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Calcium Dependence of Polycystin-2 Channel Activity Is Modulated by Phosphorylation at Ser^{812}*

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Polycystin-2 (PC-2) is a non-selective cation channel that, when mutated, results in autosomal dominant polycystic kidney disease. In an effort to understand the regulation of this channel, we investigated the role of protein phosphorylation in PC-2 function. We demonstrated the direct incorporation of phosphate into PC-2 in cells and tissues and found that this constitutive phosphorylation occurs at Ser^{812}, a putative casein kinase II (CK2) substrate domain. Ser^{812} can be phosphorylated by CK2 *in vitro* and substitution S812A results in failure to incorporate phosphate in cultured epithelial cells. Non-phosphorylated forms of PC-2 traffic normally in the endoplasmic reticulum and cilial compartments and retain homo- and hetero-multimerization interactions with PC-2 and polycystin-1, respectively. Single-channel studies of PC-2, S812A, and a substitution mutant, T721A, not related to phosphorylation show that PC-2 and S812A function as divalent cation channels with similar current amplitudes across a range of holding potentials; the T721A channel is not functional. Channel open probabilities for PC-2 and S812A show a bell-shaped dependence on cytoplasmic Ca^{2+} but there is a shift in this Ca^{2+} dependence such that S812A is 10-fold less sensitive to Ca^{2+} activation/inactivation than the wild type PC-2 channel. *In vivo* analysis of PC-2-dependent enhanced intracellular Ca^{2+} transients found that S812A resulted in enhanced transient duration and relative amplitude intermediate between control cells and those overexpressing wild type PC-2. Phosphorylation at Ser^{812} modulates PC-2 channel activity and factors regulating this phosphorylation are likely to play a role in the pathogenesis of polycystic kidney disease.

Autosomal dominant polycystic kidney disease (ADPKD)[1] consists of at least two genetically distinct disorders characterized by bilateral renal cyst formation and progressive renal enlargement. ADPKD affects more than 1 in 1000 live births and is the most common monogenic cause of kidney failure in humans. *PKD1* and *PKD2*, the genes mutated in almost all ADPKD patients, encode polycystin-1 (Pc-1) and polycystin-2 (PC-2), respectively (1).[2] PC-2 is the prototypical member of a subfamily of the TRP Ca^{2+} channel superfamily that is highly conserved in metazoan evolution (2) and functions as a non-selective cation channel (3–5). In mammalian species, PC-2 and Pc-1 have been localized to the apical primary cilia of renal tubule cells and epithelial cells in culture (6–8) as well as other cellular locations. PC-2 has also been found in monocilia at the embryonic node that play a critical role in left-right axis determination (9). The PC-2 Ca^{2+} channel is thought to be part of flow sensor complexes in both renal tubular and node cilia (8, 9).

Protein phosphorylation is a post-translational modification that often plays a role in regulation of channel function (10). Regulation of ion channels by phosphorylation is most commonly mediated either by protein kinase A and protein kinase C (10, 11). Several studies have suggested functional involvement of protein phosphorylation in the pathogenesis of ADPKD. Apical stimulation by epidermal growth factor causes epidermal growth factor receptor phosphorylation in kidney cyst cells but not in normal cells (12). Pc-1 has been found to be tyrosine-phosphorylated and phosphorylated by Protein kinase A (13, 14). Protein kinase X, a novel cAMP-dependent serine/threonine kinase that regulates epithelial migration and morphogenesis is aberrantly expressed in kidney tissues from ADPKD patients (15). Mutations in Nek-family kinases Nek1 and Nek8 underlie polycystic kidney phenotypes in a pair of naturally occurring mouse models (16, 17). These kinases may act by regulating cytoskeletal structure in epithelial cells (17).

PC-2 is predicted to have at least four serine/threonine phosphorylation sites in its cytoplasmic COOH terminus (2). In the current study, we sought to determine whether PC-2 is phosphorylated and what the functional role of such phosphorylation may be. We found that PC-2 is constitutively phosphoryl-

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[1] The abbreviations used are: ADPKD, autosomal dominant polycystic kidney disease; GST, glutathione *S*-transferase; HA, hemagglutinin; MDCK, Madin-Darby canine kidney cells; DMEM, Dulbecco's modified Eagle's medium; H-DMEM, Ham's F-12:DMEM; IF, immunofluorescent cell staining; IP, immunoprecipitation; Endo H, endoglycosidase H; ER, endoplasmic reticulum; ERD, ER retention domain; CK2, casein kinase II; MES, 4-morpholineethanesulfonic acid; CIP, calf intestinal phosphatase.

[2] A clarification: Pc-1 and Pc-2 denote the mouse proteins, whereas PC-1 and PC-2 denote the human proteins.

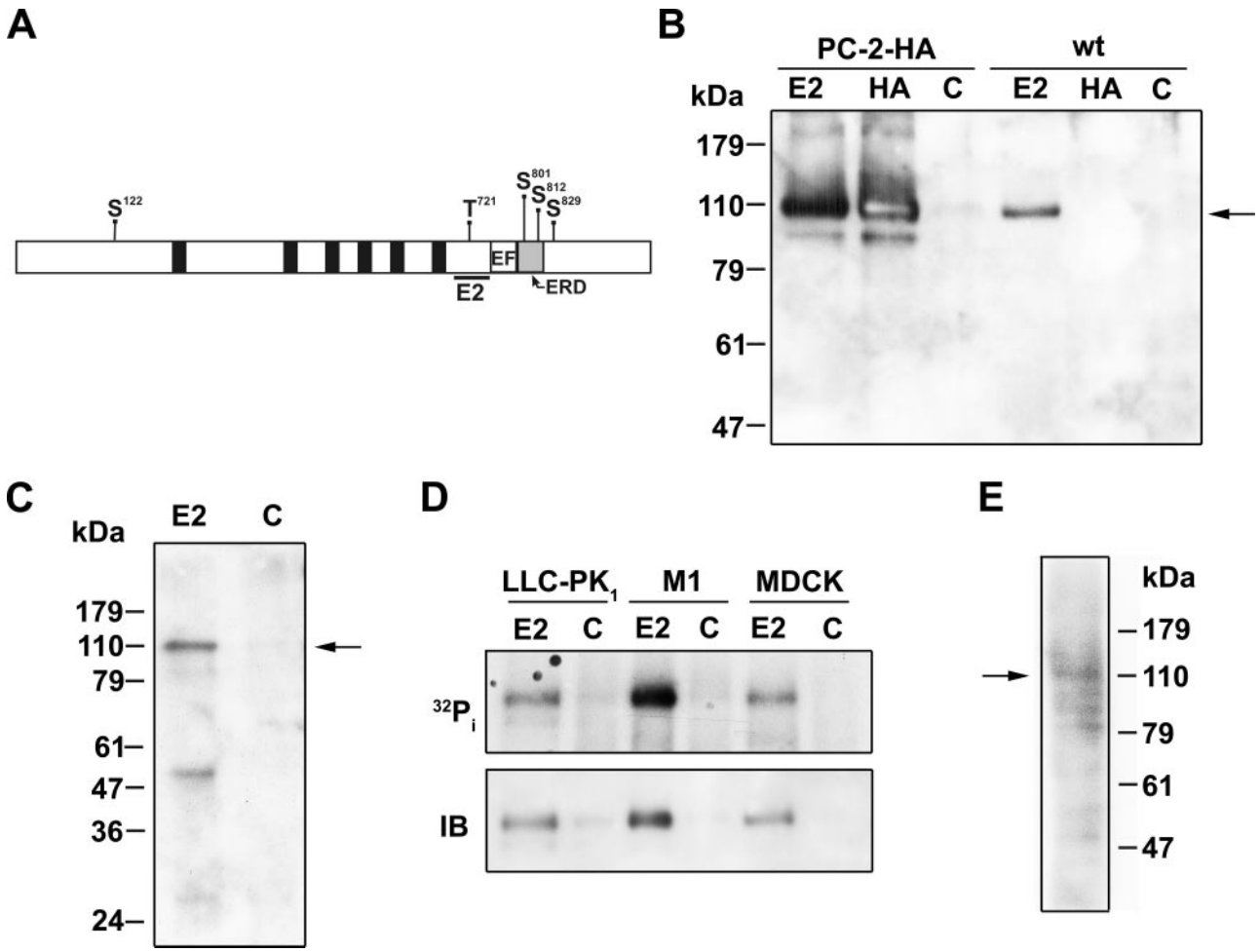

FIG. 1. **Phosphorylation of PC-2 *in vivo*.** *A*, schematic representation of PC-2 showing the relative locations of the six transmembrane segments (*black bars*), the EF-hand (*EF*), the ER retention domain (*ERD*), and five predicted phosphorylation sites. The location of the E2 epitope in the cytosolic COOH terminus is also shown. *B*, IP of PC-2 using YCE2 monoclonal antibody. Total cell lysates of LLC-PK_1 expressing HA-tagged human PC-2 (*PC-2-HA*) or wild type cells (*wt*) were immunoprecipitated by YCE2 (*E2*), anti-HA (*HA*), or control medium (*C*), and PC-2 was detected by immunoblotting with polyclonal YCC2. YCE2 can immunoprecipitate both endogenous and overexpressed PC-2. The *arrow* indicates the immunoreactive band migrating at ~110 kDa. *C*, IP native mouse Pc-2 from kidney tissue. YCE2 (*E2*) or control medium (*C*) were used for immunoprecipitated from tissue lysate; Pc-2 was detected by immunoblotting with anti-PKD2 polyclonal YCB9. *D*, phosphorylation of PC-2. Cultured cells expressing human PC-2 were labeled by $^{32}P_i$, and cell lysates were immunoprecipitated by YCE2 (*E2*) or control (*C*) medium. IP proteins were analyzed by SDS-PAGE and autoradiography ($^{32}P_i$), and the same membranes were then immunoblotted with YCC2 to confirm the identity of the phosphorylated PC-2 (*IB*). *E*, kidney tissue was dissociated, and the cells were similarly incubated with $^{32}P_i$ prior to immunoprecipitated by YCE2 and autoradiography. The *arrow* indicates a band migrating at ~110 kDa corresponding to PC-2 confirmed by immunoblotting (not shown).

ated at Ser^{812} *in vivo*. Phosphorylation at this site does not effect the subcellular localization or the interaction with Pc-1. However, single channel studies show that loss of phosphorylation at Ser^{812} results in a significant decrease in the sensitivity of the PC-2 channel to calcium stimulation. Phosphorylation of PC-2 is an important factor in regulating the activity of the channel *in vivo*.

MATERIALS AND METHODS

Antibodies—Mouse monoclonal antibody for human polycystin-2, YCE2, was generated by standard hybridoma techniques with the assistance of Susan Buhl of the Hybrydoma Facility at Albert Einstein College of Medicine. Briefly, GST fusion protein E2 containing a portion of the COOH terminus of human PC-2 (amino acids 687–754) was used to immunize BALB/C mice (Charles River Inc.). Spleen cells from an immunized mouse were fused to myeloma cells (Ag8.653), and ~1000 hybridomas were screened by enzyme-linked immunosorbent assay using GST fusion protein E2 (positive) and GST alone (negative). Three clones, 21, 156, and 223, among 30 that showed E2-positive results upon enzyme-linked immunosorbent assay were characterized further by immunoblotting, immunoprecipitation, and immunocytochemistry and found to be specific antibodies. The YCE2 antibody used in this study was obtained from cultured supernatant of clone 223, although the other clones were used as well. Polyclonal PC-2 antisera YCC2 and YCB9 have been described previously (18). Other antibodies are from commercial sources: monoclonal anti-myc (clone 9E10; Roche Applied Science (#1667149)); polyclonal anti-myc (Cell Signaling (#2272)); monoclonal anti-HA (clone 3F10; Roche Applied Science (#1867423)); polyclonal anti-HA (Zymed Laboratories Inc. (#715500)); polyclonal anti-calnexin (Stressgen (#SPA-860)); monoclonal anti-FLAG M2 agarose (Sigma (A2220)); polyclonal anti-FLAG (Sigma (#F7425)); polyclonal anti-casein kinase II (Upstate Biotech (#06-873)); goat anti-GST polyclonal antibody (Amersham Biosciences (#27-4577-01)); anti-Na^+K^+-ATPase (mab6H, kind gift from Michael Caplan).

cDNA Constructs and Reverse Transcription-PCR—Amino acid substitution mutations were introduced into wild type human PKD2 cDNA (TM4) by site-direct mutagenesis by using the QuikChange kit (Stratagene). PC-2 clones were expressed as COOH-terminal HA or myc-tagged fusion proteins in pcDNA3.1 (Invitrogen). *Pkd1* cDNA was cloned in segments by reverse transcription-PCR using mouse kidney mRNA (Clontech) as the template. Full-length *Pkd1* was assembled from the cloned cDNA fragments and confirmed by bidirectional sequencing of the entire insert. A triple HA-tag (HA) was introduced in-frame immediately prior to the stop codon. A triple FLAG-tag (FLAG) was introduced in-frame between the leader sequence and LRR domain between codons 24 and 25.

Cell Lines, Cell Culture, and Immunofluorescent Cell Staining—Cells

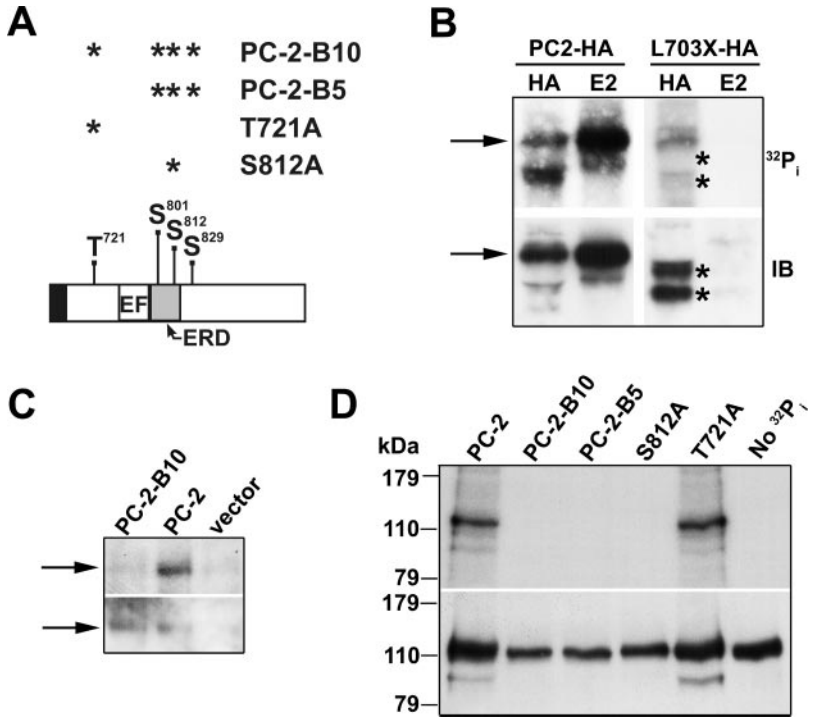

FIG. 2. **Ser^{812} is the site of constitutive phosphorylation of PC-2.** *A*, schematic diagram showing the COOH termini of constructs used for PC-2 phosphorylation analysis. The *asterisk* indicates the residue(s) substituted by alanine. Only the portion after the sixth membrane span (*black box*) is shown. *B*, phosphorylation of PC-2 occurs in the COOH terminus. The $^{32}P_i$-labeled LLC-PK$_1$ cells expressing PC2-HA or the truncated L703X-HA were immunoprecipitated by anti-HA (*HA*) or anti-PKD2 (*E2*) and detected by either autoradiography (*top panel*) or immunoblot with an anti-NH$_2$ terminus polyclonal antiserum (YCB9). The truncated L703X-HA protein was not labeled by $^{32}P_i$, although it was abundantly immunoprecipitated by anti-HA, but not YCE2, antibody (bands marked by an *asterisk*); the two bands represent different glycosylation states of the truncated protein (18). Full-length PC-2 marked by *arrows*. *C*, MDCK cells transiently transfected with PC-2-B10, PC-2, or empty vector alone, incubated with $^{32}P_i$ and immunoprecipitated by YCE2. Autoradiography (*upper panel*) shows that PC-2-B10 is not phosphorylated. Immunoblotting with YCC2 (*lower panel*) shows presence of respective proteins in the IP. *D*, LLC-PK$_1$ cells transiently transfected with myc-tagged forms of PC-2 harboring different mutations, incubated with $^{32}P_i$, and immunoprecipitated by anti-myc. Autoradiography (*top panel*) shows that a single amino acid substitution, S812A, is sufficient to prevent phosphorylation of PC-2 *in vivo*. YCC2 immunoblot (*lower panel*) controls for success of anti-myc IP.

lines stably expressing *Pkd2* and *Pkd1* constructs in MDCK, LLC-PK$_1$, and M1 cells were established as described previously (18). Cells were transfected either with PKD2-HA or full-length FLAG-Pkd1-HA cDNA followed by selection with medium containing G418 at 0.4 mg/ml for MDCK and LLC-PK$_1$ cells and 0.2 mg/ml for M1 cells. All cells were cultured in DMEM with 10% fetal bovine serum at 37 °C under 5% CO_2. Clonal stable cell lines were selected by plating the stably overexpressing cells at low density (~1000 cells per 100-mm dish), and isolated colonies were picked up by using cloning cylinders (Bel-Art Product, Pequannock, NJ). The presence of the PC-2 and Pc-1 expression in the cell lines was confirmed by immunoblotting and immunofluorescent cell staining (IF), and IF was done as described previously (18). Cells were fixed using 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Fluorescein isothiocyanate- or Cy3-laveled anti-mouse or anti-rabbit secondary antibodies were used to image cells by double indirect confocal microscopy (LSM 510, Zeiss).

Immunoprecipitation, Glycosylation, and Cell Surface Biotinylation—Immunoprecipitation (IP) of PC-2 was performed using either of YCE2, anti-HA or anti-myc; Pc-1 was IP using anti-FLAG. For immunoprecipitation, cells were lysed in radioimmune precipitation assay buffer (1% Nonidet P-40; 1% sodium deoxycholate; 0.1% SDS; 150 mM NaCl; 10 mM sodium phosphate, pH 7.2; 2 mM EDTA; and 1× protease inhibitor mixture (Roche Applied Science)) and immunoprecipitated overnight at 4 °C. IP proteins were washed five times with ice-cold radioimmune precipitation assay buffer and eluted with 2× SDS sample buffer (125 mM Tris, pH 6.8, 200 mM dithiothreitol, 6% SDS, 20% glycerol, 0.2% bromphenol blue). Endoglycosidase H (Endo H) sensitivity was determined using 1 μl (500 units) of Endo H (New England Biolabs, Beverly, MA) as previously described (18). For cell surface biotinylation studies, cells were plated at high density to allow them to reach confluence after overnight culture. These non-ciliated, living, non-permeabilized confluent cells were immediately labeled using *N*-hydroxysuccinimide-SS-biotin (Pierce, Rockford, IL) at 4 °C as described previously (18). Cells were lysed, and 20 μl was used for SDS-PAGE and immunoblotting. The remaining sample (~600–800 μl) was IP with streptavidin, and the IP proteins were analyzed by immunoblotting using either anti-PC-2-NH$_2$ terminus antisera (YCB9), anti-COOH terminus antisera (YCC2), or anti-Na^+K^+-ATPase antibody.

In Vitro and in Vivo Phosphorylation Analysis—For *in vivo* [^{32}P]orthophosphate ($^{32}P_i$) labeling, cultured cells at ~80% confluence were preincubated in phosphate-free DMEM medium (Invitrogen) with 10% dialyzed fetal bovine serum at 37 °C for 1 h, followed by incubation in medium containing $^{32}P_i$ at 1000 μCi/ml for 5 h (19). Cells were lysed with radioimmune precipitation assay buffer with phosphatase inhibitors (50 mM sodium fluoride; 0.2 mM sodium vanadate) followed by PC-2 IP as described above. Proteins eluted from immunoprecipitating antibodies were resolved by SDS-PAGE and electro-transferred onto polyvinylidene difluoride membrane. The $^{32}P_i$-labeled proteins were visualized by autoradiography. Total IP proteins were demonstrated by immunoblotting using the same blot.

The *in vivo* $^{32}P_i$ labeling of mouse kidney tissue was performed as described previously with modifications (19). Kidneys of adult C57/Bl6 mice were excised and immersed in ice-cold H-DMEM medium (Ham's F-12:DMEM, 1:1, supplemented with 2 mM heptanoic acid, 15 mM HEPES, 15 mM $NaHCO_3$, under 5% CO_2). The tissue was minced with a razor blade, suspended in 25 ml of digestion medium (H-DMEM supplemented with 150 units/ml collagenase, 2 mg/ml bovine serum albumin, and 1 unit/ml DNase), and incubated in CO_2 incubator for 1 h. Digested tissue was strained through a tissue sieve and washed three times in H-DMEM. Cells were washed an additional two times in phosphate-free DMEM, then incubated in 1 ml of phosphate-free DMEM with 1 mCi of $^{32}P_i$ in a 35-mm dish for 5 h at 37 °C. Samples were washed with ice-cold Tris-buffered saline containing protease inhibitors, and phosphatase inhibitors and lysates were made by addition of radioimmune precipitation assay buffer as described above. The $^{32}P_i$-labeled proteins were IP by YCE2. IP proteins were resolved by SDS-PAGE and detected by autoradiography followed by immunoblotting using anti-PC-2 polyclonal antisera (YCC2).

In vitro casein kinase II (CK2) assays were performed according to the methods reported by Takahashi *et al.* (20). Briefly, bacterially

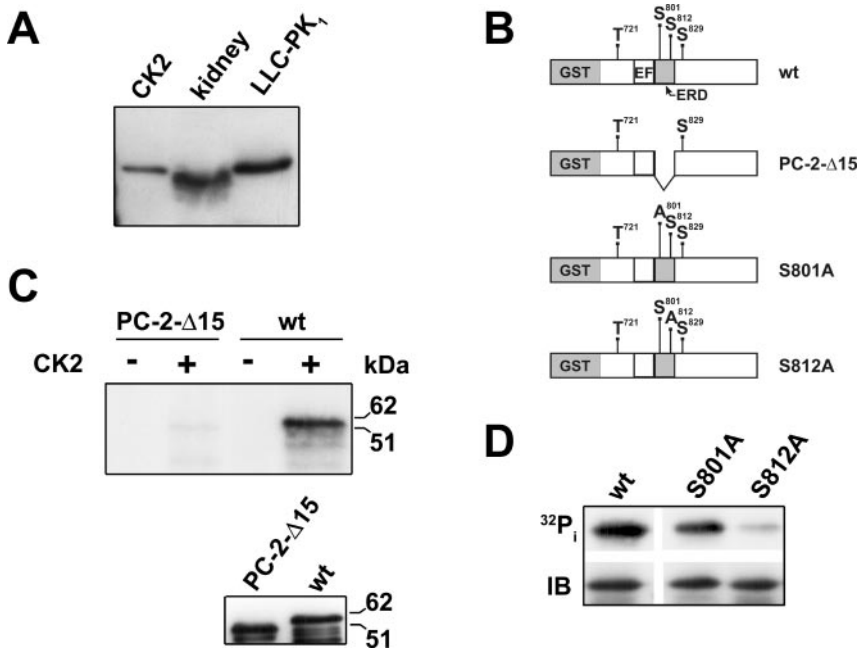

FIG. 3. **Casein kinase II can phosphorylate Ser^{812}.** *A*, immunoblot showing expression of endogenous casein kinase II (CK2) in lysates of mouse kidney and LLC-PK$_1$ cells by anti-CK2 antibody. CK2 protein serves as a positive control. *B*, schematic representation of GST fusion proteins used in *in vitro* CK2 phosphorylation analysis. *C*, *in vitro* phosphorylation by CK2 in the presence of [γ-^{32}P]ATP does not occur when the ERD is deleted (PC-2-Δ15). *Upper panel*, autoradiography; *lower panel*, immunoblot using anti-GST of the proteins used in the assay; the two samples shown were divided into equal aliquots and treated with (+) or without (−) CK2 in the *upper panel* experiments. *D*, *in vitro* phosphorylation by CK2 showing that Ser^{812} is the substrate site. *Upper panel*, autoradiography; *lower panel*, immunoblot with anti-GST of the same membrane showing equal loading of the S812A GST fusion protein that was not phosphorylated in the autoradiograph. All three lanes shown are from a single immunoblot.

expressed GST fusion proteins were pre-bound to glutathione-Sepharose 4B beads (Amersham Biosciences). The bound Sepharose beads were sedimented, washed with 400 ml of CK2 buffer (20 mM MES-KOH (pH 6.9), 130 mM KCl, 10 mM $MgCl_2$, 4.8 mM dithiothreitol), and re-suspended in 50 ml of CK2 buffer. The suspension was incubated with 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol, 10.0 mCi/ml, PerkinElmer Life Sciences) and 0.0001 unit of recombinant CK2 (Roche Molecular Biochemicals) at 37 °C for 1 h. The beads were washed five times with CK2 buffer and re-suspended in sample buffer containing 2-mercaptoethanol. Samples were resolved by SDS-PAGE and autoradiography, followed by Western blotting with anti-GST antibody.

Single Channel Recordings and Optical Imaging of Ca^{2+} Transients—Microsome fractions enriched in ER membranes we prepared from LLC-PK$_1$ cell lines stably overexpressing PC-2 mutant constructs (5). The presence of the PC-2 mutant proteins in the microsomes was confirmed by immunoblotting using epitope tag antibodies (data not shown). Microsomes were fused to lipid bilayers, and single channel studies were performed as previously described (5). To test the effects of phosphatase on channel activity, 4 μl of calf intestinal phosphatase (CIP; New England Biolabs, Beverly, MA) at 4 units/ml, was added directly to the bilayer on the *cis* (cytoplasmic) side without stirring. The dephosphorylation reaction was stopped 3 min after CIP addition by stirring the solution in the *cis* chamber for 30 s. Stirring resulted in a 350-fold reduction of CIP concentration (4 μl in a 1.4-ml dilution), reducing the rate of PC-2 dephosphorylation in the bilayer. Data were filtered at 1 kHz and digitized at 3 kHz, directly transferred to a computer and analyzed using pClamp version 8.1 software (Axon Instruments, Burlingame, CA).

For detection of whole cell Ca^{2+} transients, cells expressing PC-2 constructs were loaded with fluo-4 and imaged using a Ziess Aviovert S100 confocal microscope as described previously (5). Fluorescence intensity ratios and $t_{1/2}$ for decay back to baseline fluorescence were determined for at least 50 randomly selected cells in each experiment. Comparisons of cellular responses were carried out using one-way analysis of variance on ranks (the non-parametric Kruskal-Wallis test) with post-test multiple pair-wise comparisons done by Dunn's method.

RESULTS

Polycystin-2 Is Constitutively Phosphorylated in Vivo—PC-2 contains six predicted sites for phosphorylation, of which five are in the COOH terminus (2). Four of these five are conserved in mouse PC-2. Ser^{801}, a predicted PKC phosphorylation site, and Ser^{812}, a casein kinase II (CK2) site, are located within the previously defined ER retention domain (ERD) in the cytosolic COOH terminus of PC-2 (18). A pair of predicted protein kinase A/G sites are located upstream of the EF-hand domain and downstream of the ERD at Thr^{721} and Ser^{829}, respectively (Fig. 1*A*). To aid in the analysis of PC-2 function, we generated a mouse monoclonal antibody YCE2 against an epitope in the COOH terminus of the human protein (amino acids 687–754). YCE2 can specifically detect overexpressed human PC-2 in transfected cells by both immunoblotting and immunofluorescent cell staining (data not shown). YCE2 can immunoprecipitate (IP) both overexpressed and endogenous PC-2 from LLC-PK$_1$ cells as well as endogenous Pc-2 from mouse kidney tissue (Fig. 1, *B* and *C*).

We first examined whether PC-2 is phosphorylated in cells in culture. Cells expressing human PC-2 were incubated with $^{32}P_i$ and the lysates IP using YCE2. Autoradiography of the IP protein resolved by SDS-PAGE revealed a single band migrating at ~110 kDa. Immunoblotting of the same membrane with YCC2 polyclonal anti-PC-2 confirmed that the phosphorylated IP protein was indeed PC-2 (Fig. 1*D*). We found that phosphorylation of PC-2 was independent of the particular cell line used as three independent epithelial cell lines (LLC-PK$_1$, MDCK, and M1) all showed similar results (Fig. 1*D*). The data suggest that PC-2 is constitutively phosphorylated in renal epithelial cells. To test whether endogenous PC-2 is phosphorylated in the kidney, dissociated mouse kidney cells was incubated with $^{32}P_i$, lysed, and immunoprecipitated by YCE2 antibody. Native PC-2 from mouse kidney was phosphorylated (Fig. 1*E*), supporting the conclusion that PC-2 is constitutively phosphorylated in the kidney *in vivo*.

The Site of Phosphorylation of PC-2 Is Ser^{812}—The majority of the predicted phosphorylation sites in PC-2 are located in the COOH terminus. To determine whether the observed phosphorylation of PC-2 occurs in the COOH terminus, we examined phosphorylation of a COOH-terminal truncated form of PC-2, L703X-HA (18). This truncated form of PC-2 is stable and traffics to both the ER compartment and the cell surface (18). In contrast to full-length PC-2, L703X-HA was not phosphorylated *in vivo* (Fig. 2*B*), suggesting that the site for constitutive phosphorylation for PC-2 is located within the COOH terminus. To determine the specific residues for phosphorylation, we substituted alanine (Ala) at various combinations of four predicted phosphorylation sites of the COOH terminus, Thr^{721}, Ser^{801}, Ser^{812}, and Ser^{829} (clone PC-2-B10, Fig. 2*A*). We transiently transfected PC-2-B10, with all four predicted sites substituted by alanine, into MDCK cells, and performed *in vivo* phosphorylation analysis. PC-2-B10 was not phosphorylated

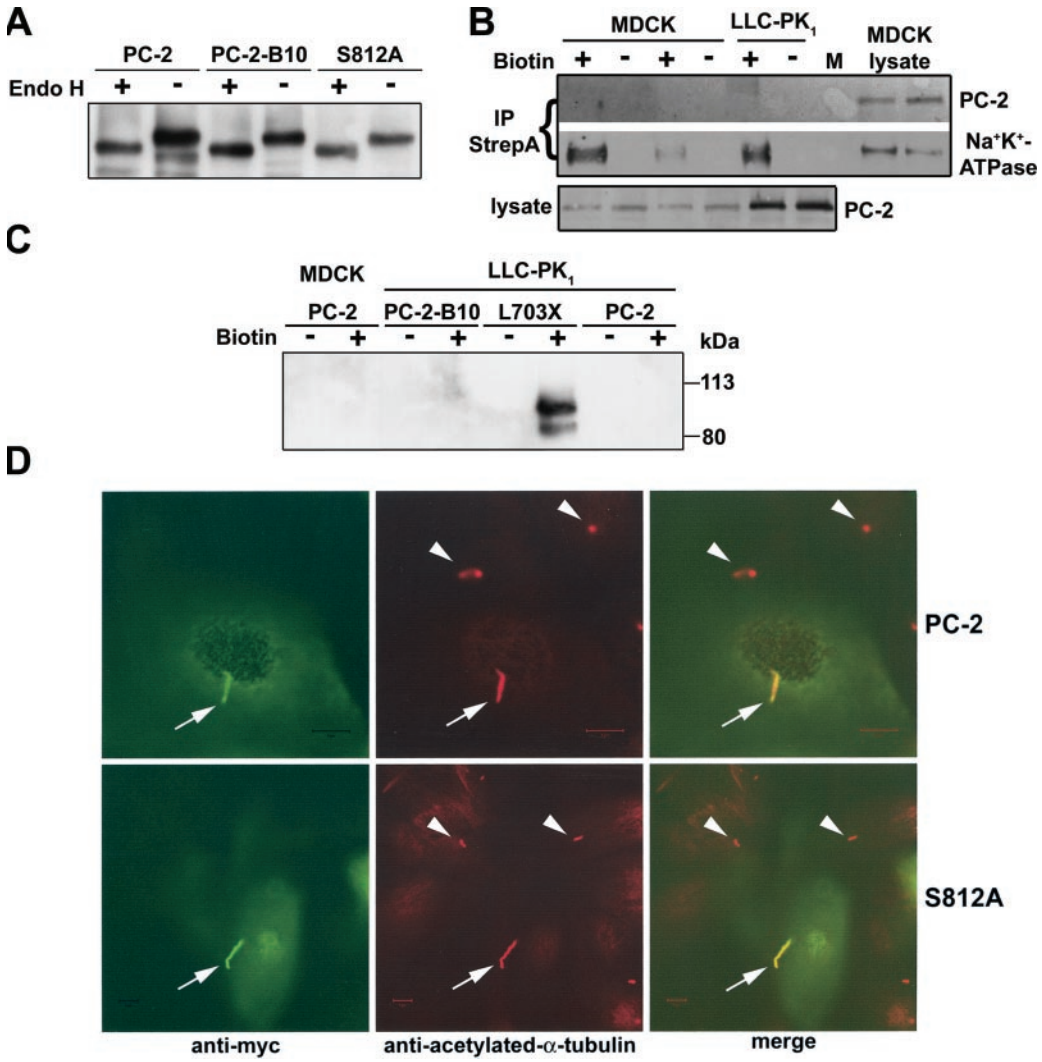

FIG. 4. **Phosphorylation does not play a role in subcellular localization of PC-2.** *A*, wild type PC-2, PC-2-B10, and S812A stably expressed in LLC-PK$_1$ cells are all completely sensitive to Endo H consistent with location in pre-middle Golgi membrane compartments in sub-confluent, non-ciliated cells. Immunoblot by YCC2. *B*, wild type (not transfected) PC-2 does not traffic to the cell surface in either MDCK or LLC-PK$_1$ cells. Biotinylated protein immunoprecipitated by streptavidin (*StrepA*; *top two panels* from duplicate gels run in parallel) shows no immunoreactive PC-2 detectable by YCC2, whereas Na^+K^+-ATPase serves as positive control for successful biotinylation and streptavidin IP; cell lysates on the same membrane serve as positive control for IB; *M*, marker lane. Cell lysates (*bottom panel*) show expression of native PC-2 in starting material. *C*, similarly, overexpressed wild type PC-2 and non-phosphorylated PC-2-B10 are not biotinylated on the cell surface; overexpressed truncated L703X is biotinylated on the cell surface as a positive control; immunodetection is by YCB9. *D*, LLC-PK$_1$ cells expressing myc-tagged wild type PC-2 (*top row*) or S812A (*bottom row*). Anti-myc IF (*green*) shows heterogenous cell population with some cells expressing PC-2 or S812A (*arrows*) and others not (*arrowheads*). Anti-acetylated-α-tubulin (*red*) marks the cilial axoneme in all cells; *size bar*, 5 μm. Overexpressed myc-tagged PC-2 and S812A both traffic to cilia in cells that express the heterologous proteins.

(Fig. 2*C*), suggesting that the one or more substrate residues for phosphorylation are among the four predicted sites in the COOH terminus. Phosphorylation analysis using PC-2-B10, PC-2-B5, and the T721A and S812A single point substitution mutant proteins (Fig. 2*A*) identified Ser^{812} as the site of constitutive phosphorylation of PC-2 (Fig. 2*D*).

Ser^{812} Can Be Phosphorylated by Casein Kinase II—Ser^{812} is a candidate substrate residue for casein kinase II (CK2) (2), a ubiquitously distributed Ser/Thr protein kinase. To confirm that CK2 is expressed in renal epithelial cells where the polycystic kidney disease phenotype manifests, mouse kidney tissue as well as LLC-PK$_1$ epithelial cells were examined by immunoblotting using anti-CK2 antibody. CK2 is expressed in mouse kidney and LLC-PK$_1$ cells (Fig. 3*A*). We next tested whether PC-2 is phosphorylated by CK2 *in vitro*. Purified GST fusion proteins containing the COOH terminus of PC-2 with or without mutations affecting the phosphorylation sites (Fig. 3*B*) were assayed for phosphorylation by CK2. Deletion of the ER retention domain (ERD) that contains both Ser^{801} and Ser^{812} resulted in loss of phosphorylation by CK2 (Fig. 3*C*). When GST fusion proteins carrying single amino acid substitutions at Ser^{801} and Ser^{812} were assayed, S801A mutants were phosphorylated normally, whereas S12A mutants were not (Fig. 3*D*). These finding are consistent with Ser^{812} being a site of phosphorylation by CK2.

Phosphorylation at Ser^{812} Is Not Required for Subcellular Localization or Protein Interaction—Although there remains some controversy in the subcellular location of PC-2, there is general agreement that PC-2 is expressed in the ER and primary cilia of epithelial cells (6, 7, 18) and that the primary cilia are a site of functional activity for the cation channel (8, 9). We examined the role of phosphorylation at Ser^{812} in the subcellular localization of PC-2. Non-phosphorylated S812A forms of PC-2 in non-ciliated cells retain complete endoglycosidase H (Endo H) sensitivity as is observed for wild type PC-2 (Fig. 4*A*) (5, 18). Phosphorylation-deficient forms of PC-2 with an otherwise intact COOH terminus capable of protein interactions (see below) cannot be biotinylated at the cell surface in confluent

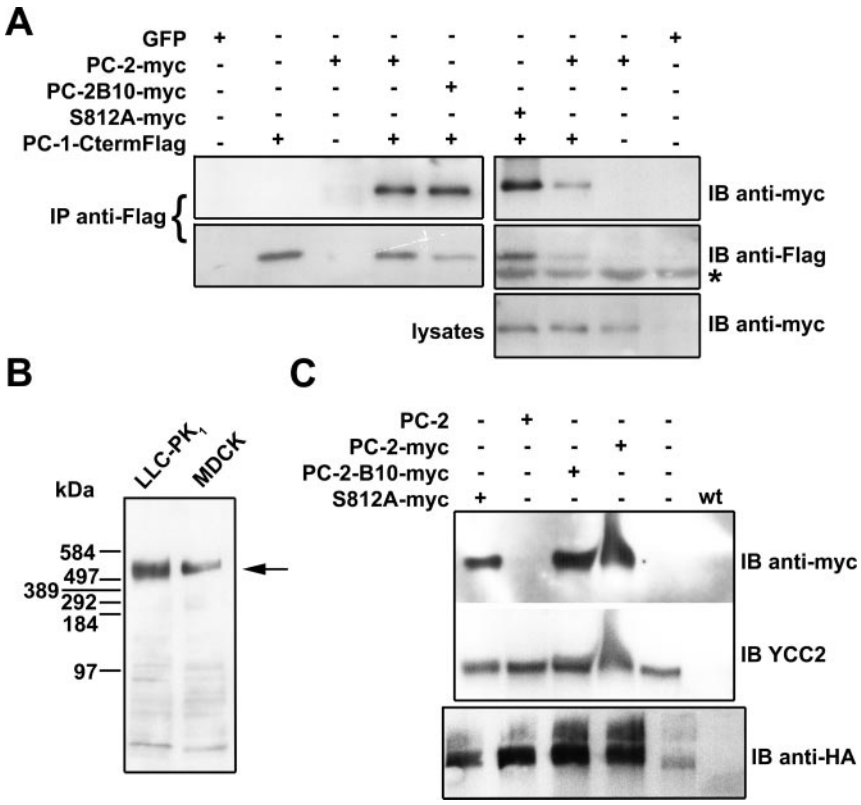

FIG. 5. **Phosphorylation does not alter interaction of PC-2 with Pc-1.** *A*, PC-2, PC-2-B10, and S812A with myc epitope tags can all be indirectly immunoprecipitated by anti-FLAG (*M2*) when they are co-expressed with the COOH terminus of Pc-1 carrying a FLAG epitope tag (*PC-1-CtermFlag*). Representative expression of myc-tagged PC-2 and S812A is shown in the *lower right panel*; *, nonspecific IgG band. *B*, full-length mouse Pc-1 detected by immunoblotting of lysates of MDCK and LLC-PK_1 cells stably overexpressing the epitope-tagged protein using anti-HA antibody. *C*, interaction of PC-2 and its phosphorylation-deficient variants with FLAG and HA epitope-tagged Pc-1 in an LLC-PK_1 cell line stably expressing the latter. IP in all lanes is by anti-FLAG monoclonal antibody. IB with anti-myc detects heterologously expressed, myc-tagged forms of PC-2, but not the untagged form. IB with YCC2 detects both heterologously expressed PC-2 (whether or not epitope-tagged) as well as endogenous PC-2 in cells not transfected with a PC-2 construct. IB with HA detects full-length PC-1 demonstrating successful immunoprecipitated with anti-FLAG. wt, LLC-PK_1 cells not expressing heterologous Pc-1, show no indirect IP of PC-2.

but non-ciliated cells (Fig. 4*B*). When the cells overexpressing the myc-tagged S812A phosphorylation-deficient form of PC-2 are cultured under conditions allowing cilia formation, S812A localizes to cilia in a manner similar to wild type overexpressed PC-2 (Fig. 4*C*). Collectively, these data suggest that loss of phosphorylation at Ser^{812} does not alter the trafficking of PC-2 within the cell.

PC-2 interacts with Pc-1, and this interaction is thought to be essential to the normal function of both proteins in kidney epithelia (4, 21, 22). We used co-transfection, co-IP assays to investigate the role of phosphorylation at Ser^{812} in PC-2 homo-multimerization and Pc-1/PC-2 hetero-multimerization (Fig. 5). Full-length PC-2, and the non-phosphorylated forms PC-2-B10 and S812A were immunoprecipitated by anti-FLAG when co-expressed with Pc-1-COOH terminus carrying a FLAG epitope tag (Fig. 5*A*). We next extended these experiments to interaction with the full-length Pc-1. We have established cell lines stably overexpressing Pc-1 with a COOH-terminal HA epitope tag and an NH_2 terminus FLAG tag (Fig. 5*B*). PC-2-B10 and S812A interact with Pc-1 in a manner similar to wild type overexpressed and endogenous PC-2 (Fig. 5*C*). Phosphorylation at Ser^{812} does not effect interaction of PC-2 with Pc-1.

Phosphorylation Alters Sensitivity of the PC-2 Channel to Activation by Ca^{2+}—Finally, we examined the effect of phosphorylation on PC-2 channel properties. Single channel recordings of wild type, PC-2-B10, T721A, and S812A forms of the PC-2 channel were carried out in planar lipid bilayers (5). Comparable levels of expression in ER-enriched microsomes from cell lines expressing wild type and mutant PC-2 were demonstrated by immunoblotting with YCC2 (data not shown). For all experiments, Ba^{2+} on the luminal side of the channel was used as the current carrier, because it does not alter channel activity when present on either side of the channel (5). Wild type PC-2 and S812A had channel activity in the bilayers (Fig. 6*A*). By contrast, PC-2-B10 and T721A had no channel activity (Fig. 6*A*). Currents were observed at all holding potentials tested for PC-2 and S812A, and the current amplitude of these channels was similar. S812A has a slope conductance of 93 pS compared with 85 pS for PC-2 (Fig. 6*B*). PC-2-B10 and T721A exhibited no channel activity at all voltages and cytoplasmic side calcium concentrations tested (Fig. 6, *B* and *C*). Although Thr^{721} is not an apparent site of phosphorylation for PC-2, it is necessary for maintaining its activity as a cation channel.

PC-2 is a Ca^{2+}-dependent cation channel (5), and we examined the effect of phosphorylation on this property of the channel (Fig. 6, *C* and *D*). We found that at 0.3 μM [Ca^{2+}] on the cytosolic side, PC-2 had a higher channel open probability (32%) than S812A (15.4%); at 3 μM [Ca^{2+}], S812A had a higher open probability (39%) than PC-2 (16%; Fig. 6, *C* and *D*). When the channel open probability was compared over the range of Ca^{2+} from 0.01 μM to 1 mM, both wild-type and S812A forms of the PC-2 channel showed a bell-shaped dependence on cytoplasmic Ca^{2+} (Fig. 6*D*). However, there was a shift in the Ca^{2+} dependence such that PC-2 had a maximum open probability of 31% at 0.3 μM [Ca^{2+}], whereas S812A had a maximum open probability of 39% at 10-fold higher Ca^{2+} concentrations (3 μM [Ca^{2+}]; Fig. 6*D*). The S812A substitution results in loss of phosphorylation of PC-2 but may also result in an alteration in

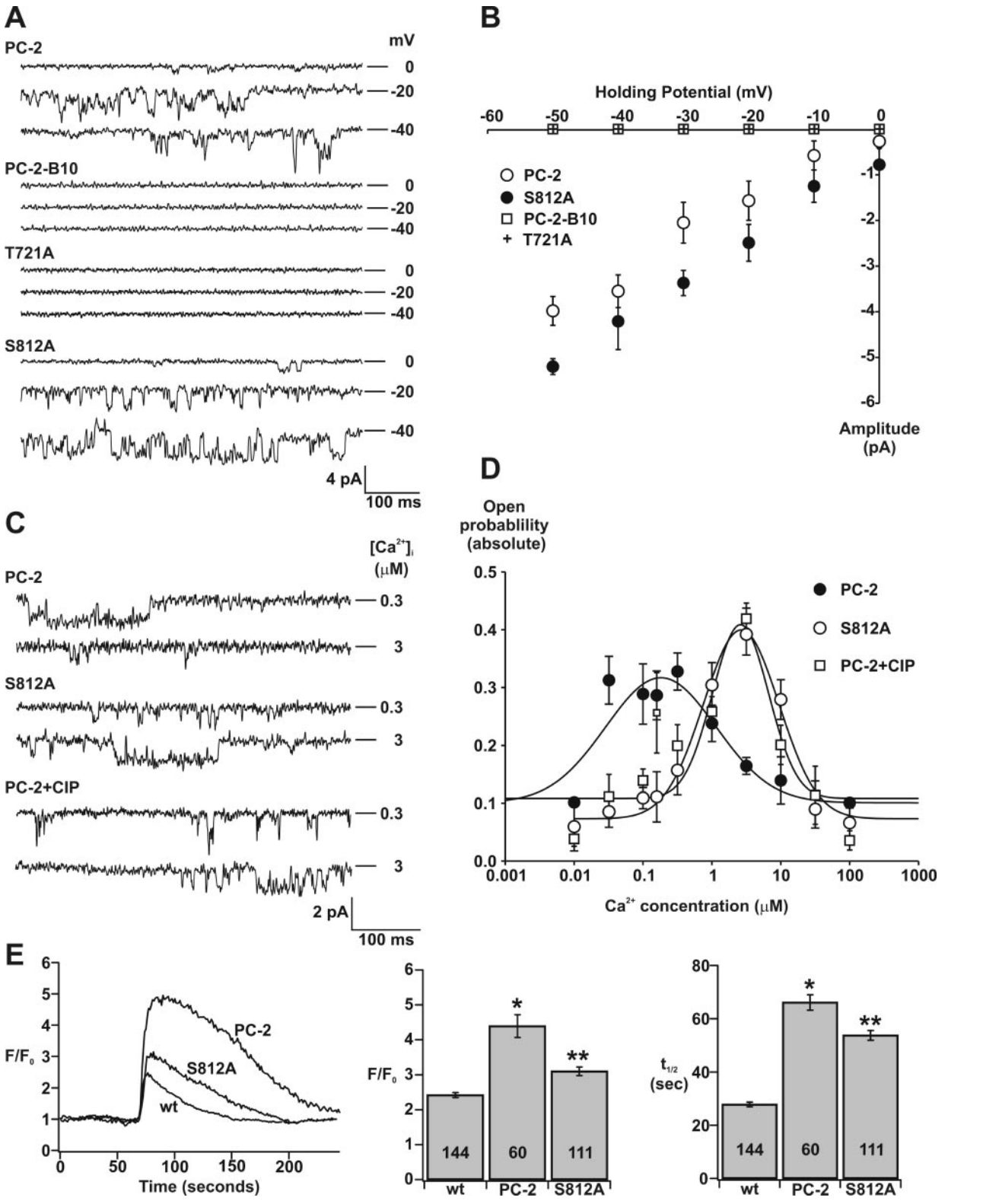

FIG. 6. **Phosphorylation of PC-2 alters the Ca^{2+} activation properties of the channel.** *A*, representative current traces of PC-2, PC-2-B10, T721A, and S812A channel activity at different holding potentials. *Solid lines* to the *right* of each *trace* indicate channel closed-state; *downward deflections* in *traces* indicate channel openings. *B*, current amplitudes for PC-2 channels (○, $n = 15$), PC-2-B10 (□, $n = 20$), T721A (+, $n = 4$), and S812A (●, $n = 20$) were measured at holding potentials ranging from 0 mV to −50 mV. Ba^{2+} was used as the conducting ion and was recorded as an inward current. The slope conductances for PC-2 and S812A between −10 and −20 mV is 85 and 93 pS, respectively. *C*, current traces showing channel open probability of PC-2, S812A, and PC-2+ (calf intestinal phosphatase) in the presence of 0.03 and 3 μM cytosolic Ca^{2+}. *D*, open probability of PC-2 without or with CIP, and S812A channels in the presence of increasing cytosolic Ca^{2+} concentration. PC-2 channels (●, $n = 3$), PC-2 channel in presence of CIP (□, $n = 3$), and S812A channels (○, $n = 3$) were tested at Ca^{2+} concentrations ranging from 0.001 μM to 1 mM. *Error bars*, S.E. *E*, representative traces of Ca^{2+} transients elicited by vasopressin (100 nM) in single LLC-PK_1 cells (*wt*), and LLC-PK_1 cell overexpressing either PC-2 or S812A shown as the ratio of Ca^{2+}-dependent fluo-4 fluorescence over pre-stimulus background fluorescence (F/F_0) (*left panel*). The mean amplitude (±S.E.) (*middle panel*) and the mean half duration ($t_{½}$ ± S.E.) (*left panel*) of vasopressin-induced Ca^{2+} transients measured in each cell line. The number of cells in each group is indicated in the *histogram bar*. *, PC-2-overexpressing cells differed significantly compared with wt and S812A cells, $p < 0.05$; **, S812A-overexpressing cells differed significantly compared with wt, $p < 0.05$.

protein folding. To determine whether the change in Ca^{2+} dependence was the result of the loss of phosphorylation or of some other change, we performed single channel studies in the presence of calf intestinal phosphatase (CIP) either on the *cis* (cytoplasmic) side of the bilayer or on the *trans* (luminal) side. Treatment with phosphatase on the *cis* side resulted in Ca^{2+} dependence identical to that observed in the S812A substitution confirming that the reduced sensitivity to Ca^{2+} activation is indeed the result of loss of phosphorylation (Fig. 6*D*). CIP on the *trans* side of the channel had no effect (data not shown). The maximum open probability of wild type PC-2 channel occurs at a Ca^{2+} concentration at which the non-phosphorylated remains closed. If Ca^{2+} activation plays an important role in PC-2 channel function *in vivo*, then phosphorylation is an essential step in the normal function of PC-2.

We have previously developed an *in vivo* assay for Ca^{2+}-activated PC-2 channel activity (5). Overexpression of wild type PC-2 in LLC-PK_1 cells results in enhanced amplitude and duration of vasopressin-induced Ca^{2+} release from intracellular pools. Consistent with Ca^{2+}-activated PC-2 activity, this enhancement is dependent on baseline Ca^{2+} release also observed in non-transfected cells. We used this system to examine Ca^{2+} release by the non-phosphorylated S812A form of PC-2. Application of vasopressin resulted in transient increase in intracellular Ca^{2+} in control cells as well as cells overexpressing either PC-2 or S812A (Fig. 6*E*). However, both the relative amplitude (Fig. 6*E*) and decay time ($t_{½}$, Fig. 6*E*) for the Ca^{2+} transients were significantly different among the three groups. Overexpression of wild type PC-2 resulted in the largest relative amplitude and duration of transients. Cells expressing only the empty vector had the lowest relative amplitude and duration of Ca^{2+} transients. Cells expressing the non-phosphorylated S812A had Ca^{2+} transients with amplitude and duration intermediate between PC-2 overexpressing and non-overexpressing cells (Fig. 6*E*). The data are consistent with the hypothesis that phosphorylation at Ser^{812} is essential for the normal response by PC-2 to changes in intracellular Ca^{2+} concentration. Collectively, these data suggest that phosphorylation at Ser^{812} modulates the Ca^{2+} dependence of polycystin-2 channel function *in vivo*.

DISCUSSION

Mutations in the PC-2 channel underlie ADPKD. Most of these mutations result in predicted premature termination of the peptide chain (23), which interferes with the functionally important interaction of PC-2 with itself and with PC-1 (24). However, at least one disease-causing mutation, D511V (25), results in loss of channel activity without interfering with COOH-terminal protein interactions (5). Therefore, the channel activity of PC-2 is essential to its function, and loss of the channel activity alone is sufficient to cause the ADPKD phenotype (5). Little is known about the regulation of PC-2 channel activity. Some studies have suggested that interaction with PC-1 is necessary for delivery of PC-2 to its site(s) of primary action (4, 8), and we have shown that cytosolic Ca^{2+} modulates PC-2 channel activity, but other factors responsible for the molecular regulation of the channel activity have not been reported. To begin to address this issue, we investigated the role of protein phosphorylation in the regulation of PC-2.

We found the PC-2 is constitutively phosphorylated *in vivo* at a single site, Ser^{812}. This putative CK2 site is the highest scoring consensus phosphorylation motif predicted by NetPhos 2.0 (available at www.cbs.dtu.dk/services/NetPhos/) in the entire 968-amino acid PC-2 sequence. CK2 can phosphorylate this site *in vitro*, and mutation of this site abolishes phosphorylation of PC-2 *in vivo* and phosphorylation by CK2 *in vitro*. Generally, phosphorylation can regulate channels by influencing their trafficking, interactions, and activity. It is noteworthy that Ser^{812} occurs in a domain previously identified as necessary for retention of PC-2 in the ER (18). Despite this, we found that neither trafficking nor interactions with PC-2 and PC-1 are affected by phosphorylation at Ser^{812}. However, the Ca^{2+}-dependent activation and inactivation of the channel was strongly influenced by phosphorylation at this site. Normal intracellular Ca^{2+} concentration is in the range of ~0.1 μM (26). Minor changes of $[Ca^{2+}]_i$ in this range will result in maximal activation of the wild type PC-2 channel but not the non-phosphorylated S812A channel. We examined this hypothesis *in vivo* using vasopressin stimulation of LLC-PK_1 cells. In non-stimulated LLC-PK_1 cells, $[Ca^{2+}]_i$ is ~0.065–0.15 μM (27–29). Vasopressin increases $[Ca^{2+}]_i$ to 0.22–0.65 μM (27–29) by activation of the V1 receptor with resultant Ca^{2+} release through the inositol 1,4,5-trisphosphate receptor (30). In this range variation of intracellular Ca^{2+}, we would expect PC-2 to be maximally activated while S812A would not be. In keeping with this, we found that the response to vasopressin stimulation on LLC-PK_1 was lower in S812A-overexpressing cells compared with cells overexpressing wild type PC-2. It is therefore likely that phosphorylation is required for the physiologic function of PC-2.

Functional regulation of channel activity by CK2 has been reported in channels expressed in neurons (31, 32). Tonically active CK2 can regulate the activity of the *N*-methyl-D-aspartate through cyclical phosphorylation/dephosphorylation where channel activity is up-regulated by spermine through activation of CK2 or down-regulated by calcineurin phosphatase in response to activation by Ca^{2+}/calmodulin (31). In the Kv3.1 potassium channel, the basal properties of the channel, including its voltage dependence of activation and inactivation, are influenced by constitutive phosphorylation by CK2 (32). Although not as acutely regulated as second messenger-activated protein kinases, CK2 can nonetheless play an essential role in regulating channel properties, and it may exert such an influence on the native PC-2 channel as well.

Finally, the current study provides some insight into the region of the PC-2 molecule responsible for the Ca^{2+} dependence of its channel activity. Initially, it was hypothesized that the Ca^{2+} binding to the EF hand in PC-2 (amino acids 754–782), as well as in the structurally related PCL and α subunit of voltage-activated Ca^{2+} channels, was the mediator of the Ca^{2+}-dependent modulation of channel activity (2, 33, 34). Although studies have not directly addressed this in PC-2, in both polycystin-α-like and the α_{1c} voltage-activated Ca^{2+} channel, this hypothesis has not been entirely borne out (33, 35). It may therefore be that, in PC-2 as well, binding of Ca^{2+} to the EF hand is not the major factor in Ca^{2+}-dependent modulation of the channel. Ser^{812} lies within an acidic amino acid subdomain of the region required for ER retention of PC-2 (amino acids 787–821) (18). Phosphorylation at this site may directly effect the conformation of PC-2 or may alter binding properties to the as yet unknown interacting partners that influence the channel activity. PC-2 is not mutated in the more common and severe form of ADPKD: that caused by mutation in PKD1. PC-2 is a therapeutic target in PKD1 patients, and understanding the mechanisms of regulation of this channel is a necessary step in exploiting the potential role played by modulating PC-2 activity in treating ADPKD.

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