Receptor for Activated C Kinase 1 (RACK1) Inhibits Function of Transient Receptor Potential (TRP)-type Channel Pkd2L1 through Physical Interaction*

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Background: Pkd2L1 is a calcium- and acid-activated channel likely involved in acid sensing, but how it is regulated remains unclear.
Results: Receptor for activated C kinase 1 (RACK1), known to regulate various receptors/channels, interacts with and inhibits the function of Pkd2L1.
Conclusion: Pkd2L1 is a novel target channel of RACK1.
Significance: Pkd2L1-RACK1 interaction may play important physiological roles through regulating channel activation by calcium or acid.

Pkd2L1 (also called TRPP3) is a non-selective cation channel permeable to Ca^{2+}, Na^+, and K^+ and is activated by Ca^{2+}. It is also part of an acid-triggered off-response cation channel complex. We previously reported roles of the Pkd2L1 C-terminal fragments in its channel function, but the role of the N terminus remains unclear. Using a yeast two-hybrid screening, we found that the Pkd2L1 N terminus interacts with the receptor for activated C kinase 1 (RACK1), a scaffolding/anchoring protein implicated in various cellular functions. This interaction requires the last two Trp-Asp (WD) motifs of RACK1 and fragment $Ala^{19}–Pro^{45}$ of Pkd2L1. The interaction was confirmed by GST pulldown, blot overlay, and co-immunoprecipitation assays. By ^{45}Ca tracer uptake and two-microelectrode voltage clamp electrophysiology, we found that in *Xenopus* oocytes with RACK1 overexpression Pkd2L1 channel activity is abolished or substantially reduced. Combining with oocyte surface biotinylation experiments, we demonstrated that RACK1 inhibits the function of Pkd2L1 channel on the plasma membrane in addition to reducing its total and plasma membrane expression. Overexpressing Pkd2L1 N- or C-terminal fragments as potential blocking peptides for the Pkd2L1-RACK1 interaction, we found that Pkd2L1 N-terminal fragment $Met^1–Pro^{45}$, but not $Ile^{40}–Ile^{97}$ or C-terminal fragments, abolishes the inhibition of Pkd2L1 channel by overexpressed and oocyte-native RACK1 likely through disrupting the Pkd2L1-RACK1 association. Taken together, our study demonstrated that RACK1 inhibits Pkd2L1 channel function through binding to domain $Met^1–Pro^{45}$ of Pkd2L1. Thus, Pkd2L1 is a novel target channel whose function is regulated by the versatile scaffolding protein RACK1.

Pkd2L1 is a homologue of Pkd2 with 54% sequence identity (1). Both proteins are Ca^{2+}-modulated cation channels permeable to Ca^{2+}, Na^+, and K^+ (2–4). Pkd2 and Pkd2L1 share high similarity in membrane topology to the transient receptor potential (TRP)[2] superfamily of cation channels and have been termed TRPP2 and TRPP3, respectively, as members of the TRP polycystin (TRPP) subfamily (5). Mutations in the *Pkd1* or *Pkd2* gene account for most cases of autosomal dominant polycystic kidney disease with an incidence of 0.1–0.2% worldwide. We previously reported that Pkd2L1 channel is activated by application of extracellular Ca^{2+} followed by an ensuing inactivation (3) and is inhibited by amiloride analogues and large monovalent cations such as tetrapentylammonium and tetrabutylammonium, which led to an estimation of its channel pore size of ~7 Å (6, 7).

Pkd2L1 channel activity is modulated by membrane potential, pH, and cell volume (3, 8, 9). We also reported that Pkd2L1 physically interacts with α-actinin, an important component of the actin filament, in brain and other tissues and is functionally stimulated by α-actinin *in vitro* (10). Although overexpressed Pkd2L1 is targeted to the plasma membrane (PM) of *Xenopus* oocytes, it mostly localizes in intracellular membranes of mammalian cells when expressed alone (10–12). Pkd2L1 co-localizes with Pkd1 in the centrosome and may function in the cell cycle (13). Interestingly, co-expression of Pkd2L1 with Pkd1 in human embryonic kidney (HEK) 293 cells resulted in Pkd2L1 trafficking to the PM where Pkd2L1 seemed to mediate Ca^{2+} entry in the presence of a hypo-osmotic extracellular solution (11). Pkd1L3, a homologue of Pkd1, is critical in the PM trafficking of Pkd2L1 because knock-out of Pkd1L3 resulted in internalization of Pkd2L1 in mouse taste bud neurons (12). How Ca^{2+} triggers the activation remains unknown. So far it is

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[2] The abbreviations used are: TRP, transient receptor potential; TRPP, TRP polycystin; PM, plasma membrane; RACK1, receptor for activated C kinase 1; NMDAR, NMDA receptor; IP, immunoprecipitation; WB, Western blotting.

known that EGTA preinjection abolishes this activation (3), indicating that an increase in the intracellular Ca^{2+} concentration is required for the activation and that deletion of the Pkd2L1 C-terminal domain Thr^{622}–Ser^{805} does not abolish Ca^{2+}-induced activation (14).

Pkd2L1 localizes to tongue taste receptor cells together with Pkd1L3, which is possibly involved in sour tasting (15–17), and to central canal neurons of the spinal cord, which is possibly involved in proton-dependent regulation of action potentials (15). Mouse Pkd2L1 and Pkd1L3 together mediate pH-dependent cation conductance (16), which was later described as the off-response (*i.e.* the channel is activated only after the low extracellular pH is removed) in a report proposing that the Pkd2L1-mediated off-response may work together with an on-response mediated by other acid receptors to account for acid sensing at large pH ranges (18). It was shown that Pkd2L1 together with a carbonic anhydrase is involved in sensing gaseous CO_2 in the tongue (19). Interestingly, in the tongue of two patients with an acquired sour ageusia (*i.e.* unresponsive to sour stimuli), the mRNA and protein of Pkd2L1, Pkd1L3, and acid-sensing ion channels were undetectable (20). Indeed, mice with Pkd2L1 knock-out (KO) have reduced responses to sour stimuli (21). However, no anomaly in tasting was observed in Pkd1L3 KO mice (21, 22), and responses in sour taste cells are associated with acid-activated proton, but not Na^+, currents (23), which challenges the implication in acid sensing of Pkd1L3 or Pkd2L1, a Na^+-permeable channel. Chang *et al.* (23) also found that these proton currents are not affected by targeted deletion of Pkd1L3, a result that is incompatible with the study by Ishimaru *et al.* (12) about Pkd1L3-dependent plasma membrane trafficking of Pkd2L1, which would let one argue whether these proton currents are indeed mediated by Pkd2L1. Thus, whether and how Pkd1L3 or Pkd2L1 is involved in acid sensing and whether there is a link between Ca^{2+}- and proton-induced channel activation remain subjects of further studies. Pkd2L1 is also present in ganglion cells and other retina neuron cells (24), several compartments of mouse brain (10), kidney, liver, and heart (14). The physiological role of Pkd2L1 in these tissues and whether it involves Ca^{2+} and/or acid dependence of the channel remain to be determined.

The receptor for activated C kinase 1 (RACK1) is a guanine nucleotide-binding protein, β2-like 1 (also called GNB2L1) and a conserved scaffolding protein that regulates a range of cell activities, including cell growth, shape, and protein translation (25, 26). RACK1 was originally identified as an anchoring protein for activated protein kinase C and a scaffolding protein that recruits PKCs (and other proteins) into a signaling complex (27, 28). RACK1 is a 36-kDa protein composed of seven repeating units of Trp-Asp 40 (WD40) motifs forming an overall β-propeller structure (27). These WD40 motifs represent structural domains formed by four antiparallel β-strands and are thought to mediate protein-protein interaction. In the last decade, several of its new cellular functions have been discovered (29). In fact, RACK1 acts as a scaffolding/anchoring protein for or regulates the expression/function of a number of binding partners, ranging from signaling proteins such as protein kinase C (27), Src tyrosine kinase (30), protein-tyrosine phosphatase PTPμ (31), cAMP-specific phosphodiesterase PDE4D5 (32), and planar plan polarity protein Vangl2 (33) to receptor/channel proteins such as TRPM6 and TRPC3 (34, 35), integrin β subunit (36), type 1 IFN receptor (37), NMDA receptor (NMDAR) NR2B subunit (38), GABA$_A$ receptor β subunits (39), G protein-coupled receptor for thromboxane A$_2$ (40), and inositol 1,4,5-trisphosphate receptors (41). In addition, recent data suggest that the binding of RACK1 plays a role in gene expression, translation, and ribosome assembly and activation (25, 26). RACK1 mediates the regulation of NMDAR by ethanol (42) and together with PKC is an integral part of the circadian feedback loop (43). RACK1 is ubiquitously expressed in the tissues of higher mammals and human, including brain, liver, and spleen, suggesting that it has an important functional role in most if not all cells (29, 38). Therefore, RACK1 is a versatile protein that regulates various biological functions.

In the present study, we first examined the physical interaction of Pkd2L1 with RACK1 using different protein-protein interaction approaches. We then investigated the effect of RACK1 on channel function and expression of Pkd2L1 and the importance of their physical interaction in the regulation of the channel by RACK1.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—We cloned a RACK1 cDNA from a human kidney cDNA library (Clontech) through a standard PCR approach with a pair of specific primers spanning the entire open reading frame: forward, 5′ <u>CTCTAGAGGATC</u>-<u>C</u>ATGACTGAGCAGATGACCC, and reverse, 5′ <u>CCATAT</u>-<u>G</u>CTAGCGTGTGCCAATGGTCACC. XbaI, BamHI, and NdeI sites (underlined) were engineered into the primers to facilitate subsequent cloning. The authenticity of all constructs was confirmed by sequencing. Human Pkd2L1 and RACK1 were subcloned into vectors pCDNA3.1 and pEGFPC2, respectively, for mammalian cell expression and vector pTLN2 (44) for *Xenopus laevis* oocyte expression. His-tagged Pkd2L1 N- and C-terminal fragments tagged with 5′ His were subcloned into vector pCHGF for oocyte expression. The pCHGF vector was generated by deleting the enhanced GFP fragment and part of the CMV promoter in vector pEGFP-C2 using restriction enzymes NheI and SmaI and then ligating in (by the same enzymes) the β-globin 5′ and 3′ untranslated regions generated by PCR from vector pTLN2.

Preparation of mRNAs and Microinjection into Oocytes—Pkd2L1 and RACK1 cDNAs in vector pCHGF were linearized with MluI followed by phenol/chloroform purification and ethanol precipitation. Linearized DNAs were used to *in vitro* synthesize capped mRNAs using the mMessageMachine™ kit (Ambion, Austin, TX). Stage V-VI oocytes were isolated from *X. laevis*. Defolliculation of oocytes was performed through incubation in Ca^{2+}-free Barth's solution (14) containing collagenase (2 mg/ml) at room temperature (RT) for 2–2.5 h. Oocytes were then incubated at 18 °C in Barth's solution for at least 3 h before injection of 50 nl of H_2O containing variable amounts of mRNAs. An equal volume of H_2O was injected into control oocytes. Injected oocytes were incubated at 16–18 °C in Barth's solution supplemented with antibiotics for 2–4 days prior to experiments.
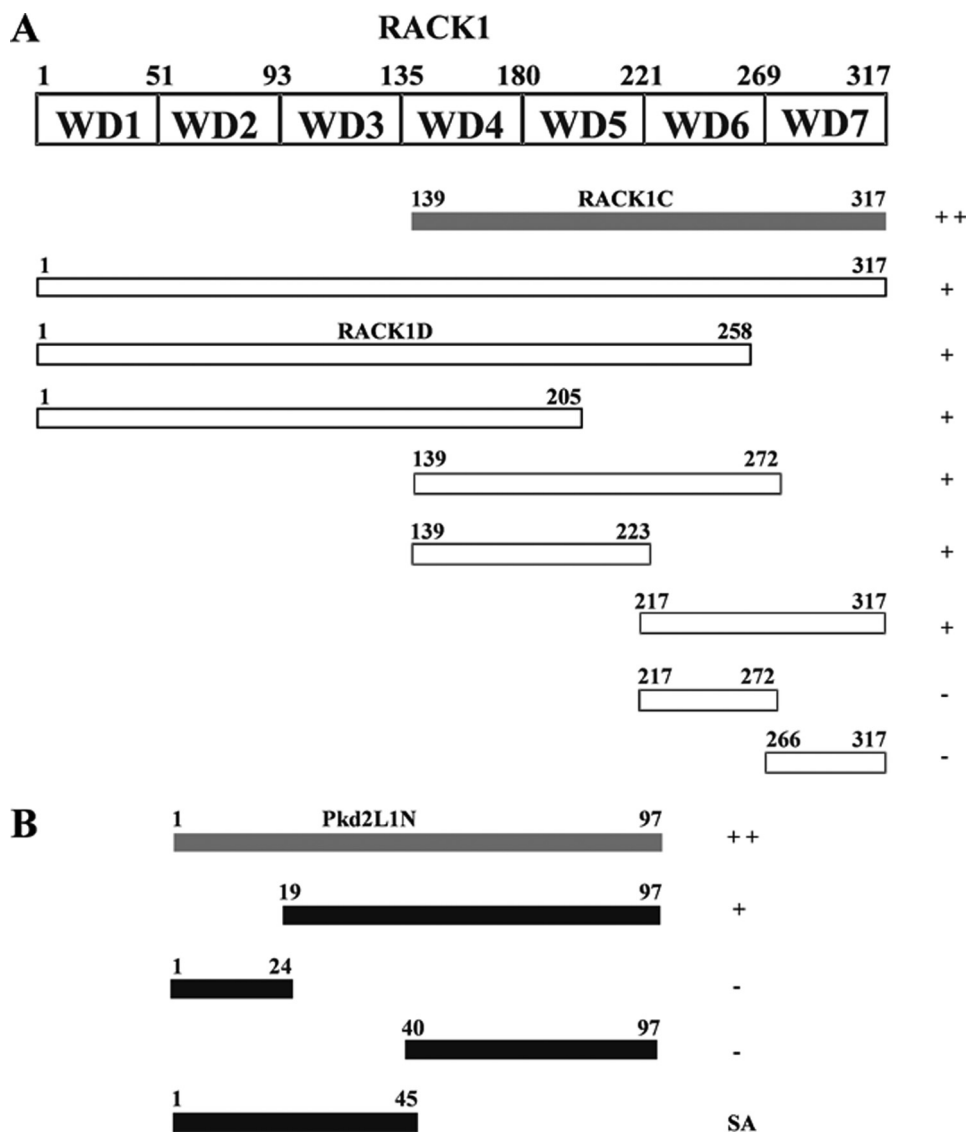
FIGURE 1. **Interaction between Pkd2L1 N terminus and RACK1 identified by yeast two-hybrid assay.** The start and end amino acid residue numbers for each domain are indicated. "++", "+," and "−" indicate the presence of a strong interaction, positive interaction, and absence of interaction, respectively. *A*, RACK1 was identified from a human kidney library by yeast two-hybrid screening using the Pkd2L1 N-terminal segment Met[1]–Ile[97] (Pkd2L1N) as bait. RACK1C and several truncated fragments of RACK1 were assessed for their interaction with Pkd2L1N. *B*, Pkd2L1 N-terminal fragments were examined for interaction with RACK1C. Domain Met[1]–Pro[45] exhibited self-activation (*SA*) and was not used to assess the interaction with RACK1.

Cell Culture—HEK293, mouse inner medullary collecting duct-3 (IMCD3), and NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells of less than 25 passages were cultured to full confluence before collection.

Yeast Two-hybrid Analysis—Human Pkd2L1 N terminus (Pkd2L1N; amino acids Met[1]–Ile[97]) was generated by PCR and subcloned in-frame into the GAL4 DNA binding domain vector pGBKT7 (Clontech). A yeast two-hybrid assay using yeast strain AH109 and Pkd2L1N as bait to screen a human kidney cDNA library constructed in vector pACT2 (Clontech) was performed according to protocols reported previously (45, 46).

GST Pulldown—Precleared mammalian cell lysates containing HA-Pkd2L1N were incubated with 2 μg of purified GST-RACK1 protein in binding buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM CaCl$_2$). The mixture was incubated at RT for 1 h

with gentle shaking followed by another hour of incubation after addition of 100 μl of glutathione-agarose beads (Sigma-Aldrich). The beads were then washed several times with 140 mM NaCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.5, and the remaining proteins were eluted using 10 mM glutathione, 50 mM Tris, pH 8.0. The protein samples were resolved by SDS-PAGE (10%) and transferred to a nitrocellulose membrane (Bio-Rad). The filters were then blocked with 3% skim milk powder, immunoblotted with HA antibody (Cell Signaling Technology, Danvers, MA), and visualized with enhanced chemiluminescence (Amersham Biosciences).

Blot Overlay Assay—Purified proteins were resolved by SDS-PAGE (10%) and transferred to a nitrocellulose membrane. The membrane was then incubated at 4 °C overnight with lysates of NIH 3T3 cells in blocking buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM CaCl$_2$, 1% BSA), washed three times (10 min each) in blocking buffer containing 0.05% Tween 20, detected with a
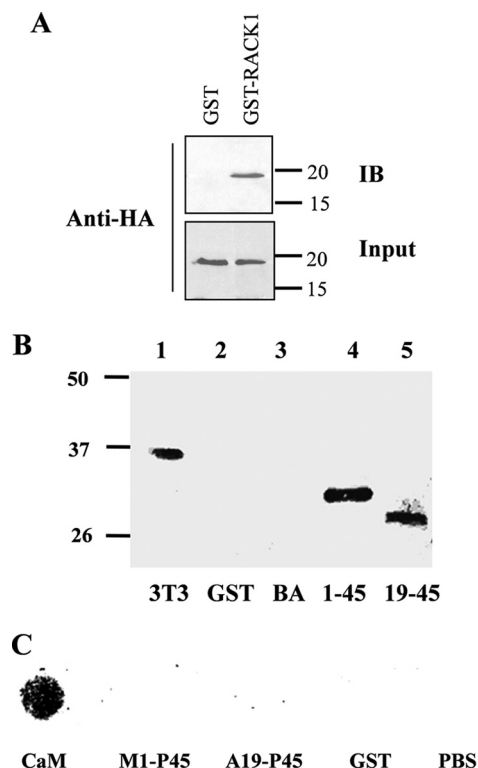
FIGURE 2. **Interaction between Pkd2L1N and RACK1 by GST pulldown and blot overlay.** *A*, GST pulldown. Precleared HEK293 cell lysates containing HA-Pkd2L1N were incubated with purified GST-RACK1 protein (2 μg) in binding buffer. *B*, blot overlay for interaction between a Pkd2L1 N-terminal fragment and endogenous RACK1. A nitrocellulose membrane containing purified GST-fused Pkd2L1 N-terminal fragments was incubated with total protein lysates of NIH 3T3 cells and immunoblotted (*IB*) by the anti-RACK1 antibody. NIH 3T3 cell lysates were used as a positive control (*lane 1*). *C*, Ca^{2+} binding of Pkd2L1 N-terminal domains Met^1–Pro^{45} and Ala^{19}–Pro^{45}. GST fusion polypeptides purified from *E. coli* were spotted on the nitrocellulose membrane strips, overlaid with ^{45}Ca in PBS buffer, and autoradiographed. Calmodulin (*CaM*) served as a positive control, whereas GST alone and bovine albumin (*BA*) served as negative controls.

FIGURE 3. **Interaction between Pkd2L1 and RACK1 by co-IP.** *A*, *Xenopus* oocytes overexpressing Pkd2L1 + RACK1 or RACK1 and control (*Ctrl*) oocytes were used for reciprocal co-IP using anti-Pkd2L1 (PR71) or anti-RACK1 antibody for IP or immunoblotting (*IB*) as indicated. *B, left panel*, cell lysates from native HEK293 cells were precipitated with PR71, non-immune rabbit IgG serum (*RIgG*; as control), or no antibody (*None*; as control). Immunoprecipitated proteins were analyzed by WB using the anti-RACK1 antibody (BD Biosciences). *Right panel*, representative data obtained from similar co-IP assays using native IMCD3 cells and mouse kidney and heart tissues with anti-Pkd2L1 and -RACK1 antibodies for IP and immunoblotting, respectively. *C*, interaction between Pkd2L1 and RACK1 in mouse brain by reciprocal co-IP. *Left panel*, cell lysates from mouse brain were precipitated with PR71 or rabbit IgG serum (*RIgG*). Immunoprecipitated proteins were analyzed by WB using the anti-RACK1 antibody. *Right panel*, reciprocal co-IP experiments were performed using RACK1 antibody B-3 or non-immune mouse IgG serum (*mIgG*) for IP and PR71 for immunoblotting.

RACK1 antibody (1:1500; catalog number 610178, BD Biosciences) and a horseradish peroxidase-coupled secondary antibody (1:1500; Chemicon International, Temecula, CA), and visualized with enhanced chemiluminescence (Amersham Biosciences).

Co-immunoprecipitation (co-IP)—Co-IP experiments using *Xenopus* oocytes co-expressing Pkd2L1 and RACK1 were performed using a modified protocol (47). Briefly, each sample contained 10 healthy oocytes that were injected with Pkd2L1 and/or RACK1 mRNAs and harvested in 500 μl of ice-cold CelLytic M lysis buffer (Sigma-Aldrich) 3 days after injection. Total proteins from the postnuclear supernatant were incubated on ice for 2 h either with anti-Pkd2L1 antibody PR71 or anti-RACK1 antibody (B-3, catalog number sc-17754, Santa Cruz Biotechnology for mammalian cells or BD Biosciences for oocytes) followed by another 2-h incubation with gentle shaking upon addition of 100 μl of protein A (for the anti-Pkd2L1 sample; Sigma-Aldrich) or protein L (for the anti-RACK1 sample; Pierce)-Sepharose. The immunoprecipitates absorbed to protein A/L-Sepharose were resuspended in 40 μl of Laemmli sample buffer, and a 20-μl aliquot of the extract was subjected to SDS-PAGE followed by immunoblotting. Co-IP using native tissues and mammalian cultured cells was conducted as described recently (48).
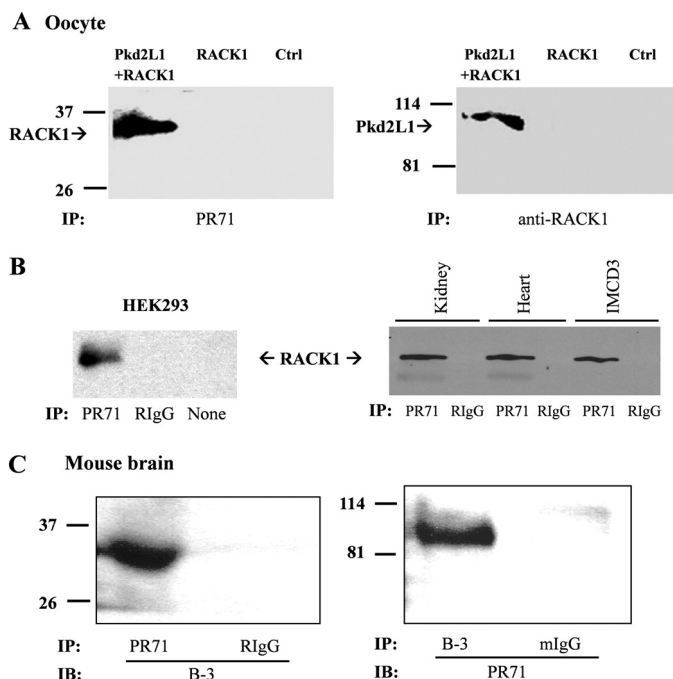
^{45}Ca *Uptake Measurements*—$^{45}CaCl_2$ (Amersham Biosciences) at 30 μM was added to uptake solution (100 mM *N*-methyl-D-glucamine, 2 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.5) plus 1 mM non-radioactive $CaCl_2$. Ten oocytes of each sample were incubated in 0.5 ml of uptake solution for 30 min, and the incubation was terminated by washing oocytes through six wells containing an ice-cold NaCl-containing solution (6). Individual oocytes were then dissolved in 250 μl of 10% SDS and mixed with 2.5 ml of scintillation mixture prior to scintillation counting. Data were compared using the paired or unpaired Student's *t* test and expressed as means ± S.E. $p < 0.05$ and $p < 0.01$ were considered statistically significant and very significant, respectively.

Electrophysiology—Current and voltage signals were measured using the conventional two-microelectrode voltage clamp technique with a commercial amplifier (TEV-200A, Dagan, Minneapolis, MN). Electrodes were fabricated from borosilicate glass (Warner Instruments, Hamden, CT) by a micropipette puller (P-87, Sutter Instruments, Novato, CA) and filled with 3 M KCl with a typical tip resistance of 0.5–3 megaohms. The Digidata 1320A converter and pClamp 8 (Axon Instruments, Union City, CA) were used for data acquisition and analysis, respectively. SigmaPlot 11 or 12 (Jandel Scientific Soft-
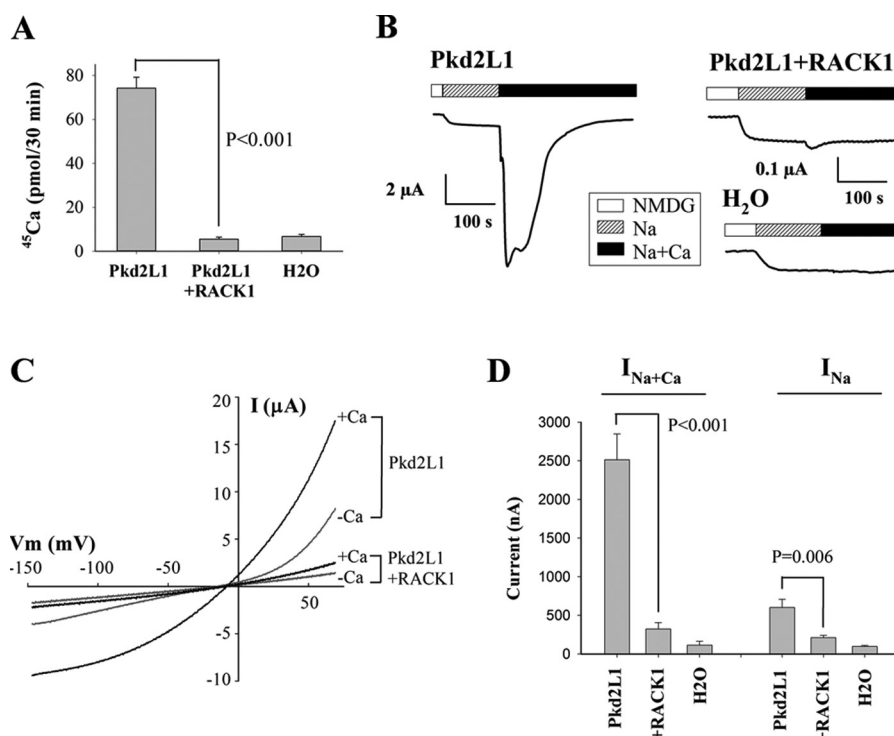
FIGURE 4. **Functional modulation of Pkd2L1 channel by RACK1.** *A*, radiolabeled [45]Ca uptake in oocytes expressing Pkd2L1 + RACK1 or Pkd2L1 alone and H_2O-injected control oocytes. Radiolabeled [45]Ca uptake was performed using uptake solution 3 days following RNA injection. Data were averaged from three experiments. The effect of RACK1 was analyzed by unpaired *t* test. *B*, representative whole-cell current tracings under voltage clamp ($V_m = -50$ mV) using the two-microelectrode voltage clamp technique. Different scales of current were used as indicated. Currents were measured using solution "*Na*" (the standard Na^+-containing solution (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.5) or "*Na+Ca*" (standard Na^+-containing solution + 5 mM $CaCl_2$) by the two-microelectrode voltage-clamp technique with a voltage ramp protocol. *C*, representative current-voltage curves in an oocyte expressing Pkd2L1 alone or Pkd2L1 + RACK1 in the presence of the standard Na^+-containing solution with (+*Ca*) or without (−*Ca*) 5 mM $CaCl_2$, as indicated. *D*, averaged currents obtained in oocytes expressing Pkd2L1 + RACK1 ($n = 17$) or Pkd2L1 alone ($n = 17$) and H_2O-injected control oocytes ($n = 6$) in the presence of Na^+-containing solution with (*Na+Ca*) or without (*Na*) addition of 5 mM $CaCl_2$. Inhibition of currents by RACK1 was very significant both with and without Ca^{2+} with $p < 0.001$ and $p = 0.006$ (unpaired *t* test), respectively. Two-way analysis of variance found that the inhibitory effect of RACK1 is very significant ($p < 0.001$), that the stimulatory effect of Ca^{2+} is very significant ($p < 0.001$), and that the effect of RACK1 is significantly different ($p < 0.001$) whether Ca^{2+} is present or not in the solution, consistent with *t* test analysis. *Error bars* indicate S.E.

ware, San Rafael, CA) was used for further data analysis, including data plotting, *t* test, analysis of variance, and regressions. In experiments using a ramp or gap-free protocol (3), current/voltage signals were digitized at 0.2 or 200 ms/sample.

Immunofluorescence—*Xenopus* oocytes expressing Pkd2L1 alone or Pkd2L1 plus RACK1 were embedded in a cryostat medium and shock frozen, and 10-μm sections were sliced on a cryostat and mounted onto Superfrost Plus microscope slides (Fisher). Sections were dried for 30 min at RT and then fixed in phosphate-buffered saline (PBS) plus 2% paraformaldehyde for 10 min. After rinses in PBS, sections were blocked in 2% normal rabbit serum and 0.1% Triton X-100 in PBS for 5 min and incubated at RT for 1 h with anti-Pkd2L1 rabbit polyclonal antibody PR71. Sections were then rinsed and incubated with a secondary FITC-conjugated anti-rabbit antibody (1:100; Chemicon International) for 1 h. After washing, sections were mounted in Vectashield (Vector Laboratories, Burlington, Ontario, Canada) for fluorescence detection with a Zeiss 510 confocal laser-scanning microscope.

RESULTS

Interaction between Pkd2L1 and RACK1 Revealed by Yeast Two-hybrid Analysis—Our previous study showed that the C-terminal EF-hand motif Glu[637]–Leu[665] acts as a negative regulator of Ca^{2+}-induced Pkd2L1 channel activation and that

truncation mutant T622X still exhibits channel function, indicating that the domain C-terminal of Thr[622] is not essential for channel function (14). In the present study, we utilized the N terminus (Pkd2L1N; Met[1]–Ile[97]) as bait to screen for interacting partner proteins by the yeast two-hybrid approach. For this, Pkd2L1N was fused into vector pGBKT7 as bait to screen a kidney cDNA library in yeast strain AH109. The C terminus of RACK1 (Lys[139]–Arg[317]; named RACK1C) was identified from the library as a Pkd2L1N binding partner (Fig. 1*A*). The physical association between the two protein fragments was confirmed by an individual pair test in yeast. To further delineate the corresponding regions within Pkd2L1N and RACK1 that mediate the association, we constructed a series of truncated cDNA fragments that encode peptides expressed as fusion proteins in yeast strain Y187 using vector pGBKT7. Upon examination for interaction with Pkd2L1N, we found that at least two domains of RACK1, including Lys[139]–Glu[223] and Met[217]–Arg[317], interact with Pkd2L1N (Fig. 1*A*) and that on the other hand fragment Ala[19]–Ile[97] of Pkd2L1N interacts with RACK1C (Fig. 1*B*). Furthermore, our yeast two-hybrid assays found that RACK1 also interacts with the Pkd2L1 C terminus (Pkd2L1C; Glu[566]–Ser[805]), indicating that more than one domain in both RACK1 and Pkd2L1 are involved in their interaction.
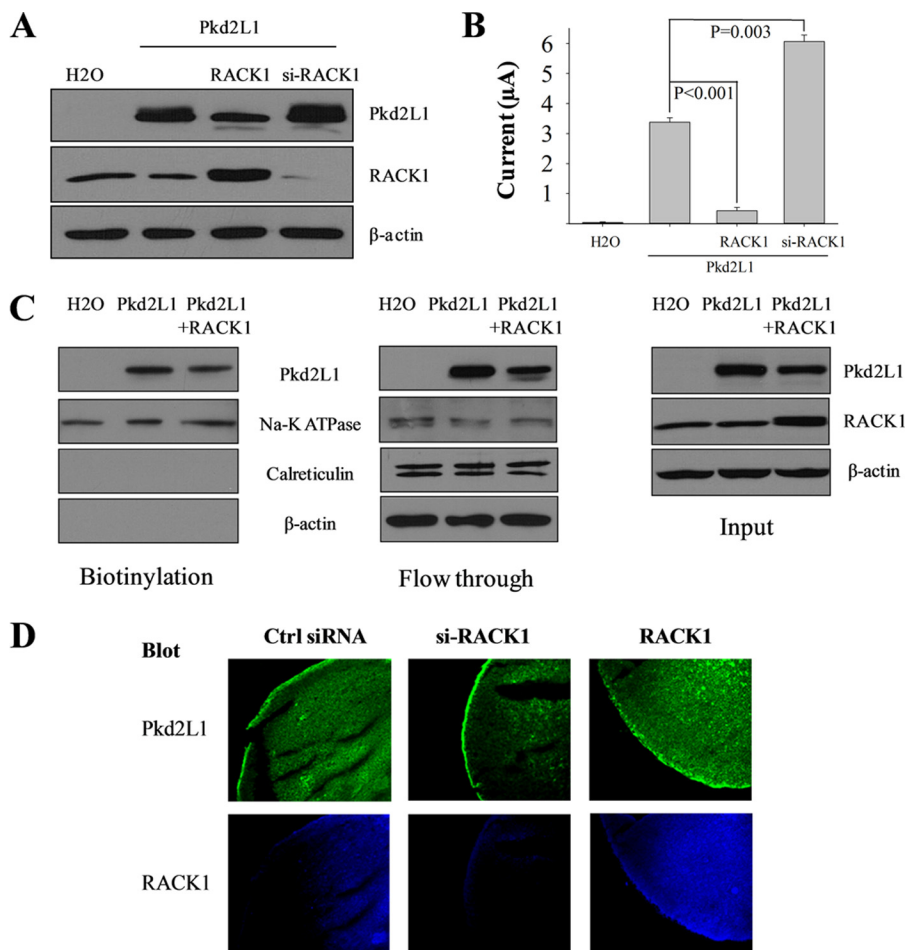
FIGURE 5. **Effects of RACK1 on channel activity and total and PM expression of Pkd2L1 expressed in *Xenopus* oocytes.** *A* and *B*, oocytes were injected with Pkd2L1 + RACK1, Pkd2L1 + si-RACK1, Pkd2L1 alone, or water (negative control). *A*, representative data on expression of Pkd2L1 and RACK1 by WB. *B*, Ca^{2+}-activated currents were measured at -50 mV. Shown are averaged values from four oocytes per group with *p* values from a paired *t* test. *Error bars* indicate S.E. *C*, *left panel*, representative data on the PM expression of Pkd2L1 by biotinylation. Na,K-ATPase (PM marker) and calreticulin and β-actin (non-PM markers) were used as controls of the biotinylation assay. *Middle* and *right panels*, flow-through and WB input data, respectively, from the same experiment. *D*, representative data on subcellular distribution of Pkd2L1 and RACK1 by immunofluorescence.

Interaction between Pkd2L1 and RACK1 under in Vivo and in Vitro Conditions—We performed other assays to further document the interaction between Pkd2L1 and RACK1. By GST pulldown, we showed that HA-Pkd2L1N expressed in HEK293 cells is able to interact with GST-RACK1 purified from *Escherichia coli* (Fig. 2*A*) with full-length human RACK1 generated by PCR. We also used a blot overlay assay to verify this interaction *in vitro*. Purified fusion proteins, including GST-Pkd2L1N, GST-Met[1]–Pro[45], and GST-Ala[19]–Pro[45], were resolved using SDS-PAGE and transferred to a nitrocellulose membrane followed by incubation with NIH 3T3 cell lysates. Immunoreactive protein bands from probing with a RACK1 antibody were observed only in the presence of GST-Pkd2L1N, GST-Met[1]–Pro[45], or GST-Ala[19]–Pro[45] but not with GST alone or bovine albumin (Fig. 2*B*), which is indicative of specific binding between Pkd2L1N and RACK1.

Furthermore, we *in vitro* transcribed complementary RNAs of Pkd2L1 and RACK1 and injected them into *Xenopus* oocytes. After 3 days, total protein extracts were used to perform co-IP assays between the two proteins. Precipitation with anti-Pkd2L1 antibody PR71 resulted in the pulldown of RACK1, and reciprocally, anti-RACK1 antibody was also able to co-precipi-

tate Pkd2L1 in oocytes (Fig. 3*A*), demonstrating that the two proteins are in the same complex in oocytes. We further substantiated the interaction between Pkd2L1 and RACK1 by co-IP using native HEK293 and IMCD3 cells and mouse kidney and heart tissues (Fig. 3*B*), indicating that the two proteins interact with each other *in vivo*. It is well known that RACK1 is abundantly expressed in brain and interacts with brain proteins such as NMDA and GABA receptors (38, 39, 49). On the other hand, Pkd2L1 was also recently found in several brain compartments such as hippocampus, thalamus, and spinal cord, although its physiological roles there remain unclear (10, 15). We thus tested their interaction in mouse brain tissues by co-IP. Indeed, we found that Pkd2L1 and RACK1 precipitate with each other and reciprocally (Fig. 3*C*). Taken together, our data demonstrated the interaction between Pkd2L1 and RACK1 under *in vitro* and *in vivo* conditions.

Inhibition of Pkd2L1 Channel Activity by RACK1—We next tested whether RACK1 has any effect on Pkd2L1 channel function. By co-expressing Pkd2L1 and RACK1 in *Xenopus* oocytes, we found that overexpression of RACK1 abolishes the Ca^{2+} transport ability of Pkd2L1 (Fig. 4*A*). Under voltage clamp conditions, RACK1 abolished both the basal conductance and

Pkd2L1 (ng): 0 50 50 25 12.5 6.2
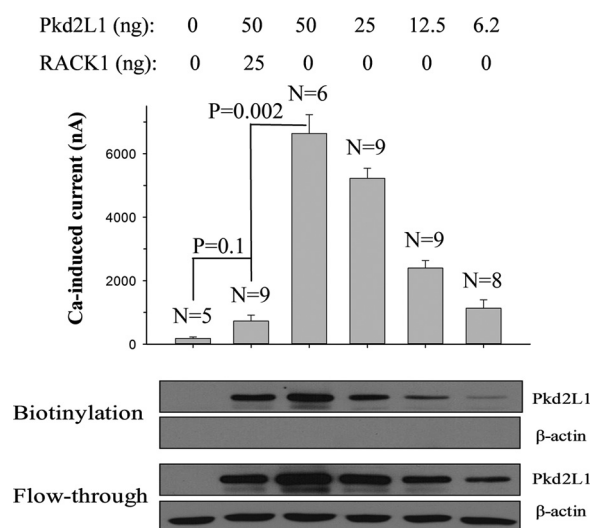RACK1 (ng): 0 25 0 0 0 0

FIGURE 6. **Inhibition of Pkd2L1 channel function by RACK1.** *Top, Xenopus* oocytes were injected with various amounts of Pkd2L1 mRNA with or without co-injection of RACK1. Shown are averaged Ca^{2+}-activated currents measured at -50 mV from different numbers of oocytes as indicated. *p* values obtained from an unpaired *t* test are indicated. Furthermore, linear and Michaelis-Menten regression analyses (current *versus* mRNA amount) indicate that current very significantly ($p < 0.001$) increases with the amount of injected mRNA. *Bottom,* the same batches of oocytes as in the *top panel* were used for biotinylation assays. Shown are representative data on the PM expression of Pkd2L1 with β-actin as control.

Ca^{2+}-induced channel activation of Pkd2L1 (Fig. 4, *B* and *C*). On average, RACK1 inhibited 65 and 87% of basal and Ca^{2+}-activated currents, respectively (Fig. 4*D*). Because *Xenopus* and human RACK1 are 95% identical and 99% similar, we assume that endogenous RACK1, if present, is detectable by our antibody and inhibits part of Pkd2L1 channel activity. Indeed, our Western blotting (WB) assays detected the endogenous RACK1 and showed that the total Pkd2L1 expression is reduced by overexpressed RACK1 (to $68 \pm 3\%$; averaged from five experiments) and increased by endogenous RACK1 knockdown by siRNA (Fig. 5*A*) with sequences 5'-CAAUCAA-ACUGUGGAACACTT-3' and 5'-GUGUUCCACAGUUUG-AUUGTT-3' synthesized by Shanghai GenePharma Co. (Shanghai, China). Consistently, RACK1 knockdown on average increased Ca^{2+}-induced channel activity of Pkd2L1 by 79% ($n = 4$) (Fig. 5*B*). We then performed biotinylation assays to examine whether abolished or substantially reduced Pkd2L1 activity by RACK1 could be accounted for by a decrease in the Pkd2L1 PM expression. We found that Pkd2L1 PM expression in the presence of RACK1 overexpression was decreased to $64 \pm 7\%$ (averaged from four experiments) (Fig. 5*C*). Thus, RACK1 reduces similarly the total and PM expression of Pkd2L1 to about two-thirds, indicating that it does not alter the Pkd2L1 trafficking. This reduction in the PM expression does not seem to be sufficient to account for the decrease in the Ca^{2+}-induced current (to 13%), indicating that RACK1 inhibits Pkd2L1 channel function. Immunofluorescence data are also in agreement with the conclusion that RACK1 overexpression and knockdown, respectively, reduce and increase the Pkd2L1 surface membrane expression (Fig. 5*D*). To provide further documentation, we injected oocytes with decreasing amounts of Pkd2L1 mRNA to identify an amount that generates channel activity comparable with that produced by 50 ng of Pkd2L1 +

25 ng of RACK1. We found that the current obtained from injection of 50 ng of Pkd2L1 + 25 ng of RACK1 is comparable with that from the injection of 6.2 ng of Pkd2L1 alone, but the latter has much less PM expression than the former (Fig. 6). In fact, although the Pkd2L1 PM expression is comparable between injection of 50 ng of Pkd2L1 + 25 ng of RACK1 and that of 25 ng of Pkd2L1 alone, the latter produced far larger channel currents than the former. In this biotinylation assay, we used Na,K-ATPase as a PM marker control and calreticulin and β-actin as non-PM marker controls. These data demonstrated that RACK1 substantially inhibits Pkd2L1 channel function.

Effects of Mutations in RACK1 and Pkd2L1 on Pkd2L1 Channel Function—Although both fragments Met[1]–Ser[205] and Met[217]–Arg[317] of RACK1 bind Pkd2L1 (Fig. 1*A*), whether they are sufficient to exhibit inhibition of Pkd2L1 channel function remains unknown. To test this, we co-expressed each of various fragments of RACK1 with Pkd2L1 in oocytes and performed the two-electrode voltage clamp experiments. We found that, similar to the full-length RACK1, fragments Met[1]–Asp[220] and Met[1]–Ile[258] significantly reduce Pkd2L1 channel activity, whereas Met[217]–Arg[317] has no significant effect (Fig. 7*A*). This indicates that, although Met[217]–Arg[317] binds Pkd2L1N, the binding is not essential for the channel inhibition. We also found that two missense mutants of RACK1, Y228F and Y246F, have inhibitory effects similar to those of WT RACK1, suggesting that the phosphorylation of RACK1 at Tyr[228] or Tyr[246] by Src tyrosine kinase (50) is not involved in its inhibition of Pkd2L1 channel function. We also conducted Pkd2L1 transport activity by means of ^{45}Ca radiotracer uptake. The results are in agreement with our data obtained from electrophysiology experiments (Fig. 7*B*).

Because our yeast two-hybrid experiments revealed that RACK1 binds fragment Ala[19]–Pro[45] of Pkd2L1 and RACK1 was reported to modulate the function of target channels through regulating their phosphorylation (34, 38), we made point mutations at potential phosphorylation sites within this fragment to determine whether these sites are important for channel function or inhibition by RACK1. The NetPhosK online program predicted that Thr[34], Thr[39], Ser[41], and Ser[42] in Pkd2L1N are candidate phosphorylation sites. We thus changed these residues to alanine and found that the resulting mutants still retain channel function comparable with that of WT Pkd2L1 (Fig. 7*C*). Furthermore, RACK1 overexpression inhibited the channel function of these mutants as well (Fig. 7*C*). This result suggests that phosphorylation within Ala[19]–Pro[45] is not critical to Pkd2L1 channel function or to its inhibition by RACK1.

Role of Pkd2L1 Fragment Met[1]–Pro[45] in Mediating Pkd2L1 Channel Inhibition by RACK1—To determine whether the physical interaction between Pkd2L1 and RACK1 through the Met[1]–Pro[45] fragment of Pkd2L1 is important for the inhibition of Pkd2L1 channel function by RACK1, we co-expressed Pkd2L1 and RACK1 with His-tagged Met[1]–Pro[45] peptide that should serve as a blocking peptide through competing with Pkd2L1 for binding RACK1. Indeed, expression of His-Met[1]–Pro[45] rescued the channel function of Pkd2L1 otherwise inhibited by RACK1 (Fig. 8*A*). In contrast, the remaining part of the N terminus (His-Ile[40]–Ile[97]) and C-terminal fragments (His-
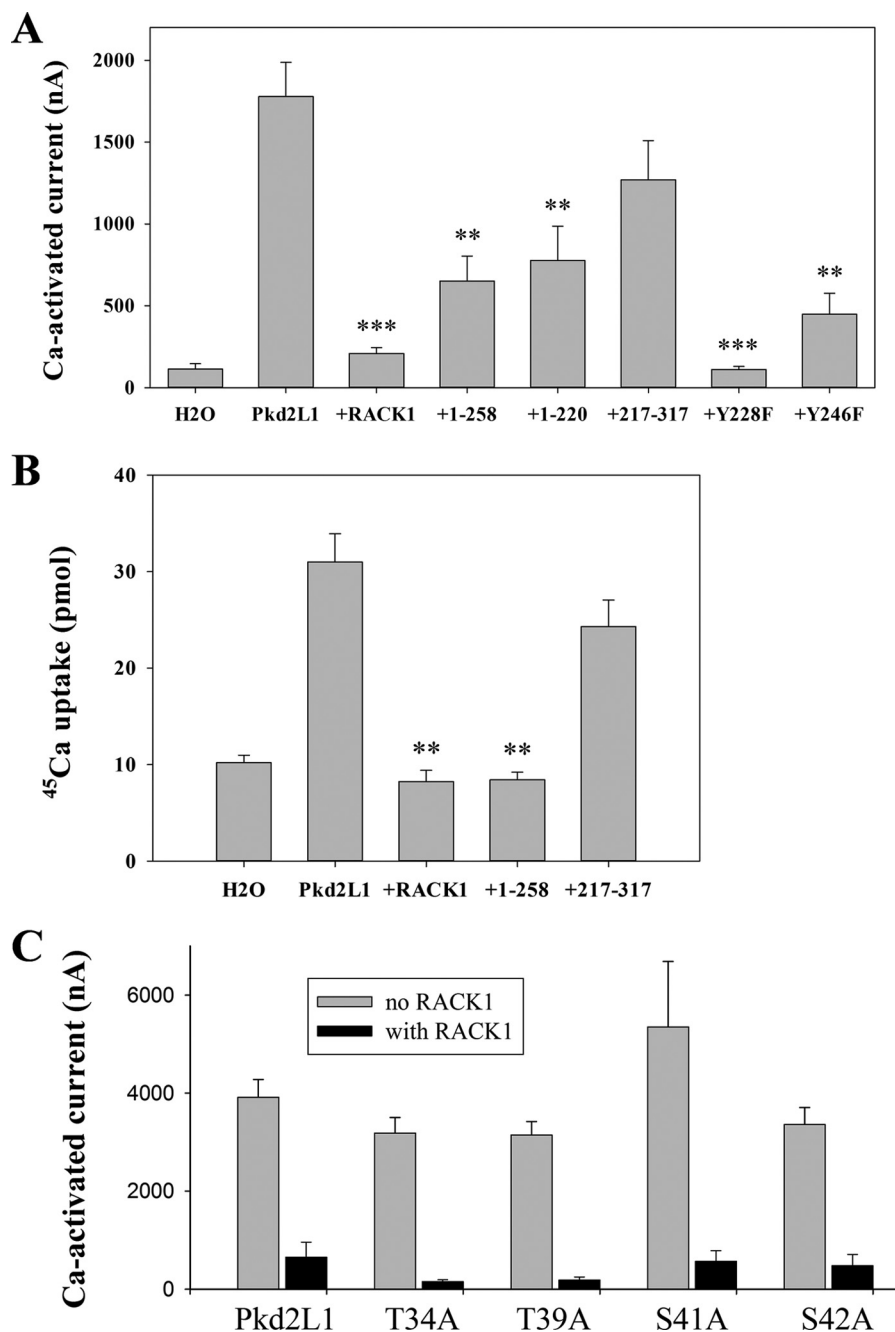
FIGURE 7. **Effects of mutations in RACK1 and Pkd2L1.** *A* and *B*, PKd2L1 was expressed in oocytes with or without co-expression of a RACK1 mutant. *A*, averaged Ca^{2+}-activated currents measured at -50 mV with each group from 5 to 16 different oocytes. ** and *** indicate $p < 0.01$ and $p < 0.001$, respectively. *B*, averaged ^{45}Ca tracer uptake from three independent experiments using uptake solution. *Error bars* indicate S.E. *C*, effect of RACK1 on the function of Pkd2L1 mutants. Pkd2L1 missense mutants were expressed in oocytes with or without co-expression of RACK1. Shown are averaged Ca^{2+}-activated currents measured at -50 mV with each group from 7 to 11 different oocytes.

tagged Glu^{566}–Thr^{622} and Gln^{617}–Ser^{805}) had no rescuing effects (Fig. 8*B*). By WB, we verified that the functional rescue by Met^1–Pro^{45} is not through decreasing RACK1 overexpression (Fig. 8*C*). Consistently, we found by co-IP that Met^1–Pro^{45} indeed substantially decreases the Pkd2L1-RACK1 interaction (Fig. 8*D*). In negative control experiments, we verified that Met^1–Pro^{45} expressed alone has no channel function (data not shown). Taken together, our data support that binding between Pkd2L1 and RACK1 through its N-terminal fragment Met^1–Pro^{45}, but not Pkd2L1C or the remaining part of Pkd2L1N, is required for inhibition of Pkd2L1 channel function by RACK1.

Because oocytes possess significant endogenous RACK1 levels, we reasoned that Met^1–Pro^{45} should block the inhibition of Pkd2L1 channel function by endogenous RACK1. Indeed, we found that overexpression of blocking peptide Met^1–Pro^{45} roughly doubles the Ca^{2+}-induced current (at -50 mV) from an average of $2.5 \pm 0.3\ \mu A$ ($n = 24$) in oocytes expressing Pkd2L1 to $4.8 \pm 0.6\ \mu A$ ($n = 20$, $p = 0.005$) in those expressing Pkd2L1 + Met^1–Pro^{45}. Furthermore, in the presence of Met^1–Pro^{45}, the Pkd2L1 expression assessed by WB was slightly decreased (Fig. 8*F*) possibly due to limited expression capability of oocytes. These data indicate that Met^1–Pro^{45} blocks the inhi-
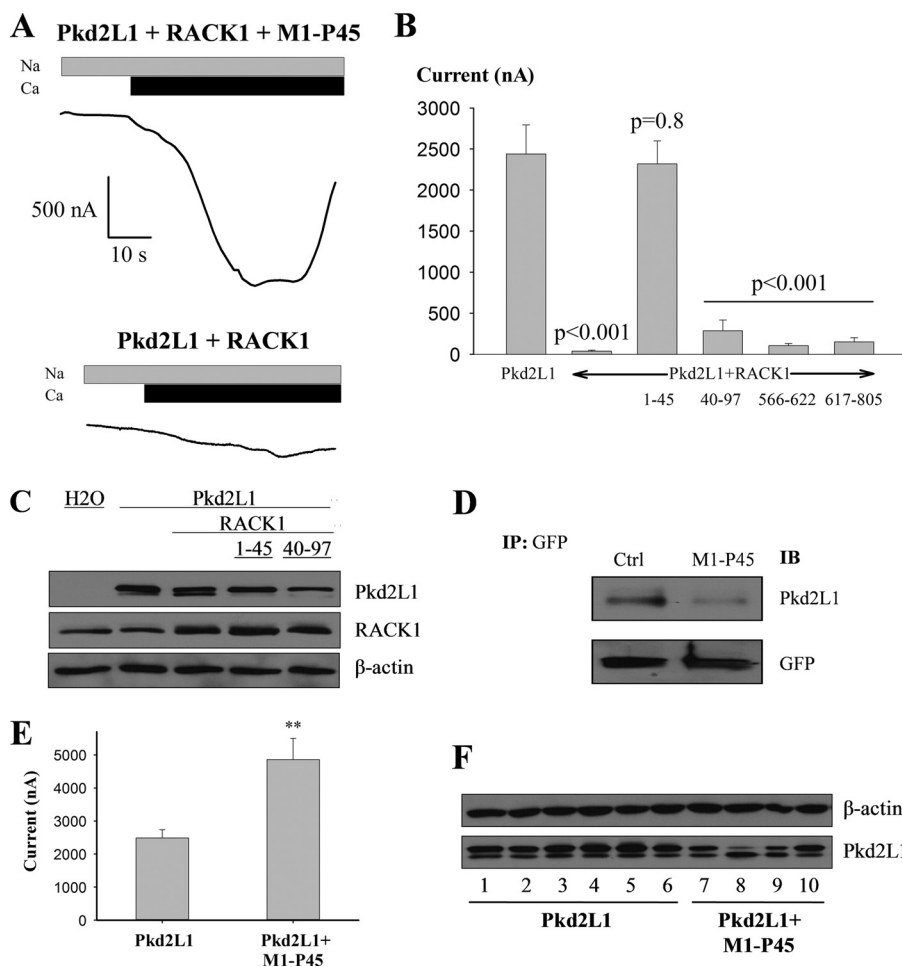
FIGURE 8. **Effects of Pkd2L1 N- and C-terminal fragments on inhibition of Pkd2L1 by RACK1 and on Pkd2L1-RACK1 interaction.** *A*, representative recordings of currents measured at -50 mV in oocytes expressing Pkd2L1 and RACK1 with or without co-expression of Met^1–Pro^{45} in the presence of Na^+-containing solution ± 5 mM Ca^{2+} as indicated. *B*, averaged Ca^{2+}-induced currents measured at -50 mV in oocytes expressing Pkd2L1 alone ($n = 15$), Pkd2L1 + RACK1 ($n = 12$), or Pkd2L1 + RACK1 + a blocking peptide (Met^1–Pro^{45}, $n = 14$; Ile^{40}–Ile^{97}, $n = 11$; Glu^{566}–Thr^{622}, $n = 9$; and Gln^{617}–Ser^{805}, $n = 6$). *p* values obtained from comparison with Pkd2L1 alone (by unpaired *t* test) are indicated. *C*, expression of RACK1 and Pkd2L1 with or without co-expression of Met^1–Pro^{45} in oocytes assessed by WB. *D*, the Pkd2L1-RACK1 interaction assessed by co-IP with or without (control (*Ctrl*)) co-expression of Met^1–Pro^{45}. Pkd2L1, GFP-RACK1, and/or His-Met^1–Pro^{45} were expressed in oocytes. Shown are representative data in which GFP antibody was used for IP and Pkd2L1 and GFP antibodies were used for immunoblotting (*IB*) as indicated. *E*, effect of overexpression of Met^1–Pro^{45} on Pkd2L1 activity. Shown are averaged Ca^{2+}-induced currents from oocytes expressing Pkd2L1 ($n = 24$) or Pkd2L1 plus Met^1–Pro^{45} ($n = 20$, $p = 0.005$; **, unpaired *t* test). *F*, representative data of Pkd2L1 expression by WB in individual oocytes expressing either Pkd2L1 (*lanes 1–6*) or Pkd2L1 plus Met^1–Pro^{45} (*lanes 7–10*). These oocytes are part of the oocytes used in generating currents in *E*. *Error bars* indicate S.E.

bition of Pkd2L1 by endogenous RACK1 in agreement with the data obtained from using overexpressed RACK1 (Fig. 8, *A* and *B*).

DISCUSSION

The mechanism of Ca^{2+}- or acid-induced Pkd2L1 channel activation has remained obscure. The C terminus of Pkd2L1 contains a number of motifs, including a putative Ca^{2+}-binding EF-hand and a coiled coil domain, but when these motifs were removed as in the truncation mutant T622X or when the EF-hand was deleted, Ca^{2+}-induced activation was still present and even increased (14). In an effort to examine the role of the Pkd2L1 N terminus in channel activation, we found in the present study that Pkd2L1N associates with RACK1 and that RACK1 inhibits Pkd2L1 channel function through binding to Pkd2L1N. RACK1 was initially identified as a receptor for activated PKC, but recent studies showed that it is also involved in many other cellular functions. For example, RACK1 acts as a

novel inhibitory scaffolding protein in complex with tyrosine kinase Fyn and its substrate, the NR2B subunit of NMDAR, and this association prevents the phosphorylation of NR2B by Fyn and inhibits NMDAR channel activity (38, 42). The presence of RACK1 in CA1 hippocampal slices resulted in a 50% decrease in NMDAR-mediated current (38).

Because RACK1 reduced both the total and PM expression of Pkd2L1, it remained to be determined whether RACK1-inhibited whole-cell Pkd2L1 channel activity is solely due to the reduced PM level of Pkd2L1 or also to the inhibited activity of individual channel molecules. First, although co-expression of RACK1 reduced the Pkd2L1 PM level by 36%, it inhibited 87% of the Ca^{2+}-activated whole-cell current, indicating that RACK1 represses the activity of each individual Pkd2L1 channel. Then we found that although the Pkd2L1 surface membrane expression from injection of 50 ng of Pkd2L1 and 25 ng of RACK1 mRNAs, is comparable with that from injection of

25 ng of Pkd2L1 mRNA alone, the whole-cell current associated with injection of 50 ng of Pkd2L1 + 25 ng of RACK1 is only comparable with that associated with injection of 6.2 ng of Pkd2L1 alone (Fig. 6). Thus, these data together showed that RACK1 substantially inhibits Pkd2L1 channel function in addition to reducing its total and surface membrane expressions.

We found that two RACK1 fragments bind Pkd2L1 and that RACK1 binds both the N and C termini of Pkd2L1. It was thus unclear whether all physical bindings are important for inhibition of Pkd2L1 function by RACK1. Although we could utilize truncation/deletion mutations in Pkd2L1 and/or RACK1 that disrupt the Pkd2L1-RACk1 interaction to examine whether a mutant RACK1 still inhibits WT channel function or whether WT RACK1 inhibits the function of a mutant Pkd2L1, the information we obtain would not be sufficient to make a conclusion because the normal function of RACK1 or Pkd2L1 may readily be affected by these mutations. In view of this, we utilized co-expression of Pkd2L1 N- and C-terminal fragments as potential blocking peptides to break down the physical association between WT Pkd2L1 and RACK1. Our data demonstrated that fragment Met^1–Pro^{45} is able to substantially reduce the Pkd2L1-RACK1 binding and abolish inhibition by RACK1, indicating that their binding through domain Met^1–Pro^{45} is strongest and critical for RACK1 to exert functional inhibition. In contrast, although Pkd2L1C also binds RACK1, overexpression of Pkd2L1C was unable to significantly affect the Pkd2L1-RACK1 binding, suggesting that their binding through Pkd2L1C is not as strong as through Met^1–Pro^{45} and is not important for functional regulation. It is interesting to note that similarly, although both Met^1–Asp^{220} and Met^{217}–Arg^{317} of RACK1 associate with Pkd2L1, only Met^1–Asp^{220} inhibited Pkd2L1 channel activity, indicating that physical interaction of Met^{217}–Arg^{317} with Pkd2L1 is not essential for the channel inhibition.

How the binding of RACK to domain Met^1–Pro^{45} of Pkd2L1 inhibits the channel function remains unclear. Because RACK1 was known as a chaperone of protein kinases to promote the phosphorylation of target proteins, it is possible that binding of RACK1 to domain Met^1–Pro^{45} prevents phosphorylation of a residue within the domain. Therefore, we mutated candidate phosphorylation residues to alanine: T34A, T39A, S41A, and S42A. However, all these mutants still possessed channel function comparable with that of WT channel and were inhibitable by RACK1, indicating that these potential phosphorylation sites are not important for channel function or binding with RACK1. On the other hand, if Ca^{2+} could bind to Met^1–Pro^{45} of Pkd2L1 then RACK1 may prevent the Ca^{2+} binding, thereby inhibiting channel activation. However, our dot blot calcium overlay assays for testing whether Met^1–Pro^{45} can directly bind Ca^{2+} ions using GST-fused peptides Met^1–Pro^{45} and Ala^{19}–Pro^{45} purified from *E. coli* revealed that GST-tagged Met^1–Pro^{45} and Ala^{19}–Pro^{45} cannot bind Ca^{2+} (Fig. 2*C*). Thus, it is possible that Ca^{2+}-induced channel activation is mediated by an unknown protein that binds Met^1–Pro^{45} and that RACK1 competitively disrupts their interaction, thereby inhibiting Pkd2L1 channel function. Further studies to identify and characterize such an unknown protein would shed light on the mechanism underlying Ca^{2+}-induced Pkd2L1 activation.

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