Polycystin-1 Interacts with Inositol 1,4,5-Trisphosphate Receptor to Modulate Intracellular Ca\(^{2+}\) Signaling with Implications for Polycystic Kidney Disease*

Yun Li‡1, Netty G. Santoso‡1, Shengqiang Yu§2, Owen M. Woodward†, Feng Qian§, and William B. Guggino*†3

From the *Department of Physiology and the ‡Division of Nephrology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

The PKD1 or PKD2 genes encode polycystins (PC) 1 and 2, which are associated with polycystic kidney disease. Previously we demonstrated that PC2 interacts with the inositol 1,4,5-trisphosphate receptor (IP\(_3\)R) to modulate Ca\(^{2+}\) signaling. Here, we investigate whether PC1 also regulates IP\(_3\)R. We generated a fragment encoding the last six transmembrane (TM) domains of PC1 and the C-terminal tail (QIF38), a section with the highest homology to PC2. Using a Xenopus oocyte imaging system, we observed that expression of QIF38 significantly reduced the initial amplitude of IP\(_3\)-induced Ca\(^{2+}\) transients, whereas a mutation lacking the C-terminal tail did not. Thus, the C terminus is essential to QIF38 function. Co-immunoprecipitation assays demonstrated that through its C terminus, QIF38 associates with the IP\(_3\)-binding domain of IP\(_3\)R. A shorter PC1 fragment spanning only the last six TM and the C-terminal tail also reduced IP\(_3\)-induced Ca\(^{2+}\) release, whereas another C-terminal fragment lacking any TM domain did not. Thus, only endoplasmic reticulum-localized PC1 can modulate IP\(_3\)R. Finally, we show that in the polarized Madin-Darby canine kidney cells, heterologous expression of full-length PC1 resulted in a smaller IP\(_3\)-induced Ca\(^{2+}\) response. Overexpression of the IP\(_3\)-binding domain of IP\(_3\)R reversed the inhibitory effect of PC1, suggesting interaction of full-length PC1 (or its cleavage forms) with endogenous IP\(_3\)R in Madin-Darby canine kidney cells. These results indicate that the behavior of full-length PC1 in mammalian cells is congruent with that of PC1 C-terminal fragments in the oocyte system. These data demonstrate that PC1 inhibits Ca\(^{2+}\) release, perhaps opposing the effect of PC2, which facilitates Ca\(^{2+}\) release through the IP\(_3\)R.

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common, life-threatening genetic diseases; it occurs in at least 1 in 1000 people (1, 2). It causes many large fluid-filled cysts in the kidney and often in the liver and pancreas as well. As these cysts grow, 50% of the time they result in kidney failure. ADPKD is associated with two alleles, PKD1 and PKD2, encoding gene products polycystins 1 and 2 (PC1 and PC2). Mutations of PKD1 and PKD2 are responsible for 85 and 15% of ADPKD cases, respectively.

PC1 is a 4,302-amino acid integral membrane protein that has 11 putative TM domains, a long extracellular N terminus, and a short intracellular C terminus (3). The large extracellular portion contains a novel combination of motifs originally found in other proteins that appear to be involved in cell-cell and cell-matrix interactions (3–7). The C terminus of PC1 contains a coiled-coil domain responsible for interacting with PC2 (8, 9). PC2 is a 968-amino acid integral membrane protein that functions as a Ca\(^{2+}\)-permeable nonselective cation channel (10, 11).

The ADPKD cysts probably are generated when either allele is inactivated in germ and somatic lines (10–14), producing insufficiently active polycystins. This lack of polycystin proteins has been linked to abnormal cell proliferation, apoptosis, and fluid/ion secretion, all of which contribute to cyst expansion (6, 15–20). Polycystin-deficient cells have significant abnormalities in basal Ca\(^{2+}\) levels, a situation that could contribute to all of the phenotypes of ADPKD (21–23). It therefore seems clear that insufficient polycystin expression can result in deregulation of intracellular Ca\(^{2+}\) homeostasis.

PC1 and PC2 are located in the primary cilium of kidney epithelial cells, where they sense fluid flow and convert mechanical stimulation into intracellular calcium signals (24). PC1 is also expressed in the adherens junction and focal adhesions of the plasma membrane (25, 26). Interestingly, significant amounts of polycystins have also been found in the endoplasmic reticulum (ER) (27–31). Although various studies have focused on the C-terminal tail of PC1 targeted to the plasma membrane and reported that it participates in several important signaling pathways (28, 32–36), the physiological significance of ER-localized PC1 has not been addressed in the literature.

In our previous work we found that ER-localized PC2 interacts with the ER Ca\(^{2+}\) channel, inositol 1,4,5-trisphosphate receptor (IP\(_3\)R), to promote ER Ca\(^{2+}\) release. We showed that...
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overexpression of PC2 significantly boosted IP$_3$-induced Ca$^{2+}$ release (37). Because PC1 and PC2 share some homology of amino acid sequences (38) and because mutations in either allele result in similar clinical phenotypes, we wished to ascertain whether PC1 interacts with IP$_3$R in a similar way.

Our methodology was to express fragments of PC1 with a variety of characteristics in Xenopus oocytes and in mammalian cells, to assess the function of PC1 in modulating IP$_3$R, and the very end of the C-terminal tail. To generate QIF38 and R4227X, we used the sequences corresponding to amino acids 3645–4302 of human PKD1 cDNA (accession HSU24497) followed by a FLAG epitope. They were digested out from AF20 (11) (for QIF38) and R2F (11) (for R4227X) by BsiWI and Sall and inserted into pCS2-MT vector between the StuI and Xhol sites. The construct QIF38-F3651A mutation was performed in a recoding buffer containing 96 mM NaCl, 2 mM EDTA, 10% glycerol, 2% Triton X-100, and inserted into pCS2-MT vector between the EcoRI and XhoI sites. The construct AESW was generated by site-directed mutagenesis. The forward and reverse primers are: 5'-GCTGAGCAG-3' and 5'-CTGTGGCTCGG-3'. The final PCR product was subcloned into a pRK5-HA vector between the EcoRI and XbaI sites. The construct encoding the N-terminal Myc-tagged and C-terminal FLAG-tagged QIF38 and its C-terminal truncation mutant R4227X were obtained from F. Qian. Compared with QIF38, R4227X lacks most of the coiled-coil domain and the very end of the C-terminal tail. To generate QIF38 and R4227X, we used the sequences corresponding to amino acids 3645–4302 and 3645–4227 of human PKD1 cDNA followed by a FLAG epitope. They were digested out from AF20 (11) (for QIF38) and R2F (11) (for R4227X) by BsiWI and Sall and inserted into pCS2-MT vector between the StuI and Xhol sites. The construct QIF38-F3651A mutation was generated by site-directed mutagenesis. The forward and reverse primers are: 5'-CGGCCACCCAGGGGCCTGAC-TCTTCTCCTGGCC and 5'-GGCCAGAGATGTCAGCGGC-GCTGGGGTGCCG. To generate constructs NNY38, we amplified by PCR from template AF20 with primers 5'-GTCATCGTCCTTGTA-3' and 5'-ACGCGTCGACGTGAGTG-ATACAAAGATGACCTTCA-3' and 5'-ATCGGCGGCGCCTATATGATGAGCTTTTCCAGC-3' and primers of 5'-ACCGGTACGGTGGAGTGA-TAACAAGATGACCTTCA-3' and 5'-ATCGGCGGCGCCTATATGATGAGCTTTTCCAGC-3' subcloned into a pRK5-HA vector between Sall and NotI.

**Experimental Procedures**

**Constructs**—Constructs encoding the N-terminal Myc-tagged and C-terminal FLAG-tagged QIF38 and its C-terminal truncation mutant R4227X were obtained from F. Qian. Compared with QIF38, R4227X lacks most of the coiled-coil domain and the very end of the C-terminal tail. To generate QIF38 and R4227X, we used the sequences corresponding to amino acids 3645–4302 and 3645–4227 of human PKD1 cDNA (accession HSU24497) followed by a FLAG epitope. They were digested out from AF20 (11) (for QIF38) and R2F (11) (for R4227X) by BsiWI and Sall and inserted into pCS2-MT vector between the StuI and Xhol sites. The construct QIF38-F3651A mutation was generated by site-directed mutagenesis. The forward and reverse primers are: 5'-CGGCCACCCAGGGGCCTGAC-TCTTCTCCTGGCC and 5'-GGCCAGAGATGTCAGCGGC-GCTGGGGTGCCG. To generate constructs NNY38, we amplified by PCR from template AF20 with primers 5'-GTCATCGTCCTTGTA-3' and 5'-ACGCGTCGACGTGAGTG-ATACAAAGATGACCTTCA-3' and 5'-ATCGGCGGCGCCTATATGATGAGCTTTTCCAGC-3' and primers of 5'-ACCGGTACGGTGGAGTGA-TAACAAGATGACCTTCA-3' and 5'-ATCGGCGGCGCCTATATGATGAGCTTTTCCAGC-3' subcloned into a pRK5-HA vector between Sall and NotI.

**Oocyte Methods and Confocal Ca$^{2+}$ Imaging with Oocytes**—Synthetic mRNAs were prepared using the SP6 MEGAScript kit according to the manufacturer's protocol. Stage V and VI defolliculated Albino Xenopus oocytes were injected with 50 ng of QIF38 mRNA or its mutants and cultured in 50% L-15 medium for 3–6 days at 16 °C (39). To perform a Ca$^{2+}$ imaging assay in oocytes, the oocytes were first co-injected with fluorescent Ca$^{2+}$ indicator Oregon Green II (final concentration, 12.5 μM) and 1,2-nitrophenyl)-ethyl-caged IP$_3$ (final concentration, 5 μM) 60 min before imaging. Ca$^{2+}$ release was initiated by photolysis of caged IP$_3$ using a UV laser (405 nm). Ca$^{2+}$ images were acquired at a rate of 0.5 s/frame on a confocal laser scanning microscope (model LSM510; Zeiss, Oberkochen, Germany) using a 10× objective (numerical aperture = 0.50) at zoom 1. Imaging was performed in a recoding buffer containing 96 mM NaCl, 2 mM KCl, 2 mM MgCl$_2$, 5 mM HEPES, pH 7.5, 1 mM EGTA.

**Cell Culture and Transient Transfection**—HEK-293 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 10% fetal calf serum and 50 units/ml penicillin and 50 μg/ml streptomycin in a humidified atmosphere of 5% CO$_2$, 95% air. When the cells became 90% confluent, the plasmids were transfected into cells by Lipofectamine 2000 according to the manufacturer's instructions. Stable MDCK$^zeo$ (F6) and MDCK$^{PKD1zeo}$ (C8/68) cell lines were cultured as described before (16). For transient transfection in MDCK cell lines, the cells were co-transfected with the GFP vector and HA-tagged IP$_3$R 226–604 plasmids several days after it became confluent to ensure complete polarization. Transfection with only GFP vector plasmid was used as control.

**Western Blot**—The oocytes were incubated on ice for 30 min with Triton X-100 lysis buffer (20 mM Tris·HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 2% Triton X-100 supplemented with a protease inhibitor mixture). After homogenization, the samples were centrifuged for 15 min at 4,500 × g, and the supernatants were collected. HEK-293 cells were solubilized in Nonidet P-40 lysis buffer (50 mM NaCl, 150 mM Tris·HCl, pH 7.4, 1% Nonidet P-40, and complete protease inhibitor). The cell lysates were spun at 14,000 × g for 15 min at 4 °C to pellet insoluble material. The protein concentrations of the supernatants were quantified with a BCA protein assay kit.

**After incubation in Laemmlı buffer at 42 °C for 30 min, the protein samples were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. Mouse anti-Myc monoclonal antibody (1:1,000) was used for QIF38 detection.**
After probing with horseradish peroxidase-conjugated sheep anti-mouse IgG secondary antibody (1:10,000), Western blots were visualized by enhanced chemiluminescence.

Endo-H and PNGase F Sensitivity Assay—Mature embryonic fibroblasts were derived from the wild type (PKD1+/+T) E12 embryo. Endogenous PC1 was detected by a combination of immunoprecipitation and Western blotting using PC1-CC antibody directed against the cytoplasmic C-terminal tail of mouse PC1 as described previously (40). Endo-H and PNGase F sensitivity assays were carried out according to the manufacturer’s instructions.

Co-immunoprecipitation Assay—For co-IP of QIF38 with IP3R-1, HEK-293 cells were transiently co-transfected with full-length IP3R (IP3R-1) and QIF38. Cells transfected with full-length IP3R-1 alone were the negative control. Note that the QIF38 is N-terminally Myc-tagged and C-terminally FLAG-tagged. The cells were solubilized in the Nonidet P-40 lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, complete protease inhibitors). For co-IP of QIF38 with HA-tagged domains of IP3R, HEK-293 cells were co-transfected with HA-tagged domains of IP3R and QIF38 or R4227X. Cells that were transfected with the various domains of IP3R (tagged with HA) alone served as the negative control. The cells were then lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, complete protease inhibitors). The whole cell lysates were spun at 14,000 g for 15 min at 4 °C to pellet the insoluble material. Total protein concentrations of the supernatants were quantified with a BCA protein assay kit and diluted into 1 mg/ml pluronics/DMSO and then diluted into 5 μM with Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium. MDCK cells were loaded with fura-2/AM at room temperature for 90–120 min. Following incubation, the cells were allowed to recover for 30 min in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium. The glass cover slips were then placed in a perfusion chamber, which was mounted on the stage of a Zeiss inverted microscope equipped with a Nikon Fluor 40× oil immersion lens, a Sutter Lambda 10-2 controller and filter wheel assembly. For ATP stimulation experiments, the cells were first perfused with a buffer containing 140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 2 mM MgCl2, and 10 mM HEPES, pH 7.4, and were then switched to be exposed to 100 μM ATP diluted in the same buffer. All of the perfusions were performed at room temperature at 2 ml/min to ensure rapid turnover of the 0.5-ml bath volume. A Zeiss FluorArc mercury lamp was used to excite the cells at 340 and 380 nm, and the emission response was measured at 510 nm. Cell fluorescence was measured in response to excitation for 100 ms at 340 nm and 20 ms at 380 nm once every 3 s. Image acquisition, image analysis, and filter wheel control were performed by IPLab software.

Imaging Analysis—Ca2+ images were analyzed using the public domain National Institutes of Health ImageJ program.

Materials—The Myc monoclonal antibody is from Santa Cruz Biotechnology (Santa Cruz, CA). FLAG monoclonal antibody conjugated beads (M2 beads) were used to pull down the immunocomplexes. After rotating for overnight at 4 °C, the beads were spun down and washed three times with lysis buffer. The immunocomplexes were then incubated in Laemmli buffer at 42 °C for 30 min and resolved by SDS-PAGE. After being transferred onto a polyvinylidene difluoride membrane, the following primary antibodies were used to detect the co-IP signal; IP3R3 monoclonal antibody (1:1000) was used to detect full-length IP3R-1, and HA monoclonal antibody (1:1000) was used to detect HA-tagged domains of IP3R. After probing with horseradish peroxidase-conjugated sheep anti-mouse or -rabbit IgG secondary antibody (1:10,000), Western blots were visualized by enhanced chemiluminescence. For co-IP of QIF38 with endogenous type-3 IP3R in HEK-293 cells, the cells were transfected with QIF38 alone and solubilized in Nonidet P-40 buffer as described above. Monoclonal anti-type-3 IP3R was used to pull down the immunocomplexes. IP3R with unrelated HA monoclonal antibody was the negative control. Mouse anti-Myc monoclonal antibody (1:1000) was used for QIF38 detection.

Immunostaining Assay—HEK-293 cells were transiently transfected with QIF38, R4227X, AESW, RWRYH, or co-transfected with QIF38 and IP3R-1. After 24 h, the cells were washed with PBS and fixed with 4% paraformaldehyde. After permeabilization with 0.3% Triton X-100, the cells were washed with PBS and subsequently blocked in 5% normal goat serum for 1 h at room temperature. The cells were incubated with the primary antibody (rabbit anti-PC1-CT, 1:100; rabbit anti-FLAG, 1:100; rabbit anti-IP3R type-1 antibody, 1:100; or mouse anti-Myc, 1:500) overnight at 4 °C. After sustained washing with PBS, the cells were probed with corresponding secondary antibodies, Cy3-conjugated donkey anti-rabbit antibody (1:500 or 1:1000 as indicated) or Alexa488-conjugated goat anti-mouse antibody (1:200) for 1 h at room temperature. After being washed thoroughly in PBS, the coverslips were mounted with anti-quenching medium, and then the slides were sealed and examined with a confocal microscope (model LSM510; Zeiss).

Fura-2 Ca2+ Imaging Assay—MDCK cells were cultured on glass coverslips for 3–5 days after becoming confluent. The cells were loaded with cell-permeant acetoxyethyl (AM) ester of the calcium indicator fura-2 (fura-2/AM). Fura-2/AM was first dissolved in 1 mg/ml pluronics/DMSO and then diluted into 5 μM with Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium. MDCK cells were loaded with fura-2/AM at room temperature for 90–120 min. Following incubation, the cells were allowed to recover for 30 min in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium. The glass coverslips were then placed in a perfusion chamber, which was mounted on the stage of a Zeiss inverted microscope equipped with a Nikon Fluor 40× oil immersion lens, a Sutter Lambda 10-2 controller and filter wheel assembly. For ATP stimulation experiments, the cells were first perfused with a buffer containing 140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 2 mM MgCl2, and 10 mM HEPES, pH 7.4, and were then switched to be exposed to 100 μM ATP diluted in the same buffer. All of the perfusions were performed at room temperature at 2 ml/min to ensure rapid turnover of the 0.5-ml bath volume. A Zeiss FluorArc mercury lamp was used to excite the cells at 340 and 380 nm, and the emission response was measured at 510 nm. Cell fluorescence was measured in response to excitation for 100 ms at 340 nm and 20 ms at 380 nm once every 3 s. Image acquisition, image analysis, and filter wheel control were performed by IPLab software.

Statistics Analysis—Statistical significance was determined by two-tail Student t test and accepted as p < 0.05. In all of the figures, the error bars represent the standard error.
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![Diagram of Polycystin-1 and IP$_3$R interaction]

**RESULTS**

**Expression of a PC1 Fragment (QIF38) Reduces IP$_3$-dependent Ca$^{2+}$ Release**—We generated a construct, QIF38, which encodes the last six TM domains of PC1 and its C-terminal intracellular tail, a PC1 segment that shares a moderate degree of sequence homology with PC2. We tagged the N terminus of QIF38 with Myc and its C terminus with FLAG. To specifically assess the role of the C terminus of PC1, we generated a truncated mutant, R4227X, that is associated with ADPKD and that lacks most of the coiled-coil domain and the very end of the C-terminal tail (38) (Fig. 1A).

We applied IP$_3$ to *Xenopus* oocytes in the absence of extracellular Ca$^{2+}$ and assessed cytosolic Ca$^{2+}$ signals with a confocal Ca$^{2+}$ imaging system (37). As can be seen in Fig. 1B, application of IP$_3$ to *Xenopus* oocytes results in a rapid increase in intracellular Ca$^{2+}$ followed by a slower decay. To understand how PC1 might modulate intracellular Ca$^{2+}$, we evaluated both the peak amplitude and the slower decay of the transient response to IP$_3$. Compared with the controls that were injected with H$_2$O, QIF38 expression significantly reduced the amplitude of the IP$_3$-induced transient increase in cytosolic Ca$^{2+}$. In contrast when R4227X was expressed, the amplitude of the transient increase in Ca$^{2+}$ did not differ significantly from the H$_2$O-injected controls (Fig. 1, B and C). In parallel, we also calculated the half-decay time ($T_{1/2}$) of the Ca$^{2+}$ transients and found that there was no statistically significant difference among the groups (Fig. 1C). We verified that the expression levels of QIF38 and R4227X are similar (Fig. 1C). In addition, there was no change in the expression of the endogenous type-1 IP$_3$R (IP$_3$R-1) that could account for the phenotype associated with QIF38 expression (data not shown).

In the oocyte system, the initial amplitude of the transient increase in cytosolic Ca$^{2+}$ is the combined effect of the release of Ca$^{2+}$ from the ER through the IP$_3$R, and the reuptake of Ca$^{2+}$ through the Ca$^{2+}$ pump. On the other hand, the $T_{1/2}$ of the slow decay is primarily determined by the reuptake of Ca$^{2+}$ back into the ER through the Ca$^{2+}$ pump. Thus, the reduced initial amplitude accompanied with an unchanged $T_{1/2}$ suggests that QIF38 reduces the IP$_3$-dependent Ca$^{2+}$ release through inhibition of IP$_3$R. The differential responses between QIF38 and R4227X indicate that the coiled-coil domain/C-terminal tail is essential for the function of QIF38.

**QIF38 Physically Interacts with IP$_3$R**—To ascertain whether QIF38 physically associates with IP$_3$R, we co-transfected HEK-293 (human embryonic kidney cells) with QIF38 and IP$_3$R-1. HEK-293 cells transfected with IP$_3$R-1 alone served as control. Compared with IP$_3$R-1 transfection alone, co-transfection of IP$_3$R-1 and QIF38 dramatically reduced the expression of the exogenous IP$_3$R-1, a phenomenon commonly observed in double transfection experiments, but nevertheless, the biochemical co-IP assay still showed a strong association between QIF38 and the endogenously expressed type-1 IP$_3$R (Fig. 2A).

Next we transfected HEK-293 cells with QIF38 alone and performed a co-IP experiment by pulling down the endogenous type-3 IP$_3$R, an abundant subtype in HEK-293 cells. Co-IP results showed that QIF38 significantly associated with the endogenously expressed type-3 IP$_3$R (Fig. 2B).

In addition, an immunostaining assay shows that QIF38 and R4227X both co-localize with IP$_3$R on a classical perinuclear ER
network (Fig. 3, A and B). We next asked whether there is any ER-resident pool of endogenous PC1 in vivo. We obtained murine embryonic fibroblasts from wild type (Pkd1/H11001/H11001) E12 embryos and investigated for the sensitivity of endogenous PC1 to Endo-H and PNGase F treatment (Fig. 3C). By a combination of immunoprecipitation and Western blot with PC1-CC antibody, we found that endogenous PC1 is extensively cleaved at the GPS site into a C-terminal fragment (CTF) band (150 kDa), as previously described (40). The remaining full-length PC1 (FL) yields a weak band in the untreated sample (500 kDa, as shown by the arrowhead in Fig. 3C, first lane) and became hardly detectable after treatment with either Endo-H.
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or PNGase F. It has been shown that CTF is the predominant functional product of PC1 (40, 41). Here, we demonstrated that Endo-H treatment resulted in the appearance of a doublet of CTF fragments of equal intensity (indicated by arrows in Fig. 3C, second lane). The upper band of the doublet has the size of CTF that is untreated (Fig. 3C, first lane) and is thus Endo-H-resistant, whereas the lower band migrates to the same position as the major product treated with PNGase F (Fig. 3C, third lane) and is thus Endo-H-sensitive. We interpret the appearance of the latter (Endo-H-sensitive CTF band) as an indication of significant fraction of polycystin-1 fragment present in the ER in vivo.

Recent reports indicate that PC1 contains a consensus Homer-binding motif PPXXXYFA and endogenous PC1 physically associates with Homer (42). Homer has been demonstrated to be the adapter protein that facilitates the physical interaction between the TRPC1 channel and IP₃R (43). To establish whether the Homer-binding motif is essential for the interaction between IP₃R and QIF38, we generated a point mutation QIF38F-A (F3651A) to destroy the consensus motif and a shorter version, NNY38, which omits the entire motif (Fig. 4A). We noticed that the mobility of point mutation QIF38F-A in the gel is different from that observed with QIF38. However, the functions of both constructs (QIF38F-A and NNY38) were similar to that of QIF38 (Fig. 4B), demonstrating that neither the mutation nor the truncation had any adverse effects. The immunoprecipitation results shown in Fig. 4B also confirm that Homer is not involved in the interaction of IP₃R and QIF38.

QIF38 Interacts with the Ligand-binding Domain of IP₃R—The next question was how the interaction of QIF38 with IP₃R inhibits Ca²⁺ release. To answer this, we tried to find the binding domain(s) of IP₃R important for this interaction. The fact that QIF38 associates with both type-1 and -3 IP₃R suggests that the possible binding domain should be in their conserved region. Type-1 and -3 IP₃R share the highest sequence similarity in their N-terminal ligand-binding region as well as the C-terminal channel-forming region (44). Interestingly, the N-terminal region of IP₃R includes a ligand-binding domain and a suppressor domain. Interference with either domain is capable of inhibiting IP₃-mediated Ca²⁺ release. Therefore, we asked whether any of these above domains is able to interact with QIF38. We generated two HA-tagged constructs: HA-IP₃R 226–604 encoding the IP₃-binding domain of IP₃R (amino acids 226–604) and construct HA-IP₃R 1–225 encoding the suppressor domain of IP₃R (amino acids 1–225) (Fig. 5A). We separately co-transfected either construct into HEK-293 cells with QIF38. Our co-IP results show that QIF38 only associates with the IP₃-binding domain of IP₃R and not with the suppressor domain (Fig. 5B).

We next asked whether the coiled-coil/C-terminal tail of QIF38 serves as the site of the physical interaction with IP₃R. We co-transfected R4227X (lacking the coiled-coil and C terminus of PC1) and the IP₃-binding domain of IP₃R (amino acids 226–604) into HEK-293 cells. Our co-IP results indicate that R4227X cannot associate with the IP₃-binding domain of IP₃R (Fig. 5C), thereby explaining why R4227X does not function as does QIF38 when expressed in Xenopus oocytes. From all of these results so far, we concluded that the C terminus of PC1 carries the essential domains for both functional and physical interaction with the IP₃-binding domain of IP₃R.

PCI-1 C Terminus Resides in the ER, and Its ER Localization Is Important for the IP₃ Inhibitory Function—To establish which portions of QIF38 are sufficient for its function, we generated a construct, AESW, which included only the last TM domain and the entire C-terminal tail (Fig. 6, A and B). As with QIF38,
expression of AESW in Xenopus oocytes significantly reduced the initial amplitude of IP_3-dependent Ca^{2+} release (Fig. 6A). This result implies that localization in the ER is essential for the function of PC1 C-terminal fragment.

Full-length PC1 Expression in Polarized MDCK Cells Reduced the Ca^{2+} Response for ATP—To confirm that QIF38 does in fact represent some physiological functions of full-length PC1, we tested whether expression of full-length PC1 in polarized kidneys epithelial cells would have the same effect as QIF38 seen in the Xenopus oocyte system. We employed a fura-2 ratiometric Ca^{2+} imaging assay to measure intracellular Ca^{2+} release in polarized MDCK cells (Madin-Darby canine kidney epithelial cells) that have stable expression of full-length PC1 (16, 45). MDCK (MDCK^{zeo}, F6) is known to form cysts when grown in a three-dimensional collagen gel and to have endogenous PC2. It has been shown that upon PC1 expression, MDCK cells (MDCK^{PKD1^{zeo}}, C8/68) will undergo spontaneous tubulogenesis (16). Bathing MDCK cells with extracellular ATP activates the purinergic receptor, which in turn increases the production of IP_3, thus causing Ca^{2+} release through IP_3R (45).

We found that in the presence of full-length PC1, the release of Ca^{2+} in response to ATP stimulation is decreased (Fig. 7, A and B). This result is consistent with that obtained with QIF38 in the Xenopus oocyte system.

Overexpression of the Ligand-binding Domain of IP_3R Rescued Full-length PC1 Inhibition of ATP-stimulated Ca^{2+} Release—To further investigate whether the reduced Ca^{2+} release in response to ATP in polarized PC1-expressing MDCK cells is mainly due to the interaction between PC1 with IP_3R, we overexpressed the IP_3R ligand-binding domain in MDCK cells and again measured intracellular Ca^{2+} release following exposure to ATP. We reasoned that if PC1 interacts with the ligand-binding domain of IP_3R, overexpressing this fragment (IP_3R 226–604) should competitively bind to PC1 and disrupt its interaction with the endogenous IP_3R. This would in turn relieve the inhibitory effect of PC1 and restore Ca^{2+} release from IP_3-sensitive stores.

We co-transfected the IP_3R ligand-binding domain (IP_3R 226–604) together with GFP into polarized MDCK cells...
and measured the release of intracellular Ca\(^{2+}\) in response to stimulation by ATP in GFP positive cells. GFP was used as a marker for transfected cells. As expected, overexpression of the IP3R fragment 226–604 in PC1-expressing MDCK cells significantly increased the initial amplitude of the ATP-induced transient increase in Ca\(^{2+}\) compared with GFP alone, the transfection control (Fig. 7C, compare last two bars, C8 G6/H11001 GFP and C8 G6/H11001 226–604). In contrast, we also noticed that overexpression of the same fragment in control MDCK cells slightly decreased the Ca\(^{2+}\) transient (Fig. 7C, first two bars, F6 G6/H11001 GFP and F6 G6/H11001 226–604), probably because of the buffering effect of the 226–604 fragment on IP3 as has been reported previously (46). Our results thus support the notion that the 226–604 fragment most likely works through the competitive binding to PC1 to interfere with interaction between PC1 and endogenous IP3R. Therefore, these data support the model that full-length PC1 can inhibit Ca\(^{2+}\) release mediated by IP3R through interaction with the ligand-binding domain of IP3R.


discussion

Previously we had found that PC2 interacts with the IP3R to enhance the IP3-induced release of ER Ca\(^{2+}\) stores (37).
Because PC1 and PC2 have some homology within their transmembrane domains and because mutations affecting either polycystin result in similar clinical phenotypes, we originally hypothesized that, like PC2, PC1 interacts with IP$_3$R to modulate Ca$^{2+}$ signaling. This present work indeed found that PC1 and PC2 both modulate Ca$^{2+}$ release through IP$_3$R, but not in the same manner. It is apparent that the regulation of Ca$^{2+}$ signaling is more subtle than previously thought.

Direct measurement of Ca$^{2+}$ release through IP$_3$R in the Xenopus oocytes shows that expression of a PC1 fragment homologous to PC2 has an inhibitory effect on Ca$^{2+}$ release. This phenotype is conserved in the mammalian cell system with heterologous expression of full-length PC1. MDCK cells stably expressing full-length PC1 consistently show reduced Ca$^{2+}$ release from the ER in response to ATP. However, a similar study done by Hooper et al. (45) yielded a different conclusion from ours. They suggest that expression of PC1 in MDCK cells enhances reuptake into the ER instead of reducing the release of Ca$^{2+}$. This discrepancy might be related to the difference in the experimental condition of MDCK polarization. Polarization of epithelial cells can change the expression and localization and thus the function of proteins (47). As for our case, we used polarized MDCK cells to mimic the more physiologically relevant conditions found in the kidney. In fact, in vivo data from polarized kidney epithelial cells derived from normal mice also exhibit a smaller increase in cytosolic Ca$^{2+}$ in response to thrombin, an IP$_3$ agonist, than that of PKD1$^{-/-}$ mice (24).

In parallel, several efforts have been made to study the function of PC1 through investigation of a fusion protein that fused the PC1-C-terminal tail with the CD7 transmembrane or other transmembrane domains to locate it into the plasma membrane (28, 32–36). The overexpression of the plasma membrane-targeted C-terminal tail of PC1 stimulates GPCR to increase Ca$^{2+}$ release (32). It has been suggested that when the C-terminal tail of PC1 is located at the plasma membrane it behaves in a dominant negative manner (28, 34, 48) to inhibit the normal functioning of the native, full-length protein. In sharp contrast to these above studies, we expressed a PC1-C-terminal tail with its own native TM domains and found that various PC1 C-terminal fragments (QIF38, NNY38, and AESW) localize predominantly to the ER and inhibit IP$_3$-induced Ca$^{2+}$ release. We further demonstrated that PC1 fragments interact with the IP$_3$R ligand-binding domain through the very end of its C-terminal tail and that ER localization is essential for this interaction. Removal of TM domains of PC1 completely abolishes its inhibitory effect by mislocalizing the fragment from the ER to the cytoplasm. Furthermore, we confirmed that full-length PC1 interacts with IP$_3$R to reduce the IP$_3$-dependent release of Ca$^{2+}$ in MDCK cells, and this inhibitory effect can be reversed by overexpression of the ligand-binding domain of IP$_3$R. Thus, we conclude that full-length PC1 functions through the same mechanism as the shorter C-terminal fragments via an interaction with the IP$_3$R ligand-binding domain to reduce the release of Ca$^{2+}$ from the ER.

Whether PC1 is located and functions in the ER is still a much debated subject. Despite the controversy, there is compelling evidence supporting the notion that a significant amount of PC1 is localized in the ER. Newby et al. (31) have demonstrated that endogenous PC1 is Endo-H-sensitive in mice and human kidney cells. Another study by Grimm et al. (49) also documents ER co-localization of PC1 with PC2 in the mouse-proximal tubule cells. These findings correlate with our immunostaining data where we observed co-localization in the ER of C-terminal fragments of PC1 with the IP$_3$R. Moreover, our Endo-H sensitivity assay with protein samples obtained from wild type murine embryonic fibroblasts has also provided evidence for the presence an ER-resident pool of endogenous CTF (the cleavage form of PC1) in vivo. Thus, it is very possible that although full-length PC1 can traffic into different cell compartments including the primary cilium and the cell surface, some of the shorter cleavage form(s) of PC1 stay in the ER and have some functional interaction with ER-resident proteins such as IP$_3$R.

In addition, it is interesting to note that the IP$_3$-binding core of IP$_3$R contains an $\alpha$-helical domain that shares great structural similarity with $\beta$-catenin (50). $\beta$-Catenin has been shown to interact with PC1 to form a macromolecular complex at the adhesion junction of cells (25). The similar characteristics of the IP$_3$R-PC1 complex and the $\beta$-catenin-PC1 complex on their distinct membranes suggest that different subcellular pools of PC1 may interact with various partners for different functions.

Polycystins also interact with other key components in Ca$^{2+}$ signaling. Recently it has been reported that PC2 interacts with the ryanodine receptor, another intracellular Ca$^{2+}$ release channel, to inhibit its channel activity (51). Therefore, the presence of both polycystins may be essential for normal regulation of intracellular Ca$^{2+}$ homeostasis. In the absence of either one, the loss of modulating inhibitory and/or enhancing effects on IP$_3$R and ryanodine receptor might contribute to abnormal basal levels of Ca$^{2+}$. Any of these misregulations of intracellular Ca$^{2+}$ signaling could eventually lead to pathological cysts. It has been shown that if the PC1 level is either too low (52, 53) or too high (54, 55), cysts can develop. In relation to our study, the loss or mutation of PC1 can increase the level of IP$_3$-induced Ca$^{2+}$ release or deregulation of Ca$^{2+}$ signaling, which might in turn induce cell proliferation (48, 56), fluid secretion (28, 34), and cell apoptosis (57). Cell overproliferation, fluid secretion, and apoptosis have all been described in the cystogenesis process (58–61).

In summary, PC1 plays a novel role in the regulation of IP$_3$R to modulate intracellular Ca$^{2+}$ signaling. Together with our previous studies, we propose that PC1 and PC2 interact with IP$_3$R in a complementary way to maintain Ca$^{2+}$ homeostasis. We observed that although PC2 facilitates IP$_3$-dependent transient increases in cytosolic Ca$^{2+}$, the coiled-coil/C terminal section of PC1 binds to the IP$_3$R ligand-binding site to inhibit such an event, perhaps through masking of the binding of IP$_3$ to IP$_3$R. PC1 can only provide its inhibitory function when its TM domain is inserted in the ER membrane. Our results provide a better understanding of how mutations in either PC1 or PC2 can result in the dysregulation of intracellular Ca$^{2+}$ homeostasis and the associated pathology of ADPKD.
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