Polycystin-1 Activates and Stabilizes the Polycystin-2 Channel*

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Autosomal dominant polycystic kidney disease (ADPKD) is a prevalent genetic disorder largely caused by mutations in the PKD1 and PKD2 genes that encode the transmembrane proteins polycystin-1 and -2, respectively. Both proteins appear to be involved in the regulation of cell growth and maturation, but the precise mechanisms are not yet well defined. Polycystin-2 has been implicated in the regulation of cell growth and maturation, but the precise mechanisms are not yet well defined. Polycystin-2 has recently been shown to function as a Ca2+-permeable, non-selective cation channel. Polycystin-2 interacts through its cytoplasmic carboxyl-terminal region with a coiled-coil motif in the cytoplasmic tail of polycystin-1 (P1CC). The functional consequences of this interaction on its channel activity, however, are unknown. In this report, we show that P1CC enhanced the channel activity of polycystin-2. R742K, a disease-causing polycystin-2 mutant lacking the polycystin-1 interacting region, fails to respond to P1CC. Also, P1CC containing a disease-causing mutation in its coiled-coil motif loses its stimulatory effect on wild-type polycystin-2 channel activity. The modulation of polycystin-2 channel activity by polycystin-1 may be important for the various biological processes mediated by this molecular complex.

ADPKD is a common genetic disorder caused by mutations in either one of the two genes, PKD1 and PKD2, that encode polycystin-1 and -2, respectively. Polycystin-1 is an 11-membrane-spanning desmosome-associated protein (2, 3) that may be involved in the regulation of cell growth (4). Polycystin-2 is a six-span membrane protein with homology to voltage-dependent (5) and transient receptor potential (TRP) channel proteins (6). Six-spanning desmosome-associated protein (2, 3) that may be involved in the regulation of cell growth (4). Polycystin-2 interacts through its cytoplasmic carboxyl-terminal region with a coiled-coil motif in the cytoplasmic tail of polycystin-1 (P1CC). The functional consequences of this interaction on its channel activity, however, are unknown. In this report, we show that P1CC enhanced the channel activity of polycystin-2. R742K, a disease-causing polycystin-2 mutant lacking the polycystin-1 interacting region, fails to respond to P1CC. Also, P1CC containing a disease-causing mutation in its coiled-coil motif loses its stimulatory effect on wild-type polycystin-2 channel activity. The modulation of polycystin-2 channel activity by polycystin-1 may be important for the various biological processes mediated by this molecular complex.

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Plasmid Constructs—The PKD2 baculovirus expression construct pVL1393-PKD2 has been described (7). To make a mammalian expression construct, the PKD2-encoding fragment between PstI (blunted) and XbaI was cut out and ligated into vector pcI (Promega, Madison, WI) between EcoRI (blunted) and NheI. The PKD2R742X truncation mutant in pCI was constructed by PCR and tagged at the COOH terminus by a FLAG epitope. To obtain the GST fusion constructs, DNA fragments corresponding to various murine polycystin-1 carboxyl tail (P1CT) regions were PCR-amplified with Vent DNA polymerase (New England Biolabs, Beverly, MA) by introducing a BamHI site at the 5′-end and a translation stop codon at the 3′-end. The PCR fragments were digested with BamHI and phosphorylated by T4 polynucleotide kinase at the 3′-end. The resulting fragments were ligated into pGEX-2T (Amersham Biosciences) between the BamHI and Smal sites. The GST-P1CCQ4215P construct was made by two steps of PCR. In the first step, two separate PCR reactions were performed on the GST-P1CC template to obtain two overlapping PCR fragments where the mutation was introduced in the overlapping region. The two PCR fragments were then used together as a template for the second step of PCR using two end primers that flank the subcloning sites in vector pGEX-2T. The oligonucleotides for the two end primers are 5′-CAGCATATATGCTGACCGTCTC-3′ (sense) and 5′-CAGGAAGCTACGAGCTCCTC-3′ (antisense). The two overlapping mutagenic primers are 5′-GGCTCA-AACGGGCAAGCAGACTTTC-3′ (antisense) and 5′-GGCTGCGCGGT- TTGAGACCACG-3′ (sense). The final PCR product was digested with BamHI and EcoRI and ligated into pGEX-2T between the same sites. The sequences of all constructs were confirmed by the DNA sequencing facility at Massachusetts General Hospital.

Protein Expression and Isolation—Plasmids for GST fusion constructs were transformed into the bacterial strain BL21(DE3) (Novagen, Madison, WI) for protein expression. The bacteria were grown in LB broth to an optical density of about 0.5 before isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.2 mM. Induction was allowed for 3–4 h at 37 °C, followed by centrifugation to collect the bacteria. To isolate the proteins, bacteria were lysed by...
sonication in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 1.5% Sarkosyl plus protease inhibitors (Roche Diagnostics). Cell debris was removed by a 30 min centrifugation at 10,000 rpm in a Sorval SLA-600C rotor. The supernatant was then incubated with glutathione beads (Amersham Biosciences) for 30 min at 4 °C. The beads were washed three times with 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and three times with the same buffer containing no Triton X-100. Proteins were eluted from the beads in a solution containing 50 mM Tris-HCl, pH 8.0, and 20 mM reduced glutathione (Sigma). The protein eluates were dialyzed against 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1 mM dithiothreitol overnight with three changes of the buffer. The final protein preparation was mixed with an equal volume of 50% glycerol and either used freshly or stored at −80 °C. Protein concentration was determined by the Bradford method (16). The expression level of GST-PCTN was low.

In Vitro Transcription-Translation of FLAG-tagged Polycystin-2—In vitro transcription-translation reactions were carried out on pC-PK2D and pC-PIK2DR2G2X for the wild-type and R742X polycystin-2, respectively, using a T7-T7 coupled reticulocyte lysate system (Promega). To determine the presence of the in vitro translation products, one set of reactions was performed using [35S]methionine (Amersham Biosciences) as a protein label. The reaction products were diluted with 400 μl of phosphate-buffered saline containing 1% Triton X-100 and 1× protease inhibitor mixture (Roche Diagnostics). Anti-FLAG M2 antibody-bound protein G beads (10 μl, Sigma) were added to the mixture to immunoprecipitate the translated products. The proteins were released from the beads in boiling Laemmli sample buffer containing 5% 2-mercaptoethanol, resolved on 4–12% SDS-PAGE, and detected by autoradiography.

Ion Channel Reconstitution—Lipid bilayers were formed with a mixture of synthetic phospholipids (Avanti Polar Lipids, Birmingham, AL) in n-decane as recently reported (7). The lipid mixture was made of 1-palmitoyl-2-oleoyl phosphatidyl-choline and phosphatidyl-ethanolamine in a 7:3 ratio. The lipid solution (~20–25 mg/ml) in n-decane was spread with a glass rod over the 250-μm diameter aperture of a polystyrene cuvette (CP13–150) of a bilayer chamber (model BCH-13, Warner Instruments Corp.). Both sides of the lipid bilayer were bathed with a solution containing 10 mM MOPS-KOH and 10 mM MES-KOH, pH 7.40, and 10–15 μM Ca2+. The final K+ concentration in the solution was ~15 mM. KCl was further added to the cis compartment where membrane vesicles were added so that final concentrations of 150 mM K+, and 135 mM Cl− were achieved in this side of the chamber.

Data Acquisition and Analysis—Holding potentials were applied from the trans chamber with either a DC voltage source or a wave function generator with the opposite, cis side, defined as virtual ground. Unless otherwise stated, a cis minus trans voltage conversion was utilized throughout the study. Bilayer formation was monitored by applying a 2.5 V peak-to-peak, 20 Hz triangular wave with a typical membrane capacitance of 100–200 pF. All the experiments were performed at room temperature (20–25 °C). Electrical signals were recorded using a current-to-voltage converter with a 10 Gohm feedback resistor. Output (voltage) signals were low-pass filtered at 700 Hz (~3 dB) with an eight pole, Bessel-type filter (Frequency Devices, Haverhill, MA). Signals were displayed on an oscilloscope, and channel recordings were simultaneously digitized with a pulse code modulator (Sony PCM-501 ES). and stored in videotapes with a video cassette recorder (Toshiba HQ). Data were later transferred for subsequent analysis at 4 kHz to a personal computer. Single channel current tracings were further filtered (see "Results") for display purposes only. Unless otherwise stated, pCLAMP Version 5.5.1 (Axon Instruments, Foster City, CA) was used for data analysis, and Sigmaplot Version 2.0 (Jandel Scientific, Corte Madera, CA) was used for statistical analysis and graphics. Single channel conductances (γ) under asymmetrical conditions were calculated by the best fitting of current-to-voltage experimental data to the Goldman-Hodgkin-Katz (GHK) equation, such that

\[ \gamma = \frac{V_{m} - E_{j}}{V_{j} - E} \]

was obtained from Equation 1,

\[ I(V_{j}) = \frac{(z_{i} + z_{j})P_{i}P_{j}V_{j}(RT)/(C_{i} + 1 - \exp(-\alpha))}{(z_{i} + z_{j})P_{i}P_{j}V_{j}(RT)/(C_{i} + 1 - \exp(-\beta))} \]

(Eq. 1)

where i (species in trans compartment) and j (species in cis compartment) represent the cation species (K+, Na+) on either side of the membrane; \( V_{j} \) is the holding electrical potential in mV; \( z_{i} \) and \( z_{j} \) the charge for species i and j, respectively; \( C_{i} \) and \( C_{j} \) are the trans and cis concentrations of i and j, respectively, and \( \alpha = \frac{RT}{z_{i}F} \) and \( \beta = \frac{RT}{z_{j}F} \) are the permeability coefficient for either species i or j, respectively. Whenever a single salt was present in the preparation (i.e. KCl), i and j correspond to the cation and anion, respectively, F, R, and T have their usual meanings. Each reconstituted lipid-protein membrane preparation contained at least three variables, namely, the number of active ion channels, different single channel currents (due to multiple substates (7)), and distinct open probabilities under each condition (17). Thus, the data were analyzed as follows: the mean membrane current for each membrane preparation was determined prior to averaging data for each condition separately. The averaged data represented \( I = Np_{o} \), encompassing \( N \), the total number of active channels in the preparation, i, the average single channel current for the channel species, and \( p_{o} \), the open probability of the open channel at a given holding potential. Unless otherwise stated, data were obtained at a holding potential of 40 mV. Whenever indicated, statistical significance was obtained by unpaired t test comparison of sample groups of similar size (18). Average data values were expressed as the mean ± S.E. (n) under each condition, where n represents the total number of experiments analyzed. Statistical significance was accepted at p < 0.05.

RESULTS

Protein Preparations and in Vitro Translation Products—To study modulation of the channel properties of polycystin-2 by P1CT, different portions of the murine polycystin-1 cytoplasmic region were expressed as GST fusion proteins and purified for functional evaluation (Fig. 1, A–C). These fusion constructs included GST-P1CN, harboring the 84 N-terminal amino acids of the polycystin-1 carboxyl-tail, and GST-P1CC, containing the 77 amino acids encompassing the coiled-coil motif known to interact with polycystin-2. GST-P1CC-Q4215P, which harbors the mutation equivalent to an ADPKD-causing mutation in human polycystin-1, Q4224P (19), was also constructed. The Gln to Pro mutation in amino acid 4215, equivalent to the ADPKD-causing mutation Q4224P in human polycystin-1. C, two representative preparations of GST fusion proteins were labeled with Coomassie Blue G-250. More than three independent protein preparations were tested for each construct. The numbers indicate molecular mass in kDa. The full-length P1CT fusion protein was subject to degradation and not tested. D, [35S]methionine-labeled in vitro translation products of the wild-type and R742X mutant polycystin-2.
helix. Full-length wild-type polycystin-2 and its ADPKD-caus-
ing truncation mutant R742X were in vitro translated (Fig. 1
D) and reconstituted in a lipid bilayer system as reported recently (7, 17).

Single Channel Currents of Wild-type and R742X Poly-
cystin-2—Wild-type and R742X-polycystin-2 ion channel activity
was assessed in the presence of a chemical gradient with 150 mM KCl in the cis side and 15 mM KCl in the trans side of a reconstitution chamber (7). Wild-type polycystin-2 showed single-channel activity as previously reported (7, 17) (Fig. 2A). The R742X mutant also exhibited spontaneous channel activity in agreement with a recent report (15). Similar to the wild-type, R742X showed two most common single conductance substates of 77.2 ± 1.19 pS (n = 36) (Fig. 2B). However, the high conductance state, occasionally seen in the in vitro translated wild-type polycystin-2 and most frequently in the endogenous channel of human syncytiotrophoblast (hST) (7), was not observed in R742X. R742X polycystin-2 showed a smaller conductance state of 16.5 ± 0.87 pS (n = 24, Fig. 2B), which was not obvious in the wild-type channel. R742X also tended to close more frequently than the wild-type channel.

**Fig. 2. Single channel currents of the wild-type and R742X mutant polycystin-2.** A, single channel currents of the wild-type polycystin-2 (top) and R742X mutant (bottom). In vitro translated wild-type and R742X mutant polycystin-2 were reconstituted in a lipid bilayer system. Data were obtained in the presence of asymmetrical KCl (150 mM) in the cis side and 15 mM KCl in the trans side and are representative of 24 and 36 experiments, respectively. Dashed lines in between asterisks indicate substates. B, I-V relation for the wild-type and R742X mutant polycystin-2. Current-to-voltage relationships were obtained from single channel tracings at different holding potentials and fitted to the Goldman-Hodgkin-Katz equation as indicated under "Materials and Methods." The two main single channel conductance substates observed in R742X polycystin-2 were 77.2 pS, and 25.2 pS (thin lines), comparable to the conductance levels observed in the wild-type polycystin-2 (dashed lines). A third, smaller conductance state of 16.5 pS was unique to the mutant.

**Fig. 3. Effect of P1CC fusion protein on the wild-type polycystin-2 channel activity.** Top panel shows the strategy for testing the effect of P1CC on polycystin-2 channel function. Polycystin-2 single channel activity was observed at the beginning of the experiment (top tracing, left). Inactivation occurred either spontaneously (shown at the beginning of the top right tracing) or after holding the membrane to negative potentials. Addition of GST-P1CC restored polycystin-2 channel activity (top tracing, right). The middle panel shows representative expanded tracings for each condition, including spontaneous channel activity (a), spontaneous inactivation (b), and two subsequent channel reactivation levels after the addition of P1CC (c and d). Dashed lines indicate substates. The bottom panel shows the corresponding all-point histograms from tracings a through d in the middle panel. Data are representative of seven experiments.
All in vitro translated wild-type polycystin-2 spontaneously inactivated, in contrast to the stable channel function observed with the human syncytiotrophoblast native protein (7). Spontaneous ion channel inactivation of wild-type polycystin-2 occurred in 151 ± 29 s (n = 15) with varying times ranging from 15 to 440 s. Interestingly, ion channel inactivation could be induced, in most cases, by switching the holding potential to negative values. Voltage-induced inactivation occurred in 27.4 ± 5.54 s (n = 8). The time needed to elicit voltage inactivation was statistically shorter (p < 0.05) than that for spontaneous inactivation. In the only two cases in which channel activity was not completely inactivated by voltage, channels later inactivated spontaneously. Inactivated channels (by either method) failed to restore ion channel activity within 30 min after returning to positive holding potentials. A similar pattern of inactivation was observed in R742X polycystin-2 (data not shown).

Modulation of Polycystin-2 Channel Activity by P1CC—To examine the regulatory role of the polycystin-1 carboxyl-tail on the channel function of polycystin-2, the following experimental protocol was adopted. Single channel currents of wild-type polycystin-2 in a lipid bilayer membrane were first examined at the beginning of the experiment. Membranes showing spontaneous channel activity were inactivated either by voltage switch or spontaneously and then tested for response to various P1CT fusion proteins. During this period, a GST-P1CC fusion protein was added to the cis (but not trans) side of the reconstitution chamber. Proteins were added either to the bulk of the solution or, occasionally, directly to the surroundings of the chamber orifice holding the lipid bilayer membranes (“painting”). Fig. 3 shows the restoration of wild-type polycystin-2 ion channel activity by painting GST-P1CC from the cis side of the chamber. The addition of the GST-P1CC-Q4215P to inactivated wild-type polycystin-2 did not reactivate channel activity, thus establishing the specificity of the stimulatory effect by wild-type P1CC (Fig. 4).

The addition of GST-P1CC induced a 5,070 ± 2,110% (n = 5, p < 0.001, Fig. 5) increase in the mean currents compared...
with those of the inactivated membranes. In contrast, the addition of either GST-P1CTN, GST-P1CC-Q4215P, or GST alone had no effect. The painting of either GST or GST-P1CC to membranes lacking polycystin-2 did not produce any channel activity (data not shown). Wild-type polycystin-2 channel activity was restored by P1CC in 22.3 ± 8.34 s (mean ± S.E., n = 5, Figs. 3 and 5), except for two instances where channel activity was restored in 225 and 350 s, respectively. Polycystin-2 channel activity restored by GST-P1CC did not inactivate for up to 15 min, suggesting that the peptide prevented the spontaneous inactivation of polycystin-2 channel function (not shown).

To further confirm the stimulatory effect of P1CC on wild-type polycystin-2, a different experimental protocol was carried out. Equal volumes of a polycystin-2 in vitro translation product were incubated with equimolar amounts of the different PICT fusion proteins. Polycystin-2 ion channel activity was then measured as the mean current per membrane integrated over the initial 12.5 s after membrane reconstitution (Fig. 6). Typically, most reconstituted complexes showed no channel activity (Fig. 6, A and B). The polycystin-2-GST-P1CN complex had a small increase of 206 ± 46.3% over the GST (control) complexes (mean ± S.E., n = 52, p < 0.05). The lipid bilayers reconstituted with the wild-type GST-P1CC mixture, however, had a 2,730 ± 730% (n = 30, p < 0.02) increase in channel activity compared with that reconstituted with the mixture containing GST alone. Complexes containing P1CC Q4215P were without effect (Fig. 6). The data indicate that the interaction between polycystin-1 and -2 facilitates the formation of active polycystin-2 channels.

Modulation of Polycystin-2 Channel Activity by Polycystin-1 Requires the Respective Interacting Segments in Both Proteins—To further substantiate the importance of polycystin-1/-2 interaction on the channel activity of the latter, R742X polycystin-2 was tested for its response to P1CC after channel inactivation. Although displaying spontaneous channel activity, R742X failed to reactivate following the addition of GST-P1CC after spontaneous inactivation (Fig. 7, A and B), indicating that the cytoplasmic tail of polycystin-2 mediates the activation and stabilization by P1CC.

**DISCUSSION**

The present findings indicate that an interaction with polycystin-1 activates and stabilizes the channel activity of wild-type polycystin-2 when reconstituted in a lipid bilayer system. In contrast to the stable channel function of the native protein in the human syncytiotrophoblast (7), the in vitro translated protein spontaneously inactivates, a phenomenon that is also induced by negative potentials. The polycystin-1/-2 interaction reversed both spontaneous and voltage-induced inactivation of polycystin-2. This regulation is largely achieved by the membrane-distal segment of polycystin-1, which contains the polycystin-2-interacting region. The proximal segment had little effect, and the disease-causing Q4215P mutant completely lacked this effect. The polycystin-2 R742X mutant, lacking most of the cytoplasmic tail, displayed spontaneous channel activity as reported previously (15) but failed to reactivate after the addition of the wild-type polycystin-1 carboxyl tail.

A number of functional consequences of polycystin-1/-2 interaction have been suggested. It has been reported that polycystin-1 may be required for the trafficking of polycystin-2 to the cell surface of Chinese hamster ovary cells overexpressing both proteins (13). Recently, an interaction between the two proteins was linked to the Jak-2-mediated anti-proliferative activity of polycystin-1 (4), and the interaction between polycystin-1 and -2 was found to block G protein signaling by polycystin-1 heterologically expressed in neurons (14). The data in this report present the first direct demonstration of the electrophysiological significance of this interaction, namely the activation and stabilization of polycystin-2 channel activity by polycystin-1, which may underlie some of the above effects. The resulting increase in channel activity by the polycystin-2/-1 complex may be critical for the activation of cation-dependent signaling pathway(s) normally associated with various cell functions including cell cycle, vesicle trafficking, and ion transport. Functional interruption of this interaction may account for abnormalities in protein targeting, cell growth, and ion transport, characteristic of ADPKD.

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