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Human ADPKD primary cyst epithelial cells with a novel PKD1 mutation exhibit defective ciliary polycystin localization and loss of flow-induced Ca^{2+} signaling

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Abstract

ADPKD gene products polycystin-1 (PC1) and polycystin-2 (PC2) colocalize in the apical monocilia of renal epithelial cells. Mouse and human renal cells without PC1 protein show impaired ciliary mechanosensation, and this impairment has been proposed to promote cystogenesis. However, most cyst epithelia of human ADPKD kidneys appear to express full-length PC1 and PC2 in normal or increased abundance. We show that confluent primary ADPKD cyst cells with the novel PC1 mutation ΔL2433 and with normal abundance of PC1 and PC2 polypeptides lack ciliary PC1 and often lack ciliary PC2, whereas PC1 and PC2 are both present in cilia of confluent normal human kidney (NK) epithelial cells in primary culture. Confluent NK cells respond to shear stress with transient increases in $[\text{Ca}^{2+}]_i$ dependent upon both extracellular Ca^{2+} and release from intracellular stores. In contrast, ADPKD cyst cells lack flow-sensitive $[\text{Ca}^{2+}]_i$ signaling and exhibit reduced ER Ca^{2+} stores and store-depletion-operated Ca^{2+} entry, but retain near-normal Ca^{2+}_i responses to angiotensin II and to vasopressin. Expression of wildtype and mutant CD16.7-PKD1(115-226) fusion proteins reveals within the C-terminal 112 aa of PC1 a coiled-coil domain-independent ciliary targeting signal. However, the coiled-coil domain is required for CD16.7-PKD1(115-226) expression to accelerate decay of the flow-induced Ca^{2+} signal in NK cells. These data provide evidence for ciliary dysfunction and polycystin mislocalization in human ADPKD cells with normal levels of PC1.

Keywords

Autosomal dominant polycystic kidney disease; monocilium; shear stress; protein trafficking; Fura-2

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Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is the most common life-threatening monogenic human renal disease, with a prevalence between 1:400 and 1:1000. It is characterized by progressive development and enlargement of fluid-filled cysts originating from only ~3% of nephrons, leading ultimately to renal failure in 50% of affected individuals. More than 85% of ADPKD cases are caused by mutations in the *PKD1* gene, with almost all remaining cases associated with *PKD2* gene mutations. The *PKD1* polypeptide gene product, polycystin-1 (PC1/TRPP1), is a 4302 amino acid (aa) polypeptide with an N-terminal extracellular domain of ~3000 aa, ~11 transmembrane domains, and a ~200 aa C-terminal cytoplasmic domain interacting with polycystin-2 (PC2/TRPP2) (45,62), heterotrimeric G-proteins, and the regulator of G-protein signaling RGS7, among many other proteins. The C-terminal tail of PC1 also upregulates several transcriptional pathways, in part by regulated proteolysis (24), and activates endogenous Ca^{2+} -permeable cation channels of 20-30 pS in *Xenopus* oocytes and HEK-293 cells (64,65). The *PKD2* gene product, PC2, is a 968 aa polypeptide believed to function as a Ca^{2+} -permeable cation channel in the endoplasmic reticulum and/or at the plasma membrane, independently or in complex with PC1 (33,11,26) or other proteins. The cellular functions of PC1, PC2, and the PC1/PC2 complex remain incompletely understood, but likely include roles in epithelial cell proliferation, differentiation and tubulogenesis, matrix interaction, Ca^{2+} signaling, and determination of developmental asymmetry in the embryonic ventral node.

Nearly all polarized epithelial cell types express a central apical monocilium with a “9 + 0” axoneme structure, long considered vestigial but periodically proposed to function as a mechanosensor. Several recent findings from diverse fields have converged to suggest a central role for the primary cilia of renal tubular epithelial cells in the cystogenesis of polycystic kidney disease (40,8,12). After the MDCK cell cilium was shown to respond to mechanical bending and to flow by transducing an increase in cytoplasmic $[\text{Ca}^{2+}]_i$ ($[\text{Ca}^{2+}]_i$) (41,42), several genes encoding intraflagellar transport proteins of the green alga, *Chlamydomonas*, were noted to encode cystic kidney disease genes and to localize to the renal epithelial cilium (74,39). These findings promoted the discovery of altered ciliary morphology in the *orpk* mouse (39), and provided additional insight into ciliary localization of the ADPKD gene homologs *lov-1* and *pkd2* in sensory neurons of *C. elegans*, and ciliary or basal body functions of glomerulocystic disease genes from zebrafish.

Nauli et al. soon proposed that the PC1/PC2 complex functions as a flow-sensing mechanoreceptor in the primary cilia of primary cultures of mouse embryonic renal epithelial cells, and showed that cells from *pkd1*(-/-) mice are deficient in this proposed sensing function (30). The partially defective flow-sensing in the *orpk* mouse (21,54) further supported the proposed central role of defective ciliary sensation of and/or response to tubular flow to the cystogenesis of ADPKD (30). The *pkd1*(-/-) mouse embryonic renal epithelial cells in which flow-induced signaling defects were observed completely lacked PC1 and PC2 polypeptides. A recent report appearing after completion of the work presented here extended this observation to human ADPKD cyst cells expressing little or no PC1 polypeptide (31). However, human ADPKD is almost always characterized by normal or increased renal levels of apparently full-length PC1 polypeptide (36,37,32), despite the significantly truncated proteins encoded by most *PKD1* germline mutations (35). Indeed, phenotypically similar murine polycystic kidney disease results from knockout and from overexpression of the wildtype *pkd1* gene (43,59).

Therefore, we compared ciliary expression of PC1 and PC2 and flow-sensitive Ca^{2+} signaling in primary human renal epithelial cells derived from normal kidneys (NK cells) or from ADPKD cysts (PKD cells). We report that NK cells and PKD cells with a novel

heterozygous in-frame single codon deletion mutation in the *PKD1* gene exhibit equivalent abundance of PC1 and PC2 polypeptides, but differ in their ciliary localization of PC1 and PC2. Exposure to low shear stress increased $[Ca^{2+}]_i$ in a minority of NK cells, but shear stress at the higher levels achieved during diuresis increased $[Ca^{2+}]_i$ to higher peak values in most NK cells. In contrast, PKD cyst cells exhibited no flow-sensitive elevation of $[Ca^{2+}]_i$ at any level of shear stress. PKD cyst cells were also characterized by reduced endoplasmic reticulum Ca^{2+} stores, reduced capacitative Ca^{2+} entry, and near-normal hormone-induced Ca^{2+} -signaling. We also report that aa 115-226 of the PC1 C-terminal tail modulated flow-induced NK cell Ca^{2+} signaling through its coiled-coil domain, and contains a coiled-coil domain-independent ciliary targeting sequence.

Methods

Cell culture

Human renal cyst epithelial cells (PKD) were harvested from multiple superficial cortical cysts of kidneys resected from ADPKD patients PKD 10-27-98 and PKD 3/14/00, in both of whom disease progression, family histories, and pathologic examination suggested germline *PKD1* mutations. Cortical tubules were dissected from one freshly harvested cadaveric human kidney not used for transplant (NK 6-1-99) and from the grossly normal lower poles of two human kidneys (NK57M03 and NK 11-7-02) resected for renal cell carcinoma. The epithelial cells from both sources were grown in primary culture and passaged as described (4, 5). Cells for experiments were transferred to glass coverslips coated with Vitrogen collagen (Conhesion Technologies Inc., Palo Alto, CA), fed every other day with Clonetics Renal Epithelial Cell Media (REBM, Clonetics), and grown to confluence 5-6 days after plating. Only cells between passages 2 and 6 were used for experiments. Experiments presented in Figs. 3-7 and in Suppl. Figure 2 were replicated with and include cells from all donor lines. PKD cyst cell results presented in Figs. 8-10 and Suppl. Figures 1 and 3 are from donor PKD 10/27/98. All discarded tissue was harvested according to CCI/IRB protocols reviewed and approved at Indiana University School of Medicine and Beth Israel Deaconess Medical Center.

Human pancreatic adenocarcinoma cells (HPAC), HeLa, and HEK 293 human embryonic kidney cells from ATCC were grown in DMEM supplemented with 10% fetal calf serum.

Genomic DNA analysis

Genomic DNA was prepared from NK cells from individual NK57M03 and cyst cells from individual PKD 10/27/98. All coding exons of the *PKD1* and *PKD2* genes were amplified by PCR from genomic DNA as previously reported (50,51) or with newly developed primers (available on request). Amplicons (300-600 ng) were analyzed by Denaturing High-Pressure Liquid Chromatography (DHPLC) performed at two temperatures on a DNasep Cartridge HT with the Wave System 3500HT (Transgenomic, Inc., Omaha, NE, USA), and eluted through a 2.5-minute linear gradient of buffer A [5% triethylammonium acetate (TEAA)] and buffer B (5% TEAA and 25% acetonitrile). Samples with abnormal elution chromatograms compared to a normal control were subjected to DNA sequencing. The DNA sequencing allowed identification of heterozygous variation in gene sequence.

Antibodies

Rabbit polyclonal anti-PC1 antibody NM005 raised against the 223 aa recombinant PC1 C-terminal cytoplasmic domain aa 4070-4302 (66) was used as an Ig fraction, with specificity confirmed by fusion protein antigen preadsorption as previously described (49) and by siRNA knockdown as described below. Rabbit polyclonal antibody LRR (14) and monoclonal antibody 7e12 (36,37), both raised against the PC1 leucine-rich repeat domain,

were previously described. Rabbit polyclonal antibody NM002 was raised against PC1 aa 3619-3631 (63), affinity-purified by peptide antigen column, and specificity tested by siRNA knockdown. Anti-PC2 antibody (64) raised against a GST-PC2 fusion protein encoding the PC2 C-terminal cytoplasmic aa 687-968 was originally obtained from Dr. Oxana Beskrovnaya-Ibraghimova (Genzyme). Anti-CD16 monoclonal antibody (mAb) 3G8 was used as an Ig fraction as described (64,65). Anti-N-acetylated- α -tubulin was obtained from Sigma, anti-calnexin from Stressgen, and anti-GM130 from BD Biosciences Pharmingen. Fluorescein isothiocyanate-labeled lectins were from Vector Laboratories.

Immunoblots

Cells scraped in ice-cold PBS in the presence of Complete™ protease inhibitor (Roche Diagnostics, Mannheim, Germany) were pelleted, rinsed in the same medium, then suspended and boiled briefly in 1% SDS, 10 mM Tris-HCl, pH7.4, or in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris-HCl, pH7.5). Samples were suspended in Laemmli sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.1% bromophenol blue, 5% β -mercaptoethanol) and incubated 30 min at 37 °C prior to loading Nupage 3-8% polyacrylamide-SDS Tris-Acetate gels (Invitrogen, Carlsbad, California) or Criterion 5% polyacrylamide-SDS gels (BioRad) for PC1 analysis, and 7.5% acrylamide-SDS gels for PC2 analysis. Proteins separated by electrophoresis were transferred at 100 V for 1 hr to nitrocellulose, blocked with 5% milk in BLOTTO buffer (20 mM Tris, 0.9% NaCl, 0.03% TWEEN-20, pH 7.4) for 30 min at 37 °C, probed with primary antibody for 1 h at 37 °C followed by horseradish peroxidase-conjugated goat anti-rabbit-Ig for 1 h at 20 °C. Bound secondary antibody was detected by enhanced chemiluminescence (Boehringer) on SB5 X-ray film (Kodak).

cDNA Transfection

Lipofection was ineffective for transfection of NK and PKD cyst cells. Therefore, cells were trypsinized for 7 min at 37°C, pelleted, and resuspended at $1.5\text{--}2.5 \times 10^6$ cells/100 μL in basic primary mammalian epithelial cell Nucleofector™ solution (Amaxa Biosystems, Koeln, Germany). After addition of 10 μg plasmid DNA, the mixture was placed in a 2 mm cuvette and electroporated at room temperature in the Nucleofector device (Amaxa) with program T-05. 500 μL of pre-warmed REBM medium containing 10% FBS was immediately added to the cuvette, and the suspension was plated to a 35-mm coverslip in a 60-mm² culture dish. The transfected cells were incubated at 37°C for 5 days or longer in REBM containing 10% FBS. Apparent transfection efficiency assessed cytologically by GFP fluorescence 6 days post-electroporation was up to 70% in both cell types. Transfection efficiency assessed by expression of CD16.7-PKD1(115-226) was 15% in NK cells and 5% in PKD cyst cells.

Confocal Immunofluorescence Microscopy

Cell monolayers grown on coverslips were fixed for 30 min at room temperature with PBS containing 3% (wt/vol) paraformaldehyde. Fixed cells were extensively rinsed with PBS, quenched with 50 mM lysine HCl, pH 8.0, exposed to 1% SDS for 15 min, and blocked for 15 min in PBS with 1% bovine serum albumin and 0.05% saponin. After 4 °C overnight incubation with primary antibody against PC1 or PC2 (1:100), coverslips were incubated with Cy3-conjugated donkey anti-rabbit Ig secondary antibody (1:500) for 2 h at room temperature. Some coverslips immunostained as above for PC1 or PC2 were then co-stained with antibodies to the ciliary marker N-acetylated α -tubulin (1:10,000), the ER marker calnexin (1:500), or the Golgi marker GM130 (1:200 dilution).

Costaining with two rabbit polyclonal antibodies against PC2 and calnexin (Supplemental Figure 3) was performed by the microwave denaturation method (61). After completion of

PC2 staining, coverslips were microwaved 10 min in 10 mM citrate, pH 6.0, to denature bound antibody molecules and prevent cross-reaction during subsequent calnexin staining with rabbit polyclonal antibody and FITC-coupled goat anti-rabbit Ig secondary antibody. The same microwave procedure was used to costain with two mAbs against CD16 and N-acetylated α -tubulin (Figure 9). In both cases, control incubations with secondary antibody post-microwave treatment alone confirmed successful denaturation of previously bound Ig (not shown). Immunostained cells were imaged with a Bio-Rad MRC-1024 laser-scanning confocal immunofluorescence microscope. Pixel fluorescence intensity of cilia and cell bodies in X-Z sections of single cells was measured with Bio-Rad software.

RNA knockdown

Six human PC1 siRNA sequences of 21 nt in length complementary to several PC1 domains of the 14135 pb coding sequence of *PKD1* (Genbank NM000296) were selected with Ambion's "siRNA Target Finder tool (www.ambion.com). The most effective sequence targeted nt 584-605 within the leucine-rich repeat (LRR) domain. Blast search confirmed the lack of significant homology with other human genes. Sense and antisense oligodeoxynucleotides (Integrated DNA Technologies, Coralville, IA) were used to generate templates and synthesize siRNA according to manufacturer's instructions. PC1 siRNA, GAPDH siRNA, or control scrambled siRNA (Ambion control template set #4800) were transfected into 50% confluent human pancreatic adenocarcinoma (HPAC) cells (50-100 pmol per well of a 6-well plate) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) per manufacturer's instructions. Knockdown efficacy was evaluated by immunoblot of cells lysed in SDS-PAGE sample buffer containing 5% 2-mercaptoethanol and resolved on pre-cast 4-15% gels (BioRad Criterion), using NM005 anti-PC1 antibody or monoclonal anti-GAPDH antibody (Ambion). Chemiluminescent signal was quantitated with a phosphorimager (Molecular Dynamics) using ImageQuant software. Fold decrease was calculated relative to the scrambled siRNA control. Maximal knockdown was observed 48 hrs post-transfection.

Measurement of cytosolic $[Ca^{2+}]_i$

NK and PKD cyst cells cultured to confluence on collagen-coated 35-mm glass coverslips were loaded with 5 μ M Fura 2-AM (Molecular Probes) in Hepes-buffered Hank's Balanced Salt Solution (HBSS, pH 7.2) at 37°C for 30 min. Extracellular Fura 2-AM was removed by washing twice with Hepes-HBSS. The coverslip was then mounted into the bottom of a parallel-plate flow chamber (GlycoTech) 0.5 cm in width and 0.0254 cm in depth, and perfused at room temperature with a Harvard Syringe pump. In some experiments, cells were perfused at 37°C using a calibrated, WPI in-line heater, with monitoring of inflow and outflow temperatures. Perfusion medium composition was (in mM) 127 NaCl, 5.4 KCl, 1.27 $CaCl_2$, 1 $MgCl_2$, 5.6 glucose, and HEPES 11.6, final pH 7.2. Intracellular free $[Ca^{2+}]_i$ concentration ($[Ca^{2+}]_i$) was measured by fluorescence ratio imaging with a Metafluor digital imaging system (Universal Imaging, West Chester, PA) equipped with an Olympus IMT-2 inverted microscope, and a CoolSNAP CCD camera (Photometrics, Tucson, AZ). Fura 2 emission images were monitored at 510-nm with alternating excitation at 340 and 380 nm. For each coverslip, one visual field was selected as a region of interest, recorded before and during imposition of a uniform rate of fluid flow. Shear stress (τ_w) was calculated as: $\tau_w = 6\mu Q/a^2b$, where: μ = apparent viscosity of superfusate (1.00 for H_2O at 20°C; 0.70 at 37°C),

Q = flow rate (ml/sec), and

a and b = flow chamber depth and width.

All cells within the visual field were analyzed as a single region of interest. On some coverslips individual cells were analyzed separately as noted. For each coverslip of Fura2-

loaded, cDNA-transfected cells, individual dsRed-positive and dsRed-negative cells in a visual field were preselected as regions of interest, then recorded before and during fluid flow. Pharmacological characterization in Figure 8A, C, and D was carried out in a coverslip chamber of 1 ml volume to which 100× drug stock solution was added by pipet under “no flow conditions” which in the absence of drug elicited no increase in $[Ca^{2+}]_i$. Ca^{2+} readdition in Figure 8D was performed by superfusion at 0.75 dyn/cm², or (as noted) by gentle manual replacement of half the cell chamber volume. Fluorescence ratio emission values and images were calculated on a pixel-by-pixel basis and processed with Metamorph software (Universal Imaging).

Fura 2 fluorescence ratio values determined by in situ calibration in immortalized epithelial cells did not differ from values determined by in vitro calibration for $[Ca^{2+}]_i$ (29). Therefore, Fura 2 fluorescence ratios were calibrated in vitro (29) with the same experimental settings for the imaging system, using the Ca^{2+} calibration buffer kit no. 2 (Molecular Probes) with concentrations between 36 nM and 4 μM $[Ca^{2+}]$. The minimal fluorescence ratio (R_{min}) was determined at “zero Ca^{2+} ” (free $Ca^{2+} < 10$ nM) and the maximal fluorescence ratio (R_{max}) at 4 μM total Ca^{2+} . The equilibrium constant (K_b) was determined by fitting experimental fluorescence ratio R values at various free Ca^{2+} concentrations with the equation

$$\left[Ca^{2+}\right]_{free} = K_b (Sf2/Sb2) [(R - R_{min}) / (R_{max} - R)],$$

where the factor Sf2/Sb2 corrects for Fura 2 ion selectivity at 380 nm.

GsMTx-IV was obtained from Peptides International (Osaka, Japan). All other drugs were from Sigma.

Results

A novel ADPKD mutation

Genomic DNA was prepared from cyst cells of individual PKD10-28-98 and from normal cortical tubular epithelial cells of individual NK57M03. No mutations of the *PKD1* or *PKD2* genes were detected in the NK cell sample. In contrast, the PKD cyst cell genome revealed a heterozygous in-frame deletion of a tgc trinucleotide in exon 18 (7509_7511delTGC), encoding the predicted mutant polycystin-1 polypeptide PC1 ΔL2433 (p.Leu2433del) lacking a leucine in the receptor-for-egg-jelly (REJ) domain (Figure 1). The deleted Leu residue encoded by the human gene is identical in chicken, *Xenopus*, and *Fugu*, and is conserved as Val in mouse and rat. Two additional noncoding polymorphisms were also detected in cis: IVS31-38C-G, and IVS44+22delG. Since neither the coding deletion mutation nor the two IVS mutations were present in 100 chromosomes from 50 normal control individuals, the deletion is likely the germline disease mutation. However, the anonymity constraints under which kidneys were obtained prevented confirmation by genotyping of family members. No *PKD2* mutations were found in this patient.

PC1 and PC2 polypeptide expression in NK and PKD cyst cells

PC1 was detected by immunoblot of confluent NK cell lysate with three distinct antibodies (7e12, NM005, and LRR) as a polypeptide of Mr >400 kDa (Figure 2A). The total cell abundance of PC1 in confluent PKD cells was no less than that in confluent NK cells (Figure 2B). Immunoblot specificity of the previously characterized NM005 PC1 antibody (49) was confirmed by the 85% reduction in the >400 kDa PC1 immunoblot band in PC1 siRNA-treated HPAC cells (Figure 2C). A similar PC1 band of >400 kDa was detected by all three antibodies in HEK 293 cells, HeLa cells, and HPAC cells (not shown). PC1 immunolocalization with the NM005 antibody revealed a predominantly intracellular distribution in both NK cells and PKD cells, with some PC1 present at lateral cell membranes in NK cells (Supplemental Figure 1) as previously described (49).

PC2 migrated with $M_r \sim 110$ kDa, and was detected at equal abundance in NK and PKD cyst cells (Figure 2B). PC2 localized predominantly to endoplasmic reticulum in NK and PKD cells, as evidenced by colocalization with calnexin (Supplemental Figure 2), and consistent with previous reports (2,18).

PKD cyst cells form primary cilia devoid of PC1

PC1 and PC2 have been colocalized, along with other ciliary gene products linked to cystic kidney disease, to the primary cilium in mouse kidney, in primary cultured mouse kidney cells, and in several mammalian kidney cell lines (57,67,74). Human kidney cells in culture also exhibit primary cilia (39, 31). Figure 3 shows the presence of cilia in both NK and PKD cyst cells as detected by immunostaining of the ciliary axoneme marker, N-acetylated α -tubulin. γ -tubulin is also localized to cilia in both cell types (not shown). Neither cell type expressed a primary cilium at day 1 or 2 after splitting (<50% confluence), but by day 3, NK cells within confluent islands expressed short primary cilia of $2.7 \pm 0.1 \mu\text{m}$ in length ($n = 32$). At confluence 6 days after splitting, PKD cyst cell ciliary length was $3.1 \pm 0.1 \mu\text{m}$ ($n = 73$), slightly shorter than that of NK cells ($4.2 \pm 0.1 \mu\text{m}$ ($n = 81$; $p < 0.01$)).

PC1 polypeptide in NK cells colocalized in the central cilium with N-acetylated- α -tubulin in all 37 NK cells examined but in none of 38 PKD cyst cells immunostained with anti-PC1 antibody NM005 (Figure 4). Ciliary immunostaining intensity exceeded that of the cell body. Use of anti-PC1 antibody NM002 similarly revealed ciliary PC1 localization in all 24 examined NK cells, but in none of 17 additionally examined PKD cyst cells (not shown). PC2 polypeptide also co-localized with the ciliary marker in all 41 cilia examined in NK cells (Figure 5A), with ciliary PC2 immunofluorescence intensity again exceeding that of the cell body. However, PKD cyst cells exhibited two patterns of ciliary PC2 expression. Although bright ciliary PC2 staining was evident in 11 of 30 PKD cyst cells (Figure 5C), ciliary PC2 was undetectable in 19 of the 30 PKD cyst cells examined (Figure 5B). The length of PC2-positive cilia in these 30 PKD cyst cells was $2.8 \pm 0.2 \mu\text{m}$, whereas PC2-negative cilia were consistently shorter ($2.2 \pm 0.1 \mu\text{m}$; $p < 0.05$), of greater width, and less orthogonal in the fixed state than PC2-positive cilia (Figure 5C). These data demonstrate localization of PC1 and PC2 to primary cilia in NK cells, consistent with the recent observations of Nauli et al (31). In contrast, PKD cyst cells with normal PC1 abundance lack ciliary PC1, and PC2 is absent from most cilia of cyst cells.

ADPKD cyst cells lack the flow-induced $[\text{Ca}^{2+}]_i$ increase observed in NK cells

Confluent, ciliated MDCK cells (42) and confluent primary mouse embryonic kidney cells (30) responded to low-level shear stress with increased intracellular $[\text{Ca}^{2+}]_i$. However, primary mouse embryonic kidney cells from $\text{Pkd1}(\text{del}^{34}/\text{del}^{34})$ mice lacked this response to fluid flow. Although flow-sensitive Ca^{2+} signaling has been measured in isolated perfused collecting ducts from rabbit (71) and mouse (21), flow-sensitive Ca^{2+} signaling had until recently (31) not been reported in human renal epithelial cells.

In NK cells, room temperature basal $[\text{Ca}^{2+}]_i$ without flow was 149 ± 6 nM ($n = 61$ coverslips). Abrupt application of 0.75 dyne/cm^2 shear stress (Figure 6A), increased NK cell $[\text{Ca}^{2+}]_i$ by 56 ± 10 nM above basal levels ($p < 0.01$) in 22 of the 65 coverslips examined (34%). Supplemental Figure 3 depicts the distribution of peak $[\text{Ca}^{2+}]_i$ elevations induced by this low shear stress (0.75 dyn/cm^2) among all individually imaged cells studied on several representative “responsive” coverslips. An increase in shear stress from 0 to 2.3 dyne/cm^2 similarly increased NK cell $[\text{Ca}^{2+}]_i$ by 48 ± 9 nM above basal levels ($P < 0.01$) in 13 of 40 coverslips examined (33%). Individual cells on “responsive” coverslips exhibited $[\text{Ca}^{2+}]_i$ elevations which peaked at ~ 10 sec and returned to baseline within 40 sec while flow was maintained (Figure 6A). NK cells on the other $\sim 65\%$ of coverslips studied showed no

detectable $[Ca^{2+}]_i$ elevation after onset of flow at 0.75 or 2.3 dyne/cm²). Flow-induced $[Ca^{2+}]_i$ increase in NK cells was abolished in nominally Ca^{2+} -free medium at both 0.75 and 2.3 dyn/cm² (Figure 6A, open symbols ; $p = 0.007$, Fisher Exact test applied to all studied coverslips, “responsive” and “nonresponsive”). At these levels of shear stress, neither the magnitude of the flow-induced Ca^{2+} elevation nor the proportion of “responsive” coverslips was increased at 37°C. In 2 of 10 coverslips of NK cells perfused at 1.6 dyne/cm² at 37°C, $[Ca^{2+}]_i$ increased by 53 and 60 nM, but the 8 other coverslips were “unresponsive” (not shown).

Shear stress values of 20 dyn/cm² or more have been estimated in rat collecting ducts during maximal diuresis (summarized in 3). As shown in Figure 6C (filled circles), increasing shear stress to 10 dyne/cm² increased $[Ca^{2+}]_i$ in NK cells by 207 ± 65 nM ($n = 13$ coverslips) after 20 sec of perfusion. After 60 sec the falling $[Ca^{2+}]_i$ level remained 63 ± 22 nM above the initial basal level. Abrupt increase in shear stress to the high level of 35 dyne/cm² increased $[Ca^{2+}]_i$ by 252 ± 43 nM ($n = 15$ coverslips) within 10 sec, and $[Ca^{2+}]_i$ remained 64 ± 12 nM above the basal level after 60 sec of perfusion. At both these higher shear levels, 80% of NK cell coverslips exhibited flow-induced $[Ca^{2+}]_i$ elevations, and 80% of individual cells on each “responsive” coverslip exhibited this $[Ca^{2+}]_i$ response. High shear stress-induced elevations of $[Ca^{2+}]_i$ were similarly abolished in the nominal absence of extracellular Ca^{2+} (Figure 6C, open squares; $p = 0.00002$, Fisher Exact test applied to all studied coverslips, “responsive” and “non-responsive”). The NK cell Ca^{2+}_i response to flow at 20°C exhibited a refractory period of >30 min following cessation of flow at all tested shear stress values. Supplemental Figure 3 depicts the distribution of peak $[Ca^{2+}]_i$ elevations induced by 35 dyne/cm² among all individual cells studied on several representative “responsive” coverslips.

Tests of flow-sensitivity at 37°C shortened both the time-to-peak values of $[Ca^{2+}]_i$ and the refractory period without increase in peak magnitude. 10 of 11 coverslips of NK cells exposed to 10 dyne/cm² shear stress at 37°C exhibited flow-stimulated increases in $[Ca^{2+}]_i$ to 107 ± 6 nM over baseline, peaking between 5 and 10 sec in 9 cells, and by 20 sec in 1 cell. Rechallenge of these 10 “responsive” coverslips after 30 min of stasis elicited identical elevations in $[Ca^{2+}]_i$ in response to the same shear stress. Similar magnitudes and kinetics of $[Ca^{2+}]_i$ increase were elicited in 8 additional coverslips by exposure to 24 dyne/cm² shear stress at 37°C. Thus, increasing temperature from 20°C to 37°C increased the rate of rise of the flow-induced $[Ca^{2+}]_i$ and reduced the refractory period of the Ca^{2+} signal to <30 min, but did not increase signal magnitude (not shown).

PKD cyst cells exhibited basal (static) $[Ca^{2+}]_i$ of 137 ± 4 nM ($n = 38$), not different from the basal $[Ca^{2+}]_i$ level in NK cells. Increasing shear stress to 0.75 or to 2.3 dyne/cm² produced minimal elevation of $[Ca^{2+}]_i$ in PKD cyst cells, with respective $\Delta[Ca^{2+}]_i$ after 10 sec perfusion of 6 ± 3 nM ($n = 17$) and 2 ± 2 nM ($n = 10$) (Figure 6B). These increments differed significantly from the peak flow-induced $[Ca^{2+}]_i$ elevations in NK cells subjected to the same shear stress ($p < 10^{-4}$, Fisher Exact test applied to all studied coverslips). At the higher shear stress levels of 10 and 35 dyne/cm², PKD cyst cell $[Ca^{2+}]_i$ increased 10 ± 12 nM ($n = 6$) or 12 ± 7 nM ($n = 17$), respectively, (Figure 6D), and again differed from flow-induced $[Ca^{2+}]_i$ elevations in NK cells at the same shear stress values ($p < 10^{-6}$, Fisher Exact test applied to all studied coverslips). Thus, $[Ca^{2+}]_i$ signaling in PKD cyst cells was unresponsive to flow across a wide range of shear stress in the conditions tested.

Flow-induced Ca^{2+} signal in normal cells requires both Ca^{2+} influx and Ca^{2+} release from ryanodine-sensitive intracellular Ca^{2+} stores

We characterized the inhibitor pharmacology of flow-induced Ca^{2+} signaling at the high shear stress of 35 dyne/cm². Praetorius and Spring (41) showed that bending of the MDCK

cell primary cilium with a micropipette led to $[Ca^{2+}]_i$ elevation reflecting both Ca^{2+} entry and release from inositol-1,4,5-triphosphate (IP_3)-sensitive stores. However, Nauli et al.(30) found that flow-induced, cilium-dependent elevation of $[Ca^{2+}]_i$ in embryonic mouse collecting duct epithelial cells was insensitive to inhibitors of phospholipase C and IP_3 receptors.

In NK cells, the phospholipase C inhibitor U73122 (10 μ M) was without effect on flow-induced elevation of $[Ca^{2+}]_i$ (Figure 7A), with peak flow-induced $[Ca^{2+}]_i$ increase of 132 ± 20 nM ($n = 5$) in U73122-treated cells and 146 ± 22 nM ($n = 6$) in untreated cells. In contrast, 30 μ M ryanodine nearly abolished the flow response, supporting a role for ryanodine receptor-regulated Ca^{2+} stores (Figure 7C) similar to that proposed for embryonic mouse collecting duct epithelial cells (30). The flow-induced elevation of $[Ca^{2+}]_i$ (224 ± 34 nM, $n=5$), was also inhibited nearly completely (to 12 ± 8 nM, $n=8$) by 20 μ M 2-aminophenylborate (2-APB, Figure 7B). Since 2-APB inhibits not only IP_3 receptors, but also store-operated Ca^{2+} entry channels, we examined additional inhibitors of Ca^{2+} entry. The NK cell peak response to initiation of flow (236 ± 18 nM, $n = 4$) was reduced to 28 ± 24 nM ($n = 9$) by 10 min preincubation with 3 μ M GsMTx-IV (Figure 7D), a potent blocker of mechanosensitive channels isolated from tarantula venom (56). In contrast, the nonspecific cation channel inhibitor SKF-96365 (50 μ M before and during flow) attenuated and delayed both activation and inactivation of the flow-induced Ca^{2+} signal (Figure 7E). After a 30 sec lagtime following onset of flow, an increase in $[Ca^{2+}]_i$ in a few cells gradually propagated throughout the NK population, increasing after ~ 150 sec to a modest peak of 67 ± 14 nM by ~ 150 sec, with a greatly prolonged decay time ($n = 6$). In contrast, the rapid increase in $[Ca^{2+}]_i$ in untreated NK cells (186 ± 25 nM, $n = 4$) was almost completely reversed by the time Ca^{2+} elevation was evident in cells exposed to SKF-96365.

Figure 8 shows that PKD cyst cells sustained intact or partial activities of other Ca^{2+} signaling pathways, despite their lack of flow-responsive Ca^{2+} signaling. The peak response to 10 μ M thapsigargin was smaller in PKD cyst cells (148 ± 29 nM) than in NK cells (648 ± 245 nM, $n=8$, $p<0.01$) (Figure 8A), suggesting that releasable ER Ca^{2+} stores of PKD cyst cells are reduced. "Store-operated" or "capacitative Ca^{2+} entry (CCE) was also evaluated in the two cell types (Figure 8B). Cells were pretreated 10 min with 10 μ M thapsigargin in nominally Ca^{2+} -free bath to deplete intracellular Ca^{2+} stores. Superfusion of this Ca^{2+} -free bath at 0.75 dyne/cm² for 2 additional min (during which no $[Ca^{2+}]_i$ was unchanged; see Figure 6A) was followed at $t=0$ by addition of 10 mM $CaCl_2$ to the superfusate (Figure 8B). The peak CCE response of PKD cyst cells was reduced (64 ± 9 nM, $n=11$) compared to the larger peak in NK cells (180 ± 23 nM, $n=11$; $p < 0.01$). The peak $[Ca^{2+}]_i$ values in PKD cyst cells exposed (under "no flow conditions") to 1 μ M angiotensin II (Figure 8C) and to 1 μ M arginine vasopressin (Figure 8D) were equivalent in magnitude to those of NK cells, but exhibited slightly slower activation and substantially slower decay rates. Thus the nearly complete abrogation of flow-sensitivity in PKD cyst cells did not reflect a global loss of Ca^{2+} signaling responses.

CD16.7-PKD1(115-226)localizes to cilia of both NK and PKD cyst cells

Heterologous expression of the tripartite fusion protein CD16.7-PKD1(115–226)increases endogenous Ca^{2+} -permeable cation channel activity in *Xenopus* oocytes (64) and in EcR-293 cells (65). We examined the effects of CD16.7-PKD1(115-226) expression on flow-induced Ca^{2+} signaling in NK and PKD cyst cells. Five days after transfection, unfixed cells were stained for cell surface expression of the CD16 epitope, then fixed as described by Vandorpe et al. (64,65). After microwave denaturation of bound anti-CD16 mAb 3G8, coverslips were stained for N-acetylated α -tubulin and studied by confocal immunofluorescence microscopy. CD16.7PKD1-(115-226)was detected throughout the cilium in all 9 transfected NK cells examined (an example is shown in Figure 9A-F), and

was similarly present throughout the cilium of all 6 transfected PKD cyst cells examined (Figure 9M-R). We tested the dependence of this localization on the integrity of the PC1 C-terminal tail coiled-coil domain, which can mediate interaction with PC2 (62,45). The PC1 coiled-coil domain mutant CD16.7PKD1-(115-226)L152P (65) was similarly expressed throughout the cilium in 6 of 6 transfected NK cells examined (Figure 9G-L) and in 16 of 16 transfected PKD cyst cells examined (Figure 9S-X). Thus, the terminal 112 amino acids of the PC1 C-terminal cytoplasmic tail can target a transmembrane fusion protein to the cilium by a mechanism which does not require integrity of the fragment's coiled-coil domain.

Expression of CD16.7-PKD1(115-226) in NK cells shortens the duration of flow-induced $[Ca^{2+}]_i$ increase

Five to six days after cotransfection of NK or PKD cyst cells with CD16.7-PKD1(115-226) and dsRed cDNAs, the consequences to flow-induced Ca^{2+} signaling were assessed. Transfected and nontransfected cells on single coverslips were identified by the respective presence or absence of dsRed fluorescence (Figure 10E, upper left panel) which colocalized with cell surface CD16 expression (not shown). Expression of DsRed alone in NK cells changed neither resting $[Ca^{2+}]_i$ (170 ± 6 nM in 21 single transfected cells vs. 186 ± 7 nM in 141 untransfected cells), peak flow-induced $[Ca^{2+}]_i$ increase (212 ± 36 nM in 21 DsRed cells vs. 140 ± 12 nM in 141 untransfected cells), nor the rate of post-peak decline in $[Ca^{2+}]_i$ (Figure 10C).

Resting $[Ca^{2+}]_i$ in CD16.7-PKD1(115-226)-transfected (164 ± 9 nM, $n = 41$) and untransfected NK cells (167 ± 3 nM, $n = 219$) was indistinguishable, as in EcR-293 cells expressing this construct (65). The flow-induced peak $[Ca^{2+}]_i$ increase in CD16.7-PKD1(115-226) expressing NK cells (124 ± 39 nM) also did not differ from that of untransfected cells (187 ± 21 nM; Figure 10A). However, 25 sec after initiation of flow, $[Ca^{2+}]_i$ in NK cells expressing CD16.7-PKD1(115-226) had fallen to lower values (66 ± 13 nM) than in untransfected cells (116 ± 11 nM, $p < 0.05$). In contrast, expression in NK cells of the coiled-coil domain mutant CD16.7-PKD1(115-226)L152P changed neither the peak flow-induced $[Ca^{2+}]_i$ increase (237 ± 76 nM vs. 179 ± 17 nM in untransfected cells) nor its rate of post-peak decrease (Figure 10B). Expression of CD16.7-PKD1(115-226) failed to rescue flow-sensitive $[Ca^{2+}]_i$ signaling in PKD cyst cells (Figure 10D). Thus, heterologous expression of the terminal 112 amino acids of the PC1 C-terminal cytoplasmic tail accelerates post-peak decay of flow-induced elevation in $[Ca^{2+}]_i$ in NK cells by a mechanism that requires integrity of the PC1 coiled-coil domain.

Discussion

In the present study we have shown that confluent primary cultures of normal human renal cortical tubular (NK) cells elevate $[Ca^{2+}]_i$ in response to fluid flow. In contrast, confluent primary cultures of human ADPKD cyst epithelial (PKD) cells harboring a novel heterozygous in-frame single codon deletion in the PC1 gene completely lack this response. The defect is not generalized, since PKD cyst cells retain near-normal Ca^{2+} signaling induced by angiotensin II and by vasopressin, as well as reduced capacitative Ca^{2+} entry and a reduced thapsigargin response. The flow-induced $[Ca^{2+}]_i$ elevation in NK cells requires extracellular Ca^{2+} and release from ryanodine-sensitive intracellular stores. The NK and PKD cyst cells studied here express equivalent whole cell levels of PC1 and PC2, and both develop monocilia upon achievement of confluency. However, cilia of confluent PKD cyst cells lack detectable PC1, whereas PC1 and PC2 are both present in cilia of confluent NK cells. PC2 is expressed in cilia of only 30% of confluent PKD cyst cells. The C-terminal PC1 fusion protein, CD16.7-PKD1(115-226), localizes to the cilium of both NK and PKD cyst cells by a mechanism independent of the PC1 coiled-coil domain. However, accelerated decay of the flow-induced Ca^{2+} signal in NK cells associated with overexpression of

CD16.7-PKD1(115-226) requires integrity of that coiled-coil domain. These results both confirm and extend the recent study by Nauli et al. (31) published after completion of these experiments.

A novel in-frame single codon deletion in PC1 is associated with loss of ciliary PC1 and (in some cells) of ciliary PC2 without reduction in total PC1 or PC2

The novel heterozygous *PKD1* gene mutation Δ L2433 reported here is likely a germline mutation, since the epithelial cells from which genomic DNA was isolated were harvested from multiple renal cysts of a single ADPKD donor. The failure to detect additional mutation(s) in the *PKD1* gene is consistent with the second hit hypothesis of cystogenesis (46). The deleted residue L2433 resides in the REJ domain of PC1. Overexpression in MDCK cells of engineered PC1 missense mutants in the REJ domain has been associated with loss of AP-1 activation and of tubulogenesis induced by wildtype PC1 overexpression (44).

The heterozygous PC1 Δ L2433 mutant allele is associated with normal cellular levels of PC1 and PC2 (Figure 2). Expression of PC1 and PC2 polypeptides in the cell body of all PKD cyst cells as noted in the present study resembles human ADPKD kidney and *Pkd1*(+/-) mouse kidney (66,72), but differs from the mosaic (all-or-none) PC2 expression observed in renal cysts of the *cy/+* rat, and from the mosaic or complete loss of PC2 expression in the *cy/cy* mouse (34) and in the *Pkd2*(+/-) and *Pkd1*(+/-)/*Pkd2*(+/-) mice (72). These findings suggest that the PC1 Δ L2433 mutant polypeptide may be present in PKD cyst cells.

PC1 antigen has been detected at tight junctions, adherens junctions, desmosomes, focal adhesions, intracellular cytoplasmic vesicles (summarized in 55, 49), and nuclei (24), as well as in cilia. These many PC1 localizations may reflect examination of varied cell types and differentiation states, as well as post-translational processing or covalent modification of PC1, and (at least in rodents) alternative splicing of PC1 transcripts. PC2 is predominantly localized in the endoplasmic reticulum (2). Presence of the heterozygous PC1 Δ L2433 mutation is associated with complete loss of ciliary PC1 expression, and by loss of ciliary PC2 expression in 70% of cells (Figures 4 and 5). The continued ciliary expression of PC2 in 30% of cells with the novel PC1 Δ L2433 mutation contrasts with the reported absence of ciliary PC2 in all SV40 large T-transformed, DBA-positive human 9-12 cyst cells and in (genetically uncharacterized) human primary cyst epithelial cells (31). Ciliary localization of PC1 and PC2 was not described in a study of tsSV40 large T-immortalized human ADPKD cells with the E1537X germline mutation, which express normal PC2 levels with very low levels of PC1 (55).

Ciliary length was slightly shorter in PKD cyst cells than in NK cells of equivalent confluency, and cyst cilia lacking PC2 tended not to be orthogonal in orientation. This ciliary phenotype of human cyst epithelial cells in primary culture is intermediate in severity between the severely shortened, dysmorphic cilia of cells cultured from the *orpk* mouse with a hypomorphic polaris mutation (39), and the normal ciliary length in cells from the *pkd1*^{del34/del34} mouse (30).

The flow-sensitive Ca²⁺ signaling response of NK cells is absent from PKD cyst cells which retain other Ca²⁺_i responses

Flow-sensitive Ca²⁺-signaling is a property of isolated, perfused rabbit and mouse collecting ducts (22,21), and has been reported also in isolated, perfused mouse medullary thick ascending limb (19). In the *orpk* mouse model of recessive polycystic kidney disease, flow-evoked Ca²⁺ signaling in CCD remained normal during the first postnatal week, but was

slightly reduced compared to wildtype CCD at age two weeks (21), a difference replicated in CCD cells grown in primary culture (54).

Our observation of the complete absence of flow-sensitive Ca^{2+} signaling in human primary cultures of PKD cyst cells resembles those previously reported in immortalized CCD cells from the *pkd1^{del34/del34}* mouse and in immortalized and primary human cyst cells (30,31). Our results extend this earlier work in cyst cells lacking PC1 polypeptide by showing that the lack of flow-induced Ca^{2+} signaling in human cyst cells occurs also in the presence of normal total cell polypeptide levels of PC1 and PC2, including the 30% of cyst cells which retain PC2 expression in the cilium. The results further show that human cyst cells fail to elevate $[\text{Ca}^{2+}]_i$ in response to flow at all tested levels of shear stress, and at both 20°C and 37°C. However, tests of the centrality of defective ciliary flow sensing to early cystogenesis or cyst growth in ADPKD will require further study.

The current results also present an initial pharmacological characterization of other Ca^{2+} signaling responses of human PKD cyst cells, indicating that the absence of flow-induced elevation in $[\text{Ca}^{2+}]_i$ in PKD cyst cells does not represent a global Ca^{2+} signaling defect. Thus, thapsigargin-induced Ca^{2+} release, and capacitative Ca^{2+} entry (CCE) are both preserved in PKD cyst cells, although decreased in magnitude compared to NK cells (Figure 8). These diminished signals resemble those observed in aortic vascular smooth muscle cells of *pkd2(+/-)* mice (47). Unlike the reduced thapsigargin and CCE responses, PKD cyst cell Ca^{2+} elevations in response to AII and to AVP are of normal magnitude, although slightly delayed in onset. The components of the local renin-angiotensin system are overexpressed in human ADPKD (23), with possible consequences to flow-induced regulation of apical exocytic insertion of AT1a receptors in proximal tubular cells (16). The slower rate of Ca^{2+} signal decay in PKD cyst cells treated with angiotensin II or vasopressin recalls the prolonged Ca^{2+} entry suggested to occur in ATP-stimulated M1 CCD cells overexpressing a PC1 C-terminal cytoplasmic tail fusion protein (69). A slowed Ca^{2+} signal decay may represent the converse of the accelerated Ca^{2+} signal decay following ATP stimulation of MDCK cells overexpressing full-length PC1, attributed to enhanced ER Ca^{2+} reuptake with inhibition of CCE (13). CCE inhibition by either overexpression or loss of PC1 function in PKD cyst cells parallels the ability of both underexpression and overexpression of PC1 to cause polycystic kidney disease in mice (59).

Range of flow sensitivity of NK cell flow-induced Ca^{2+} signaling

Resting $[\text{Ca}^{2+}]_i$ measured in the absence of flow at room temperature and at 37°C was indistinguishable in confluent, serum-replete NK and PKD cells, and was equivalent to that reported in confluent embryonic CCD cells from wildtype and *pkd1^{del34/del34}* mice and in human normal and cyst cells by Nauli et al (30,31). Both sets of these resting $[\text{Ca}^{2+}]_i$ values substantially exceed those reported by Yamaguchi et al. (73) in subconfluent primary human epithelial cells subjected to 48 hrs of progressively increasing degrees of serum starvation. In such low serum conditions, favorable for testing effects of growth regulators, human cyst cell $[\text{Ca}^{2+}]_i$ at 57 nM was significantly lower than the 77 nM measured in normal human cells (73).

The current results with human primary NK cells differ somewhat in flow-sensitivity from those reported for immortalized mouse CCD cells, or for immortalized human cells with lectin-staining properties consistent with collecting duct origin, and for primary human cortical epithelial cells of unspecified segment of origin. Mouse CCD cells elevated $[\text{Ca}^{2+}]_i$ in response to shear stress of 0.75 dyne/cm², but failed to respond to the higher shear stress of 15 dyne/cm² (30). Normal human kidney RCTE cells responded optimally (in ~60% of tested coverslips) to shear stress of 1.2 dyne/cm² (31), results which we have reproduced (Xu and Alper, unpublished results). In contrast, NK cells from three individuals in the

current study responded to shear stresses 0.75 or to 2.3 dyne/cm² with modest elevations in [Ca²⁺]_i in only 30% of coverslips. However at 10 dyne/cm² or at higher values, the robust [Ca²⁺]_i responses observed in 80% of tested coverslips were of magnitude comparable to those reported by Nauli et al (31). The NK cell response to graded further elevation of shear stress with further increased elevation of [Ca²⁺]_i, rather than by diminution or loss of responsiveness corresponds to the reported MDCK cell response to flow (41). These responses of NK cells were not altered in magnitude or shear sensitivity by increasing temperature from 20° to 37°C. However, the higher temperature accelerated the time-to-peak [Ca²⁺]_i, and shortened the refractory period.

Thus, NK cells in the current study were weakly responsive to low shear stress, and exhibited more robust responses to shear values associated with diuresis (3,68) or with shear stress values close to those present in the central axis of the initial S1 proximal tubule. These differences in flow sensitivity may reflect differences in ciliary length (8-12 μm vs. 4 μm in the current study), in conditions of initial tubule cell outgrowth or of subsequent primary cell culture, and likely reflect heterogeneity of the cultured population, and/or in nephron segment of origin. 90% of NK cells in the current study were Lotus tetragonolobus agglutinin (LTA)-positive, whereas only 10% of NK cells expressed dolichos biflorus agglutinin (DBA). Although all NK cells expressed E-cadherin, strong staining characterized only 10%. Thus, proximal tubular markers predominated among the NK cells used in the present study.

Marker studies and cyst fluid composition have suggested possible proximal tubular origin of up to 30-44% of closed cysts in human ADPKD (reviewed in 60, 70). Moreover, late-onset renal cysts in the *Pkd1*^{+/-} mouse expressing LTA were twice as frequently observed as cysts expressing DBA, although most cysts expressed neither lectin marker (25). The laminar shear stress predicted for the central axis of a human initial S1 proximal tubule of 22 μm diameter is predicted to be 7.6 dyn/cm² for a 125 ml/min GFR (with the oversimplifying assumptions of an inelastic tubule without brush border). For a vertical cilium of length 4.2 μm, the experienced shear stress under parabolic flow in this 22 μm diameter tubule would be 4.7 dyne/cm² at the ciliary tip. These values might plausibly increase 2-fold in the oligonephronia proposed in essential hypertension (15), or after uninephrectomy. Thus, the shear stresses of 7-10 dyne/cm² at which our NK cells exhibit a maximal peak elevation of [Ca²⁺]_i are not far outside reasonable physiological estimates of shear stress experienced by epithelial cells of the human initial S1 proximal tubule. Nephron segment of origin must be considered in interpretation of cultured cell sensitivity to imposed flow.

Pharmacological properties of flow-induced elevation of [Ca²⁺]_i in NK cells

The pharmacology of flow-induced Ca²⁺ signaling in human NK cells resembled mouse CCD and MDCK cells in some ways and differed in others. The flow response in all these cell types required both extracellular Ca²⁺ and Ca²⁺-induced Ca²⁺ release from internal stores. Although IP₃-sensitive Ca²⁺ stores were implicated in MDCK cells (13) and in the perfused rabbit CCD (22), ryanodine-sensitive stores seemed to predominate in the flow response in human NK cells and in mouse embryonic CCD cells. However, RyR inhibitors can reduce IP₃-mediated Ca²⁺ signaling in colonic smooth muscle cells (27). The reported ability of PC2 to modulate IP₃R function by direct interaction (20) appeared not to contribute to NK cell flow sensitivity. Whereas 2-APB (10 μM, 45 min) was without effect in mouse embryonic kidney cells, the NK cell flow response in NK cells was completely inhibited (20 μM, 30 min). However, interpretation of this inhibition is complicated by the drug's dual inhibitory effects on IP₃R and TRP channels, and by its ability to activate TRPV1-3 channels (6). The novel NK cell response to SKF96365, with reduced peak [Ca²⁺]_i and slowed kinetics of activation and decay, may represent weak agonist activity for Ca²⁺ store release (7) rather than atypical inhibition of Ca²⁺ entry.

Flow-activated Ca^{2+} entry clearly requires integrity of the PC1/PC2 complex, but the identity of the Ca^{2+} entry pathway in NK cells remains unknown. The density and diversity of ion channels in monocilia may be very high (48). Complete block of flow-induced Ca^{2+} entry in NK cells by 3 μM GsMTx-IV, an inhibitor of stretch-activated cation channels, may provide a path towards channel identification, but a nonspecific lipid bilayer intercalation effect remains possible (56). TRPC1, previously shown to bind to PC2 in vitro, has been recently implicated as a mechanosensitive channel in *Xenopus* oocytes (28). TRPV4 colocalizes with PC2 (58) and may bind and modulate its activity (17). Since 30% of PKD cyst cells retain normal ciliary localization of PC2, the presence of PC2 in cilia apparently does not suffice for normal flow-induced Ca^{2+} signaling in the absence of ciliary PC1. However, primary cultures of cells isolated from a mature cyst of end-stage ADPKD kidney may be de-differentiated compared to NK cells in ways not specifically related to ADPKD.

The pharmacological properties of flow-induced Ca^{2+} signaling in normal human kidney tubular epithelial cells in primary culture were not reported by Nauli et al (31). Nevertheless, the similarities between our NK cells and the wildtype mouse CCD cells studied by Nauli et al. (30) include a requirement for extracellular Ca^{2+} entry, sensitivity to ryanodine, the kinetics of Ca^{2+} signal onset and decline, and the time course for recovery from the post-flow refractory period at 37°C. Thus, cells expressing markers suggesting different nephron segments of origin nonetheless share some properties of flow-induced elevation of $[\text{Ca}^{2+}]_i$.

Overexpression of the PC1 C-terminal tail in NK cells alters the decay kinetics of the flow-induced Ca^{2+} signal

CD16.7-PKD1(115-226) overexpressed in NK cells accelerated decay kinetics of the flow-induced Ca^{2+} signal, in contrast to prolongation of the ATP-induced Ca^{2+} signal in M1 cells by a similar fusion protein (69). A disease mutation disrupting the coiled-coil domain and blocking cation current activation in oocytes and EcR-293 cells prevented the accelerated decay of the flow-induced Ca^{2+} signal in NK cells. This result suggests that CD16.7-PKD1(115-226) interacts with an endogenous protein to modulate flow-induced Ca^{2+} signaling. The inhibitory effect of CD16.7-PKD1(115-226) might therefore reflect competition with mechanosensitive full-length PC1 for binding to or regulation of PC2 channel activity or signaling, as proposed by Low et al. (24) to explain cystic dilation of the zebrafish pronephric duct. However, the mechanism of mechanosensation by ciliary apical PC1/PC2 complex exposed to fluid flow may differ from that by basolateral PC1 or PC1/PC2 complex exposed to a neighboring cell or to matrix. Moreover, Ca^{2+} entry pathways induced by flow in NK cells may differ from those induced by ATP in M1 cells.

A coiled-coil domain-independent ciliary targeting sequence in the PC1 C-terminal tail

The C-terminal tail of PC1 suffices to confer ciliary targeting on a heterologous transmembrane fusion protein (Figure 9). The ciliary localization of transiently transfected CD16.7-PKD1(115-226) did not require integrity of the coiled-coil domain in the C-terminal PC1 cytoplasmic tail which interacts with the C-terminal cytoplasmic tail coiled-coil domain of PC2. Nor was the PC1 G-protein binding motif required (as it is absent from the fusion protein construct). Thus, a ciliary targeting motif in the second half of the PC1 C-terminal cytoplasmic tail is functional without the presence of adjacent sequences known to bind PC2 and heterotrimeric G proteins. Our results also show that ciliary localization of PC2 does not absolutely require the presence of immunocytochemically detectable levels of ciliary PC1, consistent with recent findings on ciliary targeting of PC2 (10).

Conclusion

Flow sensing has long been thought to contribute to proximal tubular perfusion-absorption balance, to tubuloglomerular feedback (9), to CCD K^+ secretion (71) and Na^+ reabsorption

(52), and to NO release by TAL (38) and IMCD (3). The cilium, with its apparent concentration of receptors and signaling molecules, is an attractive candidate to integrate these signals controlling tubular epithelial cell differentiation and function, perhaps through $[Ca^{2+}]_i$ -mediated regulation of B-raf (73), regulation of mTOR (53), or other pathways. However, the role of ciliary flow sensing in prevention of cystogenesis in the normal state remains in question, as evidenced by *orpk* mice in which *polaris* transgene rescue failed to prevent cystic disease despite normalization of ciliary structure and left-right asymmetry (1). Thus, comparative studies of flow sensitivity in human renal cells and PKD cyst epithelial cells should play an important and continuing role in defining the place of defective ciliary mechanosensation in the pathogenesis of dysregulated growth and secretion in human ADPKD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ADPKD	autosomal dominant polycystic kidney disease
PC1	polycystin-1
PC2	polycystin-2
NK	normal kidney
PKD	polycystic kidney disease
REJ domain	receptor for egg jelly domain
GSMTx-IV	Grammastola spatulata mechanotoxin-IV
CCE	capacitative calcium entry, or store depletion-induced calcium entry
2-APB	2-aminophenylborate

Figure 1. Novel heterozygous missense mutation in the human *PKD1* gene

A. Aligned nucleotide and predicted polypeptide sequences from exon 18 of the wildtype and mutant *PKD1* alleles in primary cyst epithelial cells. The mutant allele has a three nucleotide deletion producing an in-frame deletion of a single codon, and encodes the polycystin 1 mutant polypeptide, PC1 Δ L2433. B. Aligned genomic DNA sequence traces from a representative wildtype genome (upper) and from the heterozygous mutant cyst cell genome (lower). Underscored automated sequence call starts at the deletion site. C. Schematic of the PC1 polypeptide locating the Leu2433 deletion within the REJ domain.

Figure 2. ADPKD cyst cells express normal or elevated levels of polycystin-1 (PC1) and polycystin-2 (PC2)

(A) Immunoblot of normal kidney cell (NK) lysates with three anti-PC1 antibodies 7e12, NM005 and LRR. Representative of 6 similar experiments. (B) Immunoblot of NK and ADPKD (PKD) cell lysates containing equivalent amounts of protein were probed with anti-PC1 antibody NM005. One of 2 similar experiments. (C) PC1 polypeptide abundance in HPAC cells is reduced by transfection of specific PC1 siRNA, whereas GAPDH is unaffected. Transfection of control siRNA had no effect on either PC1 or GAPDH bands (not shown). Representative of 6 similar experiments. (D) PC2 immunoblot of cell lysates separated on 7.5% polyacrylamide gels, and probed with anti-PC2 antibody. PC2 was detected in cell lysates prepared in RIPA buffer (lanes 1 and 3) or in SDS buffer (lanes 2 and 4). The same blot was reprobed with antibody to β -actin as a loading control. Representative of three similar experiments.

Figure 3.
Identification of primary cilia in human renal epithelial cells in culture by localization of N-acetylated α -tubulin in NK (left) and PKD cyst cells (right). The single focal planes were chosen for x-z plane reconstruction to reveal the monocilia (arrows). Scale bar: 10 μ m.

Figure 4. Colocalization of PC1 with N-acetylated α -tubulin is altered in PKD cells

Confocal x-z plane reconstructions show immunofluorescence colocalization of PC1 with N-acetylated α -tubulin in NK cells (A) but not in PKD cyst cells (B). PC1 was detected in NK cell cilia (white arrows) by antibody NM005. PC1 was absent from the PKD cyst cell cilium, but present in the cell body. Results were the same with NM002 detection of PC1 (not shown). Scale bar: 10 μ m.

Figure 5. Colocalization of PC2 and N-acetylated α -tubulin is altered in PKD cyst cells
 Confocal x-z plane reconstructions showing immunofluorescence colocalization of PC2 with N-acetylated α -tubulin in NK cells (A) and in PKD cyst cells (B, C). PC2 co-localizes with acetylated α -tubulin in the cilia (white arrows) of NK cells (a) but in only 30% of PKD cyst cells (B). In the other 70% of PKD cyst cells, PC2 was not detected in cilia (C). Scale bar: 10 μ m.

Figure 6. PKD cyst cells do not elevate $[Ca^{2+}]_i$ in response to flow

Graded increases in shear stress induce graded, transient increases in $[Ca^{2+}]_i$ in NK cells (A,C) but not in PKD cyst cells (B,D), as measured by Fura-2 fluorescence excitation ratio. Fura-2-loaded NK cells (A) or PKD cyst cells (B) were imaged during initiation of superfusion with low calculated shear stresses of 0.75 or 2.3 dyn/cm², in the presence (filled symbols) or absence of added perfusate Ca^{2+} (open symbols). Fura-2-loaded NK cells (C) or PKD cyst cells (D) were imaged during initiation of flow with high calculated shear stresses of 10 or 35 dyn/cm², in the presence (filled symbols) or absence of added perfusate Ca^{2+} (open symbols). Parentheses indicate number of “responsive” NK coverslips in the presence of perfusate Ca^{2+} (30% of total studied at low shear in panel A, 80% of total studied at high shear in panel C), the total number of NK coverslips studied in the absence of Ca^{2+} , and the total number of PKD coverslips studied in the presence or absence of Ca^{2+} . F. Time sequence of fluorescence ratio images of a representative NK coverslip exposed to calculated shear stress of 10 dyn/cm² as in panel C. Photographed with 20× objective. Pseudocolor scale at left of each panel.

Figure 7. Pharmacology of flow-induced Ca^{2+} signaling in NK cells

A. Fura 2-loaded cells were preincubated for 30 min in the absence (control, open circles) or presence of 10 μM U73122, then in the continued absence or presence of drug were subjected to flow at a calculated shear stress of 35 dyn/cm^2 . B-E. Flow-sensitive Ca^{2+} signaling was similarly tested in the absence or presence of 20 μM 2-aminophenylborate (2-APB) (B), 30 μM ryanodine (C), or 50 μM SKF96365 (E). Cells were also preincubated in the absence (control) or presence of 3 μM Grammastola spatulata toxin IV (GsMTx) for 30 min, then subjected to fluid shear stress in the absence of the toxin (D). Numbers in parentheses indicate number of “responsive coverslips” (80% of total studied) in panels A and E, and for control coverslips (open circles) in panels B-D. Numbers in parentheses indicate total number of drug-treated coverslips studied (filled circles) in panels B-D.

Figure 8. Comparison of other Ca^{2+} signaling pathways in NK and PKD cyst cells

A. Fura 2-loaded NK (open circles) and PKD cyst cells (filled circles) were exposed at $t=0$ to $10 \mu\text{M}$ thapsigargin (TG), gently added in “no-flow conditions” from a 100-fold concentrated stock. Vehicle alone was without effect (not shown). B. After a 10 min preincubation with $10 \mu\text{M}$ thapsigargin in the nominal absence of Ca^{2+} , cells were exposed to 10 mM extracellular Ca^{2+} to elicit capacitative Ca^{2+} entry (CCE). C,D. Cells were exposed at $t=0$ to $10 \mu\text{M}$ angiotensin II (C) or $10 \mu\text{M}$ arginine vasopressin (AVP) (D). Numbers in parentheses are total number of coverslips studied.

Figure 9. A C-terminal cytoplasmic tail fragment of PC1 localizes to cilia

Transiently transfected CD16.7-PKD1(115-226) is expressed in monocilia of NK cells (A-F) and in monocilia of transiently transfected PKD cyst cells (M-R)). Transiently transfected CD16.7-PKD1(115-226)L152P is similarly expressed in monocilia of NK cells (G-L) and in monocilia of PKD cyst cells (S-X)). Unfixed cells (AD, GJ, MP) were stained for surface expression of CD16 with mAb 3G8 (red), then fixed, microwave-denatured as described in Methods, and stained for N-acetylated α -tubulin (green). Cells in panels S-X were fixed before staining with mAb 3G8. Large panels show confocal immunofluorescence x-y images of ciliary planes, and small panels immediately below show x-z images of the cells immediately above. Arrows mark cilia. Insets in panels A, G, and M show intraciliary x-y plane images of transfected cells (aligned with the higher x-y plane ciliary image of the same cell within each panel) and demonstrate generalized CD16 surface staining not restricted to cilia. Scale bars: 10 μ m in x-y images; x-z images are magnified in the z plane.

Figure 10. A C-terminal fragment of PC1 modulates flow induced calcium signaling
 Transient expression of CD16.7-PKD1(115-226), but not of the mutant CD16.7-PKD1(115-226)L152P, modulates the decay rate of the flow-induced increase in $[Ca^{2+}]_i$ elicited by imposition of 35 dyn/cm² shear stress. A, Time course of $[Ca^{2+}]_i$ response in CD16.7-PKD1(115-226)-transfected and untransfected NK cells on 8 coverslips. Transfected cells were identified by dsRed expression. B, Time course of $[Ca^{2+}]_i$ response in CD16.7-PKD1(115-226)L152P- transfected and untransfected NK cells on 5 coverslips. Transfected cells were identified by dsRed expression. C, Time course of $[Ca^{2+}]_i$ response in dsRed-transfected and untransfected NK cells on 3 coverslips. D, $[Ca^{2+}]_i$ response of CD16.7-PKD1(115-226)-transfected and untransfected PKD cyst cells on 7 coverslips. Numbers in parentheses are total numbers of single cells evaluated. E, Fura 2 fluorescence ratio image time course of a representative coverslip of NK cells cotransfected with dsRed and CD16.7-PKD1(115-226). dsRed-expressing cells are outlined in white. Photographed with 20× objective. Pseudocolor scale at left of each panel.