Loss of primary cilia results in deregulated and unabated apical calcium entry in ARPKD collecting duct cells

Brian J. Siroky, William B. Ferguson, Amanda L. Fuson, Yi Xie, Attila Fintha, Peter Komlosi, Bradley K. Yoder, Erik M. Schwiebert, Lisa M. Guay-Woodford, and P. Darwin Bell

Departments of 1Physiology, 2Cell Biology, 3Medicine, and 4Genetics and Translational Medicine, University of Alabama at Birmingham, Birmingham, Alabama

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Siroky, Brian J., William B. Ferguson, Amanda L. Fuson, Yi Xie, Attila Fintha, Peter Komlosi, Bradley K. Yoder, Erik M. Schwiebert, Lisa M. Guay-Woodford, and P. Darwin Bell. Loss of primary cilia results in deregulated and unabated apical calcium entry in ARPKD collecting duct cells. Am J Physiol Renal Physiol 290: F1320–F1328, 2006.—Recent genetic analysis has identified a pivotal role of primary cilia in the pathogenesis of polycystic kidney disease (PKD). However, little is known regarding how cilia loss/dysfunction contributes to cyst development. In epithelial cells, changes in apical fluid flow induce cilia-mediated Ca2+ entry via polycystin-2 (PC2), a cation channel. The Oak Ridge Polycystic Kidney (orpk) mouse contains a mutated Tg737 gene that disrupts expression of polaris, a protein required for ciliogenesis. These studies examine the effect of cilia malformation on Ca2+ entry in orpk cilia(−) collecting duct principal cells, and in orpk cells in which wild-type Tg737 was reintroduced, orpk cilia(+). [Ca2+]i was measured in confluent cell monolayers using fluorescence microscopy. Intrinsic apical Ca2+ entry was measured by Mn2+ quenching and Ca2+ depletion/readout under flow conditions below the threshold for stimulation. We found that unstimulated apical Ca2+ entry was markedly increased in cilia(−) cells and was sensitive to Gd3+, an inhibitor of PC2. Electrophysiological measurements demonstrate increased abundance of an apical channel, consistent with PC2, in cilia(−) cells. Immunofluorescence studies revealed that PC2, normally expressed on and at the base of cilia in orpk cilia(+) cells, was observed throughout the apical membrane in cilia(−) cells. Furthermore, cilia(−) cells displayed elevated subapical Ca2+ levels measured with the near-membrane Ca2+ indicator FFP-18. We propose that cilia exert a tonic regulatory influence on apical Ca2+ entry, and absence of cilia results in loss of spatial organization of PC2, causing unregulated Ca2+ entry and elevations in subapical [Ca2+], a factor which may contribute to cyst formation.

Oak Ridge Polycystic Kidney; autosomal recessive polycystic kidney disease; polycystin-2; Ca2+ permeability

WHILE GREAT STRIDES HAVE BEEN made in understanding the genetics of polycystic kidney disease (PKD), the cellular and molecular processes that underlie disease progression and cyst formation are not well understood (11). Recent work has focused on the role of apical primary cilia in the pathogenesis of the autosomal dominant (ADPKD) and autosomal recessive (ARPKD) forms of PKD (10, 16, 37). This has been centered in the seminal observation that the principal proteins implicated in human PKD, polycystin-1 (PC1), polycystin-2 (PC2), and fibrocystin/polyductin, localize to the apical primary cilium of ductal epithelial cells (9, 27, 33, 46, 50). Thus loss of proper cilia structure, function, and/or defects in cilia-associated proteins may be a primary causative factor in tubular expansion and fluid-filled cyst development in PKD.

Primary cilia are long, narrow projections that originate from basal bodies at the apical membrane of polarized epithelial cells and are nearly ubiquitously expressed (37). These nonmotile cilia appear to serve as environmental sensors that both detect and transmit information and are thought to regulate multiple and diverse cellular processes (7, 32, 34, 37). In epithelia, deformation of apical cilia in response to changes in luminal fluid flow causes transient increases in [Ca2+]i (25, 36). Presumably, this response is triggered by Ca2+ entry through PC2, a nonselective cation channel (12). In addition, there is a loss of flow-dependent elevations in [Ca2+]i with removal of apical cilia in renal epithelial cells (31, 35). This has led to the suggestion that absence of functioning cilia leads to diminution of apical membrane Ca2+ entry in the context of PKD (39). The present studies were performed to directly determine whether loss of apical primary cilia, in a mouse model of PKD, causes alterations in apical membrane Ca2+ permeability and [Ca2+]i homeostasis.

The Oak Ridge Polycystic Kidney (orpk) mouse has a mutation in the Tg737 gene that disrupts expression of polaris, an intraflagellar transport protein found to be necessary for ciliogenesis (42, 49). This defect causes complete loss or severe stunting of the length of apical primary cilia. These mice closely resemble the human ARPKD phenotype with cysts arising in proximal tubule and collecting duct segments of kidney (29). A rescued orpk mutant mouse was developed in which wild-type Tg737 was reintroduced resulting in correction of the PKD phenotype as well as the ciliary defect (47, 48). The orpk Tg737 mutant mouse was cross bred with the ImmortoMouse carrying the H-2Kb-tsA58 transgene, and a temperature-sensitive immortalized orpk cilia(−) collecting duct cell line was established (49). The wild-type Tg737 gene was also reintroduced into this cell line, which corrected the ciliary defect, creating the orpk cilia(+) immortalized collecting duct cell line (49). The ciliary phenotype displayed by each of these cell types has been well characterized. However, the physiological manifestations of cilia malformation at the cellular level have not been adequately addressed and are the focus of the present studies.

METHODS

Materials. Reagents were obtained from Sigma (St. Louis, MO) unless otherwise indicated.

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Culture of orpk cilia(+) and cilia(−) cells. Orpk cilia(+) and cilia(−) cells were generated as described previously (49). Cells were cultured under permissive conditions for SV40 large-T antigen expression (33°C, 10 U/ml interferon-γ) in defined collecting duct media (DMEM/F-12, 10% FBS, 1.3 μg/l sodium selenite, 1.3 μg/l 3,3',5'-triiodo-thyronine, 5 mg/l insulin, 5 mg/l transferrin, 2.5 mM glutamine, 5 μM dexamethasone, 100 U/ml penicillin, 100 mg/ml streptomycin, 5% CO2). Four days before an experiment, cells were seeded onto Corning Snapwell tissue culture supports. To promote differentiation and SV40 large-T antigen inactivation, cells were cultured under nonpermissive conditions at 39°C in the absence of interferon-γ. After 4 days, cells were confluent and well differentiated.

Live cell fluorescence imaging. We performed fluorescence imaging on confluent cell monolayers grown on permeable supports using a specially constructed microscope chamber which allowed discrete perfusion of apical and basolateral cell surfaces. Clear polyester (0.4-μm pore size) permeable supports emitted little or no background fluorescence, and cells were clearly imaged. Cells on filters were loaded with fura 2 (10 μM fura 2/AM; Teflabs, Austin, TX) transferred to the chamber on the microscope, and the apical and basolateral sides were perfused with Ringer solution. One millimolar probenecid was added to both the apical and basolateral solutions to prevent dye leakage, and temperature was maintained at 38°C.

We performed dual-excitation wavelength fluorescence microscopy (Photon Technologies) with a Nikon microscope, ×20 S Fluor long-working distance objective, and a cooled SenSys charged-coupled camera (Photometrics). Fura 2 was excited at wavelengths of 340 and 380 nm and emitted fluorescence was collected at 510 nm. Mn2+ quenching of fura 2 fluorescence was measured with excitation at 359 nm and emitted fluorescence was collected at 510 nm. The near membrane Ca2+ indicator FFP-18/AM (Teflabs) was used to measure subapical [Ca2+]i. Loading procedures and excitation and emission spectra were similar to those described for fura 2. Data were obtained in each experiment from a grid of 20 regions of interest containing ~8–10 cells.

Measurement of flow-induced [Ca2+]i response in fura 2-loaded cells. Cells were maintained in the absence of apical flow for 10 min followed by an abrupt increase to a flow rate of 5 ml/min for 30 s. Subsequently, flow rate was reduced to 1.5 ml/min and back to zero for the remainder of the experiment. The basolateral flow rate remained constant at 1.5 ml/min.

Measurement of apical membrane divalent cation permeability by Mn2+ quenching in fura 2-loaded cells. A baseline was established in nominally Ca2+-free Ringer solution (no EGTA). Five hundred micromolar MnCl2 was added at the indicated time point. Mn2+ entry was measured as the rate of decline (quenching) of fura 2 intensity measured at its isosbestic (Ca2+ insensitive) wavelength, 359 nm. Experiments were performed in the presence or absence of 30 μM Gd3+.

Measurement of apical membrane Ca2+ permeability by Ca2+ depletion/readdition in fura 2-loaded cells. A baseline was established in a Ringer solution, followed by removal of apical and basolateral Ca2+ (in the presence of 1 mM EGTA) and treatment with 100 nM thapsigargin (TG). Ca2+ was then added back to only the apical surface. The magnitude of the resultant [Ca2+]i increase was used as a measurement of apical membrane Ca2+ entry/permeability. Experiments were performed in the presence or absence of 30 μM Gd3+ and/or 50 μM 2-APB (Calbiochem, San Diego, CA).

Measurement of subapical membrane Ca2+ levels with FFP-18. Cells were cultured on permeable supports that were folded so that cells at the apex of the fold could be viewed in the xz plane using fluorescence microscopy. FFP-18 intensity measurements were obtained from the intracellular side of the apical and basolateral membrane in nonstimulated live cells.

Patch clamp. High-resistance seals (1–4 gΩ) were obtained for all recordings, and experiments were performed at room temperature (20–22°C). Data were recorded using P-Clamp 9 software (Axon Instruments) and an EPC-7 amplifier (List-electronics) with gain set at 100 mV/pA and total filtering was 2.3 kHz. Recordings were later filtered at 1 kHz to better evaluate single-channel activity. Single-channel amplitudes were measured using cursors placed at closed and open channel levels. Bath and pipette solutions contained (in mM) 140 NaCl, 5 KCl, 10 HEPES, 1.5 CaCl2, 1 MgCl2, pH 7.4.

Western blot and immunofluorescence. Western blotting was performed using a polyclonal PC2 antibody. Cytosolic and membrane protein fractions were isolated using the CNM Compartment Protein Extraction Kit (BioChain). Proteins were separated using polyacrylamide-amelectrohoresis and transferred onto PVDF membranes. Proteins labeled with specific primary and secondary antibodies were visualized with enhanced chemiluminescence. β-Actin was probed as a control to ensure equal protein loading.

Immunofluorescence studies were performed using confocal microscopy. Polyclonally stained cells, grown on permeable supports, were washed with PBS and fixed with 4% paraformaldehyde in PBS at room temperature. Cells were permeabilized with 0.2% Triton X-100 and subsequently washed with PBS. Blocking of nonspecific binding was accomplished by incubating in 1% BSA in PBS. Cells were incubated for 1 h with primary antibodies and subsequently incubated with Alexa Fluor 488-conjugated and Alexa Fluor 594-conjugated secondary antibodies (1:2500, Molecular Probes, Eugene, OR) for 45 min. Cells were washed and the membrane was cut out and mounted with Vectashield media, containing 4,6-diamino-2-phenylindole (DAPI), for nuclear staining (Vector Laboratories, Burlingame, CA). Filters were mounted on slides for en face imaging of the monolayer as well as in a folded orientation allowing side-view imaging of cells in the xz plane at the apex of the fold. Slides were observed with a Leica scanning laser confocal microscope and image analysis was performed using Leica software.

Statistical analysis. Data are expressed as means ± SE. Statistical significance was determined using ANOVA.

RESULTS

[Ca2+]i regulation in polarized monolayers of orpk cilia(+) and cilia(−) cells was examined using a specially designed microscope chamber that accommodates cells cultured to confluence on permeable supports (Fig. 1, A and B). In examining the effects of cilia loss on cellular Ca2+ regulation, it was important to first demonstrate that orpk cilia(+) cells followed the paradigm of flow-induced, cilia-mediated Ca2+ entry that has been established in other renal cell models. Fura-2-loaded orpk cilia(+) cells cultured on permeable supports responded to an abrupt increase in apical flow rate with a transient elevation in [Ca2+]i, while both the initial rate of increase and magnitude of [Ca2+]i responses to increased flow were greatly attenuated in orpk cilia(−) cells (Fig. 2, A–C). Neither cell type reacted to abrupt changes in basolateral flow (data not shown) with alterations in [Ca2+]i. These data indicate that loss or malformation of apical cilia in orpk cilia(−) cells impairs [Ca2+]i responses to flow stimulation. Furthermore, in cilia(+) cells the initial rate and magnitude of flow-induced [Ca2+]i elevation were markedly attenuated by the addition of Gd3+ (Fig. 2, A–C), an observation that coincides with previous findings in Madin-Darby canine kidney cells by Praetorious et al. (34).

To determine whether this impairment of [Ca2+]i responses to abrupt changes in flow, in the absence of cilia, reflects an overall diminution in apical membrane Ca2+ permeability, we assayed apical Ca2+ entry at a constant low flow rate (beneath the threshold determined to elicit a flow-induced Ca2+ elevation in our system) using either a Mn2+ influx assay or apical...
Ca2⁺ readdition. Ca2⁺ entry pathways are permeable to the divalent cation Mn2⁺, and emitted fura 2 fluorescence is quenched on binding Mn2⁺. Thus the rate at which fura 2 is quenched by Mn2⁺ is indicative of Ca2⁺ entry (28). Surprisingly, we found that orpk cilia(−) cells displayed a markedly enhanced rate of Mn2⁺ quenching compared with cilia(+) cells (Fig. 3A) suggesting that apical Ca2⁺ permeability is greater, not less, in orpk cells that lack cilia. To confirm this finding using an alternate approach, apical Ca2⁺ permeability was assessed in fura 2-loaded orpk cells after removing Ca2⁺ from both apical and basolateral solutions and depleting intracellular Ca2⁺ stores with thapsigargin. On apical Ca2⁺ readdition alone, the increase in [Ca2⁺], was also greater in orpk cilia(−) compared with cilia(+) cells (Fig. 3B). When similar experiments were performed with Ca2⁺ readdition to the basolateral surface only, there was no significant difference in the magnitude of increase in [Ca2⁺], between orpk cilia(−) compared with cilia(+) cells suggesting that the difference in permeability was specific to the apical membrane (data not shown).

We observe that cilia-dependent, flow-induced Ca2⁺ entry in orpk cilia(+) cells occurs via a Gd3⁺-sensitive nonselective cation channel (Fig. 2, A-C). Work by Nauli et al. (31) demonstrated in studies of mouse epithelial cells that flow-induced Ca2⁺ entry occurs through PC2. Furthermore, Cantiello et al. (15) showed that PC2 channel activity is sensitive to Gd3⁺ blockade. Mn2⁺ quenching and Ca2⁺ readdition studies were therefore performed to determine whether the difference in Ca2⁺ permeability between cilia(+) and cilia(−) cells was due to a Gd3⁺-sensitive pathway. Addition of Gd3⁺ significantly attenuated the increased rate of Mn2⁺-induced fura 2 quenching in cilia(−) cells while Gd3⁺ had little effect in cells that possessed normal cilia (Fig. 3C). In the presence of Gd3⁺, the rate of apical divalent cation entry in cilia(+) and cilia(−) cells was equivalent. It should be emphasized that these studies were performed in cells that were exposed to a constant, minimal apical flow rate and were not subject to abrupt flow that would activate flow-induced Ca2⁺ entry. For this reason, we assert that, under these experimental conditions, the absence of cilia results in elevated Ca2⁺ entry across the apical membrane via a pathway which is consistent with PC2.

Ca2⁺ permeability of the apical membrane of renal epithelial cells is, by in large, the product of transient receptor potential channels (13). Classically, with extracellular/intracellular Ca2⁺ depletion there is activation of store-operated Ca2⁺ channels (SOCC) (38). As shown in Fig. 3, B and D, apical Ca2⁺ readdition resulted in large increases in [Ca2⁺], that were markedly inhibited in both cilia(+) and cilia(−) cells by 2-aminoethyldiphenylborate (2-APB), an agent that has been reported to block SOCCs (19). However, in the presence of 2-APB alone, the increases in [Ca2⁺], with apical Ca2⁺ readdition remained larger in cilia(−) compared with cilia(+) cells. This difference was abrogated by addition of Gd3⁺ in the presence of 2-APB. These results indicate that the absence of cilia does not affect SOCC activity but rather lends further support to the notion of an increased Gd3⁺-sensitive pathway in cilia(−) cells.

**Fig. 1.** Chamber design for live cell imaging of polarized cell monolayers. A: diagram of the microscope chamber designed to fit Corning Snapwell permeable supports. Design allows discrete perfusion of apical and basolateral compartments. B: optically clear polyester membranes do not interfere with imaging of fura 2-loaded cells.

**Fig. 2.** Flow-induced Ca2⁺ responses in Oak Ridge Polycystic Kidney (orpk) cilia(+) and cilia(−) cells. A: representative tracings of fura 2-loaded orpk cilia(+) and cilia(−) cells. B: graphical summary of the change in fura 2 ratio on flow stimulation [Ctl denotes control; *cilia(+) vs. cilia(−) control groups, \( P < 0.001 \); #cilia(+) control vs. Gd3⁺ treated, \( P < 0.001 \)]. Estimated \( \Delta [\text{Ca}^{2+}] \) in response to flow was 80–100 nM in cilia(+) cells. C: graphical summary of the rate of increase of [Ca2⁺], due to abrupt increase in apical flow rate [*cilia(+) vs. cilia(−) control groups, \( P < 0.001 \); #cilia(+) control vs. Gd3⁺ treated, \( P < 0.001 \)].
Next, we performed patch-clamp studies in orpk cilia(+) and cilia(−) cells (Fig. 4). The presence of apical channel activity was assessed using the cell-attached configuration using Ringer solution [(in mM) 140 NaCl, 5 KCl, 10 HEPES, 1.5 CaCl2, 1 MgCl2, pH 7.4], both in the pipette and bath, in the absence of flow. We were able to obtain high-resistance seals in 80% of attempted patches for both cell types. Although experiments were performed at room temperature, we have obtained preliminary measurements of membrane potential suggesting that cells maintain a hyperpolarized state (data not shown). We identified a small (10 pS) channel in the majority of patches from both cell types (data not shown). We were also able to identify a channel of 80 pS, according to the slope of the linear fit of the I-V plot (Fig. 4C), although its activity was relatively infrequent. The reversal potential of the observed channel is approximately zero suggesting that it is a nonselective cation channel. Notably, the size of this larger channel is consistent with the conductance of PC2 (15, 18, 26, 45). Importantly, we found that spontaneous channel activity of this 83-pS conductance was much greater in the cells that lacked cilia compared with cells expressing properly formed cilia (Table 1). Because of the infrequent occurrence of this channel, we were not able to perform additional work to characterize the identity of this channel. Nevertheless, the size of this channel is consistent with PC2 and the increased frequency of channel activity in cilia(−) cells supports the assertion of an increase in apical Ca2+ entry in the absence of cilia.

Because cilia serve in cellular structural organization, it is possible that one consequence of the loss of the primary cilium may be a disruption in the normal spatial distribution of apical proteins. Therefore, we measured total protein levels of PC2 and examined the pattern of distribution of PC2 at the apical

Table 1. Assessment of channel frequency in orpk cilia(+) and cilia(−) cells

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<th>Seals w/Channels</th>
<th>Total Seals</th>
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<tr>
<td>orpk cilia (−)</td>
<td>18</td>
<td>23</td>
<td>78</td>
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<tr>
<td>orpk cilia (+)</td>
<td>3</td>
<td>26</td>
<td>12</td>
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The number of cell-attached patches in which the 83 pS nonselective channel was observed (seals w/channels) vs. the total number of patches obtained (total seals) in orpk cilia(+) and cilia(−) cells.
cell membrane of cilia(+) and cilia(–) cells. Western blot analysis using a polyclonal PC2 antibody (YCC2, graciously provided by Dr. S. Somlo) demonstrates that PC2 expression levels in plasma membrane and cytosol appear equivalent between the two cell types (data not shown). Utilizing laser confocal microscopy, we performed immunofluorescence studies to evaluate PC2 distribution at the apical membrane of cilia(+) and cilia(–) cells. With the use of the YCC2 antibody, observed in the xz plane, PC2 localizes to primary cilium and was enriched at the base of orpk cilia(+) cells, whereas staining was uniformly distributed across the apical membrane of cilia(–) cells (Fig. 5, A–D). In a second series of studies using a polyclonal PC2 antibody (graciously provided by Dr. R. Sandford), serial confocal microscopy optical sections in the xy plane revealed a strong pattern of PC2 staining at the base of the cilium in orpk cilia(+) cells while PC2 staining was observed throughout the apical surface in cilia(–) cells (Fig. 6, A–F). No staining pattern was observed in the absence of primary PC2 antibodies (data not shown). These findings provide evidence that loss of cilia results in altered spatial distribution of PC2, which could explain the differences in apical Ca2+ permeability between the cilia(+) and cilia(–) cells.

One prediction of increased apical Ca2+ permeability in orpk cilia(–) vs. cilia(+) collecting duct cells is a chronic elevation in [Ca2+]i. In our experience, there is no appreciable difference in bulk [Ca2+]i between these two cell types. However, an increase in apical Ca2+ permeability might cause localized increases in [Ca2+]i only in the subapical membrane domain. To assess the functional consequence of altered apical Ca2+ permeability in this model of PKD, we employed a technique to measure Ca2+ levels directly beneath the plasma membrane using fluorescence microscopy. The ratiometric near-membrane Ca2+ fluorescent indicator FFP-18 is a Ca2+-sensitive fluorophore that is anchored to the inner surface of the plasma membrane by a hydrophobic tail (14). We measured FFP-18 fluorescence at the intracellular surface of the apical and basolateral membrane of individual cells using a folded-filter technique for live cell imaging (Fig. 7, A and B). Measurements using this indicator demonstrate that subapical membrane Ca2+ levels are higher in orpk cilia(–) vs. cilia(+) cells when compared directly or when normalized in individual cells to basolateral membrane values (Fig. 7, C and D).

**DISCUSSION**

The loss of apical cilia structure/function has now been implicated as a primary factor in the pathophysiology of PKD. However, the physiological consequences of ciliary dysfunction are only now being elucidated. An important step in understanding the physiological relevance of epithelial primary cilia has been the demonstration that fluid flow across the apical surface of ductal epithelia causes cilia deformation inducing Ca2+ entry through PC2. The loss of apical flow-induced increases in [Ca2+]i in the absence of cilia, has led to the suggestion that apical Ca2+ entry might be attenuated in the context of pathologies in which cilia structure and/or function are impaired, such as PKD.

Our assessment of flow-induced, cilia-mediated [Ca2+]i elevations in the orpk cells yielded results which coincide with previously published studies in that a maximal [Ca2+]i response requires structurally and functionally competent cilia (24, 31, 34, 35). The exciting new finding in our studies is that the intrinsic (nonflow stimulated) apical Ca2+ entry rate is, in fact, higher in the absence of cilia. At first blush, this may appear to contradict studies that investigated Ca2+ dynamics in various models of PKD. However, previously published work has focused on (nonplasma membrane) ER Ca2+ responsiveness demonstrating that rate of release, total amount released, and SOCC activity are attenuated in various cell models in which PC2 expression is genetically modified and that ER Ca2+ release is augmented in PC1-deficient cells (1, 22, 31, 39, 43). However, this is the first study that has directly assessed apical membrane Ca2+ permeability in the absence of cilia. The findings of our present study advocate an alternate paradigm by which normal epithelial function is compromised and cyst development occurs in PKD. That is, cilia dysfunction causes increased apical Ca2+ entry, which may contribute to

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**Fig. 5.** Side-view immunofluorescence images of cilia and polycystin-2 (PC2) at ×100 magnification. A–D: confocal images of orpk cilia(+) (A and B) and cilia(–) (C and D) cells labeled with a monoclonal acetylated α-tubulin antibody (A and C green) and a polyclonal PC2 antibody (B and D red). Apical and basolateral surfaces are indicated by white arrows. PC2 is expressed on cilia (white arrowhead, B). PC2 is more diffuse at the apical membrane of cilia(–) cells (D). No staining pattern was observed in the absence of primary PC2 antibodies (data not shown).
cyst formation. Because primary cilia have been described as a prominent site of action and regulation of PC2, one would expect PC2 activity to be affected by cilia loss. This same logic, taken in the context of our findings, lends support to our proposition that PC2 mediates the increased Ca\textsuperscript{2+} entry in the orpk cilia(−) model. Furthermore, the observation that this affected Ca\textsuperscript{2+} entry pathway in orpk cilia(−) cells is Gd\textsuperscript{3+} sensitive is consistent with a role for PC2 in mediating the enhanced Ca\textsuperscript{2+} entry. The finding that store-operated Ca\textsuperscript{2+} entry pathways are similar in cilia(+) vs. cilia(−) cells indicates that not all Ca\textsuperscript{2+} entry pathways are affected by the loss of cilia. Thus cilia appear to exert a tonic inhibitory influence on, at least, one specific Ca\textsuperscript{2+} entry mechanism that is released in the absence of cilia.

Given the direct evidence for enhanced Ca\textsuperscript{2+} entry obtained via live-cell fluorescence imaging, one would predict that there is enhanced channel activity in the apical membrane of cilia(−) cells. We performed patch-clamp studies using the cell-attached configuration to directly ascertain spontaneous single-channel activity at the apical membrane of unstimulated orpk cilia(+) and cilia(−) cells. We found that the abundance of an 83 pS channel (similar in conductance to PC2) was much greater in orpk cells that do not express cilia. The presence of an apical cilium did not appear to influence our ability to patch these cells, as we were able to obtain high-resistance seals in ~80% of attempted patches of both cilia(+) and cilia(−) cells. Thus the observed difference in channel frequency was not due to inability to successfully patch the cells. One of the confounding aspects of these electrophysiological studies is that since we measured native channel activity, and not that of an overexpression system, we observed a very low open probability (\(P_o\)) of this channel. Thus we were unable to obtain specific information on channel properties such as relative ion selectivity or inhibitor sensitivity, and therefore we cannot directly identify the channel based on biophysical properties. However, we assert that the conductance and low \(P_o\) eliminate either small- or large-conductance K\textsuperscript{+}-selective channels such as ROMK or MaxiK as candidates (2, 44). The fact that the reversal potential is approximately zero given the asymmetric Na\textsuperscript{+}, K\textsuperscript{+}, and Cl\textsuperscript{−} gradients (pipette solution vs. intracellular electrolyte concentration) would strongly suggest either a non-selective cation channel or a Cl\textsuperscript{−} channel. In other words, if this channel did display marked ion selectivity, we would expect the reversal potential to be either positively or negatively shifted when measured under the given experimental conditions. Although one could argue that the observed channel is secreting Cl\textsuperscript{−}, the conductance is not consistent with what is described for Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels or the cystic fibrosis transmembrane regulator that might be expressed apically in collecting duct cells (3, 6, 17). We submit that these findings are consistent with increased activity of a nonselective cation entry channel, such as PC2, which may mediate the enhanced entry of Ca\textsuperscript{2+} across the apical membrane in cilia(−) cells.

A significant observation in terms of understanding how primary cilia function in both a normal and pathological context has been the discovery of PC2 channel expression in this cellular structure. While its colocalization with cilia has received much recent attention, PC2 is also reportedly expressed in ER and plasma membrane (22, 26). In our investi-
further credence to the notion that PC2 is mediating the regulation of PC2 channel activity. We suggest that cilia tonically regulate the PC2 Ca\(^{2+}\) channel. With abrupt changes in flow there is ciliary deformation allowing regulated Ca\(^{2+}\) entry across the apical membrane through PC2. In the absence of primary cilia, this tonic regulation is released resulting in unregulated and continuous Ca\(^{2+}\) entry across the apical plasma membrane.

Interestingly, many of the abnormalities in cell function, structure, and proliferation, which are hallmarks of PKD have been suggested, in the context of other diseases, to be a consequence of elevated or deregulated [Ca\(^{2+}\)]. (40). Furthermore, the importance of Ca\(^{2+}\) microdomains within the cell has garnered increasing attention as a rationale for explaining the specificity of signaling pathways influenced by Ca\(^{2+}\) despite its promiscuity as a second messenger (5). In our studies we were able to show that loss of cilia resulted in enhanced entry of Ca\(^{2+}\) across the apical membrane and elevated subapical membrane [Ca\(^{2+}\)]. Therefore, in PKD a loss of cilia structure/function leads to elevated levels of Ca\(^{2+}\) in the subapical membrane domain which may have profound consequences. For instance, Ca\(^{2+}\) plays an important role in cytoskeletal organization and function. We speculate that chronic elevations in subapical membrane [Ca\(^{2+}\)] acting through alterations in cytoskeletal organization could affect processes such as protein distribution and cellular transport. Additionally, a strong association has been made implicating Ca\(^{2+}\) in the regulation of signaling cascades which govern cell growth and proliferation (4, 21). Specifically, sustained elevations in [Ca\(^{2+}\)] have been shown to promote cellular proliferation (30). This, coupled with the fact that abnormal cell proliferation is a prominent feature of PKD, underscores the importance of our finding of elevated steady-state subapical [Ca\(^{2+}\)] in orpk cells. Thus enhanced Ca\(^{2+}\) entry could play a pivotal role in the pathogenesis of PKD.

These findings represent a significant advancement in the understanding of cilia-ion channel interaction and provide new insight into the pathogenesis of cyst formation in ARPKD. Although the orpk mouse is considered a model for ARPKD and does not display genetic defects in PC1 or PC2, a clear understanding the functional relationship between the cilia structure, other ciliary proteins, and PC2 may have implications in the study of ADPKD. Mutations in PC1 and PC2, in models used to study ADPKD, have been elegantly proven to reduce, if not abolish, flow-induced, cilia-mediated increases in [Ca\(^{2+}\)]. However, these studies have not examined the effects of these mutations on the overall Ca\(^{2+}\) permeability as is described in the present studies. Obviously, one would not expect to observe an increase in PC2-mediated apical Ca\(^{2+}\) permeability in a model in which PC2 was deleted. But, in the context of a model in which PC1 (a putative regulator of PC2 function and location) is mutated, or PC2 possesses mutations that cause activation rather than deletion, one might expect to see a loss of regulation and resultant increase in Ca\(^{2+}\) permeability as stated in our hypothesis. For this reason, the current studies may prove valuable in understanding the pathogenesis of both recessive and dominant forms of PKD and other cilia-associated disease.

GRANTS

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