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Association of Mammalian Trp4 and Phospholipase C Isozymes with a PDZ Domain-containing Protein, NHERF*

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Yufang Tang‡, Jisen Tang‡, Zhangguo Chen‡, Claudia Trost§, Veit Flockerzi§, Min Li¶, Vijaya Ramesh‖, and Michael X. Zhu‡**

From the ‡Neurobiotechnology Center and Department of Neuroscience, Ohio State University, Columbus, Ohio 43210, §Institut für Pharmakilogie und Toxikologie der Universität des Saarlandes, D-66421 Homburg, Germany, ¶Department of Physiology and Neuroscience, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, and ‖Molecular Neurogenetic Unit, Massachusetts General Hospital, Charlestown, Massachusetts 02129

Mammalian homologues of *Drosophila* Trp have been implicated to form channels that are activated following the depletion of Ca^{2+} from internal stores. Recent studies indicate that actin redistribution is required for the activation of these channels. Here we show that murine Trp4 and Trp5, as well as phospholipase C β1 and β2 interact with the first PDZ domain of NHERF, regulatory factor of the Na^+/H^+ exchanger. We demonstrated the association of Trp4 and phospholipase C-β1 with NHERF *in vivo* in an HEK293 cell line expressing Trp4 and in adult mouse brain by immuno-coprecipitation. NHERF is a two PDZ domain-containing protein that associates with the actin cytoskeleton via interactions with members of ezrin/radixin/moesin family. Thus, store-operated channels involving Trp4 and Trp5 can form signaling complexes with phospholipase C isozymes via interactions with NHERF and thereby linking the lipase and the channels to the actin cytoskeleton. The interaction with the PDZ protein may constitute an important mechanism for distribution and regulation of store-operated channels.

Ca^{2+} plays very important roles in regulating cell functions ranging from contraction and secretion to proliferation and differentiation. In many cells, stimulation of phospholipase C (PLC)[1] by cell surface receptors is accompanied by an increase in intracellular Ca^{2+} concentrations. This occurs via Ca^{2+} release from internal stores and Ca^{2+} influx from extracellular space (1, 2). While the mechanism of Ca^{2+} release from the endoplasmic reticulum is well understood, the channel(s) and mechanism(s) that mediate Ca^{2+} influx remain to be elucidated. It is currently believed that most, if not all, Ca^{2+} influx is mediated through a set of plasma membrane Ca^{2+}-permeable channels that open in response to internal Ca^{2+} store depletion (3, 4). These channels are referred to as store-operated channels (SOCs). Molecular cloning and functional expression have revealed that the products of the *Drosophila transient receptor potential (trp)* gene as well as a number of its mammalian homologues form SOCs (5–11). Other Trp homologues form Ca^{2+}-permeable cation channels that do not appear to be store-operated (12–16), as they do not respond to treatment by thapsigargin, an intracellular Ca^{2+}-ATPase inhibitor that induces passive store depletion by preventing the reuptake of Ca^{2+} to the endoplasmic reticulum. However, in all cases, the Trp-formed channels are activated by signaling events downstream from receptor activation and PLC stimulation. Moreover, a store depletion-insensitive Trp subunit may heteromultimerize with a store depletion-sensitive Trp to form a SOC (17, 18). Therefore, store-operated Ca^{2+} influx is most likely mediated by channels formed by Trp proteins.

In *Drosophila* eyes, Trp is organized in a supramolecular complex along with other phototransduction proteins, such as PLC and protein kinase C, through association with a multi-PDZ domain-containing protein, INAD (inactivation no after potential D) (19–21). PDZ domains were first identified as 80–100 amino acid repeated sequences in the neuron-specific post-synaptic density protein (PSD-95/SAP-90), the *Drosophila* septate junction protein disc-large (dlg) and the epithelial tight-junction protein zona occludens-1 (ZO1) (22). Growing evidence suggests that these domains are important protein-protein interaction sites for clustering and organization of signaling molecules, particularly those involved in ion transport (22–24). INAD contains five PDZ domains, each of which interacts with a particular target protein and thus serves as a scaffold to bring PLC, Trp, protein kinase C, and G protein together in a signaling complex. Although INAD is not directly involved in transmitting the signal of light perception, it plays a critical role in localizing PLC and Trp at the proper sites of the rhabdomere of *Drosophila* photoreceptors (19, 20). Since PLC-mediated signaling in mammals is very similar to phototransduction cascade of insects, an INAD-like scaffold may exist in mammalian cells. Although several multi-PDZ domain-containing proteins distantly related to INAD have been cloned from human and rodents (25–27), none has been shown to interact with mammalian Trp or PLC.

The interaction between a target protein and a specific PDZ domain is often determined by the last three C-terminal residues of the target (28). Because the C-terminal sequences of mammalian Trps are very different from their *Drosophila* counterpart and also very diverse among themselves (Fig. 1*a*), each Trp may bind to different PDZ domains and the PDZ

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF011543, AF019663, AF060107, AF288407, and AF288408.

** To whom all correspondence should be addressed: The Ohio State University, Neurobiotechnology Center, 168 Rightmire Hall, 1060 Carmack Rd., Columbus, OH 43210. Tel.: 614-292-8173; Fax: 614-292-5379; E-mail: zhu.55@osu.edu.

[1] The abbreviations used are: PLC, phospholipase C; ECL, enhanced chemiluminescence; ERM, ezrin/radixin/moesin; GST, glutathione *S*-transferase; HA, hemagglutinin; INAD, inactivation no after potential D; mTrp4, murine Trp4; NHERF, Na^+/H^+ exchanger regulatory factor; PDZ, PSD95/DLG/ZO-1; SOC, store-operated channel; Trp, transient receptor potential; PAGE, polyacrylamide gel electrophoresis.

domains that bind to the mammalian Trps may be quite different from that of INAD. Here we show that murine Trp4 (mTrp4) and Trp5 bind to the first PDZ domain of the Na^+/H^+ exchanger regulatory factor (NHERF). We additionally show that the same PDZ domain binds to the C termini of PLC-β1 and PLC-β2, indicating that NHERF is capable of bringing together the signaling molecules involved in the PLC-mediated pathway in mammalian cells and therefore, may play a role similar to that of INAD in insect phototransduction. We demonstrate the physical association of PLC-β1, Trp4, and NHERF in an HEK293 cell line stably expressing mTrp4 and in adult mouse brain by immuno-coprecipitation experiments.

EXPERIMENTAL PROCEDURES

Materials—Phenylmethanesulfonyl fluoride, benzamide, aprotinin, soybean trypsin inhibitor, and leupeptin were purchased from Sigma. Nitrocellulose membranes and protein markers were purchased from Bio-Rad. Protein A-Sepharose (4 Fast Flow), glutathione-Sepharose 4B, and the Enhanced Chemiluminescence (ECL) detection system were purchased from Amersham Pharmacia Biotech. TNT (transcription and translation)-coupled rabbit reticulocyte lysate system was from Promega. ^{35}S-Expressed protein labeling mixture (11 Ci/liter) was from PerkinElmer Life Sciences. Lubrol and IGEPAL were obtained from ICN Biochemicals. Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin/streptomycin, and trypsin-EDTA were purchased from Life Technologies. All other chemicals were from Fisher Scientifics.

Antibodies—The rabbit polyclonal anti-NHERF antibody IC270 was raised against the C-terminal synthetic peptide of human NHERF as described previously (29). A rabbit polyclonal anti-Trp4 antibody, T4cAb, was raised against a synthetic peptide representing the last 13 residues of bovine Trp4 as described (30). Another rabbit polyclonal anti-Trp4 antibody, T4nAb, was raised against the synthetic Trp4 N-terminal peptide, CYYKRNVNAPYRDR (Quality Controlled Biochemicals, Hopkinton, MA). The rabbit polyclonal antibodies for PLC-β1 (G-12) and His-tag (H-15) were from Santa Cruz Biotechnologies (Santa Cruz, CA). The monoclonal antibody 12CA5 for hemagglutinin (HA) epitope was from Babco (Berkeley, CA).

cDNAs and Expression Constructs—Full-length cDNAs for murine Trp4 and Trp5 was obtained from a mouse brain cDNA library for rapid amplification of complimentary ends by polymerase chain reaction made according to the protocol provided by the Marathon cDNA Amplification kit (CLONTECH). Rapid amplification of complimentary ends by polymerase chain reaction primers were designed and the reactions were carried out following the previous description (7). DNA sequences were determined by the dideoxy chain termination method (31). The sequences for murine Trp4α, Trp4β, and Trp5 have been deposited in GenBank data base with accession numbers AF011543, AF019663, and AF060107, respectively. For *in vitro* synthesis of ^{35}S-labeled proteins, the cDNAs were subcloned into the expression vector pAGA (32), which contains a T7 promoter that allows transcription initiation by T7 RNA polymerase. An ATG codon contained within an *Nco*I site serves as a translation initiation site. The C-terminal constructs for Trp4α and Trp5 were made by subcloning *Hin*dIII/*Eco*RI fragments encoding the corresponding sequences into pAGA at the *Nco*I site via blunt-end ligation after each fragment had been filled-in by the Klenow fragment of DNA polymerase I in the presence of dNTP. cDNAs for bovine PLC-β1 and PLC-β2 were kindly provided by Dr. Peter Gierschik. Their C-terminal constructs were made by subcloning a *Pvu*II/*Bam*HI fragment of PLCβ1 or a *Pvu*II/*Xba*I fragment of PLCβ2 into pAGA at the *Nco*I site via blunt-end ligation. HA-tagged *trp*4 constructs contain the coding nucleotide sequence for the HA epitope, "YPYDVPDY," immediately after the coding sequence for the C-terminal end of Trp4 "VTTRL." A stop codon was added after the codon for the last "Y" of the HA-epitope. For expression in HEK293 cells, the cDNA for the full-length mTrp4α or the C-terminal HA-tagged mTrp4α was subcloned into pcDNA3 (Invitrogen). Constructs for preparing glutathione *S*-transferase (GST) fusion proteins for PDZ1 and PDZ2 of NHERF were as described (28).

GST Pull-down Experiments—GST and GST fusion proteins were produced in *Escherichia coli* BL-21 cells after transformation with corresponding plasmids. Synthesis of recombinant proteins was induced by the addition of 100 μM isopropyl-1-thio-D-galactopyranoside to log-phase cultures (A_{600} ≈0.6) at 37 °C. After 3–5 h of incubation with constant shaking, the bacteria were recovered by centrifugation at 4 °C for 15 min and either used immediately or stored at −20 °C. GST and GST fusion proteins were purified, using glutathione-Sepharose 4B, from bacterial lysates prepared by incubating with 20 mM lysozyme and 1% Sarcosyl and sonication. The glutathione beads with the bound proteins were washed three times with washing buffer (20 mM Tris-Cl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.5% IGEPAL) and then resuspended in a binding buffer containing 20 mM Tris-Cl, pH 8.0, 100 mM KCl, 2 mM $MgCl_2$, and 0.5% Lubrol. ^{35}S-Labeled Trp4, Trp5, or the C-terminal portions of Trp4, Trp5, PLC-β1, and PLC-β2 were made *in vitro* using the TNT-coupled rabbit reticulocyte system. GST protein (2 μg) bound to glutathione-Sepharose and 10 μl of ^{35}S-labeled protein were added to 250 μl of the binding buffer and were incubated on a platform shaker at room temperature for 30 min. The bound proteins were collected by centrifugation at 5,000 rpm for 2 min and washed three times with the binding buffer. After the last wash, 10 μl of 2 × Laemmli buffer (1 time = 62.5 mM Tris-Cl, 1% SDS, 10% glycerol, 10% β-mercaptoethanol, pH 6.8) was added to the pellet and the samples were incubated at room temperature for 5 min. Aliquots (10 μl) of each sample were subjected to SDS-PAGE and the gels were stained with Coomassie Blue, dried, and exposed to x-ray films overnight.

Cell Lines and Cell Culture—HEK293 cells were grown in Dulbecco's modified Eagle's medium containing 4.5 mg/ml glucose, 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. For transfection, 2.8×10^6 cells were cultured in a 10-cm tissue culture dish for 20 h and 5 μg of m*trp4α*/pcDNA3 or m*trp4α-HAc*/pcDNA3 was transfected into the cells by the calcium phosphate precipitation method as described (34). After 24 h, cells were harvested, suspended in the same medium supplemented with 400 μg/ml G418, and transferred to wells of 96-well plates in three serial dilutions of 1:4. G418-resistant transformants that appeared to arise from single colonies were transferred to 12-well plates for growth and further analysis. Clonal cell lines expressing Trp4α or C-terminal HA-tagged Trp4α were identified by immunocytochemistry and confirmed by immunoprecipitation as detailed before (34) using an affinity purified polyclonal anti-Trp4 N-terminal antibody, T4nAb. The stable cell lines were diluted twice weekly and maintained in medium supplemented with 400 μg/ml G418.

Immuno-coprecipitation and Immunoblotting—HEK293 cells were washed twice in phosphate-buffered saline (phosphate-buffered saline: 136 mM NaCl, 1.4 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.4), scraped off from the dishes in 1 ml of ice-cold phosphate-buffered saline supplemented with protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 1 μg/ml aprotinin, 10 μM leupeptin, 1 μM soybean protease inhibitor), and pelleted by centrifugation at 5,000 rpm in a microcentrifuge at 4 °C for 2 min. Cells were sonicated in RIPA buffer containing 150 mM NaCl, 50 mM Tris-Cl, pH 8.0, 0.5% sodium deoxycholate, 1% IGEPAL, 0.1% SDS, 5 mM EDTA, and the protease inhibitors listed above. Mouse brain samples were homogenized using a Polytron homogenizer in 1 ml of RIPA buffer containing protease inhibitors followed by sonication. All procedures for immunoprecipitation were carried out at 4 °C. The crude lysate was centrifuged at 14,000 rpm in a microcentrifuge for 10 min and the supernatant was transferred to a new Eppendorf tube. Nonspecific bindings were removed by a preincubation with 1/10 volume of protein-A Sepharose for 1 h followed by a low speed centrifugation at 5,000 rpm for 2 min. The supernatant was then incubated with appropriate dilutions of the desired antibody for 10–16 h. The dilutions for the antibodies were 1:200 for anti-Trp4, 1:100 for anti-NHERF and anti-PLCβ1. Immunocomplexes were purified by incubation with 1/10 volume of protein A-Sepharose for 1 h, centrifuged, and washed three times in RIPA buffer. Bound immunocomplexes were eluted by incubating the Sepharose beads in 50 μl of 2 × Laemmli buffer at 95 °C for 10 min. The samples were centrifuged briefly at 14,000 rpm and a 10-μl aliquot of the supernatant was subjected to SDS-PAGE. The proteins were then transferred to nitrocellulose membranes using a Semi-dry Transfer Blotter (W. E. P. Co., Concord, CA) at 120 mA for 2 h and immunoblotted with antibodies as indicated. For immunoblotting, dilutions of antibodies were 1:2,000 for anti-Trp4, 1:1,000 for anti-NHERF and anti-PLC-β1. Horseradish peroxidase-conjugated goat anti-rabbit IgG was used as secondary antibody and all blots were developed using the ECL system.

RESULTS

Among the seven mammalian Trp homologues currently known, Trp4 and Trp5 are the most closely related. The first 754 residues of mTrp4, encompassing the N terminus, the transmembrane regions, and the first 133 residues of the C terminus, share 73% identity (82% similarity) with the equiv-

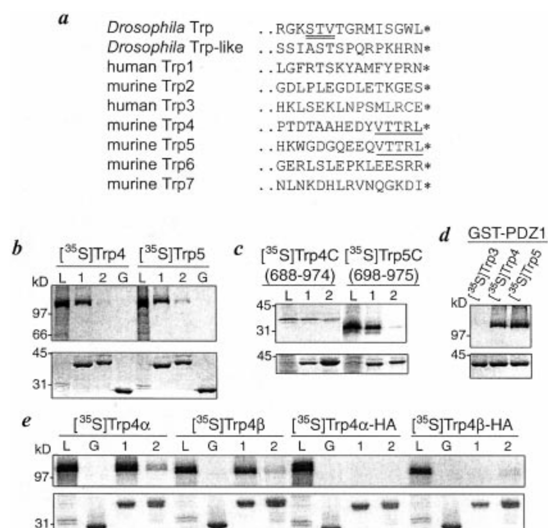

FIG. 1. **Trp4 and Trp5 interact with the first PDZ domain of NHERF.** *a*, C-terminal sequences of *Drosophila* Trp, Trp-like, and the seven mammalian Trp homologues. Shown are the last 15 residues for each Trp. Except for the last five amino acids (*underlined*) of Trp4 and Trp5, the C termini of Trp proteins are not conserved. *Double underlined* sequence in *Drosophila* Trp had been shown to be critical for the interaction between the Trp and INAD (*35*). *b*, interaction of full-length Trp4 and Trp5 with PDZ domains of NHERF. ^{35}S-Labeled mTrp4 and mTrp5 were incubated with GST or GST fusion of NHERF PDZ1 or PDZ2 bound to glutathione-Sepharose and bound proteins were separated by SDS-PAGE (8%) as described under "Experimental Procedures." *c,* interaction of the C-terminal portions of Trp4 and Trp5 with NHERF PDZ domains. The ^{35}S-labeled proteins contained portions of mTrp4 and mTrp5 as indicated by the positions of the amino acids shown in *parentheses*. Proteins were separated by 12% SDS-PAGE. *d*, NHERF PDZ1 interacts with Trp4, Trp5, but not Trp3. *e*, NEHRF PDZ1 interacts with Trp4α and Trp4β but not C-terminal HA-tagged Trp4 isoforms. Experiments were performed as in *b*. For *b-e, upper panels* show autoradiograms of input ^{35}S-labeled proteins and those retained by GST fusion proteins. *Lower panels* show the amount of GST or GST fusion proteins used as revealed by Coomassie Blue staining. Sizes (in kDa) of molecular weight markers are indicated to the *left*. Lane labels in *b, c, e*: *L*, total lysate; *G*, GST; *1*, GST-NHERF PDZ1; *2*, GST-NHERF PDZ2.

alent regions of mTrp5 (761 residues). However, the last 220 residues of Trp4 are divergent from the last 214 residues of Trp5 with an identity score of 20% and a similarity score of 38%. Strikingly, both proteins end with the same C-terminal sequence, VTTRL (Fig. 1*a*). Since the ligand specificities of PDZ domains are often defined by the last few residues of target proteins, this sequence conservation suggests that Trp4 and Trp5 might interact with a common PDZ domain. Random peptide library screening has revealed that peptides terminating with the amino acid sequence "TRL" bound with high affinity to the first PDZ domain of NHERF (28). Therefore, we tested whether Trp4 and Trp5 interact with NHERF in GST pull-down experiments. Fig. 1*b* shows that the GST fusion protein containing PDZ1 of NHERF interacted with ^{35}S-labeled full-length mTrp4 and mTrp5. PDZ2 of NHERF interacted with Trp4 and Trp5 only very weakly. In agreement with the notion that the C-terminal sequences of the target proteins are responsible for the interaction with PDZ domains, NHERF PDZ1 also interacted with the C-terminal portions of Trp4 or Trp5 (Fig. 1*c*). In contrast, human Trp3 terminates with "MLRCE" and did not interact with NHERF PDZ1 (Fig. 1*d*). Molecular cloning has revealed the existence of at least two forms of mTrp4: a longer form (Trp4α) that contains 974 amino acids and a shorter form (Trp4β) that contains 890 amino acids and lacks residues 781–864 corresponding to Trp4α. Because the C-terminal ends remain the same, both Trp4α and Trp4β interact with PDZ1 of NHERF (Fig. 1*e*). However, when the C

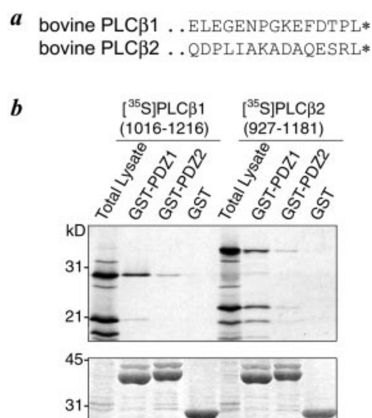

FIG. 2. **PLC-β1 and PLC-β2 interact with the first PDZ domain of NHERF.** *a*, C-terminal sequences of bovine PLC-β1 and PLC-β2. Shown are the last 15 residues for each protein. *b*, interaction of PLC-β1 and PLC-β2 with PDZ domains of NHERF. ^{35}S-Labeled C-terminal portions of PLC-β1 and PLC-β2 were incubated with GST or GST fusion of NHERF PDZ1 or PDZ2 bound to glutathione-Sepharose and bound proteins were separated by 12% SDS-PAGE. *Upper panel* shows the autoradiogram of input ^{35}S-labled proteins and those retained by GST fusion proteins. *Lower panel* shows the amount of GST or GST fusion proteins used as revealed by Coomassie Blue staining. Molecular weights are indicated to the *left*.

terminus was masked by the addition of an HA epitope, neither Trp4 isoform interacted with NHERF (Fig. 1*e*). These data indicate that the presence of TRL motif at the C-terminal end is essential for the interaction between Trp4 and NHERF PDZ1.

While the VTTRL sequence is found at the C-terminal end of bovine, human, and murine Trp4, it has been reported that rat Trp4 terminates with "HKIMI" (GenBank™ accession number AB008889). In order to confirm this, we isolated total RNA from the brain of an adult Harlan Sprague-Dawley rat and performed rapid amplification of complimentary ends by polymerase chain reaction using primers that would anneal to both mouse and rat *trp4*. Products corresponding to *trp4α* and *trp4β* were obtained and sequenced. Despite the 99.4% identity with the published rat *trp4* (AB008889) at the nucleotide level, our polymerase chain reaction products predict proteins with VTTRL at the C termini (GenBank™ accession numbers: AF288407 and AF288408). Therefore, the VTTRL sequence and hence the association with NHERF is conserved among Trp4 orthologues in mammalian species.

Bovine PLCβ1 and PLCβ2 terminate with "TPL" and "SRL," respectively (Fig. 2*a*). Results of the random peptide library screening (28) indicated that these sequences might also interact with PDZ1 of NHERF. Therefore, we tested the binding of PLC-β1 and PLC-β2 with the two PDZ domains of NHERF. As shown in Fig. 2*b*, ^{35}S-labeled C-terminal fragments of PLC-β1 and PLC-β2 bound to GST fusion protein containing PDZ1 but only very weakly to that containing PDZ2 of NHERF. Thus, the first PDZ domain of NHERF is capable of interacting with PLC-β1, PLC-β2, Trp4, and Trp5, suggesting that NHERF may serve as a scaffold for the clustering of PLC signaling molecules in mammalian cells.

In order to test whether Trp4 associates with NHERF *in vivo*, we expressed mTrp4α in HEK293 cells and established a stable cell line, T4-60. In immunoblotting, a rabbit polyclonal antibody (T4cAb) raised against a peptide representing the last 13 amino acids of Trp4 (30) recognized a 110-kDa polypeptide in membranes prepared from T4-60 cells but not that from either untransfected HEK cells or a cell line stably expressing human Trp3 (Fig. 3*a*). The expression of endogenous NHERF in HEK293 cells was detected in total cell lysates using an

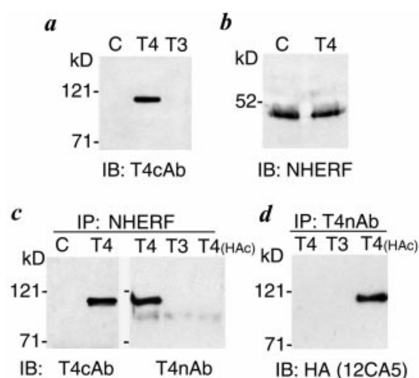

FIG. 3. **Interaction of Trp4 with endogenous NHERF in HEK293 cells expressing mTrp4α.** *a,* expression of Trp4 was detected in T4-60 (T4) but not untransfected control (*C*) or Trp3 transfected (*T3*) cells by immunoblotting using the anti-Trp4 C-terminal antibody, T4cAb. Crude membrane preparations were made according to Levy *et al.* (*36*) and 2 μg of total protein was used in each lane. *b*, NHERF was detected in control and T4-60 cells by immunoblotting using the anti-NHERF antibody, IC270. Aliquot of 10 μl of cell lysate containing 1 mg of total protein was added to each lane. *c*, cell lysates (100 μl) from control, T4-60 cells, and cells expressing Trp3 (T3) or C-terminal HA-tagged Trp4 (T4(HAc)) were immunoprecipitated with the anti-NHERF, IC270. The precipitates were separated by 8% SDS-PAGE and then probed with the anti-Trp4 C-terminal, T4cAb (*left*) or the anti-Trp4 N-terminal, T4nAb (*right*), to reveal coprecipitation of Trp4 with NHERF. *d*, cell lysates (100 μl) were immunoprecipitated with anti-Trp4 N-terminal antibody and the presence of the HA-tagged Trp4 was determined by immunoblotting using the anti-HA monoclonal antibody, 12CA5. Molecular weights as revealed by prestained protein markers are indicated to the *left*.

anti-NHERF specific antibody, IC270 (Fig. 3*b*). Moreover, the association of Trp4 with NHERF was examined by first immunoprecipitating cell extracts using IC270 followed by the separation of precipitated proteins by SDS-PAGE and immunoblotting using the anti-Trp4, T4cAb. Trp4 was detected in the precipitates obtained from T4-60 but not untransfected cells (Fig. 3*c*). No Trp4 signal was found in the blot made from immunoprecipitation of T4-60 cell lysate using a nonspecific antibody, anti-HA 12CA5 (not shown). In addition, an anti-Trp4 N-terminal antibody (T4nAb) also detected Trp4α in the anti-NHERF immunoprecipitates from the T4-60 cells but not from cells expressing Trp3 or the C-terminal-tagged Trp4α (Fig. 3*c*). The expression of the HA-tagged Trp4α in this cell line was confirmed by immunoprecipitation with T4nAb followed by immunoblotting with the anti-HA antibody. These data demonstrated that Trp4 interacts with NHERF *in vivo* and that the presence of TRL at the C-terminal end of Trp4 is essential for this interaction.

Endogenous PLC-β1 can be detected in HEK293 cells using a polyclonal anti-PLC-β1 antibody (Fig. 4*a*). Immunoblotting with the antibody revealed the presence of PLC-β1 in immunoprecipitates obtained using the anti-NHERF antibody (Fig. 4*b*) and thereby demonstrating that endogenous PLC-β1 and NHERF associate with each other *in vivo* in HEK293 cells. Moreover, in T4-60 cells, PLC-β1 coprecipitated with Trp4 in immunoprecipitates obtained using the anti-Trp4 antibody (Fig. 4*b*). Interestingly, the amount of PLC-β1 coprecipitated by the anti-Trp4 antibody was similar to that precipitated by the anti-NHERF antibody (Fig. 4*b*). Assuming that the two antibodies had the same efficiency for the precipitation of the NHERF·Trp4 complex, this result indicates that most likely, PLC-β1 is part of the same complex that contains Trp4 and NHERF. Thus, the three proteins appear to coassemble in HEK cells expressing Trp4.

In order to determine whether the three proteins also coassemble in animal tissues that normally express them, we per-

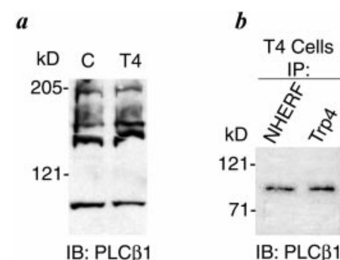

FIG. 4. **Coassembly of PLC-β1, Trp4, and NHERF in T4-60 cells.** *a,* detection of PLC-β1 in control (*C*) and T4-60 (*T4*) cells by immunoblotting using the anti-PLCβ1 antibody. 100- and 150-kDa forms are commonly detected in cell lines and freshly isolated tissues by this antibody and probably represent alternatively spliced forms of PLC-β1 or proteolytic degradation during sample processing (37). The higher molecular weight bands are common for the HEK cells as they were also seen by others using the same antibody (37). An aliquot of 10 μl of total cell lysate was used in each lane. *b*, PLC-β1 immuno-coprecipitated with NHERF and Trp4. T4-60 cell lyaste (100 μl) in RIPA buffer was subjected to immunoprecipitation by the anti-NHERF, IC270, or the anti-Trp4, T4cAb. The precipitates were separated by 8% SDS-PAGE, transferred to nitrocellulose membrane, and probed with the anti-PLC-β1 antibody.

formed immuno-coprecipitation experiments using adult mouse brain. Significant amounts of PLC-β1 and NHERF were detected in mouse brain by immunoblotting using the respective specific antibodies (Fig. 5*a, left* and *middle*). The expression of Trp4 in adult mouse brain was difficult to detect by immunoblotting alone using the anti-Trp4, T4cAb. However, two bands, 110 and 100 kDa, presumably representing the α and β isoforms of Trp4, were detected by immunoprecipitation followed by immunoblotting using the same antibody (Fig. 5*a, right*). Immuno-coprecipitation experiments showed that the anti-NHERF antibody also precipitated the two isoforms of Trp4 from the brain lysate (Fig. 5*b*) and that a similar amount of PLC-β1 was precipitated by the anti-NHERF, IC270, and the anti-Trp4, T4cAb (Fig. 5*c*). An unrelated rabbit polyclonal antibody, anti-His tag, did not precipitate Trp4 or PLC-β1. Thus, PLC-β1, Trp4, and NHERF coexist as a molecular complex in adult mouse brain.

DISCUSSION

Our data suggest that NHERF is a molecular scaffold that brings PLC isozymes and Trp4 or Trp5 into a signaling complex. Similar function is known to be carried out by INAD in the *Drosophila* phototransduction system (19–21). The association with INAD appears to be crucial for the proper localization of the cascading enzymes as well as for the precision of the spatial and temporal control of signaling events, such as those involved in the recruitment of G protein upon stimulation (38) and the inactivation of Trp by protein kinase C-mediated phosphorylation (39, 40). NHERF was first isolated as a cofactor required for protein kinase A-mediated inhibition of type 3 Na^+/H^+ exchanger found on the renal brush-border membrane (41, 42) and later determined to be the same as the phosphoprotein that binds members of the ezrin/radixin/moesin (ERM) proteins (33, 43). The ERM proteins are known to link cell surface receptors to actin cytoskeleton (44) and in many cases such link is mediated through NHERF. The two PDZ domains of NHERF bind to target proteins while its C terminus binds to the N terminus of ERMs (33, 43). It has been shown that the first PDZ domain of NHERF interacts with a number of G-protein-coupled receptors and ion transporting proteins, such as the cystic fibrosis transmembrane conductance regulator, sodium bicarbonate transporters, and H^+-ATPase (28, 45–48). Analysis of the sequence requirement for NHERF PDZ1 binding also suggested PLC-β1 as a potential ligand (45). Indeed, we show here that the C termini of both PLC-β1 and PLC-β2

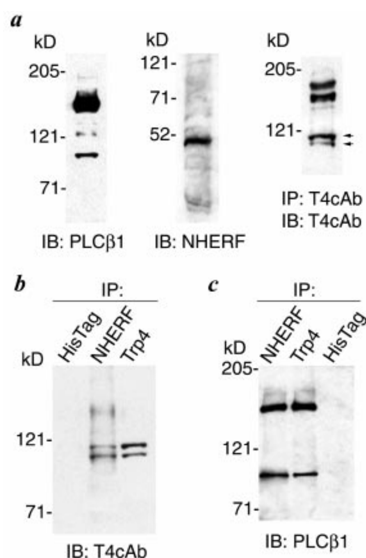

FIG. 5. **Coassembly of PLC-β1, Trp4, and NHERF in mouse brain.** *a,* expression of PLC-β1 (*left*), NHERF (*middle*), and Trp4 (*right*) in mouse brain. A whole brain from an adult mouse was homogenized in 1 ml of RIPA buffer as described under "Experimental Procedures." An aliquot of 10 μl (1.4 mg of total protein) of lysate was subjected to 8% (for PLC) or 10% (for NHERF) SDS-PAGE. Proteins were transferred and probed with antibodies against PLC-β1 or NHERF. Under the same conditions, the anti-Trp4 antibody failed to detect any signal at the expected size range. Therefore, 200 μl of whole brain lysate was immunoprecipitated with the anti-Trp4, T4cAb, and the precipitates were separated by 8% SDS-PAGE, transferred, and then probed again with T4cAb. *Arrowheads* indicate the two bands corresponding to the sizes of Trp4α and Trp4β. The identities for the higher molecular weight bands are not known as they appeared in some but not all blots. *b*, association of Trp4 with NHERF in brain. Brain lysate (200 μl) was immunoprecipitated with antibodies against His-tag, NHERF, or Trp4. The presence of Trp4 in the immunoprecipitates was examined by immunoblotting using the anti-Trp4, T4cAb. Anti-His-tag was used as a negative control for immunoprecipitation. *c*, PLC-β1 immuno-coprecipitated with Trp4 and NHERF from brain. Brain lysate was immunoprecipitated with antibodies against His-tag, NHERF, or Trp4. The presence of PLC-β1 in the immunoprecipitates was examined by immunoblotting using the anti-PLC-β1 antibody.

bind NHERF PDZ1. In addition, this PDZ domain also binds Trp4 and Trp5. Our immuno-coprecipitation studies further demonstrated the association of PLC-β1, Trp4, and NHERF in the same complex in HEK293 cells expressing mTrp4 and in mouse brain. Because the ligand specificity of the PDZ domain interaction is determined, in most cases, by the last 3 to 4 C-terminal amino acids of the target proteins, it is predictable that only a subset of mammalian Trps can interact directly with NHERF. Thus, NHERF is considered one of the functional analogs of INAD. Several other PDZ domain-containing proteins may exist for the clustering of other Trps.

There are differences between NHERF and INAD. First, INAD has five PDZ domains with PLC-β binding to PDZ1 and PDZ5 (19, 21), whereas Trp binds to PDZ3 (19). In contrast, NHERF has only two PDZ domains and PLC-β1/2 and Trp4/5 all bind to PDZ1. It is thus unlikely that a single NHERF molecule binds simultaneously to PLCs and Trps. Rather, it is possible that the molecules are clustered via NHERF's self-association (49) or its association with ERMs, which oligomerize among themselves (44, 50). In such a case, the stoichiometry for PLC-β, NHERF, and Trp4 in the molecular complexes may vary greatly. Second, unlike INAD, which is a photoreceptor-specific protein primarily involved in scaffolding and modulating the photosensing signaling molecules, NHERF appears to participate in multiple signaling pathways, as shown by its interaction with multiple types of receptors, ion channels, transporters, and exchangers. Therefore, questions remain as to how NHERF selects among its many potential partners and what the physiological significance of each association is. The answer to the first question may lie partly on the tissue-specific distribution of each target protein. However, in the case of PLC-β and Trp4, some kind of sorting mechanism would have to be incorporated so that the binding with one partner does not overwhelm the association with the others. For the second question, although the specific physiological implication for each association is not known, there are functions that may be common to all target proteins. These include preferential distribution to the apical membrane of polarized cells (51, 52), facilitation of phosphorylation by protein kinase A (53), which associates with ERM (54), and by non-receptor tyrosine kinase, *e.g.* c-Yes, through interaction of NHERF PDZ2 with Yes-associated protein 65 (51).

It was recently shown that actin redistribution plays a critical role in activating SOCs in cultured smooth muscle cells and HEK293 cells as well as in activating Trp3 expressed in the HEK cells (55, 56). The binding of Trp4 (and Trp5) to NHERF constitutes the first physical link of these channels to actin cytoskeleton. The C terminus of NHERF interacts with the N terminus of ezrin, moesin (43), and the related protein merlin (33). The C terminus of ERM protein interacts with F-actin in a manner that is dependent on the oligomerization and the phosphorylation states of ERM (44, 57, 58). Blocking ERM dephosphorylation by the phosphatase inhibitor, calyculin A, led to condensation of F-actin in the cell periphery (59) and inhibition of SOCs (56). Conceivably, the phosphorylation state of ERMs will also affect the dynamics of association of Trp4/5 with the actin network due to their association with ERMs via NHERF. Thus, physical association of SOCs with ERM proteins may be one of the reasons that capacitative Ca^{2+} entry is dependent on the reversible redistribution of actin and is blocked by phosphatase inhibitors (55, 56). Other Trp proteins may multimerize with Trp4 or Trp5 and hence associate with ERMs. Although Trp4 has been shown to be a part of native SOCs in adrenal cells (30), it is yet to be established whether Trp4 or Trp5 also participate in the formation of SOCs in other cell types. Perhaps in the absence of Trp4/5, different PDZ domain-containing proteins are involved in linking the channels to the ERMs through a similar mechanism.

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