Troponin I Binds Polycystin-L and Inhibits Its Calcium-Induced Channel Activation†

Qiang Li,‡ Yan Liu,‡ Patrick Y. Shen,‡ Xiao-Qing Dai,‡ Shaohua Wang,§ Lawrence B. Smillie,|| Richard Sandford,⊥ and Xing-Zhen Chen*,‡

Membrane Protein Research Group, Department of Physiology, Department of Surgery, and Department of Biochemistry, University of Alberta, Edmonton, Alberta, T6G 2H7, Canada, and Department of Medical Genetics, Wellcome Trust Centre for Molecular Mechanisms in Disease, Cambridge Institute for Medical Research, Addenbrookes Hospital, Cambridge, United Kingdom CB2 2XY

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ABSTRACT: Polycystin-L (PCL) is an isoform of polycystin-2, the product of the second gene associated with autosomal dominant polycystic kidney disease, and functions as a Ca^{2+}-regulated nonselective cation channel. We recently demonstrated that polycystin-2 interacts with troponin I, an important regulatory component of the actin microfilament complex in striated muscle cells and an angiogenesis inhibitor. In this study, using the two-microelectrode voltage-clamp technique and *Xenopus* oocyte expression system, we showed that the calcium-induced PCL channel activation is substantially inhibited by the skeletal and cardiac troponin I (60% and 31% reduction, respectively). Reciprocal co-immunoprecipitation experiments demonstrated that PCL physically associates with the skeletal and cardiac troponin I isoforms in overexpressed *Xenopus* oocytes and mouse fibroblast NIH 3T3 cells. Furthermore, both native PCL and cardiac troponin I were present in human heart tissues where they indeed associate with each other. GST pull-down and microtiter binding assays showed that the C-terminus of PCL interacts with the troponin I proteins. The yeast two-hybrid assay further verified this interaction and defined the corresponding interacting domains of the PCL C-terminus and troponin I. Taken together, this study suggests that troponin I acts as a regulatory subunit of the PCL channel complex and provides the first direct evidence that PCL is associated with the actin cytoskeleton through troponin I.

Autosomal dominant polycystic kidney disease (ADPKD)[1] is an inherited nephropathy, primarily characterized by the formation of fluid-filled cysts in the kidneys. It is one of the most frequent genetic disorders affecting approximately 0.1% of individuals and accounts for up to 10% of all cases of end-stage renal disease (*1*). *PKD1* and *PKD2* are the two known genes responsible for about 95% of ADPKD and have been mapped to chromosomes 16p13.3 and 4q21−23, respectively (*2*, *3*). Polycystin-1 (PC1), the product of *PKD1*, is a large cell surface membrane glycoprotein (460 kD) with 11 putative transmembrane domains, a short intracellular C-terminus, and a large extracellular N-terminal region containing multiple domains (*2*). Polycystin-2 (PC2), the product of *PKD2*, is a 110 kD integral membrane protein and contains six putative transmembrane spans and intracellular N- and C-termini (*3*). Polycystin-L (PCL), encoded by *PKDL*, is the third member of the polycystin family (*4*). A 7-cM deletion including the locus containing the mouse homologue of *PKDL* is associated with defects in kidney and retina (*4*). However, the precise physiological roles of PCL remain unknown. PCL is an isoform of PC2 (52% identity) and the product of *PKD2L2* (51% identity) whose function is unclear (*5*, *6*). Homologues of *PKD2*, *PKDL* or *PKD2L2* have been identified in other species, including *Caenorhabditis elegans* and *Drosophila PKD2* (*7*), which exhibit about the same sequence similarity to human *PKDL*, *PKD2*, and *PKD2L2*. *C. elegans PKD2* is involved in mating behavior while the physiological role of *Drosophila PKD2* remains elusive. PCL shares similar membrane topology and modest sequence homology with the α-subunits of voltage-gated K^+, Na^+, and Ca^{2+} channels and the transient receptor potential channels (*8*), which are involved in the transduction of sensory stimuli in vision, olfaction, thermoreception, osmoregulation, and mechanosensation (*9*−*11*). We have shown that PCL is a Ca^{2+}-permeable, Ca^{2+}-activated nonselective cation channel when expressed in *Xenopus* oocytes (*12*, *13*). The single-channel properties of PCL were strongly asymmetrical with respect to the membrane potential (*13*). We have also demonstrated that the Ca^{2+}-induced channel activation is not due to the EF-hand present in the PCL C-terminus nor to other parts of the C-terminal domain (*14*). Instead, this EF-hand domain was shown to control the Ca^{2+}-induced PCL channel activation (*14*). PC2 was also shown to be a Ca^{2+}-permeable nonselective channel with functional

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* To whom correspondence should be addressed. Tel: (780) 492-2294. Fax: (780) 492-8915. E-mail: xzchen@ualberta.ca.

‡ Membrane Protein Research Group, Department of Physiology, University of Alberta.

§ Department of Surgery, University of Alberta.

|| Department of Biochemistry, University of Alberta.

⊥ Cambridge Institute for Medical Research, Addenbrookes Hospital.

[1] Abbreviations: ADPKD, autosomal dominant polycystic kidney disease; *PKD1*, polycystic kidney disease-1 gene; PC1, polycystin-1; *PKD2*, polycystic kidney disease-2 gene; PC2, polycystin-2; PCL, polycystin-like; *PKDL*, polycystic kidney disease-like gene; TnI, troponin I; GST, glutathione-*S*-transferase; co-IP, co-immunoprecipitation.

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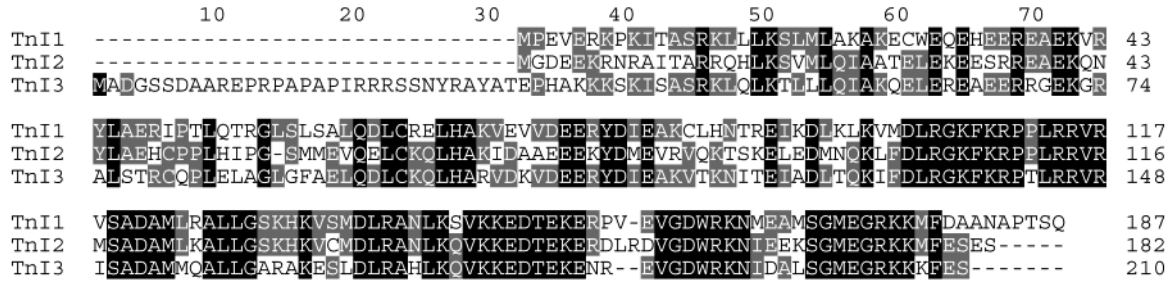

FIGURE 1: Alignments of three troponin I isoforms. Two skeletal (TnI1, 187 amino acids and TnI2, 182 amino acids) and cardiac (TnI3, 210 amino acids) troponin I were compared.

characteristics and membrane targeting distinct from PCL (*15*−*20*). PC2 was reported to interact with polycystin-1 (*18*, *21*−*24*) and several other proteins, including CD2AP, Hax-1, tropomyosin-1, and troponin I (*25*−*28*). No interacting partner of PCL has so far been reported.

Troponin I (TnI) is an important regulatory component of the actin microfilament complex, and together with troponin-C (TnC) (a Ca^{2+} receptor) and troponin T (TnT) (a tropomyosin-binding protein), forms the troponin complex that is attached to the thin filament of striated muscles that provide a calcium-sensitive switch for muscle contraction (*29*, *30*). As the inhibitory subunit of the troponin complex, TnI binds to actin. It inhibits actomyosin Mg^{2+}-ATPase in the presence of tropomyosin, and the inhibition is removed by TnC in the presence of Ca^{2+} (*29*). Multiple isoforms of TnI are found in birds and mammals and are encoded by at least three distinct genes: slow-twitch skeletal isoform (*TnI1* or *ssTnI*), fast-twitch skeletal isoform (*TnI2* or *fsTnI*), and cardiac isoform (*TnI3* or *cTnI*) (*31*−*33*). The three isoforms of troponin I consist of 182−210 amino acid residues and share high sequence homology; the cardiac isoform is larger than the two skeletal isoforms because of the presence of an additional 30 amino acids on its N-terminus (see Figure 1) (*29*). Recently, several reports demonstrated that TnI plays various functional roles other than being a constituent part of the troponin complex, such as modulation of calcium channels (*34*) and inhibition of angiogenesis (*35*) that was reported to be associated with ADPKD (*36*).

In the present study, we studied functional modulation of the PCL channel by cardiac and skeletal TnIs using a *Xenopus* oocytes expression system in conjunction with electrophysiologic analysis. We also investigated the physical interaction between PCL and TnIs using a variety of techniques and under various in vitro and in vivo conditions.

EXPERIMENTAL PROCEDURES

Preparation of RNAs and Oocytes. The *TnI1*, *-2*, and *-3* cDNAs were recently cloned through a standard PCR approach (*28*). The *PKDL* and *TnI* cDNAs in vectors pTLN2 (*37*) and/or pCDNA3.1 (Invitrogen, Toronto, ON) were linearized and purified by phenol/chloroform extraction and ethanol precipitation. Capped, complimentary RNAs were synthesized in vitro from linearized cDNAs using the mMessageMachine Kit (Ambion, Austin, TX). Stage V−VI oocytes were extracted from *Xenopus laevis* and defolliculated by collagenase type I (2.5 mg/mL, Sigma-Aldrich Canada, Oakville, ON) in the Barth's solution (*13*, *14*) at room temperature for 2 h. Oocytes were then injected with 50 nL of water containing 20 or 40 ng of each RNA 3−24 h following defolliculation. An equal volume of water was injected into control oocytes. Injected oocytes were incubated at 16−18 °C in the same solution supplemented with antibiotics for 2−5 days prior to experiments.

Two-Microelectrode Voltage-Clamp Technique. Experimental approaches were similar to those previously described (*13*, *14*). Briefly, the two electrodes impaling *Xenopus* oocytes were filled with 3 M KCl to form a tip resistance of 0.5−3 MΩ. Oocytes whole-cell currents were measured through Pentium III PC computers and the commercial amplifiers TEV-200A and CA-1B (Dagan Co., Minneapolis, MN). The Digidata 1320A AD/DA converter and the pClamp 8 software (Axon Instruments, Union City, CA) were applied for data acquisition and analysis. Currents and voltages were digitally recorded at 200 μs/sample and filtered at 2 kHz through a Bessel filter. SigmaPlot 8 (Jandel Scientific Software, San Rafael, CA) was used for data fitting and plotting.

Expression in Mouse Fibroblast NIH 3T3 Cells. Full-length *PKDL* and *TnI1*, *-2*, or *-3* were subcloned into pCDNA3.1 for mammalian cell expression. Mouse fibroblast NIH 3T3 cells were cultured in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum. Cells were cultured to 40% confluency for transient transfection using Effectene Transfection kit (Qiagen, Mississauga, ON) as we described previously (*27*).

Co-Immunoprecipitation (co-IP). Co-IP experiments using NIH 3T3 cells were performed 48 h following transient transfection with *PKDL* and *TnI1*, *-2*, or *-3*, based on our recent protocol (*28*). Briefly, cell monolayers were grown in 100 mm dishes and lysed in 500 μL of an ice-cold lysis buffer. Total proteins (400 μL each) from postnuclear supernatant were incubated on ice for 1 h either with the PCL antibody, which was raised from rabbit against the C-terminal tail (ALEERRLSRGEIPTLQRSC) of PCL, or with a TnI antibody (cardiac TnI from Fitzgerald, Concord, MA; skeletal TnI from Biodesign, Saco, ME), followed by another 1 h incubation with gentle shaking upon addition of 50 μL of protein G-sepharose (Sigma-Aldrich Canada). The immunoprecipitates absorbed to protein G-sepharose were resuspended in 50 μL of Laemmli's sample buffer, and a 25 μL aliquot of the extract was subjected to SDS−PAGE, followed by immunoblotting. The membranes were incubated with the TnI or PCL antibody, and the signal was visualized with enhanced chemiluminescence (Amersham, Baie d'Urfe, QC). A similar method was employed for co-IP using *Xenopus* oocytes coexpressing PCL and a TnI. Reciprocal co-IP of native PCL and TnI was performed as described

above using human adult heart tissues (15 mg of total protein) collected from the University of Alberta Hospital.

GST Pull-Down Assay. The bacterial expression vector pGEX-5X-3 (Pharmacia, Piscataway, NJ) was used to produce a glutathione-*S*-transferase (GST) fusion protein in *Escherichia coli.* The C-terminus of PCL (amino acids D562−V805, named PCLC) was fused in frame into pGEX-5X-3 and transformed into bacterial strain BL21 (DE3). Bacterial culture was induced by 1 mM IPTG and allowed to express proteins for 5 h at 30 °C. The protein extracts were purified using a GST purification kit (Clontech, Palo Alto, CA) or directly performed GST pull-down after cell lysis. Precleared bacterial protein extracts (250 μL) containing GST-PCLC or GST alone were incubated with 4 μg of purified human cardiac TnI (*38*) or skeletal TnI protein (Calbiochem, San Diego, CA) in the binding buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$). The mixture was incubated overnight at 4 °C for subsequent affinity binding to glutathione-agarose beads (Sigma-Aldrich Canada) at room temperature for 1 h. The beads were washed several times with the binding buffer, and the resulting protein samples were prepared for Western blots. The immunoblots were detected by the cardiac or skeletal troponin I antibody.

Microtiter Assay. Purified cardiac or skeletal TnI protein (0.2 μg/well) was covalently immobilized onto 96-well microtiter plates using 1-cyclohexyl-3-(2-morpholinoethyl) carbodimide metho-*p*-toluenesulfonate (1.25 mg/mL, Sigma-Aldrich Canada). The wells were washed three times with the PBS buffer and blocked for 2 h at room temperature in the PBS buffer supplemented with 2% BSA. After washing three times with an antibody buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 0.5 mM Ca^{2+}, 1% BSA, 0.05% Triton X-100), the wells were incubated with various concentrations (0−1600 nM) of purified GST-PCLC or GST, overnight at room temperature. Bound proteins were detected by incubating the wells with a monoclonal GST antibody (1:1500, a kind gift of Larry Fliegel) and a horseradish-peroxidase-coupled IgG secondary antibody (1:1500, Chemicon International, Temecula, CA). This was followed by incubation with the peroxidase substrate *o*-phenyldiamine (Sigma-Aldrich Canada) for 5 min, addition of 3 M sulfuric acid to stop the reaction, and detection of enzymatic activity at 450 nm in a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA). Binding curves were fitted to the Michaelis−Menten equation ($y = y_0 + (y_{max}x)/(x + K_{1/2})$) using Sigmaplot 8. Values were expressed as mean ± standard error (SE).

Yeast Two-Hybrid Analysis. The MATCHMAKER 3 yeast two-hybrid system was purchased from Clontech. The yeast two-hybrid experiments were performed based on our recent protocol (*27*). Briefly, the *PCLC* and its various truncated fragments were subcloned in frame into the GAL4 DNA binding domain of vector pGBKT7 as bait, while the full-length *TnI3* and its various truncated fragments were constructed in the GAL4 activation domain of vector pGADT7 (*28*) as prey. The plasmid pGADT7-TnI3 was co-introduced in the yeast strain Y187 with pGBKT7-PCLC or a negative control, including the pGBKT7 vector alone, pGBKT7-PC1C (PC1C refers to the C-terminal domain G4088−T4302 of polycystin-1), pGBKT7-LamC (human lamin C), and pGBKT7-p53 (murine p53). Transformants were grown on synthetic dropout medium lacking leucine, tryptophan, and then assayed to verify the real interaction. A liquid culture assay was used to quantify β-galactosidase activity using *o*-nitrophenyl β-D-galactopyranoside (Sigma-Aldrich Canada) as substrate.

RESULTS

Inhibition of Calcium-Induced PCL Channel Activation by TnIs. Polycystin-2 has been shown to interact with both cardiac and two skeletal TnIs through its cytoplasmic C-terminus (*28*). These three TnI isoforms share high sequence similarity (56−63% identity). However, the cardiac TnI3 has a longer N-terminus as compared with two skeletal TnI1 and TnI2 (Figure 1). To determine whether PCL, an isoform of PC2, interacts with TnIs as well, we coexpressed PCL with the skeletal TnI1 or cardiac TnI3 in *Xenopus* oocytes and examined the PCL channel activities under different conditions. Upon coexpression of a TnI, the PCL-mediated calcium transport, assessed by radiolabeled ^{45}Ca uptake, was substantially decreased (Figure 2A). When coexpressed with the skeletal TnI1, the Ca^{2+}-activated peak channel current (I_{Na+Ca}) was substantially decreased while the PCL-mediated resting Na^+ current (I_{Na}) at −50 mV, measured in the absence of extracellular Ca^{2+}, did not significantly change (Figure 2B). As the result, the PCL channel activation ability (*14*) assessed by the ratio of I_{Na+Ca}/I_{Na} was substantially reduced (by 60%) (Figure 2C). Cardiac TnI3 exhibited similar effects and decreased the PCL channel activation ability by 31%. Thus, the observed decreases in the Ca^{2+}-activated PCL channel activity in the presence of a TnI are not due to decreased PCL expression in the plasma membrane, which should result in an unchanged ratio of I_{Na+Ca}/I_{Na}. When current−voltage curves were obtained for membrane potentials between −140 and +90 mV, we observed no significant effect of TnI1 on the PCL resting permeability to Na^+ (Figure 3A). On the other hand, upon application of 5 mM Ca^{2+} to the external solution, significant reduction of the PCL channel activation was seen at negative membrane potentials, in oocytes coexpressing TnI1 (Figure 3B). The inhibition of the activated current (I_{Na+Ca}) seemed to be inward rectified. The mechanism of this rectification remained unclear.

In Vivo Interaction between PCL and TnIs in 3T3 Cells and Oocytes. To determine the possibility that PCL physically interacts with TnIs, as suggested by our data on functional modulation of the PCL channel by TnIs, we performed co-IP experiments using overexpressed mouse fibroblast NIH 3T3 cells and *Xenopus* oocytes. Total proteins extracted from 3T3 cells transiently transfected with *PKDL* and *TnI* were used for co-IP using the PCL antibody. The precipitates were subjected to SDS−PAGE, blotted onto membranes, and probed with the antibody against the cardiac or skeletal TnI. TnI3 signal was observed in cells cotransfected with *PKDL* and *TnI3* but not in those transfected with *PKDL* alone or nontransfected (Figure 4A). Similarly, TnI1 and TnI2 signals were also detected in cells cotransfected with *PKDL* and *TnI1* (or *TnI2*) (Figure 4B). Likewise, in reciprocal co-IP experiments, the PCL signal was seen in cells cotransfected with *PKDL* and *TnI1*, *-2*, or *-3* (Figure 4C). This result suggests that PCL interacts with all three troponin I isoforms. In parallel, cell lysates from oocytes injected with complementary RNAs of *PKDL* and *TnI1*, *-2*, or *-3* were also tested by co-IP for their physical interaction.

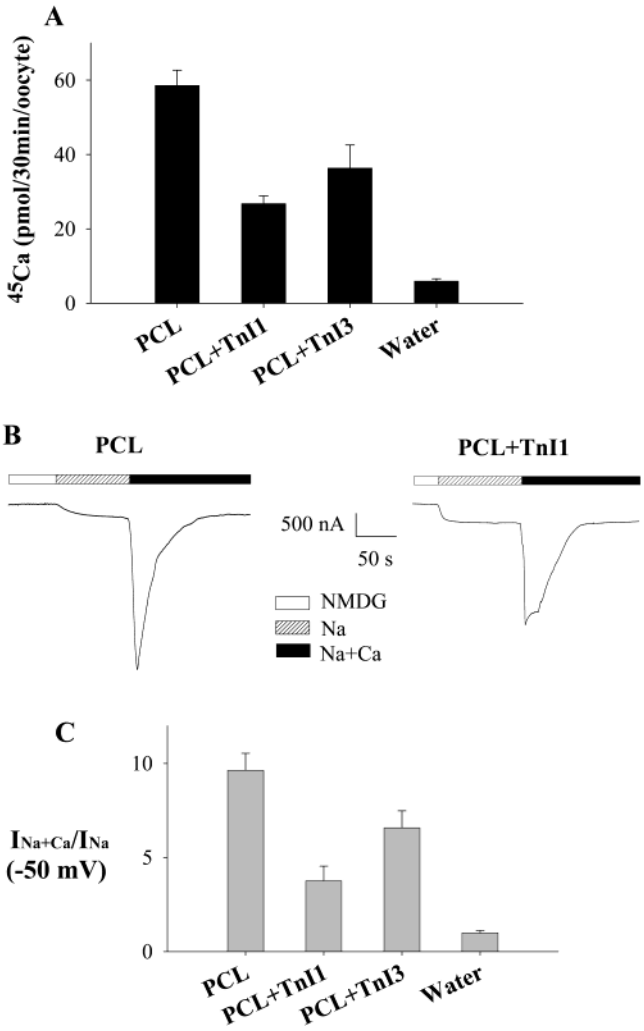

FIGURE 2: Effects of TnIs on the PCL channel activities. (A) ^{45}Ca uptake in oocytes expressing PCL, PCL + TnI1, and PCL + TnI3. Control uptake level was obtained using water-injected oocytes. (B) Representative whole-cell current recordings under voltage clamp at −50 mV, illustrating the basal Na^+ currents (in the absence of extracellular Ca^{2+}) and the Ca^{2+}-induced $Na^+ + Ca^{2+}$ currents in oocytes expressing PCL or PCL + TnI1. Na in the figure refers to the standard (Na^+-containing) solution (in mM: 100 NaCl, 2 KCl, 1 $MgCl_2$, 10 Hepes, pH 7.5). NMDG refers to the standard solution with *N*-methyl-D-glucamine (NMDG) replacing Na^+. Na + Ca refers to Na plus 5 mM $CaCl_2$. (C) Ca^{2+}-induced PCL channel activation ability assessed by the ratio I_{Na+Ca}/I_{Na} obtained at −50 mV where (I_{Na}) and activated (I_{Na+Ca}) refer to the basal Na^+ and activated $Na^+ + Ca^{2+}$ currents, respectively. The average ratios were 9.6 ± 0.9 for PCL ($N = 56$), 3.8 ± 0.8 for PCL + TnI1 (60% reduction as compared to PCL, $N = 26$, $P = 0.0001$), 6.6 ± 0.9 for PCL + TnI3 (31% reduction, $N = 49$, $P = 0.02$), and 1.0 ± 0.1 for water-injected control oocytes ($N = 23$).

Indeed, PCL was able to coprecipitate with *TnI1*, *-2*, and *-3* in oocytes, with slightly larger molecular masses than in 3T3 cells based on a slower migration on the SDS−PAGE gel, possibly because of distinct posttranslational modifications (Figure 4D, E). Conversely, three troponin I isoforms were also able to precipitate the PCL protein (Figure 4F). Taken together, our data demonstrated that PCL and TnIs associate with each other in vivo in 3T3 cells and oocytes.

In Vivo Interaction between Native PCL and TnI3 in Human Heart Tissues. To examine whether PCL and troponin I are naturally colocalized and interact with each other, we performed co-IP experiments using human adult

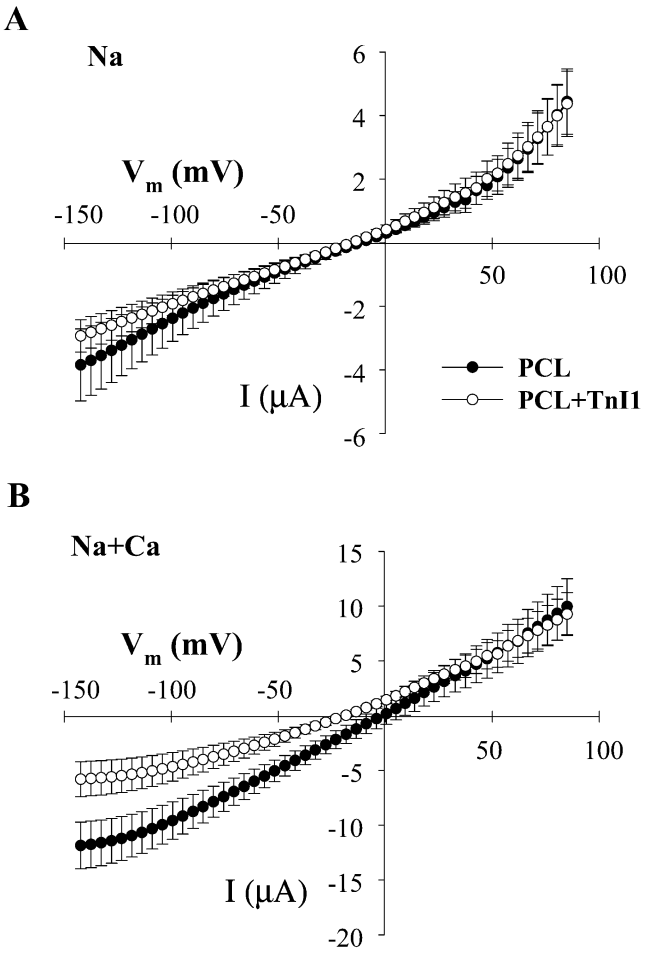

FIGURE 3: Current−voltage relationships for oocytes expressing PCL or PCL + TnI1. (A) Averaged *I*−*V* curves obtained from oocytes expressing PCL ($N = 10$) or PCL + TnI1 ($N = 12$) in the presence of the standard solution (Na). (B) Averaged *I*−*V* curves for PCL and PCL + TnI1 in the presence of the standard solution plus 5 mM Ca^{2+} (Na + Ca). A voltage ramp protocol (*12*) was employed in the experiments.

cardiac tissues. Immunoprecipitation of tissue lysates from human heart with the PCL antibody precipitated the TnI3 protein (Figure 5A). Reciprocally, TnI3 was also able to precipitate the native PCL protein from the same tissues (Figure 5B). Thus, both proteins are natively colocalized in the heart where they naturally associate with each other.

In Vitro Interaction between PCL and TnIs. Biochemical methods were applied to further investigate the interaction between PCL and TnIs. We first used a GST pull-down assay. Since TnIs associate with the intercellular C-terminus of PC2, we also utilized the C-terminus of PCL as a potential interacting target of TnIs. To test this hypothesis, the PCLC fragment was fused in frame with a GST epitope. The recombinant protein was produced in *E. coli*. Cell lysates containing fusion protein GST-PCLC or GST alone were incubated with purified human cardiac or skeletal TnI, respectively. The mixture was incubated with glutathione-agarose beads, which can couple to the GST-tagged proteins. After extensive washes, retained proteins were prepared for Western blots that were then probed with the monoclonal cardiac or skeletal troponin I antibody. GST tagged PCLC, but not GST alone (control), coprecipitated with both the cardiac (Figure 6A) and the skeletal TnI (Figure 6B). This

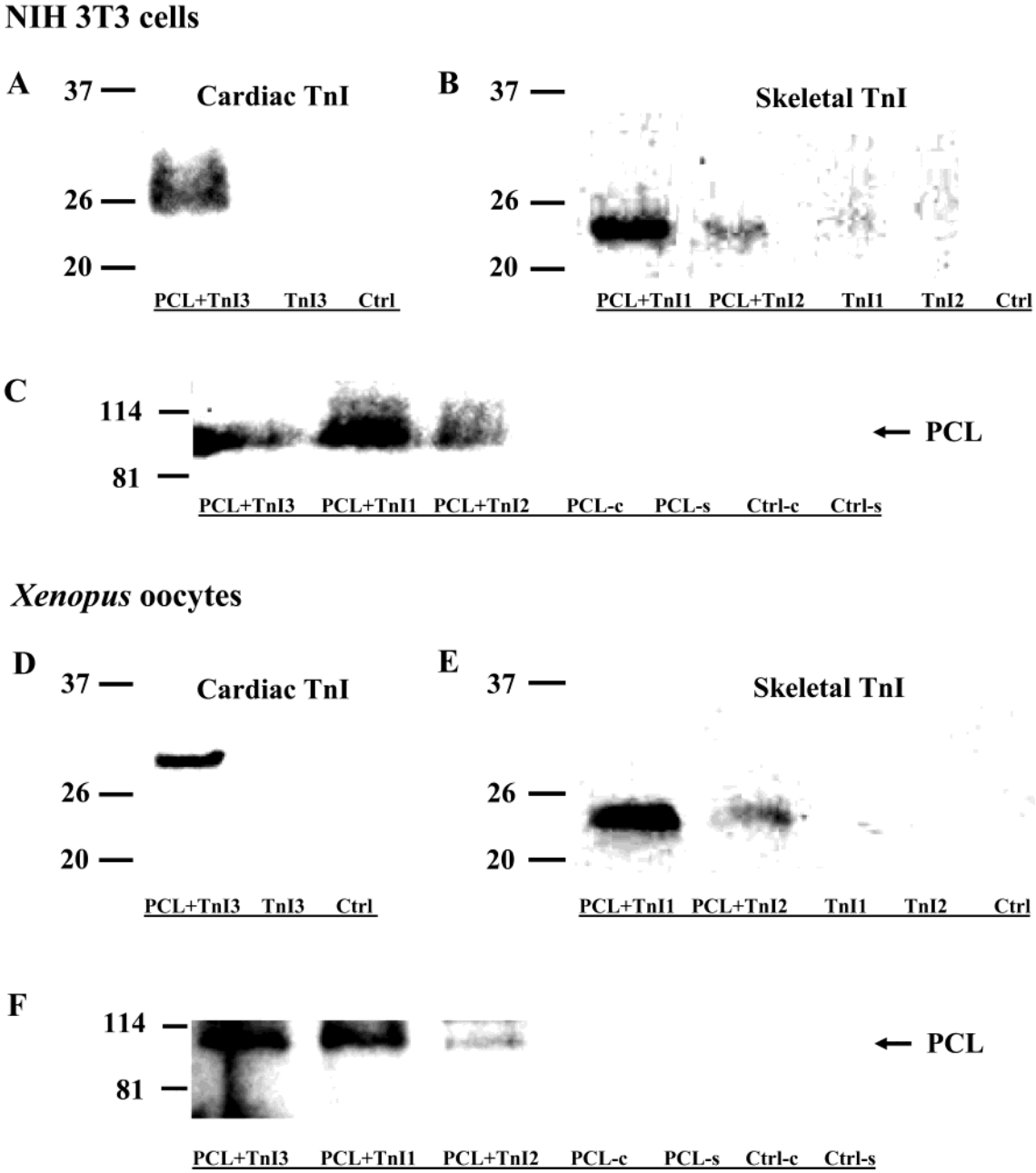

FIGURE 4: Association between PCL and TnIs in NIH 3T3 cells and *Xenopus* oocytes by reciprocal co-IP. The skeletal TnI antibody was used for precipitating or detecting TnI1 and TnI2, while the cardiac TnI antibody was used for TnI3. (A) co-IP assay using NIH 3T3 cells expressing PCL + TnI3, TnI3, or nontransfected cells (Ctrl). Protein lysates from 3T3 cells were incubated with the PCL antibody. Immunoprecipitated proteins were analyzed by immunoblotting using the cardiac TnI antibody. (B) 3T3 cells transiently expressing PCL + TnI1, PCL + TnI2, TnI1, TnI2, or nontransfected cells (Ctrl). Protein lysates were incubated with the PCL antibody, and the precipitated proteins were detected by immunoblotting using the skeletal TnI antibody. (C) Reciprocal co-IP assay using 3T3 cells expressing PCL + TnI1, PCL + TnI2, PCL + TnI3, PCL (lanes 4 and 5), or nontransfected cells (lanes 6 and 7). Protein lysates were precipitated with the TnI antibodies and detected by the PCL antibody. Lysates from control cells were precipitated with the cardiac (PCL-c and Ctrl-c) or skeletal (PCL-s and Ctrl-s) antibody. (D−F) Reciprocal co-IP assays in overexpressed *Xenopus* oocytes. Similar experimental conditions were used as in panels A−C, respectively.

experiment provided another piece of evidence for physical interaction between PCL and TnI and demonstrated that the C-terminus of PCL is the domain for the interaction.

A microtiter assay was performed to obtain a quantitative assessment of the binding between PCLC and TnI. Purified cardiac or skeletal TnI protein was immobilized on wells of microtiter plate, which was washed using the PBS buffer and blocked by 2% BSA. Purified GST-PCLC or GST alone at various concentrations was added to examine their capability of binding TnI. GST-PCLC bound to the cardiac TnI or skeletal TnI in a concentration-dependent and saturable manner, with the half-maximum binding concentration ($K_{1/2}$) of 994 ± 67 nM (N = 4) and 396 ± 117 nM (N = 7), respectively (Figure 7). These data further substantiated the PCL−TnI interaction. Michaelis−Menten relationship of the dose-dependent PCLC and TnI association suggests that one PCLC molecule associates with one or more TnI molecule(s).

Quantification of the PCL−TnI Interaction and Determination of Domains Involved in the Interaction. The PCLC fragment was constructed in vector pGBKT7 as bait, and TnI3 was chosen to fuse into vector pGADT7 as prey for

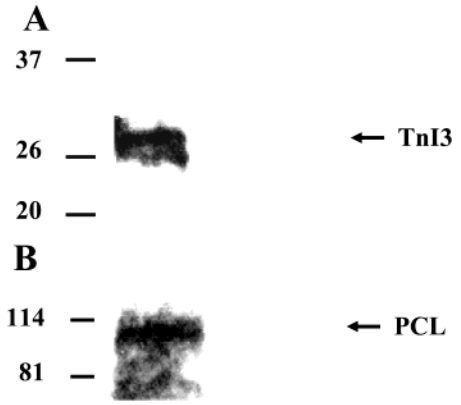

FIGURE 5: Association between native PCL and TnI3 in human heart tissues by reciprocal co-IP. (A) Tissue lysates from human adult heart were precipitated with the PCL antibody (left) or nonimmune serum (right) and detected by the cardiac TnI antibody. The TnI3 protein was present on the PCL antibody precipitates. (B) Tissue lysates from human adult heart were precipitated by the cardiac TnI antibody (left) or nonimmune serum (right) and probed with the PCL antibody. The PCL protein was present on the TnI3 antibody precipitates.

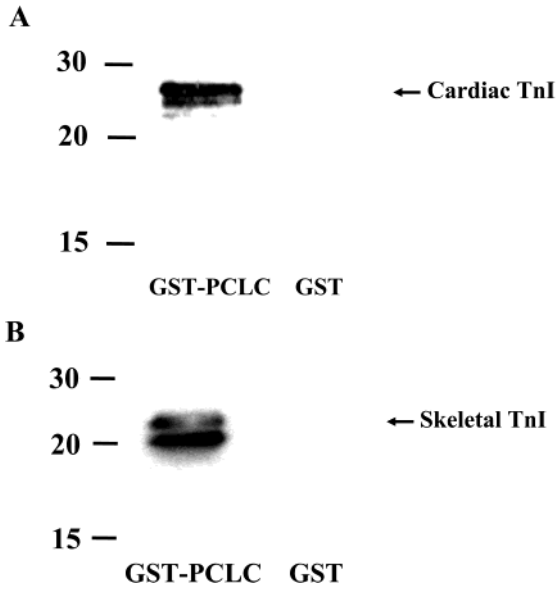

FIGURE 6: Interaction of PCL with TnIs detected by GST pull-down assay. *E. coli* extracts expressing GST-PCLC or GST alone were incubated with human cardiac (A) or skeletal TnI (B) proteins at 4 °C overnight. Glutathione-agarose beads were used to precipitate the GST-epitope binding proteins. The resultant protein samples were immunoblotted using the cardiac or skeletal TnI antibody.

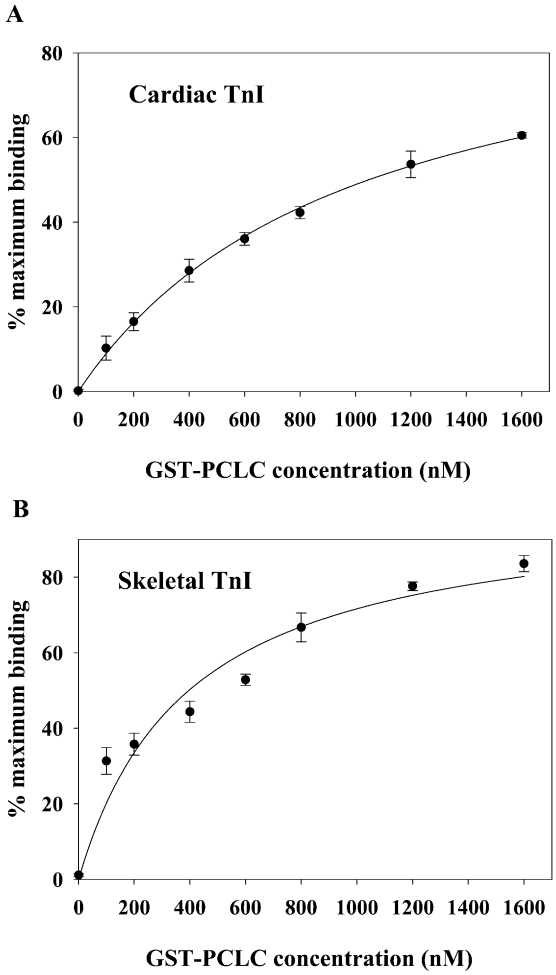

FIGURE 7: Dose-dependent binding of PCL with TnIs using microtiter plate binding assay. Human cardiac TnI (A) or skeletal TnI (B) at 0.2 μg/well was first immobilized on 96-well microtiter plates. The immobilized proteins were then incubated with different concentrations of GST-PCLC and GST alone. Bound proteins were incubated with the GST antibody and substrate *o*-phenyldiamine, followed by detection of enzymatic activity at 450 nm in a microplate reader. Shown data have been subtracted by the GST background reading and averaged from 4 to 7 replicates. The curves were generated by Michaelis−Menten fits to normalized data.

the yeast two-hybrid experiment. The colonies survived from the synthetic dropout selective medium lacking leucine and tryptophan were used in a filter lift assay to examine β-galactosidase activity for the activation of the *lacZ* reporter gene. These colonies rapidly (within 2 h) turned into blue in the presence of substrate X-gal, while no similar color change was observed when pGADT7-TnI3 was co-transformed with the empty vector pGBKT7, pGBKT7-PCLN (PCLN refers to the N-terminus of PCL, M1-I97), pGBKT7-PC1C, pGBKT7-lamC, or pGBKT7-p53. To evaluate the relative strength of the PCLC−TnI3 interaction, we quantified the β-galactosidase activity by a liquid culture assay (*27*) using *o*-nitrophenyl β-D-galactopyranoside and compared to the positive control assessed by the association between p53 and T-antigen. As illustrated in Figure 8A, the strength of the PCLC−TnI3 interaction represented approximately 52% of the positive control level and four times higher than that of the PC2C−PC2C interaction (*22*).

We also used the yeast two-hybrid system to determine the domains of PCL and TnI3 that mediate the physical association between the two proteins. Using PCR-based amplification, different portions of the *PKDL* 3′ terminus (the DNA fragment encoding PCLC) and *TnI3* were fused in frame into pGBKT7 and pGADT7, respectively, and cotransformed. As shown in Figure 8B, TnI3 interacted with domain H630−E705 of PCL but not with a smaller polypeptide (H630−E668). On the other hand, PCL interacted with two separate regions of TnI3, M1-V107, and K106-L158 (Figure 8C). Of note, the interacting domain H630−E705 of PCL includes the coiled−coil structure (K656−S686) and EF-hand Ca^{2+}-binding motif, while the interacting domain M1-L158 of TnI3 contains two coiled−coil structures. It remains to be determined whether the two proteins associate with each other through a pair of coiled−coil domains.

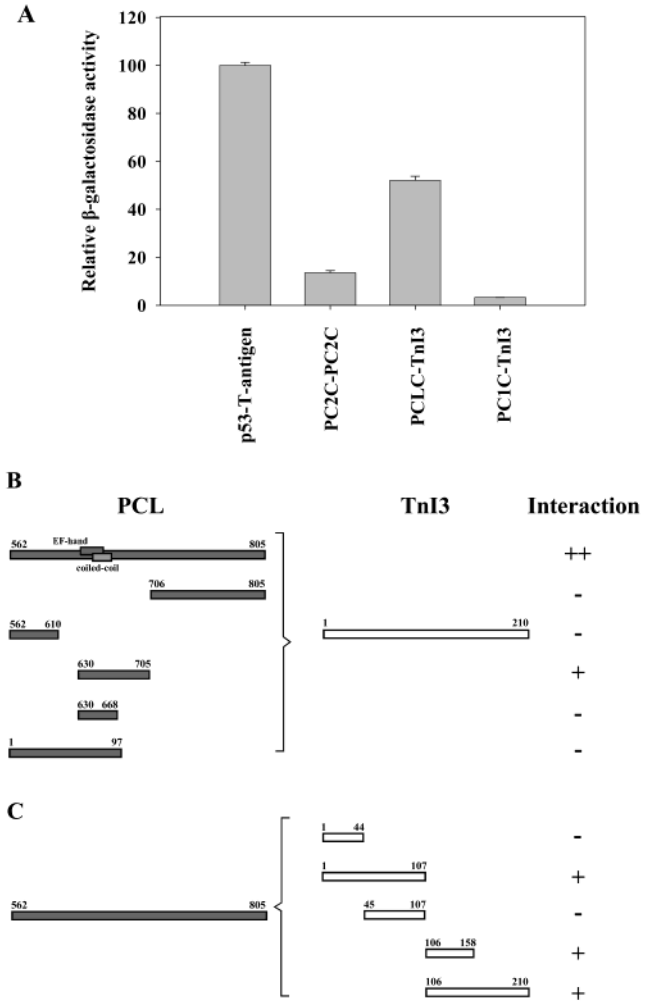

FIGURE 8: Quantification of the PCL−TnI interaction and identification of the domains of the two proteins responsible for the interaction by the yeast two-hybrid approach. (A) Quantitative interaction between PCLC and TnI3 (column 3) in a yeast liquid β-galactosidase assay was originally assessed by A_{420}/A_{600} and then normalized to the level of the p53−T-antigen interaction (column 1). Columns 2 and 4 serve as positive and negative control, respectively. The histograms represent the means ± SE from five independent experiments. (B) Schematic representation of domains responsible for the interaction between PCL and TnI3. Interaction between various PCL and TnI3 domains was examined by the yeast two-hybrid analysis. PCL domains were constructed in the pGBKT7 vector and those of TnI3 in pGADT7. The start and termination amino acid residue numbers for each domain are indicated. ++, +, and − indicate strong, weak, and no interaction, respectively.

DISCUSSION

In the present study, we have used the two-microelectrode voltage-clamp technique to investigate functional modulation of the PCL channel by troponin I, a core regulatory protein of the actin cytoskeleton system and a potent inhibitor of angiogenesis. When PCL was coexpressed with either the skeletal TnI1 or the cardiac TnI3 in *Xenopus* oocytes, we have observed significant inhibition of the PCL channel activation induced by Ca^{2+}. We have further examined the interaction between PCL and TnIs using protein−protein interaction approaches. Our co-IP experiments have revealed direct association between PCL and *TnI1*, *-2*, or *-3* upon coexpression in NIH 3T3 cells and *Xenopus* oocytes. Moreover, we have determined the presence and physical interaction of native PCL and TnI3 in human heart. Using GST pull-down and microtiter binding assays and the yeast two-hybrid method, we have further documented the PCL−TnI interaction and identified the domains of PCL and TnI3 that are responsible for the interaction. Taken together, we have concluded that TnI inhibits the Ca^{2+}-induced PCL channel activation through binding to a carboxyl fragment of PCL (H630−E705) from the intracellular compartment.

While the Ca^{2+}-induced PCL channel activation ability was substantially reduced by TnI, the resting (or basal) PCL channel activity was not significantly altered. This inhibitory effect of troponin I on the PCL channel activation may be attributed to the Ca^{2+}-binding EF-hand motif of PCL (D646−E657), which is located within the H630−E705 domain that mediates the association with TnI3. Our recent study demonstrated that this EF-hand is not essential for the channel activation but rather controls/reduces the PCL channel activation (*14*). Thus, it is interesting in a future study to examine the possibility that the interaction between TnI and domain H630−E705 increases the vitality of the EF-hand and consequently enhances the ability to control the channel activation.

PCL was found in a variety of cell types, including epithelial, neuronal, endothelial, and muscle cells. In adult kidney, PCL is predominantly localized in the principal cells of inner medullary collecting ducts (*39*). In embryonic kidney, it is found in the apical membrane of tubular epithelial cells (*39*). In heart, PCL is found in the epicardium and in the endothelial cells of the larger blood vessels. PCL signal is also detected in discrete cell types of retina, testis, liver, pancreas, and spleen (*4*, *39*). The subcellular distribution of PCL varies from one tissue to another. For example, PCL is present predominantly on or near the plasma membrane of polarized epithelial cells, whereas it is localized in intracellular compartments of retinal neurons (*39*).

Skeletal and cardiac troponin I were initially believed to be striated muscle-specific (*33*, *40*). In adults, *TnI1*, *-2*, and *-3* are exclusively expressed in slow-, fast-twitch skeletal muscle fibers and cardiac muscle, respectively (*33*, *40*). In the past decade, troponin I was found to be more widely distributed. For example, TnI1 was shown to be present in cardiac muscle during embryonic and fetal development (*33*, *41*). TnI3 antibodies specifically recognized a component in the aortic or coronary smooth muscle extracts with electrophoretic properties identical to the TnI3 protein, and this was confirmed by immunofluorescence study (*42*). TnI3 mRNA message was also found in serum samples of patients with skeletal muscle myopathies (*43*). Furthermore, TnI2 was reported to be present in nonmuscle cells, such as cartilage (*35*) and corneal epithelium (*44*). Thus, given the presence of TnIs in cardiac and skeletal muscles, our finding of the PCL−TnI interaction suggests that the PCL channel plays a yet-to-be-identified role in muscle cells. The presence of both PCL and TnI in retina and perhaps in cartilage, together with a previous finding that *Krd* mice with deletion of the *PKDL* gene is associated with defects in retina, indicates that the PCL−TnI interaction may play a currently unknown distinct role in nonmuscle cells.

Troponin I is a core regulatory protein of the actin microfilament and plays important roles in modulating striated muscle contraction. Troponin complex and tropomyosin together not only confer the Ca^{2+} sensitivity to the actin−myosin interaction but also modulate myofilament

activity through covalent or noncovalent modifications that alter the Ca^{2+} sensitivity and maximum activity (*29*, *45*, *46*). Recent findings indicate that troponin I proteins also play new functional roles. For example, troponin I was shown to associate with skeletal muscle ryanodine receptor and to convert the channel into an outward rectifying calcium release channel (*34*). More interestingly, troponin I acts as a potent angiogenesis inhibitor in nonmuscle cells, such as in cartilage (*35*, *47*). Further studies demonstrated that this inhibitory effect occurs, at least in part, via a cooperative interaction of TnI with the cell-surface basic fibroblast growth factor receptor in both endothelial and nonendothelial cells (*48*). On the other hand, angiogenesis that occurs in ADPKD (*36*) may represent an indicator of cystic cell growth and of increased vascular permeability. Thus, on the basis of the current study and our recent observation that PC2 physically associates with TnI (*28*), it would be interesting to determine in a future study whether/how TnI regulates the channel function of PC2.

REFERENCES

1. Gabow, P. A. (1993) *N. Engl. J. Med. 329*, 332−342.
2. The International Polycystic Kidney Disease Consortium (1995) *Cell 81*, 289−298.
3. Mochizuki, T., Wu, G., Hayashi, T., Xenophontos, S. L., Veldhuisen, B., Saris, J. J., Reynolds, D. M., Cai, Y., Gabow, P. A., Pierides, A., Kimberling, W. J., Breuning, M. H., Deltas, C. C., Peters, D. J., and Somlo, S. (1996) *Science 272*, 1339−1342.
4. Nomura, H., Turco, A. E., Pei, Y., Kalaydjieva, L., Schiavello, T., Weremowicz, S., Ji, W., Morton, C. C., Meisler, M., Reeders, S. T., and Zhou, J. (1998) *J. Biol. Chem. 273*, 25967−25973.
5. Veldhuisen, B., Spruit, L., Dauwerse, H. G., Breuning, M. H., and Peters, D. J. (1999) *Eur. J. Hum. Genet. 7*, 860−872.
6. Guo, L., Schreiber, T. H., Weremowicz, S., Morton, C. C., Lee, C., and Zhou, J. (2000) *Eur. J. Hum. Genet.*
7. Moy, G. W., Mendoza, L. M., Schulz, J. R., Swanson, W. J., Glabe, C. G., and Vacquier, V. D. (1996) *J. Cell Biol. 133*, 809−817.
8. Xia, X. M., Fakler, B., Rivard, A., Wayman, G., Johnson-Pais, T., Keen, J. E., Ishii, T., Hirschberg, B., Bond, C. T., Lutsenko, S., Maylie, J., and Adelman, J. P. (1998) *Nature 395*, 503−507.
9. Zuhlke, R. D., and Reuter, H. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 3287−3294.
10. Birnbaumer, L., Zhu, X., Jiang, M., Boulay, G., Peyton, M., Vannier, B., Brown, D., Platano, D., Sadeghi, H., Stefani, E., and Birnbaumer, M. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 15195−15202.
11. Kiselyov, K., Xu, X., Mozhayeva, G., Kuo, T., Pessah, I., Mignery, G., Zhu, X., Birnbaumer, L., and Muallem, S. (1998) *Nature 396*, 478−482.
12. Chen, X. Z., Vassilev, P. M., Basora, N., Peng, J. B., Nomura, H., Segal, Y., Brown, E. M., Reeders, S. T., Hediger, M. A., and Zhou, J. (1999) *Nature 401*, 383−386.
13. Liu, Y., Li, Q., Tan, M., Zhang, Y.-Y., Karpinski, E., Zhou, J., and Chen, X.-Z. (2002) *FEBS Lett. 525*, 71−76.
14. Li, Q., Liu, Y., Zhao, W., and Chen, X.-Z. (2002) *FEBS Lett. 516*, 270−278.
15. Chen, X. Z., Segal, Y., Basora, N., Guo, L., Peng, J. B., Babakhanlou, H., Vassilev, P. M., Brown, E. M., Hediger, M. A., and Zhou, J. (2001) *Biochem. Biophys. Res. Commun. 282*, 1251−1256.
16. Gonzalez-Perret, S., Kim, K., Ibarra, C., Damiano, A. E., Zotta, E., Batelli, M., Harris, P. C., Reisin, I. L., Arnaout, M. A., and Cantiello, H. F. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 1182−1187.
17. Yokoshiki, H., Katsube, Y., Sunugawa, M., Seki, T., and Sperelakis, N. (1997) *Pflugers Arch. 434*, 203−205.
18. Hanaoka, K., Qian, F., Boletta, A., Bhunia, A. K., Piontek, K., Tsiokas, L., Sukhatme, V. P., Guggino, W. B., and Germino, G. G. (2000) *Nature 408*, 990−994.
19. Koulen, P., Cai, Y., Geng, L., Maeda, Y., Nishimura, S., Witzgall, R., Ehrlich, B. E., and Somlo, S. (2002) *Nat. Cell Biol. 4*, 191−197.
20. Vassilev, P. M., Guo, L., Chen, X. Z., Segal, Y., Peng, J. B., Basora, N., Babakhanlou, H., Cruger, G., Kanazirska, M., Ye, C., Brown, E. M., Hediger, M. A., and Zhou, J. (2001) *Biochem. Biophys. Res. Commun. 282*, 341−350.
21. Qian, F., Germino, F. J., Cai, Y., Zhang, X., Somlo, S., and Germino, G. G. (1997) *Nat. Genet. 16*, 179−183.
22. Tsiokas, L., Kim, E., Arnould, T., Sukhatme, V. P., and Walz, G. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 6965−6970.
23. Newby, L. J., Streets, A. J., Zhao, Y., Harris, P. C., Ward, C. J., and Ong, A. C. (2002) *J. Biol. Chem. 277*, 20763−20773.
24. Tsiokas, L., Arnould, T., Zhu, C., Kim, E., Walz, G., and Sukhatme, V. P. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 3934−3939.
25. Lehtonen, S., Ora, A., Olkkonen, V. M., Geng, L., Zerial, M., Somlo, S., and Lehtonen, E. (2000) *J. Biol. Chem. 275*, 32888−32893.
26. Gallagher, A. R., Cedzich, A., Gretz, N., Somlo, S., and Witzgall, R. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 4017−4022.
27. Li, Q., Dai, Y., Guo, L., Liu, Y., Hao, C., Wu, G., Basora, N., Michalak, M., and Chen, X.-Z. (2003) *J. Mol. Biol. 325*, 949−962.
28. Li, Q., Shen, Y., Wu, G., and Chen, X.-Z. (2003) *Biochemistry 42*, 450−457.
29. Filatov, V. L., Katrukha, A. G., Bulargina, T. V., and Gusev, N. B. (1999) *Biochemistry (Mosc.) 64*, 969−985.
30. Perry, S. V. (1999) *Mol. Cell Biochem. 190*, 9−32.
31. Corin, S. J., Juhasz, O., Zhu, L., Conley, P., Kedes, L., and Wade, R. (1994) *J. Biol. Chem. 269*, 10651−10659.
32. Vallins, W. J., Brand, N. J., Dabhade, N., Butler-Browne, G., Yacoub, M. H., and Barton, P. J. (1990) *FEBS Lett. 270*, 57−61.
33. Bhavsar, P. K., Brand, N. J., Yacoub, M. H., and Barton, P. J. (1996) *Genomics 35*, 11−23.
34. Varsanyi, M., Sarkozi, S., Szegedi, C., Herzog, A., and Jona, I. (2002) *FEBS Lett. 515*, 155−158.
35. Moses, M. A., Wiederschain, D., Wu, I., Fernandez, C. A., Ghazizadeh, V., Lane, W. S., Flynn, E., Sytkowski, A., Tao, T., and Langer, R. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 2645−2650.
36. Bello-Reuss, E., Holubec, K., and Rajaraman, S. (2001) *Kidney Int. 60*, 37−45.
37. Lorenz, C., Pusch, M., and Jentsch, T. J. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 13362−13366.
38. Dargis, R., Pearlstone, J. R., Barrette-Ng, I., Edwards, H., and Smillie, L. B. (2002) *J. Biol. Chem. 277*, 34662−34665.
39. Basora, N., Nomura, H., Berger, U. V., Stayner, C., Guo, L., Shen, X., and Zhou, J. (2002) *J. Am. Soc. Nephrol. 13*, 293−301.
40. Hastings, K. E. (1996) *J. Mol. Evol. 42*, 631−640.
41. Huang, X., Pi, Y., Lee, K. J., Henkel, A. S., Gregg, R. G., Powers, P. A., and Walker, J. W. (1999) *Circ. Res. 84*, 1−8.
42. Zanellato, A. M., Borrione, A. C., Saggin, L., Giuriato, L., Schiaffino, S., and Sartore, S. (1991) *Circ. Res. 68*, 1349−1361.
43. Messner, B., Baum, H., Fischer, P., Quasthoff, S., and Neumeier, D. (2000) *Am. J. Clin. Pathol. 114*, 544−549.
44. Kinoshita, S., Adachi, W., Sotozono, C., Nishida, K., Yokoi, N., Quantock, A. J., and Okubo, K. (2001) *Prog. Retin. Eye Res. 20*, 639−673.
45. Pyle, W. G., Sumandea, M. P., Solaro, R. J., and De Tombe, P. P. (2002) *Am. J. Physiol Heart Circ. Physiol. 283*, H1215−H1224.
46. Farah, C. S., and Reinach, F. C. (1995) *FASEB J. 9*, 755−767.
47. Klagsbrun, M., and Moses, M. A. (1999) *Chem. Biol. 6*, R217−R224.
48. Feldman, L., and Rouleau, C. (2002) *Microvasc. Res. 63*, 41−49.

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