Polycystin-1 Activates the Calcineurin/NFAT (Nuclear Factor of Activated T-cells) Signaling Pathway*

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Sanjeev Puri‡§, Brenda S. Magenheimer‡, Robin L. Maser‡, Erin M. Ryan‡, Christopher A. Zien‡, Danielle D. Walker‡, Darren F. Wallace‡, Scott J. Hempson‡, and James P. Calvet‡

From the ‡Department of Biochemistry and Molecular Biology, §Department of Internal Medicine, and the Kidney Institute, University of Kansas Medical Center, Kansas City, Kansas 66160 and the ‡Department of Biochemistry, Panjab University, Chandigarh 160014, India

Regulation of intracellular Ca²⁺ mobilization has been associated with the functions of polycystin-1 (PC1) and polycystin-2 (PC2), the protein products of the PKD1 and PKD2 genes. We have now demonstrated that PC1 can activate the calcineurin/NFAT (nuclear factor of activated T-cells) signaling pathway through Go₃-mediated activation of phospholipase C (PLC). Transient transfection of HEK293T cells with an NFAT promoter-luciferase reporter demonstrated that membrane-targeted PC1 constructs containing the membrane proximal region of the C-terminal tail, which includes the heterotrimeric G protein binding and activation domain, can stimulate NFAT luciferase activity. Inhibition of glycogen synthase kinase-3β by LiCl treatment further increased PC1-mediated NFAT activity. PC1-mediated activation of NFAT was completely inhibited by the calcineurin inhibitor, cyclosporin A. Cotransfection of a construct expressing the Go₃ subunit augmented PC1-mediated NFAT activity, whereas the inhibitors of PLC (U73122) and the inositol trisphosphate and ryanodine receptors (xestospongin and 2-aminophenylborate) and a nonspecific Ca²⁺ channel blocker (gadolinium) diminished PC1-mediated NFAT activity. PC2 was not able to activate NFAT. An NFAT-green fluorescent protein nuclear localization assay demonstrated that PC1 constructs containing the C-tail only or the entire 11-transmembrane spanning region plus C-tail induced NFAT-green fluorescent protein nuclear translocation. NFAT expression was demonstrated in the M-1 mouse cortical collecting duct cell line and in embryonic and adult mouse kidneys by reverse transcriptase-PCR and immunolocalization. These data suggest a model in which PC1 signaling leads to a sustained elevation of intracellular Ca²⁺ mediated by PC1 activation of Go₃ followed by PLC activation, release of Ca²⁺ from intracellular stores, and activation of store-operated Ca²⁺ entry, thus activating calcineurin and NFAT.

Autosomal dominant polycystic kidney disease (PKD)¹ is one of the most common genetic disorders worldwide, affecting 1 in 200–1,000 individuals (1–7). The primary pathology in autosomal dominant polycystic kidney disease involves the formation and growth of numerous fluid-filled cysts in the kidney. Other manifestations include hepatic and pancreatic cysts, cardiac valve defects, intracranial and aortic aneurysms, inguinal hernia, and colonic diverticulae (8–12). Approximately 85% of individuals with autosomal dominant polycystic kidney disease carry mutations in the PKD1 gene, with the remaining cases caused by mutations in the PKD2 gene.

The product of the PKD1 gene, polycystin-1 (PC1), is a plasma membrane protein consisting of a large extracellular N-terminal region followed by 11 membrane-spanning domains and a cytoplasmic C terminus of ~200 amino acids (6, 13, 14). PC1 is thought to be a signaling receptor involved in cell-cell or cell-matrix interactions (15–17); however, its large size and multimembrane-spanning structure would suggest that it has multiple functions. The PKD2 gene product, polycystin-2 (PC2), has been shown to be a Ca²⁺-sensitive nonspecific cation channel whose activity may be regulated by direct or indirect interaction with the C-terminal cytoplasmic tail of PC1 (5, 18–22). Recently, PC1 and PC2 have been shown to have a role in ciliary mechanosensory Ca²⁺ entry (23).

Numerous studies have suggested that defects in the regulation of epithelial cell growth are involved in the cyst-forming process, because epithelial cells that line cysts are both hyperproliferative and hyperapoptotic (1, 24–26). As cysts expand, there is also a conversion of tubular epithelial cells from a non-proliferating to a proliferating phenotype (36). It has been shown recently that vascular smooth muscle cells from Pkd2 heterozygotes have impaired intracellular Ca²⁺ regulation, which may underlie the vascular phenotype (36).

Studies employing direct pull-downs and transfected cells have shown that the PC1 C-tail binds and activates heterotrimeric G proteins (37), activates c-Jun N-terminal kinase and the AP-1 transcription factor in a G protein-dependent fashion and PC2, polycystin-1 and polycystin-2, respectively; GSK-3β, glycogen synthase kinase-3β; STAT, signal transducers and activators of transcription; NFAT, nuclear factor of activated T-cells; CSA, cyclosporin A; HEK, human embryonic kidney; DMEM, Dulbecco’s modified Eagle’s medium; aa, amino acid; HA, hemagglutinin; ANOVA, analysis of variance; GFP, green fluorescent protein; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; RT, reverse transcriptase; IP3, inositol trisphosphate; PLC, phospholipase C; 2-APB, 2-aminophenylborate; GAD, gadolinium; 11TM, 11 transmembrane.

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| To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology and the Kidney Institute, University of Kansas Medical Center 3030, 3901 Rainbow Blvd., Kansas City, KS 66160. Tel.: 913-588-7424; Fax: 913-588-7440; E-mail: jcalvet@kumc.edu.

| The abbreviations used are: PKD, polycystic kidney disease; PC1 and PC2, polycystin-1 and polycystin-2, respectively; GSK-3β, glycogen synthase kinase-3β; STAT, signal transducers and activators of transcription; NFAT, nuclear factor of activated T-cells; CSA, cyclosporin A; HEK, human embryonic kidney; DMEM, Dulbecco’s modified Eagle’s medium; aa, amino acid; HA, hemagglutinin; ANOVA, analysis of variance; GFP, green fluorescent protein; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; RT, reverse transcriptase; IP3, inositol trisphosphate; PLC, phospholipase C; 2-APB, 2-aminophenylborate; GAD, gadolinium; 11TM, 11 transmembrane.

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Polycystin-1 Ca$^{2+}$ Signaling

(38–40), activates protein kinase C (38), and modulates Wnt signaling by inhibiting glycogen synthase kinase-3β (GSK-3β) and stabilizing β-catenin (41). PC1 overexpression has also been shown to activate the JAK2/STAT1 pathway leading to the up-regulation of p21(waf1) and potentially to cell cycle arrest (42).

Although it is currently thought that the polycystins are involved in regulating intracellular Ca$^{2+}$ levels (16, 18–23), neither the ability of the polycystins to lead to sustained Ca$^{2+}$ increases nor the identity of downstream targets of these Ca$^{2+}$ signals has been demonstrated. Transient intracellular Ca$^{2+}$ increases are associated with cellular functions such as muscle contraction, synaptic transmission, or neuroendocrine secretion. In contrast, sustained Ca$^{2+}$ signals are known to affect transcriptional events leading to adaptive cellular changes and to changes in cell proliferation and cell differentiation (43–47). A cellular target for sustained increases in Ca$^{2+}$ is calcineurin, a ubiquitous serine-threonine phosphatase (48). An important intracellular substrate for calcineurin is NFAT (nuclear factor of activated T-cells) (49). In its inactive hyperphosphorylated form, NFAT is sequestered in the cytosol. Signals causing sustained Ca$^{2+}$ increases result in calcineurin activation, dephosphorylation of NFAT, and translocation of NFAT to the nucleus where it regulates target genes, often at composite NFAT/AP-1 elements (49, 50).

The termination of NFAT signaling occurs through rephosphorylation of NFAT by GSK-3β (51, 52), resulting in its return to the cytoplasm. The immunosuppressive drugs, cyclosporin A (CSA) and FK506, inhibit calcineurin and thus nuclear translocation of NFAT (50, 53). Five NFAT isoforms have been isolated, of which four (NFAT cl–c4) are calcineurin-sensitive (49, 54). Activation of calcineurin/NFAT signaling has been shown to regulate cell differentiation, apoptosis, and cellular adaptation in a wide variety of cell types and tissues (55) and to regulate the non-canonical Wnt/Ca$^{2+}$ pathway during embryonic development (56). In attempting to identify a connection between polycystin function and Ca$^{2+}$ signaling, we determined that PC1 is able to activate a pathway that leads to calcineurin activation and translocation of dephosphorylated NFAT to the nucleus. The activation of NFAT by PC1 suggests that polycystin-1 may function to integrate Ca$^{2+}$ signals at NFAT target genes.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and DNA Constructs—**HEK293T cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5% glucose and L-glutamine supplemented with 10% heat-inactivated fetal bovine serum (Hyclone) and penicillin/streptomycin. M-1 mouse cortisol-collagen duct cells (57) were maintained in DMEM/F12 medium supplemented with 5% fetal bovine serum. Mouse PC1 C-tail constructs were subcloned downstream of sIg (CD5 signal peptide, CH2-CH3 domains) in pcDNA1.1 and pcDNA3.1 (Invitrogen) as described earlier (37, 39). As shown in Fig. 1, the C-tail constructs included the C-terminal 222 aa of PC1 (PC1-LT), the C-terminal 193 aa of PC1 (PC1-HI), the C-terminal 120 aa of PC1 (PC1-AT), which lacks the heterotrimeric G protein activation domain but lacks the coiled-coil region. A control construct (sIgG-0) consisted of sIgG only. The PC1–11TM construct (Fig. 1) was constructed from the 3' region of a mouse PC1 cDNA (13, 39, 58) containing all of the 11 transmembrane domains and the C-tail (aa 3010–4293). This region was subcloned downstream of the Cd5 signal sequence and the CH2-CH3 IgG domains in pcDNA1.1 and pcDNA3.1 (13, 39). As a control for HA-PC2, a stop codon was introduced into a pcDNA3.1, just past the first transmembrane domain (sIgG-stop). HA-tagged and Myc-tagged PC2 constructs (59) were obtained from Dr. L. Tsiokas (University of Oklahoma Health Sciences Center). As a control for HA-PC2, a stop codon was introduced, giving rise to a construct encoding aa 1–379 (PC2-stop). EE-tagged Goa in pcDNA1.3 was obtained from Guthrie cDNA Resource Center (39). cis-acting 4× pNFAT-luciferase and pBlueScript were from Stratagene. The pNFAT-luciferase construct has a TATA box and four 30-bp repeats containing the composite ARRE-2 site from the human interleukin-2 promoter sequence: 5-GGAGGAAA-

**NFAT Luciferase Assay—**HEK293T cells were plated at a density of ~7.5 × 10^5 cells/well in a six-well plastic plate in DMEM plus 10% heat-inactivated fetal bovine serum. 24 h after plating, the cells were transiently transfected with the Ca$^{2+}$-phosphate precipitation method (61). A total of 3 μg of plasmid DNA was used to transfect each well, which contained 50–500 ng of PC1 DNA or control construct, 100 ng of the ARRE-2 NFAT/AP-1 promoter-reporter construct (firefly luciferase), 1.5–5 ng of Renilla luciferase-null, pBlueScript as filler DNA, and, where indicated, 50 ng of EE-tagged Goa. 6 h post-transfection, the medium was replaced with serum-free DMEM and the cultures were incubated for an additional 20 h. Inhibitors were added 4 h before harvesting. The cells were lysed in Passive lysis buffer (Promega), and 20 μl of cell lysate was used with the dual luciferase assay kit (Promega) using an EG&G Berthold 9507 Lumimeter. Data were analyzed by one-way ANOVA using GraphPad software (GraphPad Software, Inc., San Diego, CA).

**Western Blot Analysis—**HEK293T cell lysates were boiled in the presence of 2× sample buffer, fractionated by SDS-PAGE, and transferred to a nylon membrane. (Millipore), was performed using anti-HA antibody (Roche Applied Science) and anti-PC1 C-terminal peptide (antibody A19) (62). Anti-human IgG Western blots were performed as described earlier (13, 39, 62). Secondary antibodies conjugated to alkaline phosphatase were used to detect the immobilized antibodies by chemiluminescence with CDP-Star substrate (Amersham Biosciences) according to the manufacturer's instructions.

**Western Blot Quantification of Intracellular Ca$^{2+}$**—HEK293T cells were plated at ~5 × 10^5 cells/well/six-well plate on type I collagen-coated glass coverslips 24 h prior to co-transfection with 450 ng of PC1-LT or sIgG-0 and 22.5 ng of a cytomegalovirus-enhanced GFP construct (per 3 wells) (Invitrogen) to identify transfected cells plus 9 μg of BlueScript DNA. 4 h post-transfection, the medium was replaced with DMEM plus 0.5% heat-inactivated serum and the cultures were incubated for an additional 24 h. Cells were loaded with 5 μM Fura-2/AM (Invitrogen) and then incubated for an additional 20 h. Inhibitors were added 4 h before harvesting. The cells were then fixed in 4% paraformaldehyde at room temperature. The cells were air dried, mounted with antifade (Molecular Probes), and were examined with a Nikon inverted microscope equipped with a monochromator. The chamber was continuously perfused with Ringer's solution equilibrated with 5% CO2, 95% air at 37 °C. Transfected cells were identified by their green fluorescence (FAP-1950/1951, 550/551 nm excitation and emission; filter set (490-nm excitation and 535-nm emission). Base-line Fura-2 measurements were made with dual excitation wavelengths of 340 and 380 nm. The measurement of emitted light at 510 nm was restricted to GFP-expressing cells using an adjustable iris in front of a digital photomultiplier detection system (Photon Technology International). Felix Laser Fluorescence Imaging Software, Inc., San Diego, CA).

**Monochromator and data acquisition**—the monochromator and data acquisition to generate the 340/380 fluorescence ratio (F340/F380) after a steady state (F340/F380) was established, 10 mM caffeine was added to release Ca$^{2+}$ from ryanodine-sensitive stores. At the end of each experiment, cells were permeabilized with 2 μm ionomycin in Ringer's solution containing 2 mM Ca$^{2+}$ to determine the maximum ratio (Rmax). Background correction for the glass coverslips and GFP fluorescence was measured in several cells expressing GFP at the same intensity and was subtracted from the experimental values. F340/F380 ratios were converted to [Ca$^{2+}$] using the equation [Ca$^{2+}$] = Kd × (R - Rmax)RFmax - R) × (Scontrol/Ssample) where the dissociation constant (Kd) of Fura-2 for Ca$^{2+}$ is 224 nm, Rmax and Rmin are F340/F380 ratios for Ca$^{2+}$-saturating and Ca$^{2+}$-free conditions, and Scontrol and Ssample are fluorescence signals at 380 nm for free Ca$^{2+}$ and bound Ca$^{2+}$, respectively (63). A significant difference in intracellular [Ca$^{2+}$] between cells transfected with sIgG-0 and PC1-LT was determined using a parametric Student's unpaired t test. Values are represented as the mean ± S.E.

**NFAT Nuclear Translocation Assay—**HEK293T cells were seeded in Lab-TekII chamber slides (Nunc) and were co-transfected with 100 ng of an HA-NFAT1-GFP expression vector (64) and 200 ng of PC1-LT or PC1–11TM or their respective controls. 20 h post-transfection, the cells were washed three times with PBS and fixed for 10 min with freshly prepared 4% paraformaldehyde at room temperature. The cells were rinsed with three changes of PBS for 5 min each and counterstained with DAPI for an additional 5 min. The slides were re wicked with PBS, air dried, mounted with antifade (Molecular Probes), and were exam-
NAT Activation Requires the G Protein Binding Region of the PC1 C-tail—To identify potential signaling pathways from polycystin-1 that can modulate intracellular Ca\(^{2+}\) homeostasis, we co-expressed various PC1 C-tail deletion constructs (see Fig. 1) with an NFAT promoter-luciferase reporter construct that has four composite NFAT/AP-1 binding sites from the human interleukin-2 promoter (49). As shown in Fig. 2A, the full-length PC1 C-tail construct (PC1-LT) produced significant activation of the NFAT reporter over the control construct lacking PC1 sequences (slg-0). Removal of the membrane proximal region, including a portion of the heterotrimeric G protein binding domain (PC1-HT) (37), resulted in only weak activation of the NFAT reporter (~2.5-fold over the control), which was considerably less than that seen with PC1-LT. A C-tail fusion protein that completely lacks the G protein binding and activation region (PC1-AT) was not able to activate this promoter. In contrast, a construct that contains the G protein activation region but lacks the coiled-coil domain (PC1-LS) was able to activate NFAT, albeit less so than the full-length C-tail (Fig. 2A). The expression levels of the various PC1 fusion constructs were monitored by Western blot analysis using anti-human IgG (Fig. 2A, bottom). These results suggest that PC1-induced NFAT activation is dependent on an intact G protein binding and activation region and does not require the coiled-coil region.

To further test the involvement of G proteins in PC1-mediated NFAT activation, HEK293T cells were co-transfected with a Goq expression construct, PC1-LT, and the NFAT reporter (Fig. 2B). Co-expression of the Goq expression vector with the PC1 cDNA resulted in further stimulation of PC1-LT-induced NFAT activation. This augmentation of NFAT activity was not observed when Goq was co-expressed with the slg-0 control DNA or when expressed alone. Co-expression of Go12, but not...
Results suggest that the initial events resulting in PC1-mediated NFAT activation may be, at least in part, dependent on Gq signaling to PLC followed by Ca2+ release from intracellular stores. NFAT activation has been shown to require sustained increases in intracellular Ca2+ that are dependent on Ca2+ entry via store-operated calcium release-activated Ca2+ channels (74). To determine whether the PC1-mediated NFAT activation is dependent on Ca2+ entry, we treated PC1-LT and NFAT luciferase-transfected HEK293T cells with gadolinium hydrochloride (GAD) at 4 h before harvesting. As shown in Fig. 3D, GAD treatment completely abolished PC1-mediated NFAT activation. Similar results were obtained when PC1-transfected cells were treated with the extracellular Ca2+ chelator, EGTA (data not shown). These results suggest that NFAT activation by PC1 is dependent both on Ca2+ release from internal stores and on Ca2+ entry from the extracellular pool.

The PC1 C-tail Causes Sustained Ca2+ Increases and Calcineurin-dependent NFAT Activation—Activation and nuclear translocation of NFAT is known to depend on the activity of the Ca2+/calmodulin-dependent protein phosphatase, calcineurin, which requires sustained increases in intracellular Ca2+ levels for NFAT activation (74). To test whether PC1-mediated signal transduction can lead to a sustained elevation in intracellular Ca2+, HEK293T cells were co-transfected with PC1-LT or the control construct, sIg-0, and a GFP expression construct to identify transfected cells. After 24 h, the cells were loaded with Fura-2/AM and both basal and caffeine-stimulated intracellular Ca2+ levels were determined. As shown in Fig. 4A, both steady-state intracellular Ca2+ levels (76.3 ± 17.8 nM (±S.E., n = 7) for sIg-0 versus 137.1 ± 19.5 nM (±S.E., n = 8) for PC1-LT (p < 0.05)) and peak caffeine-induced Ca2+ release were higher in PC1-LT-transfected cells. Thus, it appears that the PC1-LT construct can give rise to significant long-term elevations in basal intracellular Ca2+.

To test whether this PC1-mediated elevation in intracellular Ca2+ can activate calcineurin, we used the calcineurin inhibitor CSA. HEK293T cells transfected with PC1-LT and the NFAT reporter construct were treated with 100 ng/ml CSA 4 h before harvesting. Fig. 4B shows that CSA treatment completely abolished the PC1-mediated NFAT activation. As a control (data not shown), we found that PC1-mediated AP-1 (7× AP-1, Stratagene) activation was not inhibited by CSA treatment. The inhibition by CSA suggests that the PC1-mediated activation of the composite ARRE-2 NFAT/AP-1 promoter is strongly dependent on calcineurin activation and therefore is an NFAT-dependent process.

PC2 Does Not Activate NFAT—The co-assembly of PC1 and PC2 through an interaction involving the C-tails of both proteins has been shown to create a novel Ca2+ current (4, 5, 18, 19, 75). To determine whether PC1 activation of NFAT involves PC2, cells were transfected with a full-length PC2 construct.
Fig. 5 shows that transfection of 50 ng/well of a full-length HA-tagged PC2 construct did not activate NFAT (amounts of up to 2 μg/well did not significantly activate NFAT; data not shown). Furthermore, PC1-mediated NFAT activation was not augmented by co-transfection with 50 ng/well HA-tagged PC2 construct but instead it appeared to be somewhat inhibited. These results suggest that it is PC1 and not PC2 that is responsible for the PC1-LT activation of NFAT.

**PC1-mediated Dephosphorylation and Nuclear Translocation of NFAT**—NFAT requires dephosphorylation by calcineurin for its nuclear translocation (74). To determine whether NFAT is dephosphorylated following transfection of PC1-LT, we assayed the relative amounts of phosphorylated and dephosphorylated forms of a co-transfected HA-tagged NFATc1-GFP fusion protein by Western blot analysis using an anti-HA antibody to determine their relative electrophoretic mobilities (64). As shown in Fig. 6A, the predominant form of HA-NFATc1-GFP in sIg-0 control-transfected cells was the slower migrating phosphorylated (cytosolic) band (lanes 1 and 2). The treatment of these cells with the Ca²⁺ ionophore A23187 shifted most of the HA-NFATc1-GFP to the faster migrating dephosphorylated (nuclear) form (lanes 3 and 4). Co-transfection with PC1-LT also resulted in a shift to the faster migrating dephosphorylated form (compare lanes 1 and 2 with lanes 5 and 6), which was prevented by CSA treatment (lanes 7 and 8). Intracellular localization of HA-NFATc1-GFP was assessed by visualization of the GFP fluorescence. Co-transfection with sIg-0 resulted in predominantly cytoplasmic localization of HA-NFATc1-GFP (Fig. 6B, upper left), whereas co-transfection with PC1-LT resulted in significant nuclear localization of HA-NFATc1-GFP (upper right), in many cases in a characteristic punctate pattern (76). Co-transfection with HA-NFATc1-GFP and PC1–11TM, which contains all of the 11 transmembrane domains as well as the C-tail (13) (see Fig. 1), also resulted in nuclear translocation of HA-NFATc1-GFP (Fig. 6B, lower left versus lower right). The 11TM construct was also...
The PC1 C-tail causes sustained Ca\textsuperscript{2+} increases and calcineurin-dependent NFAT activation. A, basal and caffeine-induced intracellular Ca\textsuperscript{2+} levels. HEK293T cells were transfected with sIg-0 and PC1-LT as described under “Experimental Procedures” and loaded with Fura-2/AM for single-cell Ca\textsuperscript{2+} measurements (n = 7 for sIg-0 and n = 8 for PC1-LT). After establishing steady-state intracellular Ca\textsuperscript{2+} levels, the cells were treated with 10 mM caffeine to release intracellular stores. Shown are representative measurements for sIg-0 and PC1-LT-transfected cells. B, inhibition by the calcineurin inhibitor CSA. Cells were transfected as in Fig. 3 with either sIg-0 or PC1-LT. 4 h before harvesting, the cells were treated with either vehicle or CSA (100 ng/ml) and were harvested 24 h post-transfection. The values represent mean ± S.D. of a representative experiment of three individual experiments, each done in triplicate. Below are anti-human IgG Western blots demonstrating expression levels of the fusion proteins.

PC1-LS-mediated NFAT Activation Is Enhanced by LiCl—PC1 has been shown to inhibit GSK-3β, leading to the stabilization of β-catenin and activation the β-catenin target gene, siamois (41). The distal C-tail region of PC1, which contains the coiled-coil domain, was shown to have this activity. This region of the PC1 C-tail corresponds to the PC1-AT construct used in our experiments (see Figs. 1 and 2). Because GSK-3β is known to phosphorylate nuclear NFAT, thus causing it to translocate back to the cytoplasm (52), it is possible that there are two activities in the PC1 C-tail that can enhance NFAT activity, one mediated by the proximal C-tail leading to a sustained elevation in intracellular Ca\textsuperscript{2+} and the other mediated by the distal C-tail leading to an inhibition of GSK-3β and thus to the nuclear retention of NFAT. Consistent with this finding, the PC1-LT construct (which contains both domains) gave rise to a higher level of NFAT activation than did the PC1-LS construct, which lacks the GSK-3β inhibitory domain (see Figs. 1 and 2A). If this difference is the due to the inability of PC1-LS to inhibit GSK-3β, it should be possible to increase PC1-LS-mediated NFAT activity to the same level achieved by PC1-LT by inhibiting GSK-3β with LiCl (77). As shown in Fig. 7, NFAT activity was significantly lower with PC1-LS than with PC1-LT in the absence of LiCl but was increased to the same level in the presence of LiCl.

NFAT Localization in the M-1 Mouse Cortical Collecting Duct Cell Line and in Renal Tubules—An analysis of the temporal and spatial patterns of PC1 expression in normal and polycystic kidney tissues has suggested that PC1 functions in epithelial differentiation and maturation (78–81). To understand the role of PC1-mediated NFAT signaling in the kidney, it will be important to establish that NFAT is expressed in renal epithelial cells. To do this, M-1 mouse cortical collecting duct cells were examined by immunofluorescence using an anti-NFATc1 antibody that recognizes all of the NFAT family members. As shown in Fig. 8A (upper panels), untreated M-1 cells express NFAT, which appears to be largely cytoplasmic but also nuclear under basal cell growth conditions (green fluorescence). Treatment of the cells with the Ca\textsuperscript{2+} ionophore A23187 caused nuclear accumulation of NFAT (Fig. 8A, middle panels), whereas treatment of the cells with CSA resulted in cytoplasmic retention (Fig. 8A, lower panels). RT-PCR (Fig. 8B) showed that the NFAT c1–c4 genes are expressed in M-1 cells.

NFAT was also localized in embryonic and adult mouse kidneys. Kidneys from late gestation embryos or 3-month-old BALB/c mice were prepared for immunohistochemistry with the NFATc1 pan-antibody. In embryonic kidneys (Fig. 8C, top and middle panels), the most prominent immunostaining was found in the nephrogenic zone in vesicles (V) and S-shaped bodies with less staining in the ureteric buds (UB) except at the ureteric bud tips (arrows). NFAT staining was retained in early tubular (T) and glomerular structures in the embryonic kidney.
**Experimental Procedures.** Cells were treated with 10 ng/ml A23187, or with 100 ng/ml CSA. The cells were lysed with 1× passive lysis buffer, and Western blotting was performed using an anti-HA antibody. Shown are the results from one of two experiments. Asterisk represents data significant at p < 0.02 compared with PC1-LT (first bar). Below are anti-human IgG Western blots demonstrating expression levels of PC1-LT and PC1-LS.

**FIG. 6.** Dephosphorylation and nuclear translocation of NFAT by PC1-LT and PC1-11TM. A, NFAT dephosphorylation. HEK293T cells were co-transfected with an HA-NFATc1-GFP expression vector (64) in the presence of either PC1-LT or slg-0 as described under “Experimental Procedures.” Cells were treated with 10 μM calcium ionophore, A23187, or with 100 ng/ml CSA. The cells were lysed with 1× passive lysis buffer, and Western blotting was performed using an anti-HA antibody. Shown are the results from one of two experiments. Phos-NFAT, phosphorylated NFAT; Dephos-NFAT, dephosphorylated NFAT. B, NFAT nuclear localization. HEK293T cells were co-transfected with 100 ng/well HA-NFATc1-GFP expression vector and 200 ng of either PC1-LT (upper) or PC1–11TM (lower) and their respective controls, either slg-0 or slg-stop. 20 h post-transfection, the cells were washed and fixed, and the nuclei were counterstained with DAPI. C, reporter constructs (100 ng/well/six-well plate) and control Renilla luciferase, and 500 ng/well PC1 deletion constructs, PC1-LT or PC1-LS. 16 h prior to harvesting, the cells were treated with either vehicle or LiCl (25 mM) and the cells were harvested 24 h post-transfection. The bars represent fold activation ± S.D. for four individual experiments, each done in triplicate. Asterisk represents data significant at p < 0.02 compared with PC1-LT without LiCl (first bar). Below are anti-human IgG Western blots demonstrating expression levels of PC1-LT and PC1-LS.

**DISCUSSION**

This report demonstrates that the C-tail of PC1 can activate calcineurin/NFAT signaling, a process known to be dependent on sustained elevations in intracellular Ca^{2+}. Unlike transient increases in Ca^{2+}, which are responsible for short-term events such as muscle contraction or synaptic transmission, sustained increases in cytosolic and nuclear Ca^{2+} can give rise to long-lasting outcomes such as adaptive cellular responses involving changes in cell differentiation and cell death, which are dependent on changes in gene expression (44, 82). Based on our results, we propose that PC1-mediated heterotrimeric G protein signaling triggers PLC activation followed by an initial IP3-dependent release of Ca^{2+} from internal stores, which then causes Ca^{2+} entry through store-operated Ca^{2+} release-activated Ca^{2+} channels, thus activating calcineurin and NFAT.

Support for the idea that PC1-mediated NFAT activation involves G protein signaling comes from experiments using C-tail deletion constructs with complete or partial G protein binding ability (37). The PC1 construct that completely lacks the G protein binding domain (PC1-AT) was not able to activate NFAT, and the construct that partially lacks the G protein binding domain (PC1-HT) had diminished ability to activate NFAT. In addition, the Gαq gain-of-function studies showed that co-transfection of Gαq augmented PC1-mediated NFAT activation. These results suggest that the PC1 C-tail can couple with Gαq to activate NFAT. Previous studies from our laboratory showed that the PC1 C-tail can couple with Gαq as well as with Gαq and G12/13 to activate c-Jun N-terminal kinase and AP-1 (39). Others (83) have corroborated the observation that PC1 can activate Gαq.

It is widely known that stimulation of Gαq-coupled receptors activates PLCβ (66–68). Thus, the possibility exists that PC1 activates the β isoforms of PLC in HEK293T cells, which can then initiate the release of Ca^{2+} to activate NFAT. Indeed, we showed that a PLC inhibitor, U73122, significantly decreased PC1-mediated NFAT activation. It has been demonstrated that
HEK293 cells express all of the phosphoinositide-specific PLCβ isoforms (PLCβ1–β4) (84). The sensitivity of these isoforms to G_{q} activation differs with the order β1 > β3 > β4 > β2 (85). As with the Go_{q} subunits, co-transfected Go_{q2} subunits were also able to augment PC1-mediated NFAT activation (data not shown). Go_{q2} is known to activate PLCε (86), which can also initiate Ca^{2+} release and subsequent NFAT activation. In two of the well characterized animal models of PKD (87, 88), the activity of PLCγ has been shown to be increased (89). These observations, although not directly involving PC1, suggest the possible importance of signaling events leading to PLC activation in the pathophysiology of PKD.

Inhibition by 2-APB and xestospongin suggests that Ca^{2+} release is involved in PC1-mediated NFAT activation. The complete inhibition of NFAT activation by GAD, which blocks Ca^{2+} entry (90), suggests that PC1-mediated Ca^{2+} release triggers Ca^{2+} entry through store-operated or Ca^{2+} release-activated Ca^{2+} channels. The addition of EGTA to the extracellular medium also abolished PC1-mediated NFAT activation (data not shown), supporting this contention. Although the identity of plasma membrane Ca^{2+}-release-activated Ca^{2+} channels has not yet been established, their role has been postulated in sustained Ca^{2+} elevations (90–92). Other mechanisms for Ca^{2+} entry may also be possible, potentially involving PC2. However, the expression of PC2 alone did not result in NFAT activation and co-expression of PC2 with PC1-LT did not augment PC1-mediated NFAT activation. PC2 has been demonstrated to be constitutively active when co-assembled with PC1 and targeted to the plasma membrane (19). These observations suggest that PC2 is not involved in NFAT activation and further suggest that PC1 alone is capable of generating Ca^{2+} signals.

The NFAT-responsive reporter used in these studies contains four copies of a composite NFAT/AP-1 response element that requires cooperative binding of both NFAT and AP-1 (60). This ARRE-2 element has a weak AP-1 site (TGTTTCA), which binds Fos-Jun dimers, such that c-Jun makes direct contact with NFAT bound to its adjacent DNA element. Previously, we demonstrated that the PC1-LT C-tail construct can activate another Fos-Jun-responsive AP-1 reporter, the 7× AP-1 promoter-reporter (pAP-1-luciferase; Stratagene), which contains the AP-1 site, TGACTAA (39). We and others (40) also found that the PC1-HT construct only weakly (if at all) activates the 7× AP-1 promoter. Thus, the very poor activation of the ARRE-2 NFAT/AP-1 reporter by PC1-HT (Fig. 2A) may reflect an inability of this truncated C-tail construct to activate G protein signaling to both NFAT and AP-1.

The strong response of the NFAT reporter to PC1-LT relative to PC1-LS (see Fig. 2A) may be due to the ability of the distal C-tail region to inhibit GSK-3β. In fact, the distal C-tail region of PC1 has been implicated as a positive effector of the Wnt/β-catenin pathway by its inhibition of GSK-3β, stabilization of β-catenin, and activation of β-catenin target genes (41). It has also been shown that GSK-3β can phosphorylate NFAT, causing its nuclear export and thus its inactivation (51, 93), and that inhibition of GSK-3β with LiCl can increase NFAT activity (77, 94). As such, the inhibition of GSK-3β by PC1-LT could promote the nuclear retention of NFAT. The observation (see Fig. 7) that LiCl treatment further increased the responses of both PC1-LT and PC1-LS suggests that GSK-3β is active in HEK293T cells and can attenuate the NFAT response to the degree to which it is inhibited by the PC1 distal C-tail region. Taken together, these results suggest that the PC1 C-tail can serve to integrate both Ca^{2+}-dependent and Ca^{2+}-independent pathways, leading to the activation of NFAT targets.

The temporal and spatial patterns of its kidney expression have suggested that PC1 functions in renal epithelial differentiation and maturation (78–81). To understand the role of PC1-mediated NFAT signaling in the kidney, it will be important to establish that NFAT is expressed in renal epithelial cells. We have now shown that NFAT is expressed in HEK293T cells (data not shown) and in mouse M-1 cortical collecting duct cells (Fig. 8, A and B). We have also demonstrated that NFAT is expressed in tubular epithelial cells of the developing and adult mouse kidney (Fig. 8C), which corresponds temporally with PC1 expression (78–81), suggesting the possibility that PC1 and NFAT function together during renal development, perhaps in differentiating tubules, and in
the adult kidney, perhaps in cellular adaptive responses. Recent observations have demonstrated the presence of NFATc1 in tubu-
lar epithelial cells of the rat kidney (95), which was localized in nuclei following streptozotocin-induced diabetic renal glomerular and tubular hypertrophy (94). CSA treatment abolished the renal hypertrophy in parallel with a reduction in calcineurin activity and NFAT relocalization to the cytoplasm (95). As such, NFAT activation may be associated with adaptive cellular hypertrophy in the kidney (95) as it is in the heart (96). It has also been reported that NFAT5 is highly expressed in the renal medulla and that NFAT5 knock-out mice have severe renal dysfunction with impaired activation of osmoprotective genes and microcystic tubular dilation (97).

Most mutations in the PKD1 gene are loss-of-function mutations such as nonsense, splicing, and frameshift mutations or deletions, most of which would be expected to cause the loss of expression of the PC1 C-tail. As such, our tail-less control construct, sIg-0, should mimic most of these disease-causing mutations. Thus, if PC1 normally functions in regulating NFAT target genes (60), a loss of PC1 expression by mutation would be expected to cause the abnormal (decreased or increased) expression of these target genes during renal development or in the adult kidney in response to adaptive signals, thus causing or contributing to cyst growth in autosomal dominant polycystic kidney disease.

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