Mechanoregulation of intracellular Ca\(^{2+}\) in human autosomal recessive polycystic kidney disease cyst-lining renal epithelial cells

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Am J Physiol Renal Physiol 294: F890–F899, 2008. First published February 6, 2008; doi:10.1152/ajprenal.00341.2007. —Mechanoregulation of intracellular Ca\(^{2+}\) in human autosomal recessive polycystic kidney disease cyst-lining renal epithelial cells. Am J Physiol Renal Physiol 294: F890–F899, 2008. First published February 6, 2008; doi:10.1152/ajprenal.00341.2007. —Mutations of cilia-expressed proteins are associated with an attenuated shear-induced increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in renal epithelial cell lines derived from murine models of autosomal recessive polycystic kidney disease (ARPKD). We hypothesized that human ARPKD cyst-lining renal epithelial cells also exhibited dysregulated mechanosensation. To test this, conditionally immortalized cell lines derived from human fetal ARPKD cyst-lining (pool and clone 5E) cell lines with low levels of fibrocystin/polyductin expression and age-matched normal collecting tubule [human fetal collecting tubule (HFCT) pool and clone 2C] cell lines were grown in culture, loaded with a Ca\(^{2+}\) indicator dye, and subjected to laminar shear. Clonal cell lines were derived from single cells present in pools of cells from cyst-lining and collecting tubules, microdissected from human kidney. Resting and peak [Ca\(^{2+}\)]\(_i\) were similar between ARPKD 5E and pool, and HFCT 2C and pool; however, the flow-induced peak [Ca\(^{2+}\)]\(_i\) was greater in ARPKD 5E (700 ± 87 nM, n = 21) than in HFCT 2C (315 ± 58 nM, n = 12; P < 0.01) cells. ARPKD 5E cells treated with Gd\(^{3+}\), an inhibitor of nonselective cation channels, inhibited but did not abolish the shear-induced [Ca\(^{2+}\)]\(_i\) transient. Cilia were ~20% shorter in ARPKD than HFCT cells, but no difference in ciliary localization or total cellular expression of polycystin-2, a mechanosensory Gd\(^{3+}\)-sensitive cation channel, was detected between ARPKD and HFCT cells. The intracellular Ca\(^{2+}\) stores were similar between cells. In summary, human ARPKD cells exhibit an exaggerated Gd\(^{3+}\)-sensitive mechanosensory Ca\(^{2+}\) response compared with controls; whether this represents dysregulated polycystin-2 activity in ARPKD cells remains to be explored.

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Autosomal recessive polycystic kidney disease (ARPKD) affects ~1:20,000 live births (5, 12, 50). Mortality during the perinatal period is due primarily to respiratory insufficiency; however, in those patients who survive the neonatal period, ~40% develop chronic kidney disease, with a majority developing hypertension during childhood and adolescence (12). Histopathologically, ARPKD kidneys are characterized by ectatic collecting ducts (CDs), which remain contiguous with the filtering nephron (26, 42). ARPKD is caused by mutations in pkhd-1, which encodes the protein product fibrocystin/polyductin (25, 39).

In contrast to ARPKD, autosomal dominant polycystic kidney disease (ADPKD), the most common genetic renal disorder with an incidence of 1:1,000 (36), is characterized by progressive renal disease with a majority of patients developing end-stage renal disease by the 5th decade of life (42). Pathologically, ADPKD kidneys are distinguished by the presence of multiple noncommunicating discrete saccular cysts, which can arise from any segment of the nephron (42). ADPKD is primarily caused by mutations in pkd-1, but in a minority of cases, are related to mutations of pkd-2 (21).

The mechanisms underlying cystogenesis in ADPKD and ARPKD are incompletely understood. However, cumulative evidence suggests that the central cilium of renal epithelial cells, which is believed to act as a flow sensor (29), may be functionally abnormal in polycystic kidney disease (PKD). In fact, many of the protein products of genes associated with cystic kidney disease localize, at least partially, to the cilium; these include polycystin-1 and polycystin-2, which are encoded by pkd-1 and pkd-2, respectively, polars which is encoded by Tg737, and fibrocystin/polyductin (1, 2, 11, 21, 25, 34, 39, 40, 48).

Bending of the central cilium of an Madin-Darby canine kidney cell, a model of a distal nephron epithelial cell, either directly by application of suction via a micropipette or indirectly by laminar fluid flow, generates an increase in the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) (29). Homozygous exonic disruption of pkd-1 (pkd-1\(^{+64/64}\)) mutant) abrogates this shear-induced [Ca\(^{2+}\)]\(_i\) transient (23). Mutations of pkd-1 prevent normal expression of polycystin-1 protein in the cilium and also inhibit ciliary expression polycystin-2, a gadolinium (Gd\(^{3+}\))-sensitive, Ca\(^{2+}\)-conducting cation channel, in both murine and human models of ADPKD renal epithelia (23, 24, 47). Polycystin-2 localization to the primary cilium, in association with polycystin-1, is necessary for the flow-mediated [Ca\(^{2+}\)]\(_i\) transient in normal renal epithelia (23). The loss of the shear-induced [Ca\(^{2+}\)]\(_i\) response in murine and human models of ADPKD is not a generalized defect in the stimulus-mediated [Ca\(^{2+}\)]\(_i\) response. In fact, ADPKD cells retain their ability to respond to vasopressin, angiotensin II, and thrombin with an increase in [Ca\(^{2+}\)]\(_i\) (23, 47).

The orpk murine model of human ARPKD is associated with mutations in Tg737, whose gene product is important in ciliogenesis (49). CDs from orpk mice microperfused in their native geometry and in monolayers of renal epithelia isolated from orpk mice grown in culture exhibit an attenuated [Ca\(^{2+}\)]\(_i\) response to increases in apical flow rate (19, 33). The attenuated [Ca\(^{2+}\)]\(_i\) response in these cells has been hypothesized to be related to partial redistribution of polycystin-2 from the cilium to the apical membrane (33). In contradistinction to the...
above data, monolayers of renal epithelial cells derived from the inv/inv mutant mouse, a model of nephronophthisis, show a normal shear-induced [Ca²⁺], response, even though mutant inversin, the protein product of inv, is a cilia-associated protein (27, 28, 32). The balance of these studies in mouse and human models of genetic cystic kidney disease suggests that mutations of many, but not all, cilia-associated genes lead to a specific defect in the normal laminar shear stress (LSS)-induced increase in [Ca²⁺].

To date, the effect of laminar shear on [Ca²⁺], in human ARPKD cyst-lining cells has not been examined. We hypothesized that human ARPKD cyst-lining renal epithelial cells exhibit abnormal mechanosensation to LSS. The purpose of this study was to test this hypothesis and begin to identify the cellular and molecular pathways involved in this response to LSS by utilizing human ARPKD and age-matched control renal epithelial cells.

MATERIALS AND METHODS

Human Renal Epithelial Cell Immortalization

The National Disease Research Interchange (Philadelphia, PA) procured, in the operating room, normal kidneys intended for transplantation, but rejected for surgical reasons or antigen mismatch; those kidneys that experienced warm ischemia before being flushed with Collins salts solution at 4°C were selected. ARPKD kidneys were procured at the time of surgical removal. Specimens were de-identified and assigned anonymous numbers and were exempted for approval by the Institutional Review Board (IRB) by Mount Sinai School of Medicine (MSSM) and National Institutes of Health. Primary cell cultures of normal collecting tubule from age-matched normal kidney or cyst-lining epithelia from ARPKD kidney were generated by microdissection, followed by attachment to type I collagen and grown in 1% serum-containing, fully defined, segment-specific, growth factor-supplemented Click/RPMI media (41, 44). Conditionally immortalized normal and PKD cell lines were derived from these primary cultures by retroviral transduction of a neomycin-resistance and temperature-sensitive T antigen (U58, permissive temperature 33°C) into monolayers during the exponential phase of growth, followed by selection for 6 wk in Geneticin (30). In addition to production of frozen stocks of transfected pools of tubule-specific segments and cyst-lining cells, dilation cloning was carried out, and individual clones were characterized and selected for suitability for study by extensive functional and marker analysis. After transfer of conditionally immortalized cells to the nonpermissive temperature (37°C), T-antigen was absent, and proliferation ceased by 3 days.

Cell Culture for Experimentation

Temperature-sensitive conditionally immortalized cells of ARPKD renal cysts (pool and clone 5E from 19-wk fetal kidney) and age-matched normal fetal collecting tubule [human fetal collecting tubule (HFCT); pool, clone 2C] were grown on rat tail type I collagen-coated 40-mm glass coverslips for perfusion and 18-mm glass coverslips for immunofluorescence microscopy. Clone cells were derived from their respective pool cells, and pool cells were derived from individually microdissected collecting tubules and cysts from freshly collected human kidney, as described above (31). Of note is that ARPKD pool and clone 5E, and HFCT pool and clone 2C, expressed markers characteristic of differentiated principal cells, including the epithelial Na channel, aquaporin-2, and apical cilia (4, 31).

Monolayers were grown to 80% confluence at 33°C in cell-type-specific supplemented medium containing 1% fetal bovine serum, 5 µg/ml human transferrin, and 10⁻⁸ M dexamethasone, as previously described (8, 43, 45). Thereafter, they were transferred to 37°C for 8–15 days to maximize differentiation and to elaborate cilia. All cells were placed in serum-free media for 20 min before perfusion or fixation for immunofluorescence microscopy.

Immunofluorescence Microscopy

Cilia length. Cell monolayers grown on 18-mm glass coverslips were fixed and placed in 12-well plastic plates, where they were permeabilized with 0.1% Triton X-100, washed with phosphate-buffered saline (PBS), and blocked with 2% bovine serum albumin (BSA) and 10% normal goat serum in PBS. Cells were incubated with a 1:500 dilution of mouse monoclonal antibody directed against acetylated α-tubulin (Abcam, Cambridge, MA) overnight at 4°C. After three washes with PBS, a 1:400 dilution of a FITC-conjugated secondary goat anti-mouse IgG (Molecular Probes, Eugene, OR), prepared in a 2% BSA/PBS solution, was applied for 60 min; thereafter, the cells were washed three times with PBS. Before fixation, cilia were flattened for optimal imaging of cilia length. Cell monolayers grown on glass coverslips were placed on a plastic tray at a 45° angle and washed with PBS and fixed with 2.5% paraformaldehyde. Labeled cells on glass coverslips were placed face down on a slide to which 5 µl of Prolong Anti-Fade solution (Molecular Probes) had been applied. Each monolayer was examined by confocal laser scanning microscopy [Leica TCS-SP (UV) microscope; Heidelberg, Germany], and cilia length was calculated with Volocity (Improvement, Lexington, MA).

Localization of polycystin-2. Cells were fixed in 100% methanol at −20°C for 40 min; blocked with 10% normal goat serum, 1% BSA, and 0.05% Triton X-100 in PBS for 1 h; incubated with mouse anti-acetylated α-tubulin antibody (1:300) and a polyclonal rabbit anti-polycystin-2 antibody (1:500; YCC-2; gift of Dr. S. Somlo) (9) overnight at 4°C; rinsed thrice with PBS; and then incubated for 2 h at room temperature with goat anti-mouse Alexa 568 and goat anti-rabbit Alexa 488 antibodies (Molecular Probes), respectively. Prolong anti-fade agent with 4,6-diamidino-2-phenylindole (DAPI) (Molecular Probes) was applied to each monolayer. Immunofluorescence confocal imaging was performed as described above.

Immunoblotting for Polycystin-2 Expression

HFCT (pool and clone 2C) and ARPKD (pool and clone 5E) cells were grown to confluence in T25 flasks, differentiated at 37°C for 9–13 days, washed with Tris-buffered saline (TBS) containing a comprehensive protease inhibitor cocktail, and then extracted in 1% Nonidet P-40 and 0.5% Triton X-100 solution (8, 31). For polycystin-2 detection, protein lysate (50 µg) from each sample was resolved on a SDS-polyacrylamide gel and electrophoretically blotted onto Immobilon-P membrane (Millipore, Bedford, MA). The membrane was incubated overnight at 4°C with a primary anti-polycystin-2 antibody [H280, Santa Cruz Biotechnology, Santa Cruz, CA; YCC-2(6)], diluted 1:1,000 in 5% milk in PBS containing Tween. Polycystin-2 was detected with peroxidase-conjugated goat anti-rabbit IgG antibody (1:5,000 dilution in 5% milk in PBS containing Tween at room temperature for 30 min) for the H280 antibody or for the YCC-2 antibody. Immunoblots were visualized with an enhanced chemiluminescence (ECL) detection kit, Lumi-Light Plus (Roche Diagnostic, Indianapolis, IN). After stripping the membrane, horseradish peroxidase-conjugated goat anti-actin antibodies (anti-actin C11, Santa Cruz Biotechnology) were used to detect actin and normalize the amount of protein loaded in each lane.

Immunoblotting for Fibrocystin/Polyductin Expression

Protein lysates of HFCT (clone 7F)1 and ARPKD (pool and clone 5E) cells were generated as described above. Fifty micrograms of protein lysate from each sample were loaded onto a 5% SDS-polyacrylamide gel and electrophoretically blotted onto Immobilon-P membrane (Millipore). The membrane was incubated for 48 h at 4°C with a primary anti-fibrocystin/polyductin monoclonal antibody [H9262, The National Disease Research Interchange (Philadelphia, PA)] and electrophoretically blotted onto Immobilon-P membrane (Millipore). The membrane was incubated overnight at 4°C with a primary anti-fibrocystin/polyductin antibody [H9262, The National Disease Research Interchange (Philadelphia, PA)] and electrophoretically blotted onto Immobilon-P membrane (Millipore). The membrane was incubated overnight at 4°C with a primary anti-fibrocystin/polyductin antibody [H9262, The National Disease Research Interchange (Philadelphia, PA)].

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1 HFCT clone 7F was used for the immunoblot and real-time PCR of fibrocystin/polyductin. HFCT clones 7F and 2C have been shown to be interchangeable in their biochemical characteristics (4, 8, 41, 45).
monoclonal mouse anti-fibrocystin antibody (a generous gift from Dr. C. Ward, Mayo Clinic), diluted 1:200 in 5% milk in TBS containing 0.1% Tween (TBST). After washing with TBST, the membrane was incubated for 45 min with an anti-mouse secondary antibody (Invitrogen, Carlsbad, CA) directed against IgG, diluted to 1:10,000 in 5% milk in TBST at room temperature, followed by a 1-h wash and visualization by ECL.

Perfusate with a nominally Ca2+-free perfusate containing 10 μM ionomycin (13). [Ca2+]i response was measured, and baseline and peak [Ca2+]i were calculated. In addition, the area under the curve was measured utilizing a standard macro in Excel (Microsoft, Redmond, WA), starting at the time that the Ca2+-free perfusate containing ionomycin was added and ending when [Ca2+]i returned to baseline. Statistics

Data are given as means ± SE (n = number of monolayers). Statistical analyses were performed using paired or unpaired t-tests, as appropriate, and/or ANOVA with multiple-range test and Bonferroni inequality (SigmaStat version 2.03; SPSS, San Rafael, CA). To measure the time required for [Ca2+]i to return to baseline, a paired t-test was utilized to compare the [Ca2+]i values detected at discrete 1-min intervals following shear induction to “time zero,” the single [Ca2+]i, just before initiating LSS. A value of P ≤ 0.05 was used to assert statistical significance.

RESULTS

Polyductin/Fibrocystin Expression in Human Cell Culture Model

In prior studies, we identified and characterized several biochemically identical clonal cell lines of HFCT renal epithelia of confirmed principal cell origin, as well as ARPKD clonal cells (4, 8, 31, 41, 45). We now sought to confirm that ARPKD cells exhibited altered expression of polyductin/fibrocystin mRNA and protein compared with age-matched controls. Western blot analysis of total cellular protein lysate of HFCT and ARPKD clone cells, immunoblotted utilizing an anti-fibrocystin monoclonal antibody (gift of Dr. Christopher Ward), identified a ~460-kDa band in HFCT cells that was much reduced in ARPKD cells (Fig. 1A). We acknowledge that our human ARPKD cells may express mutant fibrocystin/polyductin protein not identified by our antibodies. Using pkhd-1 primer-specific pairs, we carried out real-time quantitative PCR on total RNA from HFCT and ARPKD clone cells and, similarly, identified reduced pkhd-1 expression in ARPKD cells compared with HFCT cells (Fig. 1B).

Effect of LSS on [Ca2+]i in HFCT and ARPKD Cells

Murine and human models of ADPKD, as well as the genetically nonorthologous orpk model of ARPKD, exhibited blunted flow-induced increases in [Ca2+]i, in cyst-lining cells (19, 23, 24, 33, 47). To test whether human ARPKD cells were similarly limited in their ability to transduce mechanical forces
peak [Ca$^{2+}$], in ARPKD clone 5E was greater than peak LSS-induced peak [Ca$^{2+}$], detected in HFCT clone 2C (Fig. 2, A and B; P < 0.05).

The time course of the [Ca$^{2+}$], response to shear was similar in HFCT clone 2C and HFCT pool cells (Fig. 2C), and ARPKD clone 5E and HFCT pool cells (Fig. 2D). Following an increase in flow, the [Ca$^{2+}$], in ARPKD cells returned to baseline by 10 min, whereas HFCT cells had reached baseline [Ca$^{2+}$], after 6 min. Because the baseline [Ca$^{2+}$], LSS-induced peak [Ca$^{2+}$], and the time course of the LSS-induced [Ca$^{2+}$], transient were identical in clone and pool cells, we concluded that the clone cells represent a valid model of the pool cells isolated from the individually microdissected collecting tubules and cysts from which they were derived. Thus, in contradistinction to other models of ARPKD and ADPKD, human ARPKD renal epithelial cells exhibit an exaggerated [Ca$^{2+}$], response to LSS compared with controls.

Source of Ca$^{2+}$ Giving Rise to LSS-Induced [Ca$^{2+}$], Response

We next sought to determine whether the observed difference in LSS-induced peak [Ca$^{2+}$], between ARPKD and HFCT cells was due to a difference in extracellular Ca$^{2+}$ entry or internal Ca$^{2+}$ store release. To evaluate this, ARPKD clone 5E cells were treated with specific inhibitors of extracellular Ca$^{2+}$ entry and/or intracellular Ca$^{2+}$ release for 30 min before and throughout the duration of the experiment.

Pretreatment of ARPKD 5E cells with Gd$^{3+}$ (30 μM), an inhibitor of nonselective cation channels, including polycystin-2, did not affect baseline [Ca$^{2+}$], (Fig. 3), but significantly reduced the peak [Ca$^{2+}$], elicited by LSS (336 ± 58 nM; n = 6; P < 0.05 vs. control ARPKD clone 5E; Fig. 3), consistent with the notion that extracellular Ca$^{2+}$ entry contributes to the mechano-induced response. The baseline and peak [Ca$^{2+}$], elicited by shear in these Gd$^{3+}$-treated ARPKD cells was not different from that observed in untreated HFCT 2C cells (Fig. 2A, P = NS). [Ca$^{2+}$], in Gd$^{3+}$-treated ARPKD cells returned to baseline within 6 min, as did untreated HFCT clone 2C cells (Fig. 4). Gd$^{3+}$ treatment did not affect the LSS-induced peak [Ca$^{2+}$], response in HFCT clone 2C cells (275 ± 32 nM; n = 5, P = NS compared with control HFCT 2C; data not shown).

In summary, these data suggest that the exaggerated shear-induced increase in [Ca$^{2+}$], in ARPKD 5E compared with HFCT 2C cells is due to extracellular Ca$^{2+}$ entry through Gd$^{3+}$-sensitive mecanosensitive cation channels.

Removal of Ca$^{2+}$ from the perfusate (Ca$^{2+}$ free) resulted in a reduction in baseline [Ca$^{2+}$], (44 ± 7 nM; n = 3; P < 0.05 vs. control) (Fig. 3) (10). LSS induced a small, yet significant, increase in peak [Ca$^{2+}$], (82 ± 10 nM; n = 3; P < 0.05 vs. baseline [Ca$^{2+}$]) (Fig. 3) in ARPKD 5E cells studied in the nominal absence of extracellular Ca$^{2+}$, presumably reflecting internal Ca$^{2+}$ store release, although the peak [Ca$^{2+}$], elicited was less than observed in the presence of extracellular Ca$^{2+}$ (P < 0.05 vs. control) (Fig. 3). Treatment of ARPKD 5E cells with 10 μM 2-APB, an inhibitor of the inositol 1,4,5-triphosphate receptor (IP$_3$R) (23) and capacitative Ca$^{2+}$ entry (14), did not alter baseline [Ca$^{2+}$], but led to a significant reduction in peak [Ca$^{2+}$], (179 ± 3 nM; n = 6; P < 0.05 vs. control) (Fig. 3) elicited by LSS and a return to baseline [Ca$^{2+}$], by 3 min in the continued presence of LSS (data not shown). In
Fig. 2. Laminar shear stress-induced changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in HFCT and ARPKD cells. A: baseline [Ca\(^{2+}\)]\(_i\), is similar in HFCT 2C and pool cells, as is the peak [Ca\(^{2+}\)]\(_i\), after flow is initiated. Both clone and pool cells respond to flow with a significant increase in [Ca\(^{2+}\)]\(_i\). (*P < 0.05 vs. low shear). B: ARPKD 5E and pool cells have similar baseline and peak [Ca\(^{2+}\)]\(_i\). ARPKD clone and pool cells respond to flow with a significant increase in peak [Ca\(^{2+}\)]\(_i\). (*P < 0.05 vs. low shear). The shear-induced peak [Ca\(^{2+}\)]\(_i\), is greater in ARPKD 5E than HFCT 2C (#P < 0.05 vs. HFCT clone). C: HFCT 2C (solid circles) and pool (shaded circles) cells exhibit similar [Ca\(^{2+}\)]\(_i\), transients as do ARPKD 5E (shaded circles) and pool (solid circles) cells (D) in response to shear. However, the peak [Ca\(^{2+}\)]\(_i\), induced by flow is greater in ARPKD than HFCT cells (A and B).

Summary, these data suggest that the [Ca\(^{2+}\)]\(_i\), response induced by LSS in ARPKD cyst lining cells is mediated by internal Ca\(^{2+}\) store release, as well as Gd\(^{3+}\)-sensitive extracellular Ca\(^{2+}\) entry.

Internal Ca\(^{2+}\) Stores in ARPKD and HFCT Cells

Polycystin-2 is expressed not only in the cilium, but also in the endoplasmic reticulum (ER), where it can interact with IP\(_3\)R and/or ryanodine receptors to release Ca\(^{2+}\) from the ER (3, 16, 18). Dysregulated function of polycystin-2 within the ER may alter internal Ca\(^{2+}\) stores, which, in turn, may contribute to differences in LSS-mediated [Ca\(^{2+}\)]\(_i\), responses observed between ARPKD and HFCT cells. We utilized ionomycin, a Ca\(^{2+}\) ionophore, in nominally Ca\(^{2+}\)-free perfusate to mobilize cellular Ca\(^{2+}\) stores in ARPKD and HFCT cells. The peak [Ca\(^{2+}\)]\(_i\), elicited by ionomycin in ARPKD (658 ± 209 nM; n = 9) and HFCT (876 ± 258 nM; n = 6; P = NS) cells was similar. Because peak [Ca\(^{2+}\)]\(_i\), may not reflect the total Ca\(^{2+}\) in intracellular stores, we measured the area under the curve, as described in MATERIALS AND METHODS, and also found no difference between ARPKD (62.0 ± 25.3 μM * s; n = 9) and HFCT (105.4 ± 32.4 μM * s; n = 6; P = NS).

Localization of Polycystin-2 in HFCT Clone 2C and ARPKD Clone 5E

The results above suggest that extracellular Ca\(^{2+}\) entry induced by LSS is mediated by a Gd\(^{3+}\)-sensitive mechanosensory Ca\(^{2+}\) channel. One such candidate channel is polycystin-2, a protein that is normally expressed in human fetal kidney, mutations or mislocalization of which are associated with cystic kidney disease, and a channel protein that is to some extent regulated by fibrocystin/polyductin (7, 33, 37, 38, 46). To determine whether aberrant localization of polycystin-2 contributes to the difference in the shear-mediated [Ca\(^{2+}\)]\(_i\), response between HFCT and ARPKD cells, monolayers of HFCT clone 2C and ARPKD clone 5E (Fig. 5) were

Fig. 3. Mechanisms underlying the shear-induced [Ca\(^{2+}\)]\(_i\), response in ARPKD cells. ARPKD 5E cells studied in Ca\(^{2+}\)-free medium, or in the presence of 30 μM Gd\(^{3+}\) or 10 μM 2-aminoethoxydiphenyl borate (2-APB), retained their ability to respond to shear with an increase in [Ca\(^{2+}\)]\(_i\), (*P < 0.05 vs. [Ca\(^{2+}\)]\(_i\), values under low shear). However, all treatments blunted the peak [Ca\(^{2+}\)]\(_i\), elicited by flow compared with the untreated ARPKD control (#P < 0.05 vs. untreated ARPKD cells). The baseline [Ca\(^{2+}\)]\(_i\), was lower (SP < 0.05 vs. control) in ARPKD cells studied in Ca\(^{2+}\)-free media than in control cells.

Fig. 4. Shear-mediated [Ca\(^{2+}\)]\(_i\), transient in HFCT 2C control and ARPKD 5E cells treated with Gd\(^{3+}\). ARPKD 5E cells (shaded circles) treated with Gd\(^{3+}\) exhibited a similar shear-induced [Ca\(^{2+}\)]\(_i\), response to HFCT 2C controls (solid circles). In ARPKD 5E cells treated with Gd\(^{3+}\), [Ca\(^{2+}\)]\(_i\), fell to a value that was identical to that measured at baseline at 6 min, as did HFCT 2C cells.
labeled with anti-polycystin-2 antibody (YCC-2), anti-acetylated α-tubulin antibody, and DAPI to identify cilia and nuclei, respectively. Of note was that cilia length tended to be shorter in ARPKD clone 5E (6.5 ± 0.6 μm; n = 15) compared with HFCT clone 2C (8.5 ± 0.8 μm; n = 21; P = 0.05) cells. In HFCT clone 2C, polycystin-2 localized to cilia, identified by anti-acetylated α-tubulin antibody staining (Fig. 5, A–D; arrow in 5D indicates colocalization). Polycystin-2 expression was also prominently detected in cilia of ARPKD clone 5E cells (Fig. 5, E–H; arrows). Immunolocalization studies using the commercially available rabbit anti-polycystin-2 antibody (H280, Santa Cruz Biotechnology) also demonstrated identical polycystin-2 expression in cilia of ARPKD and HFCT cells (data not shown).

Polycystin-2 Expression in HFCT and ARPKD Cells

Immunoblotting of protein lysates prepared from HFCT pool and ARPKD pool cells with anti-polycystin-2 antibody H280 (Fig. 6A) or YCC-2 (Fig. 6B) identified a ~110-kDa band in all samples, which is believed to be endogenous polycystin-2 protein. Densitometric analysis of the bands identified by the H280 antibody compared with actin, and then normalized to the values obtained in HFCT cells, showed that ARPKD cells (n = 3) expressed ~40% more polycystin-2 than did HFCT cells (n = 3), but this difference was not statistically significant (Fig. 6A; P = 0.5). Similarly, densitometric analysis of the immunoblot probed with the well-characterized YCC-2 antibody (6) demonstrated a tendency for polycystin-2 expression to be greater in ARPKD than in HFCT cells (Fig. 6B; P = 0.07). Because of the greater difference in expression between ARPKD and HFCT cells observed with the YCC-2 antibody, the polycystin-2 bands in the HFCT cells were barely visible using exposure times that avoided saturation of the ARPKD signal (Fig. 6B).

DISCUSSION

The results of this present study are, to our knowledge, the first to examine mechanoregulation of renal epithelial function by shear-induced Ca²⁺ signaling in ARPKD cells.
in human ARPKD cyst-lining cells, and reveal two novel findings. First, ARPKD cells exhibit an exaggerated LSS-induced increase in \([\text{Ca}^{2+}]_i\) compared with age-matched control HFCT cells (Fig. 2). All other reported analyses of the effect of flow on \([\text{Ca}^{2+}]_i\) in murine models of ARPKD and ADPKD and human models of ADPKD reveal that these cystic cell types exhibit a blunted LSS-induced \([\text{Ca}^{2+}]_i\) response (23, 24, 33, 47). Note that baseline \([\text{Ca}^{2+}]_i\) under low-shear conditions was similar in human ARPKD and HFCT cells, as has been reported in the murine orpk model of ARPKD (19). Our observation that pretreatment of ARPKD clone 5E with Gd\(^{3+}\) "normalized" the magnitude of the shear-induced peak \([\text{Ca}^{2+}]_i\) transient and accelerated the fall to baseline to generate a \([\text{Ca}^{2+}]_i\) response that was indistinguishable from HFCT controls suggests that the fetal ARPKD cells express an upregulated mechanosensitive cation entry pathway. Our second major finding is that polycystin-2, a mechano- and Gd\(^{3+}\)-sensitive \([\text{Ca}^{2+}]_i\) channel absent from cilia of human ADPKD renal epithelial cells (24, 47), localized to the cilium of human ARPKD cells, tended to be more highly expressed in the latter cells compared with age-matched controls. The precise relationship of the robust ciliary polycystin-2 expression in human ARPKD cells to the exaggerated shear-induced Gd\(^{3+}\)-sensitive \([\text{Ca}^{2+}]_i\) transient remains to be clarified, as is the relationship of this pathway to that mediating enhanced transepithelial Na absorption in human ARPKD compared with HFCT that our laboratory has previously described (31). Similar studies to those included in this investigation have been performed utilizing renal epithelia from the orpk murine model of ARPKD, which is genetically distinct from human ARPKD, but develops cystic renal disease that is phenotypically similar to ARPKD. In a series of elegant studies per-

Fig. 6. Immunoblotting for polycystin-2 expression. Protein lysate (50 µg/lane) derived from ARPKD and HFCT pool cells was immunoblotted with anti-polycystin-2 antibody H280 (A) or YCC-2 (B). A 110-kDa protein was detected both in ARPKD and HFCT lysates probed with either anti-polycystin-2 antibody. A: densitometric analysis of the 110-kDa protein normalized to actin and then to the values obtained in HFCT cells, showed an insignificant increase in polycystin-2 abundance in ARPKD cells \((n=3)\) compared with HFCT cells \((n=3, P=0.5)\). B: densitometry of immunoblots incubated with YCC-2 showed that polycystin-2 expression in ARPKD cells \((n=3)\) tended to be greater than in HFCT cells \((n=3, P=0.07)\).
formed by Siroky et al. (33) in orpk mice, immunodetectable polycystin-2 was expressed not only in the stunted cilium, but also prominently along the apical membrane (33). Patch-clamp and Mn$^{2+}$-quenching experiments demonstrated that apically localized polycystin-2 channel was functional in orpk renal epithelial cells (33). These results suggested that apical polycystin-2 channel activity and/or number was greater in orpk than in control cells (33). Using an open-flow chamber design, as opposed to the closed chamber utilized in our experiments, the authors of the latter study detected a markedly attenuated shear-induced [Ca$^{2+}$], response in orpk mutant compared with control cells (33). Murine embryonic renal epithelial cells and murine inner medullary CD cells, in which endogenous fibrocystin/polyductin mRNA and protein were knocked down, also demonstrated an attenuated LSS-induced [Ca$^{2+}$], transient compared with controls (38); however, the knockdown studies were not able to abrogate the LSS-mediated [Ca$^{2+}$], response.

Our findings in a human ARPKD model contradict those of Siroky et al. (33) and Wang et al. (38) in that we observed an augmented [Ca$^{2+}$], response to shear in ARPKD cells compared with control renal epithelial cells. The augmented shear-induced [Ca$^{2+}$], response in our study may be related to the species studied (mouse vs. human), developmental stage (postnatal vs. fetal), or genetic differences between the murine orpk (tg737) and human ARPKD (pkhd-1) models. Although the renal disease is phenotypically similar, the orpk mouse also develops preaxial polydactyly and hydrocephalus not observed in human ARPKD (22, 35). Also, the expression of mutant protein in human cells may reflect some of the disparate results seen between our human model and the fibrocystin/polyductin knockdown model used by Wang et al. (38).

The exaggerated shear-induced [Ca$^{2+}$], response in human ARPKD cells is due, in part, to extracellular Ca$^{2+}$ entry via mechano- and Gd$^{3+}$-sensitive cation channels. Our observation, that pretreatment of cells with Gd$^{3+}$ or removal of extracellular Ca$^{2+}$ did not completely abolish the shear-induced [Ca$^{2+}$], response in ARPKD cells (Fig. 3) and that 2-APB reduced the shear-mediated peak [Ca$^{2+}$], to a value below that observed in HFCT cells, suggests that both IP$_{3}$R and/or store-operated Ca$^{2+}$ channels contribute to the [Ca$^{2+}$], response (Fig. 3). As others have suggested, shear initiates extracellular Ca$^{2+}$ entry through mechano- and Gd$^{3+}$-sensitive Ca$^{2+}$ channels, and the increase in [Ca$^{2+}$], triggers Ca$^{2+}$-activated Ca$^{2+}$ release from internal stores (20, 23, 29). Internal store release then activates store-operated Ca$^{2+}$ channels at the membrane (17). We speculate that the prolonged elevation in LSS-induced [Ca$^{2+}$], in ARPKD cells may be related to activation of store-operated Ca$^{2+}$ channels.

Although polycystin-2 is expressed in the cilium (9) (Fig. 5), the bulk of endogenous polycystin-2 is localized to the ER membrane, where it is proposed to function as an intracellular Ca$^{2+}$ release channel (15). LLC-PK cells overexpressing polycystin-2 in the ER and bathed in Ca$^{2+}$-free media respond to IP$_{3}$R activation with a twofold greater peak [Ca$^{2+}$], response of 10-fold greater duration compared with vehicle-transfected cells (15). Li et al. (18), using polycystin-2 transfected Xenopus oocytes, showed that polycystin-2 and IP$_{3}$R directly interact, and that polycystin-2 prolongs the duration of the IP$_{3}$R-activated Ca$^{2+}$ transient. The observation that overexpression of polycystin-2 in the ER can prolong the amplitude and/or duration of the IP$_{3}$R-mediated Ca$^{2+}$ transient is particularly intriguing, given the nature of our results.

Fibrocystin/polyductin, the mutant protein in ARPKD, interacts with polycystin-2 (46). Wu et al. (46) and Wang et al. (38) demonstrated that fibrocystin activates polycystin-2 channel activity through a mediator protein, kif3b, that is a component of the kinesin-2 complex. Fibrocystin-kif3b-polycystin-2 forms a heterotrimERIC complex by which fibrocystin modulates polycystin-2 channel activity in the cilium. Although we were unable to identify any significant differences in polycystin-2 expression between ARPKD cyst lining cells and age-matched control cells, we speculate that the exaggerated Ca$^{2+}$ response in the former reflects dysregulated channel expression and activity, due to aberrant interactions with mutant fibrocystin.

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