

Polycystin-2 is an intracellular calcium release channel

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Polycystin-2, the product of the gene mutated in type 2 autosomal dominant polycystic kidney disease (ADPKD), is the prototypical member of a subfamily of the transient receptor potential (TRP) channel superfamily, which is expressed abundantly in the endoplasmic reticulum (ER) membrane. Here, we show by single channel studies that polycystin-2 behaves as a calcium-activated, high conductance ER channel that is permeable to divalent cations. Epithelial cells overexpressing polycystin-2 show markedly augmented intracellular calcium release signals that are lost after carboxy-terminal truncation or by the introduction of a disease-causing missense mutation. These data suggest that polycystin-2 functions as a calcium-activated intracellular calcium release channel *in vivo* and that polycystic kidney disease results from the loss of a regulated intracellular calcium release signalling mechanism.

ADPKD affects more than 1 in 1000 live births and is the most common monogenic cause of kidney failure in man¹. Mutations in either of two genes, *PKD1* (ref. 2) or *PKD2* (ref. 3), cause virtually indistinguishable clinical presentations; most notably, growth of fluid-filled cysts in the kidney, liver and pancreas. The primary molecular genetic basis for cyst formation is homozygous loss-of-function because of somatic second hits in the setting of inherited inactivating mutations^{4,5}. The near identity of polycystic disease symptoms, irrespective of the causative gene, suggests that polycystin-1 and -2 function along a common pathway. This hypothesis is supported by a variety of observations, including the similarity of phenotypes between mouse knockouts of either gene^{6,7}, the discovery that the two proteins interact^{8,9} and the identity of the phenotypes caused by mutations in homologous genes in *Caenorhabditis elegans*¹⁰.

Polycystin-2, the product of the gene mutated in type 2 ADPKD (online MIM number, 173910)³ is the prototypical member of a subfamily of the TRP channel superfamily¹¹. Immunocytochemical colocalization, subcellular fractionation and endoglycosidase H (Endo H) sensitivity analyses have established that epithelial cells express polycystin-2 exclusively in pre-medial Golgi membranes, most notably the ER^{9,12}. In addition, the cytoplasmic tail of polycystin-2 contains signals that are necessary for ER retention^{9,12}. Cell-surface biotinylation assays also suggest that native polycystin-2 is not transported to the cell surface in epithelial cells¹². Immunohistochemical studies have defined the pattern of polycystin-2 tissue-specific expression^{13–15}, but ultrastructural studies to define the subcellular location of the protein in tissues have not, as yet, been reported. Furthermore, recent studies of cation channel activities attributed to polycystin-2 in heterologous systems have been unable to determine the site or mechanism of action of the protein in epithelial cells^{9,16,17}.

In the present study, we examined both the mechanism and site of action of polycystin-2. Single channel recordings from ER microsomes fused to lipid bilayers show that polycystin-2 is a high

conductance channel that is permeable to divalent cations. Calcium imaging studies in epithelial cells over-expressing polycystin-2 demonstrate enhanced inositol 1,4,5-trisphosphate (InsP₃)-mediated calcium release from intracellular pools. Mutation of polycystin-2 abrogates this calcium release response. Gradient fractionation and Endo H sensitivity analyses of native kidney tissue show that the distribution of polycystin-2 is indistinguishable from that of ER membrane proteins. The data suggest that polycystin-2 is highly expressed in the ER of kidney epithelial cells and functions as a calcium release channel.

Results

Single channel properties of polycystin-2. LLC-PK₁ porcine kidney cell lines stably expressing either full-length *PKD2* (ref. 12), a C-terminal truncated polycystin-2 mutant (*PKD2-L703X*) (ref. 12), or a naturally occurring missense variant of polycystin-2 (*PKD2-D511V*)¹⁸ were used to study the channel properties of the protein (Fig. 1a; see Methods). *PKD2-L703X*, which lacks several putative protein interaction domains^{3,8,12,19}, is representative of clinically relevant premature termination variants (Fig. 1a). Full-length polycystin-2 is confined to pre-medial Golgi membranes, whereas the *PKD2-L703X* mutant partially translocates to the plasma membrane because of the loss of essential ER retention signals¹². The *D511V* mutant is a pathogenic missense variant that differs from the wild-type sequence by a single amino acid in the third membrane-spanning domain¹⁸ (Fig. 1a). As the *D511V* mutant has an intact C terminus, we would predict that it will retain the cellular distribution of the wild-type protein. An immunocytochemical examination of LLC-PK₁ cells stably overexpressing *PKD2-D511V* with YCC2 antisera^{5,12,13,15,18–20}, revealed a pattern of expression that was indistinguishable from the wild-type polycystin-2 pattern (Fig. 1b), and consistent with abundant expression in ER membranes. A semiquantitative assessment of *PKD2* and *PKD2-D511V* overexpression by serial dilution and immunoblotting demonstrated that

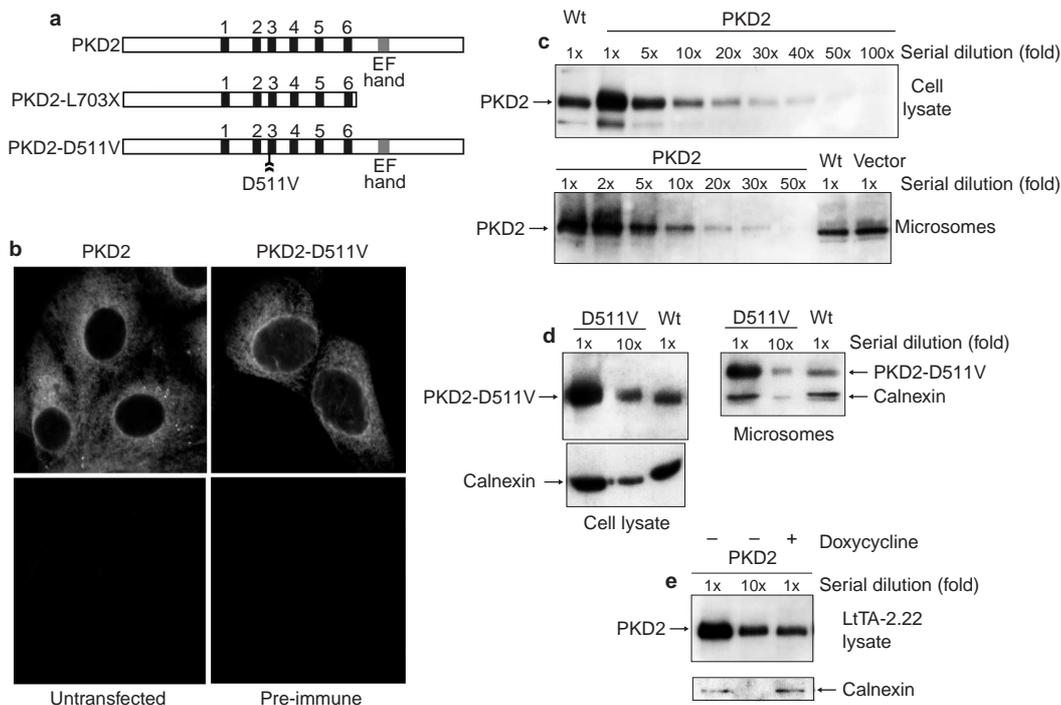


Figure 1 Expression of polycystin-2 in cell lines. **a**, Schematic representation of full length PKD2 and the PKD2-L703X and PKD2-D511V mutants. PKD2-L703X lacks the last C-terminal 265 amino acids, PKD2-D511V has a missense change in the third transmembrane-spanning domain. Dark boxes (1–6) represent membrane-spanning domains; shaded box represents the EF-hand. **b**, Confocal immunofluorescence images of LLC-PK₁ cells stained with YCC2 antiserum, showing that overexpressed PKD2 (top left) and PKD2-D511V (top right) have identical cytoplasmic reticular staining patterns. Untransfected parental cells (lower left) and PKD2-D511V over-expressing cells (lower right) were stained with YCC2 and pre-immune serum, respectively. **c**, Semiquantitative analysis of protein expression by serial dilution

shows a 5–10-fold enrichment of PKD2 expression in total lysate and microsomes from overexpressing cells. 1x represents 15 µg total protein loaded in this experiment, and also in **d** and **e**. Serial dilutions of the 15-µg samples (2–100x) were made thereof, and also apply to **d** and **e**. Wt refers to non-overexpressing cells throughout. **d**, Approximately 10-fold overexpression of PKD2-D511V in cell lysates and microsomes from the PKD2-D511V-expressing cell line. Calnexin is used as internal loading control. **e**, The inducible LtTA-2.22 cell line achieves ~10-fold overexpression of polycystin-2. Cells were incubated in the absence (–) or presence (+) of doxycycline. The same blot was also reprobbed with an anti-calnexin antibody (bottom panel).

the proteins are enriched by between 5- and 10-fold, respectively, in the stable cell lines (Fig. 1c,d). An LLC-PK₁ cell line (LtTA-2.22) that inducibly over-expresses wild-type polycystin-2 also achieved ~10-fold overexpression in the induced state (Fig. 1e). The immunostaining pattern of polycystin-2 in LtTA-2.22 cells was indistinguishable from that in stable, non-inducible, overexpressing cells (data not shown).

To investigate the function of polycystin-2 *in vitro*, we began by defining its channel properties and structure-function relationships in lipid bilayers. The presence of ER membranes in microsome fractions was examined by immunoblotting with antisera to the ER-resident protein calnexin (Fig. 1d,e). The successful fusion of microsomes to lipid bilayers was documented by recording large potassium and chloride ion channel currents (Fig. 2a, traces A,C), which disappeared after removal of potassium chloride from the *cis* side (the cytoplasmic side of the channel) of the bilayer (Fig. 2a, traces B, D). To monitor currents in the absence of potassium chloride, barium ions on the *trans* side of the bilayer (ER luminal side of the channel) were used as a current carrier. Inward barium currents were detected in ER vesicle preparations that overexpressed polycystin-2 ($n = 24/24$ channels from 8 independent ER microsome preparations; Fig. 2a, trace B). By contrast, no such currents were observed when ER vesicle preparations from wild-type LLC-PK₁ cells ($n = 0/47$ channels from 3 ER preparations; Fig. 2a, trace D) or LLC-PK₁ cells expressing the empty vector alone ($n = 0/7$ channels from 2 ER preparations, data not shown) were fused to bilayers. The possibility that the currents detected here were mediated by InsP₃

receptor (InsP₃R) channels was excluded by the absence of InsP₃ in these experiments. Any potential contribution of ryanodine receptor (RyR) channels to the current was also ruled out by the addition of the RyR inhibitor ruthenium red (2 µM), to the cytoplasmic side of the membrane during the entire course of the experiment. Channel activity under these conditions indicates the presence of a novel intracellular calcium release channel—namely, polycystin-2.

Larger negative holding potentials increased the current amplitudes for wild-type polycystin-2 (Fig. 2b). The polycystin-2 channel had slope conductances of 114, 95 and 90 pS when measured between 0 and –10 mV with barium, calcium and magnesium as the respective current carriers (Fig. 2b). Barium was used as the current carrier for the majority of subsequent bilayer experiments because it does not influence the activity of known intracellular calcium channels and generates larger current amplitudes for polycystin-2 (barium, 2 pA; calcium, 1.7 pA; magnesium, 1.5 pA; all measured at –10 mV). These electrophysiological properties of polycystin-2 parallel those described previously for InsP₃R and RyR^{21–23}, as well as other TRP-related channels²⁴, but differ from voltage-operated channels at the cell surface²⁵. The PKD2-L703X truncation mutant had reduced current amplitudes and required larger negative membrane potentials for channel activation than the wild-type protein ($n = 18/18$ channels from 4 ER microsome preparations; Fig. 2c). The slope conductance was reduced to 28 pS between –20 and –60 mV with barium as the current carrier (Fig. 2c). When ER vesicles from cells expressing the PKD2-D511V mutant were fused to bilayers, no currents were observed ($n = 0/12$

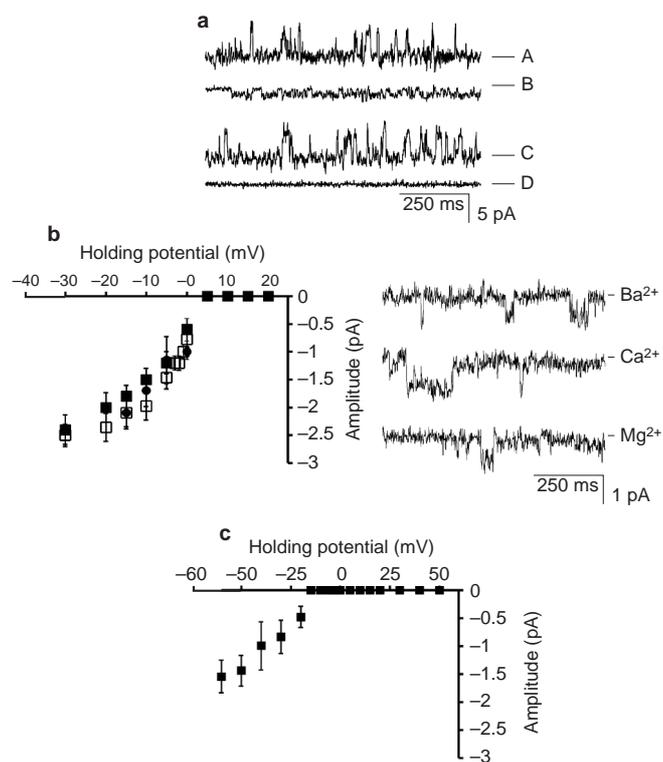


Figure 2 Polycystin-2 is a voltage-dependent ion channel that conducts divalent cations. **a**, ER-derived vesicles from LLC-PK₁ cells over-expressing polycystin-2 (**A, B**) or wild type LLC-PK₁ cells (**C, D**) were fused to lipid bilayers. Fusion was documented by the presence of potassium and chloride channels (**A, C**). After removal of potassium chloride from the medium, single channel currents generated by polycystin-2 were recorded with 55 mM barium as the sole current carrier, at a holding potential of -15 mV (**B**). Channel activity was not observed with ER vesicles from nontransfected cells (**D**). Channel openings are shown as downward deflections and bars to the right indicate zero current. **b**, Channel current amplitude-voltage relationships of polycystin-2, recorded with 55 mM barium (open square; $n = 24$), calcium (filled circle; $n = 7$) or magnesium (filled square; $n = 5$) as current carriers (left panel). Slope conductances, determined by linear regression between 0 and -10 mV, were 114 pS, 95 pS and 90 pS, respectively. Single channel currents recorded at -10 mV show that polycystin-2 conducts barium (average amplitude, 2 pA), calcium (average amplitude, 1.7 pA), and magnesium (average amplitude, 1.5 pA), (right panel). **c**, The channel current amplitude-voltage relationship of PKD2-L703X generated a slope conductance of 28 pS, determined between -20 and -60 mV.

channels from 3 ER preparations; data not shown). The latter finding provides evidence that this missense mutation in polycystin-2 results in loss of function. Coupled with the pathogenicity of this variant in patients¹⁸, the data support the hypothesis that the channel activity of polycystin-2, independent of protein interactions in the C terminus, is required for the normal functioning of the polycystin signalling pathway in the kidney.

Given the effects of intracellular calcium on the activity of intracellular calcium channels²⁶, we examined polycystin-2 channel activity in the presence of varying cytosolic calcium concentrations. The normalized open probability of polycystin-2 increased from 0.19 to 1.0 over a range of free calcium concentrations from 0.01 to 1260 μ M (Fig. 3a). These concentrations include the physiological range (0.1–10 μ M) of free cytosolic calcium. Higher free cytosolic calcium concentrations lowered the open probability (Fig. 3a). The PKD2-L703X mutant was not activated over the range of calcium concentrations tested (Fig. 3a). Whereas physiological

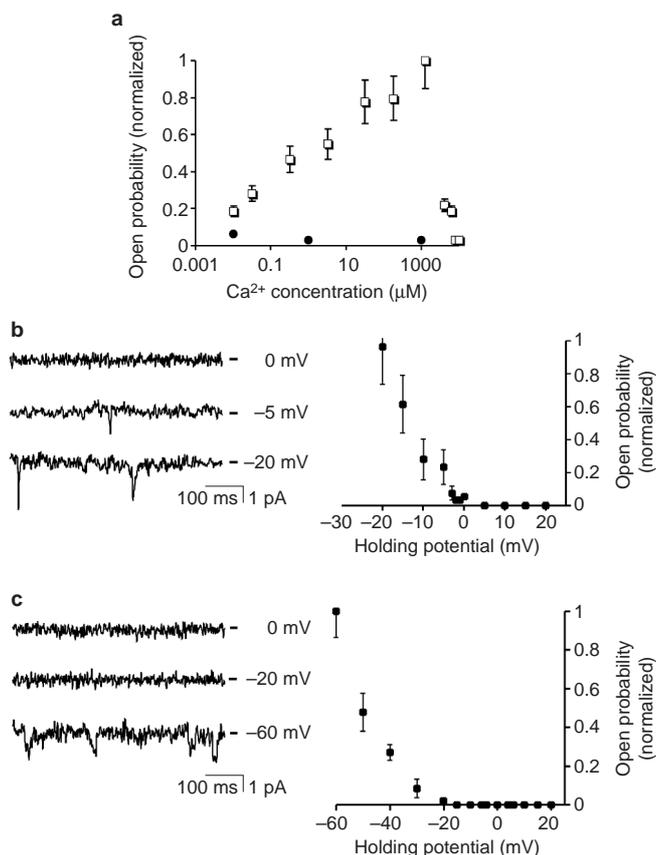


Figure 3 Dependence of polycystin-2 channel open probability on intracellular free calcium concentration and membrane holding potential. **a**, The open probabilities of PKD2 channels (open square; 0 mV; $n = 5$) and PKD2-L703X channels (filled circle; -50 mV; $n = 7$) were monitored at different free calcium concentrations on the cytosolic side of the channel. The PKD2 channel was activated by submillimolar and millimolar calcium concentrations on its cytosolic side (open probability at 100 μ M, $3.3\% \pm 1.3\%$ (S.E.M); $n = 5$). Higher cytosolic free calcium inhibited channel activity. Varying cytosolic free calcium concentrations did not effect the PKD2-L703X channel. Data were normalized to the maximum open probability. **b**, Single channel recordings of PKD2 activity (left panel) at 0 mV (P_{open} , 0.2%), -5 mV (P_{open} , 1.1%) and -20 mV (P_{open} , 2.5%). These open probabilities are for the experiment shown in the panel; the current carrier is barium. The open probability of the PKD2 channel was monitored at different membrane holding potentials ($n = 24$; right panel). Data were normalized to the open probability at -30 mV. The mean maximal open probability at -30 mV was $3.0\% \pm 0.2\%$ (S.E.M); $n = 24$). **c**, Typical recordings, as in **b**, for the PKD2-L703X channel activity (left panel) at holding potentials of 0 mV (P_{open} , 0%), -20 mV (P_{open} , 0.1%) and -60 mV (P_{open} , 2.4%) and as a function of the holding potentials ($n = 18$; right panel). The open probabilities of PKD2-L703X channel were similar to the wild-type channel, but were only achieved when the membrane potential was significantly larger. Data were normalized to the open probability at -60 mV.

concentrations of free calcium enhance the activity of polycystin-2 channels, concentrations outside this range induce channel inhibition. The absence of such modulation in the PKD2-L703X mutant suggests that this functional regulation may be mediated by sites located in the cytoplasmic tail of polycystin-2 (ref. 27). Increasingly negative holding potentials also increased the activity of polycystin-2 channels, demonstrating that the channel is voltage-dependent (Fig. 3b). The maximum open probability was achieved at holding potentials of -20 mV or greater. The mean maximal open probability at -30 mV was 3.0% ($n = 24$, $\pm 0.2\%$ S.E.M). This is comparable

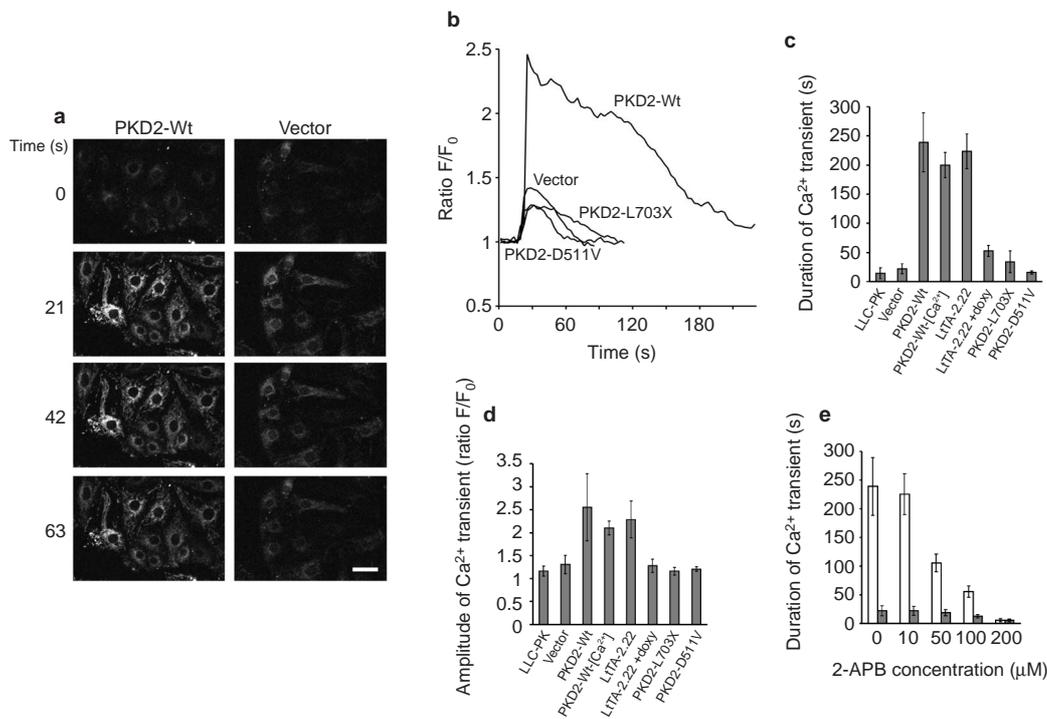


Figure 4 Overexpression of polycystin-2 enhances amplitude and duration of vasopressin-induced calcium transients. **a**, LLC-PK₁ cells stably transfected with polycystin-2 (PKD2-Wt) or the empty expression plasmid (Vector) were loaded with the fluo-3 dye. All cells respond to a vasopressin-stimulus (70 nM, applied at 0 s) with an increase in intracellular calcium, visualized by increasingly bright pixels from the fluorescence of the indicator dye with increasing time. Overexpression of PKD2 results in significantly larger calcium transients, compared to control cells. Scale bar, 25 μm. **b**, Vasopressin treatment (70 nM) induces single-peak increases in cytosolic calcium. Changes in the ratio of calcium-dependent fluorescence over prestimulus background fluorescence (F/F_0) are plotted over time in single representative LLC-PK₁ cells stably expressing full-length polycystin-2 (PKD2-Wt), the empty expression plasmid (Vector) and the mutants PKD2-L703X and PKD2-D511V.

c, The mean duration (\pm S.E.M.) of vasopressin-induced calcium transients was measured in untransfected LLC-PK₁ cells (LLC-PK₁), and in LLC-PK₁ cells stably expressing the empty expression plasmid (Vector), polycystin-2 (PKD2-Wt), polycystin-2 in calcium-free medium (PKD2-Wt-(Ca²⁺)₀), a 'tet-off' inducible polycystin-2 incubated without (LtTA-2.22) and with doxycyclin (LtTA-2.22 +doxy), PKD2-L703X, and PKD2-D511V, respectively. $n = 50$ for each condition. **d**, The mean amplitude (\pm S.E.M.) of vasopressin-induced calcium transients measured in the same group of LLC-PK₁ cell lines. **e**, Vasopressin-induced calcium transients in cells overexpressing polycystin-2 can be blocked by 2-APB, in a dose-dependent manner. Mean duration (\pm S.E.M.) of calcium transients in untransfected LLC-PK₁ cells (grey bars; $n = 50$) and in LLC-PK₁ cells overexpressing polycystin-2 (white bars; $n = 50$).

to the open probability of ~4% detected for InsP₃R in bilayers²², but much lower than that measured for RyR²¹. Furthermore, variations in membrane potential do not significantly alter the channel open probability of either InsP₃R or RyR^{23,28,29}. No significant channel activity was detected for the PKD2-L703X mutant at holding potentials up to -20 mV, which are sufficient to fully activate wild-type polycystin-2 channels (Fig. 3c). This change in the channel properties of the PKD2-L703X mutant indicates that the C-terminal domain is important in the normal channel function of polycystin-2. Pathogenic premature termination mutations in polycystin-2, if they produce stable proteins, result in channels with an altered single channel conductance and open probability, in addition to the previously described effects on protein interactions^{8,9} and alterations in subcellular localization¹².

Release of intracellular calcium by polycystin-2. The discovery that polycystin-2 is expressed in the ER of epithelial cells and that it functions as a calcium-activated calcium channel in lipid bilayers led us to hypothesize that small changes in local intracellular calcium concentration may be sufficient to activate nearby polycystin-2 channels. To test this hypothesis, LLC-PK₁ cells stably co-expressing both wild-type and mutant polycystin-2 were stimulated with vasopressin, to induce intracellular calcium release through InsP₃R³⁰. LLC-PK₁ cells express both vasopressin V1 and V2 receptors, but only V1 receptor stimulation, through activation of

InsP₃R, results in increased levels of cytosolic calcium^{29,30}. Activation of V2 receptors stimulates the production of cAMP, without inducing changes in the levels of intracellular calcium^{29,30}. LLC-PK₁ cells, loaded with the calcium-sensitive fluorescent dye fluo-3 acetoxymethyl ester (fluo-3) and expressing only endogenous polycystin-2, responded to vasopressin stimulation with a single transient increase in cytosolic calcium that declined to baseline levels within 60 s (Fig. 4a,b)³⁰. Similar results were also obtained with LLC-PK₁ cells that had been stably transfected with the empty expression vector (Fig. 4a,b). By contrast, LLC-PK₁ cells overexpressing full-length polycystin-2 generated an approximately two-fold larger increase in cytosolic calcium and an approximately ten-fold longer duration of the calcium transients (Fig. 4a-d). The increased duration and amplitude of transients persisted in the absence of extracellular calcium, suggesting that the increase in cytosolic calcium results from release of intracellular stores (Fig. 4c,d). We confirmed that InsP₃R activation is required for polycystin-2-mediated calcium release in our system by treating cells with the InsP₃R inhibitor 2-aminoethyl diphenyl borate (2-APB) (Fig. 4e). Furthermore, we confirmed that 2-APB does not directly inhibit polycystin-2 channel activity in bilayers (data not shown). We also confirmed the integrity of intracellular calcium stores, and excluded the possibility that variations in the calcium content of the ER in different cell lines accounted for the enhanced calcium transients,

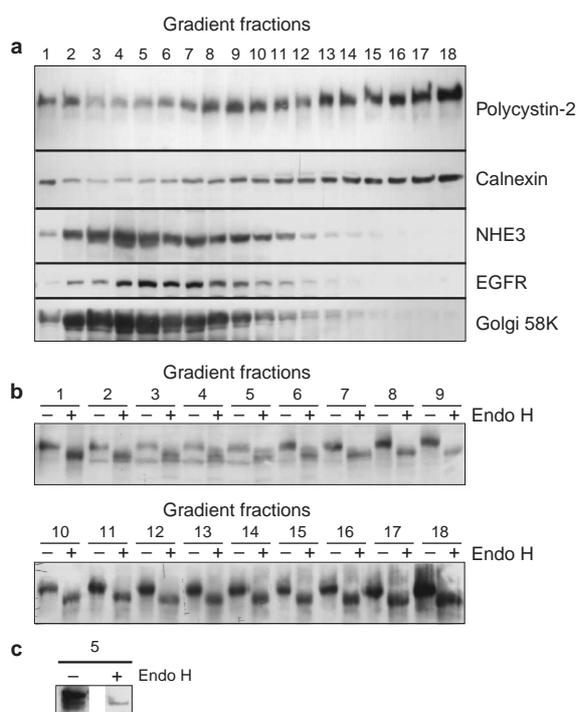


Figure 5 Polycystin-2 is expressed in the ER of native kidney. **a**, Kidney tissue lysates were fractionated by density gradient centrifugation on linear 8–34% Optiprep gradients. Equal volumes of the 18 fractions were loaded in each lane; fraction 1 is the top of the gradient, fraction 18 is the bottom. Samples were analysed by immunoblotting, with antibodies to polycystin-2, calnexin, NHE3, EGFR and the Golgi 58K protein. **b**, Equal aliquots of each fraction was digested with Endo H (+) or incubated with digestion buffer but no enzyme (-). All fractions are sensitive to Endo H; fractions 4–6 show partial Endo H resistance (see text for details). **c**, Polycystin-2 in fraction 5 is completely Endo H sensitive (+) after removal of the lower molecular weight proteins.

by showing that the addition of thapsigargin releases similar amounts of calcium in all of the cell lines tested (data not shown).

We next used the 'tet-off' inducible polycystin-2-overexpressing LLC-PK₁ cell line (LtTA-2.22) to establish that the enhanced calcium transients are directly attributable to overexpression of polycystin-2. Inducible overexpression of polycystin-2 resulted in calcium transient amplitudes and durations that were indistinguishable from polycystin-2 that had been overexpressed constitutively (Fig. 4c,d). Suppression of polycystin-2 overexpression, in turn, resulted in calcium transients of comparable amplitude and duration to transients detected in cells that do not overexpress functional polycystin-2 (Fig. 4c,d). LLC-PK₁ cells that expressed either the PKD2-L703X or PKD2-D511V mutants generated calcium transients with amplitudes and durations that were similar to wild type cells (Fig. 4c,d). The absence of enhanced calcium transients with the truncated PKD2-L703X suggests that preservation domains in the C terminus are required for the release of calcium from intracellular stores. On the other hand, the absence of enhanced calcium transients in the PKD2-D511V mutant provides evidence that polycystin-2 mediates intracellular calcium release through its own channel activity, rather than through an interaction with other channel proteins, such as InsP₃R. These findings support the hypotheses that polycystin-2 is an intracellular calcium release channel that can be activated in epithelial cells by local increases in calcium concentration.

Polycystin-2 is an ER protein in native kidney. The final arbiter of the primary subcellular site of polycystin-2 action is the location of

the protein in the tubular epithelial cells of native kidney tissue. As polycystin-2 and critical binding partners such as polycystin-1 are expressed and functional at physiological levels in native kidney, we reasoned that determination of the subcellular distribution in the kidney would establish whether polycystin-2 may function as an intracellular calcium release channel *in vivo*. We fractionated whole kidneys from C57BL/6J mice on iodixanol-based linear density gradients and compared the distribution of polycystin-2 with the distribution of known proteins from the plasma membrane, Golgi apparatus and ER (Fig. 5). The distribution of polycystin-2 on the gradient was indistinguishable from that of the ER membrane protein calnexin, and differed significantly from the distribution of epidermal growth factor receptor (EGFR) and sodium proton exchanger 3 (NHE3), a pair of proteins that are resident within basolateral and apical plasma membranes, respectively (Fig. 5a). The distribution of polycystin-2 also differed from that of the Golgi 58K protein (Fig. 5a). As the loading of the immunoblots was quantitative (see Methods), the relative intensity of the anti-polycystin-2 immunoreactive band in each lane is representative of the absolute steady state amount of polycystin-2 in each fraction (Fig. 5a). The vast majority of polycystin-2 protein in the kidney was excluded from the plasma membrane fractions altogether. Small amounts of polycystin-2 and calnexin did overlay the region of the gradient containing plasma membrane and Golgi proteins. The identical relative distribution for the known ER-restricted protein calnexin suggests that this finding results from an inherent limitation of the continuous fractionation procedure.

To refine further the biochemical data for the subcellular localization in the ER compartment, we examined the sensitivity of polycystin-2 in each fraction to Endo H. Typically, proteins that remain sensitive to Endo H are localized to the ER and *cis* portions of the Golgi apparatus. We have shown previously that wild-type polycystin-2 is confined to the ER and is completely sensitive to Endo H, whereas truncated forms of the protein that traffic to the plasma membrane acquire Endo H resistance¹². In keeping with these observations, we now find that polycystin-2 from all fractions of kidney tissue retained sensitivity to Endo H (Fig. 5b,c). The partial Endo H resistance of a minute proportion of total kidney polycystin-2 in three fractions overlying the plasma membrane region (fractions 4–6, Fig. 5b) resulted from glycosidase inhibitory factors that were concentrated in this region of the gradient. When these fractions were spin dialysed over a Centricon YM-100 membrane to remove the lower molecular weight molecules, polycystin-2 in the fractions became completely sensitive to Endo H (Fig. 5c). Thus, using whole tissue fractionation and sensitive biochemical tests, we show that polycystin-2 is an abundant ER membrane protein in native kidney tissue. We suggest that the ER may be the primary site of its physiological activity.

Discussion

Although the channel function of polycystin-2 was anticipated by its primary structure³, implied by the activity of its homologue³¹, and suggested in single channel studies^{16,17}, the function of this human disease gene in kidney epithelia has remained elusive. In the present study, we provide evidence that polycystin-2 expressed in the ER of epithelial cells is a functional calcium-activated high conductance channel that is permeable to divalent cations. Increased levels of intracellular calcium activate polycystin-2-mediated release of calcium from intracellular stores. Furthermore, a truncated polycystin-2 mutant retains channel activity, but is no longer calcium-activated or voltage-dependent, and cannot function to release calcium from intracellular stores. By contrast, a pathogenic missense mutation¹⁸ results in loss of polycystin-2 channel activity altogether. The missense mutant retains the subcellular localization and C-terminal-mediated protein interaction and regulatory domains of the wild-type protein, thus providing evidence that the loss of channel function alone is sufficient to cause polycystic kidney

disease. Polycystin-2 also shows voltage dependence in lipid bilayers. Although direct measurement of ER membrane potentials has not been reported and the *in vivo* significance of this finding is uncertain, it remains possible that localized membrane potentials in the 0–10 mV range, sufficient to inactivate polycystin-2, may occur through calcium release from the ER²⁸. Overall, the current findings suggest that in epithelial cells, polycystin-2 functions through the regulated release of calcium from intracellular stores. The loss of this intracellular calcium signalling mechanism results in the defect associated with polycystic kidney disease.

In keeping with its function as an intracellular calcium release channel, tissue fractionation studies show that polycystin-2 is expressed abundantly in the ER of native kidney tissue. The absence of any detectable polycystin-2 with resistance to Endo H digestion supports the hypothesis that polycystin-2 does not traffic past medial Golgi stacks, and therefore is not inserted into the plasma membrane. Findings in a recent study of Chinese hamster ovary (CHO) cells transfected with polycystin-1 and -2 have been interpreted as showing that polycystin-1 is required to co-assemble a polycystin complex and bring polycystin-2 to the cell surface⁹. However, direct evidence that polycystin-2 is actually inserted into the plasma membrane is lacking, and, although the authors found a novel surface cation channel activity when co-expressing polycystin-1 and -2, they were unable to demonstrate that polycystin-2 comprised the channel pore⁹. By contrast, the *C. elegans* orthologues of polycystin-1 and -2, location of vulva (LOV)-1 and PKD-2 also function in a common pathway, but localization of PKD-2 is not dependent on LOV-1 function¹⁰. Furthermore, in *C. elegans*, functional localization of the polycystin-2 orthologue is limited to intracellular membranes and the membrane of sensory cilia, and is not detected at the plasma membrane of the cell body or axon¹⁰. Interestingly, the only other instance of isoforms of an ER calcium channel existing in a cell surface membrane has been the InsP₃R in the sensory cilia of olfactory neurons in crustaceans^{32,33}. Although we did not detect any evidence of polycystin-2 in the monocilia of renal tubular cells, our study cannot exclude the presence of a minute fraction of cellular polycystin-2 in these structures.

Calcium release activity mediated by polycystin-2 did not require the co-expression of exogenous polycystin-1 in LLC-PK₁ cells. This finding, in conjunction with the bilayer data, suggests that polycystin-2 channel function does not require co-assembly with polycystin-1, although a function for low levels of native polycystin-1 in these cells cannot be excluded. The absence of intracellular calcium release in cell expressing the PKD2-D511V mutant, which retains the protein interactions of wild-type polycystin-2, argues strongly that polycystin-2 itself forms the channel. It remains possible, however, that channel activity requires association with other proteins. Polycystin-1 does associate with polycystin-2 (refs 8,9) and it is likely to be integral to the regulation of the polycystin-2 activity *in vivo*¹⁰. The interaction between these two proteins may occur either as polycystin-1 is transported through the ER and early Golgi compartments, or it may occur across closely apposed plasmalemmal and ER membranes near the cell surface³⁴. The paradigm for the association and functional interdependence of channel proteins in the plasma and ER membranes, L-type calcium channels and the RyR, respectively, is the basis of excitation-contraction coupling in muscle cells³⁵. This conformational coupling model has been extended to nonexcitable cell types, suggesting that the coupling of ER calcium release channels to cell surface channel receptors may be a generalized principle in nature^{36,37}. Indeed, the polycystin-1 and polycystin-2 receptor-channel complex may fit this paradigm³⁴. The polycystin-1-dependent channel activity at the cell surface⁹ may result in local increases in intracellular calcium that are sufficient to stimulate the release of calcium from intracellular stores through closely associated polycystin-2 channels in the ER.

Localized increases in intracellular calcium mediate a variety of subcellular processes. These processes include targeted fusion of

specialized cytosolic vesicles with discrete domains in the plasma membrane or activation of kinase cascades that stimulate cell-type specific gene expression³⁸. Either or both of these general functions could subsume the function of the polycystin pathway. Known cellular functions of polycystin orthologues include the acrosome reaction, a calcium-regulated membrane fusion event in sea urchin sperm^{39,40}, mechanosensory neuron dependent male mating behaviours in *C. elegans*^{10,41}, and recently, a defect in the late stages of endocytosis in mucopolipidosis type IV^{42,43}. Based on our current results, further studies into the function of intracellular signals and their regulation in various cell models will, in aggregate, yield crucial insights into the pathogenesis of polycystic kidney disease. □

Methods

Cell lines, cell culture and vesicle preparation

LLC-PK₁ cell lines expressing wild-type *PKD2* (GenBank Accession number U50928), the PKD2-L703X mutant and vector controls have been previously described¹². The PKD2-D511V mutant cDNA construct was made by introducing GTT (V) to replace GAT (D) at codon 511 by site-directed mutagenesis in pCDNA3.1. Cells stably overexpressing PKD2-D511V were obtained by selection with Zeocin (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. Expression of the mutant protein was evaluated by immunoblotting and immunofluorescence cell staining¹². For the tetracycline-inducible clone, LLC-PK₁ cells were transfected with pUHD15-1Neo (a gift from H. Bujard, Heidelberg, Germany) encoding a tetracycline-dependent transactivator. Clone LtTA-2.22 showed ~65-fold induction after transient transfection and was chosen to establish stable clones. PKD2-HA (ref. 12) in the expression plasmid pUHD10-3 (gift from H. Bujard) was transfected into LtTA-2.22 along with the selection plasmid pBabe-Puro. Overexpression of polycystin-2 in LtTA-2.22 cells was inhibited by the addition of doxycycline (10 ng ml⁻¹) to the culture medium. Membrane vesicles enriched for ER from LLC-PK₁ cells were isolated by differential centrifugation in the presence of protease inhibitors⁴⁴. The expression of polycystin-2 in vesicles was monitored by immunoblotting with YCC2 polyclonal antisera¹².

Single channel recordings

Vesicles enriched in ER membranes were fused to lipid bilayers containing phosphatidylethanolamine and phosphatidylserine (3:1 w/w; Avanti Polar Lipids, Alabaster, AL) dissolved in decane (40 mg lipid ml⁻¹)²⁵. A potassium chloride gradient, with 600 mM potassium chloride on the side of vesicle incorporation (*cis* side) and 0 mM potassium chloride on the opposite side (*trans* side), facilitated fusion. Experiments were performed with 250 mM HEPES-Tris at pH 7.35 on the *cis* side and 250 mM HEPES with either 55 mM Ba(OH)₂, 55 mM Ca(OH)₂, or 55 mM Mg(OH)₂, at pH 7.35, on the *trans* side of the bilayer. In some experiments, we determined the orientation of ER membrane proteins in the lipid bilayer through activation of the RyR. 2-APB (100 μM) was applied to the cytosolic side of the protein to test its effect on the polycystin-2 channel. Experiments were recorded under voltage-clamp conditions. The *trans*-side was ground (reference electrode), the *cis*-side was the input electrode. Using cellular conventions, calcium flux from the ER lumen into the cytoplasm was defined as an inward current, where inward currents are shown as downward deflections. Data were filtered at 1 kHz and digitized at 3 kHz, directly transferred to a computer and analysed with pClamp version 6.0.3 software (Axon Instruments, Burlingame, CA). The concentration of free calcium on the cytosolic side of the protein (*cis*-side of the lipid bilayer) was adjusted as described⁴⁵. In each experiment, the voltage-dependence of the polycystin-2 current was assessed and most channels were recorded under suboptimal activation conditions, by applying a holding potential of -3 to -5 mV.

Optical recordings of intracellular calcium concentrations

Wild type and stably transfected LLC-PK₁ cells were grown on glass coverslips to subconfluent density. During experiments, cells were kept at room temperature in a perfusion chamber on the microscope stage. Cells were superfused continuously with extracellular solution (ECS) at 1 ml min⁻¹. ECS contained 137 mM sodium chloride, 5 mM potassium chloride, 2 mM calcium chloride, 1 mM Na₂HPO₄, 1 mM magnesium sulphate, 10 mM HEPES and 22 mM glucose at pH 7.4. Cells were incubated in ECS containing 4 μM cell-permeant fluo-3 (Molecular Probes, Eugene, OR) with 0.04% DMSO and 0.02% pluronic acid for 15–30 minutes and washed in ECS before optical recording. Drugs applied to the bath solution were: thapsigargin (1–10 μM; Calbiochem-Novabiochem, San Diego, CA), vasopressin (0.07–70 μM; Calbiochem-Novabiochem), 2-APB (10–200 μM; Sigma, St Louis, MO). Fluo-3 fluorescence in loaded cells was measured with a BioRad MRC-1024 system (Biorad, Hercules, CA). Cells were selected randomly before each experiment and the borders of the entire cell were outlined manually to eliminate any selective contribution from nonuniform distribution of the dye within cells. Dye-loaded cells were excited at 488 nm and increases in intracellular calcium were measured at an emission wavelength of 522 nm. Images were acquired every 500 ms. Changes in fluorescence intensity were expressed as a ratio, F/F₀, of fluorescence intensity during drug application (F) and mean baseline fluorescence intensity (F₀). Transient durations were measured from the start of the calcium transients until fluorescence changes returned to the average prestimulus background. Nonstimulus-related, spontaneously occurring changes in fluorescence intensity, as well as changes after the application of control substances, were in the range of 1–5% of F/F₀.

Tissue fractionation, immunoblotting and glycosylation analysis

Tissue homogenates were prepared from eight-week-old C57BL/6J mouse kidneys. Kidneys were homogenized at 4 °C in buffer containing 10 mM Tris at pH 7.5, 120 mM sodium chloride, 20 mM potassium chloride, 1 mM EGTA, 1 mM EDTA and protease inhibitor cocktail (2 mM PMSF, 10 μg ml⁻¹ leupeptin, 10 μg ml⁻¹ pepstatin A and 1 μg ml⁻¹ aprotinin). A postnuclear supernatant (PNS) was obtained by centrifugation at 1,000g for 10 min and a microsome-rich supernatant (S20) was obtained by centrifugation of the PNS at 20,000g for 20 min. 2 ml of the S20 supernatant was layered onto

performed, precooled 8–34% linear Optiprep gradients (Life Technologies, Grand Island, NY) and centrifuged for 15 h at 100,000g. 18 equal-volume fractions (2 ml each) were collected from the top to the bottom of the gradient. Total protein from each fraction was precipitated with trichloroacetic acid and pellets were solubilized in equal volumes of Laemmli sample buffer. Equal sample volumes were separated by 7.5% SDS–polyacrylamide gel electrophoresis (PAGE) gels and transferred to PVDF membranes. Primary antibodies for immunoblotting were: anti-polycystin-2 (1:8,000; YCC2 (ref. 12)), anti-calnexin (1:2,000; Stressgen Biotechnologies Corporation, Victoria, BC); anti-EGFR (1:400; Signal Transduction Laboratories, Lexington, KY (Catalogue number E12020)), anti-NHE3 (1:500; Chemicon International, Temecula, CA (Catalogue number mAb3134)); anti-Golgi 58K protein (1:5,000; Sigma (Catalogue number G2404)). For glycosylation analysis, 500- μ l aliquots of individual fractions were digested overnight at 37 °C with 2500U of Endo H, as recommended by manufacturer's instructions (New England Biolabs, Beverly, MA). For controls, equal aliquots of each fraction were treated identically, except that no Endo H was added to the incubation. To remove potential Endo H inhibitory factors from selected fractions, the fractions were centrifuged through Centricon YM-100 columns (Millipore, Bedford, MA) at 1,000g for 30 min at 4 °C before Endo H digestion.

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