDCK cell cultures where inhibition of P2Y signaling blunts the growth of the MDCK cysts [46]. The potential role of ATP-mediated Cl^- secretion is further advanced by the observation that polycystin-1, one of the two proteins genetically linked to ADPKD, can moderate Ca^{2+} entry into ATP-stimulated epithelial cells [47].

Normal bile duct epithelial cells (i.e. cholangiocytes) also secrete ATP across their apical membranes and express specific purinergic receptor subtypes [15,18]. The purinergic signaling axis is retained, at least in part, in 'cystic' mouse and human cholangiocytes which express P2X4 receptors (Fig. 7) and have robust secretory response to apical ATP (Figs. 5 and 6). ATP signaling is likely not tonically activated since physiologic levels of ATP were detected in a subset of mouse liver cyst fluid samples (Fig. 8A) and a subset of human ADPKD liver cyst fluid samples induced an ATP-dependent secretory response from cholangiocytes (Fig. 8B). With a significant capacity to degrade extracellular ATP (Fig. 9), ATP-directed secretion by liver cyst epithelial cells likely occurs in liver cysts when the rate of apical ATP release exceeds the rate of ATP degradation and ATP accumulates to physiologically relevant levels.

4.5. Controlling cystic fluid secretion may serve as a therapeutic target

Collectively, these studies demonstrate, for the first time, that liver cyst epithelial cells have substantial capacities for Cl^- secretion and Na^+ absorption and retain a capacity to increase vectorial ion fluxes in response to elevated $cAMP_i$ or extracellular ATP. Since ADPKD liver cysts have a positive lumenal pressure [5,48], it is attractive to speculate that additional fluid secretion would increase lumenal pressure, causing epithelial cell stretching, cell proliferation and cyst expansion [6,8,29]. With no medical therapies currently available for treating ADPKD liver cyst disease, the present study suggests efforts to assess the effects of inhibiting secretion on liver cyst growth and development of the clinical manifestations of liver cyst disease are strongly warranted.

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