Spatial and temporal regulation of Ca\(^{2+}\) signaling requires the assembly of multiprotein complexes linking molecules involved in Ca\(^{2+}\) influx, sensing, buffering, and extrusion. Recent evidence indicates that plasma membrane Ca\(^{2+}\) ATPases (PMCA) participate in the control of local Ca\(^{2+}\) fluxes, but the mechanism of multiprotein complex formation of specific PMCA is poorly understood. Using the PMCA2b C-terminus tail as bait in a yeast two-hybrid screen, we identified the PSD-95, Dlg, ZO-1 (PDZ) domain-containing Na\(^+/H^+\) exchanger regulatory factor-2 (NHERF2) as an interacting partner. Protein pull-down and coimmunoprecipitation experiments using recombinant PMCA2b and PMCA4b as well as NHERF1 and NHERF2 showed that the interaction of PMCA2b with NHERF2 was specific and selective. PMCA4b did not interact with either of the NHERFs, and PMCA2b selectively preferred NHERF2 over NHERF1. Green fluorescent protein-tagged PMCA2b was expressed at the apical membrane in Madin-Darby canine kidney epithelial cells, where it colocalized with apically targeted NHERF2. Our study identifies NHERF2 as the first specific PDZ partner for PMCA2b not shared with PMCA4b, and demonstrates that PMCA splice forms differing only minimally in their COOH-terminal residues interact with unique PDZ proteins. NHERFs have been implicated in the targeting, retention, and regulation of membrane proteins including the \(\beta_2\)-adrenergic receptor, cystic fibrosis transmembrane conductance regulator, and Trp4 Ca\(^{2+}\) channel, and NHERF2 is now shown to also interact with PMCA2b. This interaction may allow the functional assembly of PMCA2b in a multiprotein Ca\(^{2+}\) signaling complex, facilitating integrated cross-talk between local Ca\(^{2+}\) influx and efflux.

The dynamic regulation of calcium ion (Ca\(^{2+}\)) homeostasis demands that a finely controlled system of Ca\(^{2+}\) transporters, channels, and Ca\(^{2+}\)-binding proteins collaborate to allow transient increases in the intracellular free calcium concentration ((Ca\(^{2+}\))\(_i\),\(^1\) while over the long term maintaining a low resting (Ca\(^{2+}\))\(_i\),\(^1\). Both the entry of Ca\(^{2+}\) into the cell and the removal of Ca\(^{2+}\) from the cytosol must therefore be under precise temporal and spatial control (3–5). Whereas substantial progress has been made over the last several years in elucidating the molecular properties, regulation, and membrane targeting of calcium channels (6), our knowledge concerning the spatial organization of Ca\(^{2+}\) extrusion mechanisms is still limited. Plasma membrane Na\(^+/Ca\(^{2+}\) exchangers and Ca\(^{2+}\) ATPases are the main systems for Ca\(^{2+}\) removal from the cell. Although their molecular characterization has advanced rapidly over the last few years (reviewed in Refs. 7–9), we still know virtually nothing about the mechanisms by which these Ca\(^{2+}\) pumps and exchangers are targeted to specific membrane domains and integrated into functional units with other Ca\(^{2+}\) signaling molecules.

The plasma membrane Ca\(^{2+}\) ATPases (PMCA) are essential for the maintenance of low resting-state [Ca\(^{2+}\)]\(_i\), but may also be involved in dynamic events such as the regulation of Ca\(^{2+}\) spikes and local Ca\(^{2+}\) signaling (reviewed in Refs. 10–12). Four nonallelic genes code for the mammalian PMCA 1, 2, 3, and 4, with additional isoform diversity arising from alternative mRNA splicing (9). The four PMCA are very similar in their predicted overall tertiary structure, but there are substantial differences in their regulation by kinases, proteases, and the Ca\(^{2+}\)-binding protein calmodulin (8, 13). In addition, the major PMCA variants “a” and “b” generated by alternative splicing differ markedly in their regulatory properties, e.g. their calmodulin sensitivity (11, 14). The alternative splice affects the protein sequence after the last transmembrane span and creates different COOH-terminal tails for the a and b variants because of a change in the translational reading frame. The last few residues of all PMCA b splice variants are highly conserved, and the final four residues match the minimal consensus sequence (E-(T/S)-X-(V/L)*, where X stands for any amino acid and the asterisk indicates the COOH-terminal residue) of protein ligands for type I PDZ (PDZ-95, Dlg, ZO-1) domains (15). PDZ protein-protein interaction domains are present in a growing list of proteins such as the membrane-associated guanylate kinase-like proteins (MAGUKs) and various other scaffolding and signaling proteins (16–18). Indeed, we previously showed that PMCA4b interacts with high affin-

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\(^{1}\) The abbreviations used are: [Ca\(^{2+}\)]\(_i\), intracellular free calcium concentration; PMCA, plasma membrane Ca\(^{2+}\) ATPase; MAGUK, membrane-associated guanylate kinase-like protein; GF, green fluorescent protein; CFTR, cystic fibrosis transmembrane conductance regulator; HA, hemagglutinin; PDZ, PDZ-95, Dlg, ZO-1; MDCK, Madin-Darby canine kidney; TBS, Tris-buffered saline; TBST, Tris-buffered saline plus Tween 20; DPBS+CM, Dulbecco’s phosphate-buffered saline + Ca\(^{2+}\)/Mg\(^{2+}\).
ity with the PDZ domains of several MAGUKs, and that this interaction is dependent on the presence of the E-T-S-V-\textsuperscript{a} COOH-terminal sequence (15). More recently, we found that PMCA4b as well as PMCA2b (which ends with the COOH-terminal sequence E-T-S-L\textsuperscript{b}) bind promiscuously to several synapse-associated protein members of the MAGUK family, but that there is also selectivity in the interaction of PMCA2b \textit{versus} PMCA4b toward some synapse-associated proteins (19).

Because PDZ domain-containing proteins are often multimodular, they display a diverse array of functions, acting as signaling protein scaffolds (20, 21) and cytoskeleton anchoring proteins (22), and playing roles in targeting and retention of transmembrane proteins (23). For any given PDZ domain containing protein, it is difficult to predict the specific target(s) of its individual domains. On the other hand, even conservative changes in the PDZ interaction domain of the ligand proteins can greatly influence target recognition by the PDZ domain. Because the PMCA b splice forms carry COOH-terminal sequences that can bind to PDZ domains, yet PMCA2b differs from PMCA4b in its COOH-terminal residue (Leu \textit{versus} Val), it may well be that these two isoforms of the PMCA recognize different subsets of PDZ proteins.

Here, we used the COOH-terminal sequence of PMCA2b as bait in a two-hybrid screen of a human brain cDNA library and isolated two identical clones coding for the COOH-terminal half of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger regulatory factor 2 (NHERF2), including its second PDZ domain. The interaction is specific for PMCA2b, as PMCA4b does not interact appreciably with NHERF2. Similarly, PMCA2b prefers NHERF2 as a binding partner, as the related NHERF1 binds with much lower affinity. We further show that NHERF2 and a GFP-tagged PMCA2b colocalize at the apical domain in polarized Madin-Darby canine kidney (MDCK) epithelial cells. These results suggest a new mechanism of PMCA regulation, specifically of PMCA2b, through a PDZ domain interaction with NHERF2.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions—** Codons for the final 72 or 71 amino acids of human PMCA2b, respectively, were cloned into EcoRI/BamHI fragments into pAS2–1 (CLONTECH, Palo Alto, CA) to produce the DNA binding fusions (pDB-CT2b and pDB-CT4b) for yeast two-hybrid screens and assays. The same codons were inserted into pGEX-2TK (Amersham Biosciences, Inc.) to produce GST fusions (GST-2b and GST-4b). Plasmids pMM2-PMCA4b and pMM2-PMCA2b for expression hybrid screens and assays. The same codons were inserted into pGEX-4b). Plasmids pMM2-PMCA4b and pMM2-PMCA2b for expression of human PMCA2b and PMCA4b, respectively, were cloned as HindIII fragments into the pGEX-2TK vector (Amersham Biosciences, Inc.).

**Yeast Two-hybrid Assays—** Yeast two-hybrid screening of a human brain cDNA library (CLONTECH) with bait plasmid pDB-CT2b was performed according to the instructions for the Matchmaker Two-hybrid System 2 (CLONTECH) and as described (19). Approximately 5 \times 10\textsuperscript{6} independent cDNA clones were screened, and initial positives were selected after 7 days of growth of the transformed yeast (strain CG1945S) on SD–Trp–Leu–His agar plates containing 5 mM 3-aminotriazole (Sigma). Plasmid inserts from positive yeast clones were rescued after picking 3-day-old yeast colonies into TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) containing 0.25 units/\textmu l lyticase (Sigma). Following 30 min at 37 °C, the yeast were subjected to one round of freeze-thaw followed by sonication and phenol/chloroform extraction (28). The aqueous fraction was precipitated with 0.1 volume of 50 mM sodium acetate, pH 5.3, and 0.7 volume of isopropanol; the nucleic acid pellet was washed in 70% ethanol and dissolved in 10 mM Tris, pH 8.0. One percent of the dissolved DNA was used as template for PCR with pACT2-specific primers (Matchmaker AD Screening Amplimers, CLONTECH). The PCR product was sequenced directly using Matchmaker 5’ AD Screen Amplimer (CLONTECH), or library plasmids were transformed into Escherichia coli strain DH5a by electroporation, and sequenced with the same primer following bacterial plasmid preparation (Qiagen).

**Combinatorial Protein Expression and Purification—** GST and GST fusion proteins were expressed as described (29) in \textit{E. coli} BL21(DE3) upon induction with 0.7 mM isopropyl-\textit{l}-thio-\textit{b}-galactopyranoside for 4 h. Cells were pelleted, resuspended in TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl), and proteases inhibitors (0.2 mM phenylmethanesulfonyl fluoride, 1 \mu g/ml pepstatin A, 2 \mu g/ml leupeptin, 0.2 \mu g/ml aprotinin, 10 mM EDTA) and 30 mM \beta-mercaptoethanol, and lysed by the addition of sarkosyl (Curtis-Matheson Scientific, Houston, TX) to a final concentration of 1.5%. After 15 min on ice, the lysate was cleared by centrifugation at 10,000 \times g and supplemented by the addition of Triton X-100 to 0.2%. This lysate was then bound to glutathione-Sepharose (Sigma) and washed with TBST (TBS + 0.1% Tween) and TBS. The quantity of bound protein was estimated by Coomassie Blue staining of SDS-polyacrylamide gels of known amounts of fusion protein-containing glutathione-Sepharose beads. For pull-down assays, all fusion proteins were adjusted to ~0.5 mg/ml.

**Production of NHERF2-specific Antibodies—** A GST fusion protein including amino acids 259–312 from human NHERF2 (see Fig. 1C) was expressed and purified as described above. The GST fusion protein was eluted from the glutathione-Sepharose and used to raise rabbit polyclonal antibodies at Cocalico Biologicals, Inc. (Reamstown, PA). After exsanguination, rabbit sera were heat-inactivated and anti-GST antibodies were removed by three rounds of affinity chromatography on GST-Sepharose (Amersham Biosciences, Inc.). Anti-NHERF2 antibodies (Ab720) in the GST-Sepharose flow-through were then purified using a GST-NHERF2 fusion protein affinity column following established procedures (29). The specificity of the antibody was confirmed by Western blot and immunofluorescence staining.

**Antibodies for Immunoblotting—** The following antibodies were the same as those used and described there, and were purchased from Caltag Laboratories, Inc. (Burlingame, CA) or used as affinity-purified polyclonal anti-NHERF2 antibody (Ab720, see above) was used at 0.5 \mu g/ml; anti-HA antibody (clone 12CA5) was from Roche and was used at 0.5 \mu g/ml; polyclonal anti-PMA2 (affinity-purified and concentrated antibody NR2) and monoclonal anti-PMA4 antibody J4A3 were obtained from Dr. John Pemstein and Aida Filoteo (Mayo Clinic, Rochester, MN) and used at a dilution of 1:5000. BSA, GPA, goat anti-mouse, or goat anti-rabbit antibodies coupled to horseshad peroxidase were purchased from Sigma and used at 1:5000 dilution.

**Pull-down Assays—** COS-1 cells were grown to ~80% confluence on six-well plates (Costar) in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, nonessential amino acids, 1 mM sodium pyruvate, glutamine, and antibiotic/antimycotic mixture (all cell culture reagents from Invitrogen). Cells were transfected with 2 \mu g of total DNA using LipofectAMINE according to the manufacturers instructions (Invitrogen). After ~48 h, the cells were rinsed, lysed, and the lysates collected as described (19). Equal amounts of lysate from COS-1 cells expressing HA-NHERF1 or HA-NHERF2 (expressed at equivalent levels) were incubated with Sepharose-bound GST-2b, and GST-4b. Beads and lysate were rocked for 90 min at 4 °C. After incubation, the beads were washed four times in 50 mM HEPES, pH 7.4, 150 mM NaCl plus 1% Triton X-100, and bound proteins were purified with 1.5× SDS sample buffer, separated on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose following standard Western blotting procedures (29). Nitrocellulose membranes were blocked in TBST + 5% milk before detection of HA-NHERF1 and HA-NHERF2 using the indicated antibodies.
clonal SF10 (a gift from Dr. John Penniston and Aida Filoteo) or anti-GST polyclonal antibody (obtained during the first step of the purification of anti-NHERF2 antibodies, see above). After 4 h of rocking at 4 °C, 50 μl of protein G-agarose (Sigma) was added to each mixture and rocking continued overnight at 4 °C. Protein G-agarose was pelleted at 4000 × g for 30 s and quickly washed twice in cold TBS-T. Bound proteins were eluted in Laemmli buffer (29). All of the bound protein and 10% of the starting lysate (input) were separated on 7.5% polyacrylamide gels, followed by transfer to nitrocellulose for Western blotting as described above.

**Immunofluorescence—**Type I MDCK epithelial cells (ATCC no. CCL-34, Manassas, VA) were grown to confluence on glass coverslips. Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and supplemented with 1% antibiotic-antimycotic mixture. Cells were transfected with a total of 1 μg of GFP-PMCA2b and HA-NHERF2 plasmid DNA using LipofectAMINE 2000™ (Invitrogen). 48 h after transfection, the cells were fixed for 15 min at room temperature in 4% paraformaldehyde (Tousimis, Rockville, MD) diluted in Dulbecco’s phosphate-buffered saline + Ca<sup>2+</sup>/Mg<sup>2+</sup> (DPBS + CM, Invitrogen). After five brief washes in DPBS + CM, coverslips were further fixed and permeabilized in pre-chilled methanol for 15 min at −20 °C. The cells were blocked in DPBS + CM containing 5% normal goat serum and 1% bovine serum albumin (blocking buffer), and then were incubated for 1 h at room temperature with affinity-purified anti-PMCA2 antibody NR2 (0.8 μg/ml) or anti-NHERF2 antibody Ab720 (0.5 μg/ml). After washing three times 5 min each time with DPBS + CM, the cells were incubated for 1 h at room temperature with anti-rabbit Alexa 594 (Molecular Probes, Eugene, OR) diluted 1:800. All antibodies for immunofluorescence were diluted in blocking buffer. After final washing, coverslips were mounted in Prolong mounting media (Molecular Probes). Confocal micrographs were taken on a Zeiss LSM510 microscope using an Apochromat 63× objective, and captured using LSM510 software (Zeiss). Images were imported and edited using Adobe Photoshop 5.0.

**Surface Biotinylation—**MDCK cells were grown as a monolayer on glass coverslips. After transfection with GFP-PMCA2b plasmid DNA as above, cells were washed three times in cold DPBS + CM and kept on ice. Cells were incubated twice for 20 min in 1 mg/ml sulfo-NHS-Biotin (Pierce) in triethanolamine, pH 8.6, 150 mM NaCl. After surface labeling, cells were washed with DPBS + CM, and excess biotin was quenched for 15 min on ice in DPBS + CM containing 100 mM glycine. For fluorescent images, the cells were fixed for 20 min in methanol at −20 °C. After extensive washing in DPBS + CM, coverslips were blocked for 1 h in blocking buffer (see above) and biotin label was detected with 3 μg/ml avidin-Alexa 594 (Molecular Probes) in blocking buffer.

**RESULTS**

**Isolation of NHERF2 in a Yeast Two-hybrid Screen Using PMCA2b as Bait—**Even conservative amino acid substitutions in the PDZ domain binding motif of a protein can strongly influence its PDZ domain selectivity (30–32). A sequence alignment of the extreme COOH termini of the PMCA b splice forms suggested that PMCA1b, 2b, and 3b would bind the same PDZ domains (Fig. 1A), whereas PMCA4b might show a somewhat different PDZ domain preference. To identify proteins that interact with the COOH-terminal sequence of PMCA1b/2b/3b, we therefore used a protein consisting of the Gal4 DNA binding domain fused to the final 72 amino acids of hPMCA2b (Fig. 1B) as a “representative” bait in a yeast two-hybrid screen of a human brain cDNA library. Approximately 30 HIS3- and β-galactosidase-positive clones were obtained from a screen of 5 × 10<sup>8</sup> clones, and after exclusion of false positives, sequencing of the cDNA inserts revealed that five clones specified proteins containing at least one PDZ domain. Of these, two strong interactors (as determined by β-galactosidase assay; data not shown) encoded the second PDZ domain and carboxyl-terminal tail of NHERF2 (Fig. 1C).

**Selectivity of the Interaction of PMCA2b and PMCA4b with NHERF1 and NHERF2—**NHERF2 belongs to a subfamily of type 1 PDZ domain proteins, comprising NHERF1 (also called EBP50) and NHERF2 (also called E3KARP, TKA-1, and SIP-1), which share ~52% sequence identity (33–35). It has been previously reported that the PDZ domains of both NHERF1 and NHERF2 recognize D-(S/T)-X motifs (where X stands for any amino acid) at the COOH terminus of target proteins (27, 36). PMCA 1b, 2b, and 3b all contain the sequence E-T-S-L at their COOH termini, whereas PMCA4b ends with E-T-S-V. We wondered whether such a conservative amino acid substitution could affect the PMCA-NHERF2 interaction. We used GST fusion proteins in “pull-down” experiments to confirm the results of the yeast two-hybrid assay, and to test the specificity of the interaction. Eukaryotic expression vectors for HA-tagged NHERF1 and NHERF2 were transfected into COS-1 cells, and the resulting lysate from these cells was presented to GST fusion proteins of PMCA2b (CT2b) and PMCA4b (CT4b) (Fig. 1B) immobilized on glutathione-Sepharose. Interestingly, only CT2b and NHERF2 demonstrated a significant interaction (Fig. 2B). By contrast, we saw little binding of NHERF1 to CT2b (Fig. 2A), and essentially no interaction of NHERF1 or NHERF2 with CT4b even though equal amounts of starting material were used in all experiments (Fig. 2C).

**Selective Coimmunoprecipitation of PMCA2b with NHERF2—**To test the ability and selectivity of full-length PMCA2b to bind NHERF2 when expressed in a cellular environment, we transiently cotransfected COS-1 cells with plasmid constructs encoding HA-NHERF1 or HA-NHERF2 and full-length human PMCA2b or full-length human PMCA4b. In agreement with the pull-down assays, NHERF2 was only coimmunoprecipitated by anti-PMCA antibodies when coexpressed with full-length PMCA2b (Fig. 3B). By contrast, NHERF1 did not coprecipitate to any significant extent with PMCA2b (Fig. 3A). Of the nHERF proteins coimmunoprecipitated with PMCA4b in these assays (Fig. 3). These results confirm that NHERF2 selectively interacts with the intact PMCA2b (but not PMCA4b) in a cellular environment, whereas the related NHERF1 interacts only weakly, if at all, with these PMCA4.

**Colocalization of GFP-PMCA2b and NHERF2 at the Apical Membrane of Polarized Epithelial Cells—**PMCA2b has recently been reported to be present in the apical plasma membrane of...
some specialized epithelial cells (e.g. in the lactating mammary gland (Ref. 37)) and in hair cells of the inner ear (38, 39). Because PMCA2 is not abundantly expressed outside of the nervous system, we expressed recombinant GFP-tagged PMCA2b (GFP2b) in a monolayer of polarized MDCK cells. GFP2b was detected in the apical membrane domain in these cells (Fig. 4A), which was confirmed by nonspecifically biotinylating the apical side of a GFP2b-transfected MDCK cell monolayer. In this assay, apical membrane proteins were identified using a fluorescent avidin conjugate and the cells were imaged in the x-z plane to differentiate apical from basolateral staining. Nearly all of the GFP2b fluorescence overlapped with the biotin-avidin label, indicating that the recombinant GFP-PMCA2b fusion protein does in fact target apically in these cells (Fig. 4B). Cotransfections with recombinant NHERF2 demonstrated that both proteins are found in the apical domain of MDCK cells (Fig. 4C). The apical localization of NHERF2 is consistent with previous reports on the membrane localization of NHERF1/2 in apical microvilli of kidney, intestinal, and airway epithelial cells (34, 35, 40, 41). GFP-PMCA2b and NHERF2 demonstrated a significant amount of co-localization at the apical plasma membrane of transfected MDCK cells, suggesting that these two proteins may interact directly in vivo.

DISCUSSION

A distinguishing feature among PMCA isoforms may be their localization in distinct membrane domains with different Ca²⁺ handling properties. Such differential localization, as well as differential regulation, may depend on isoform-specific protein interactions. In this study, we therefore sought to identify proteins that specifically interact with the b splice form of PMCA2 as a representative isoform localized to distinct membrane domains in several neuronal and epithelial cell types. Recent work has shown that PMCA4b and PMCA2b bind to PDZ domains of PSD95-like MAGUK proteins (15, 19). These protein-protein interactions may provide a means to couple the PMCas to cytoskeletal and signaling molecules. The minimal consensus sequence for type I PDZ domain binding is -T-X-(V/I/L)ₙ (where X stands for any amino acid and the asterisk denotes the terminal residue) (30). This sequence is E-T-S-Vₙ in PMCA4b and differs slightly from that of all other PMCA b splice forms, which end with the sequence E-T-S-L* (see Fig. 1A). Several recent studies have shown that even conservative changes in the PDZ ligand motif can dramatically affect the specificity of PDZ domain binding (27, 30, 42). Therefore, the three isoforms of the PMCA that end with -E-T-S-L* may have distinct PDZ protein partners compared with PMCA4b. Indeed, using yeast two-hybrid screening with a bait corresponding to the COOH-terminal tail of PMCA2b, we isolated the PDZ domain protein NHERF2 as a specific interactor of this PMCA isoform.

**FIG. 2.** Protein pull-down experiments show that PMCA2b binds NHERF2 in a specific manner. A and B, aliquots of lysates from COS-1 cells transfected with HA-NHERF1 or HA-NHERF2 constructs (indicated on top) were incubated with glutathione-Sepharose beads containing equal amounts of GST alone (GST), the COOH-terminal 72 amino acids from PMCA2b fused to GST (GST-2b), or the COOH-terminal 71 amino acids from PMCA4b fused to GST (GST-4b). After washing, bound proteins were eluted, separated by SDS-PAGE, and transferred to nitrocellulose membrane. The membranes were probed with an anti-HA antibody to detect bound NHERF1 or NHERF2. To demonstrate equal expression, 10% of each starting lysate was also loaded (Input). A, NHERF1 does not bind to GST alone or to GST-4b. A weak interaction is observed with GST-2b. B, like NHERF1, NHERF2 does not bind to GST alone or to GST-4b. A strong interaction is seen with GST-2b. C, Coomassie Blue-stained polyacrylamide gel demonstrating equal amounts of GST fusion proteins used for NHERF pull-down experiments. Approximately 3 µg of glutathione-Sepharose bound GST, GST-2b, or GST-4b were eluted with 2× Laemmli sample buffer, separated on a 10% polyacrylamide gel, and stained with Cooomassie Blue. Molecular size markers are shown on the left with their mass indicated in kDa.

**FIG. 3.** Coimmunoprecipitations demonstrate selective in vivo interaction of NHERF2 with PMCA2b but not with PMCA4b. COS-1 cells were cotransfected with different protein expression constructs as indicated on the top of each lane. After ~48 h, cells were lysed and 10% of the protein lysate was used directly as input (IN) for SDS-polyacrylamide gel electrophoresis. The remaining lysate was immunoprecipitated (IP) with anti-PMCA antibody 5F10 as described under “Experimental Procedures” prior to SDS-polyacrylamide gel electrophoresis. Western blots (WB) of the separated proteins were then probed with appropriate antibodies (indicated on the left) to detect the recombinant proteins as indicated on the right of each panel. Note that NHERF1 does not coprecipitate with either PMCA2b or 4b (A), whereas NHERF2 coprecipitates with PMCA2b but not with 4b (B).
PMCA2b and NHERF2 colocalize at the apical membrane of MDCK cells. A, recombinant GFP-PMCA2b (green) is transiently expressed in MDCK cells and can be recognized by a PMCA2-specific antibody (red). No PMCA2 staining is seen in untransfected cells. In x-z sections (lower panel), GFP-PMCA2b appears to be concentrated in the apical plasma membrane. B, surface labeling of membrane proteins in a monolayer of polarized MDCK cells with sulfo-NHS-biotin, followed by detection with avidin-Alexa 594 conjugate. Biotin labeling (red) is performed prior to fixing and permeabilizing the cells while tight junctions are maintained, and selectively labels the apical membrane (bottom). GFP-PMCA2b fluorescence (green) is seen in a single transfected cell (middle). Overlap of GFP-PMCA2b and biotin labeling is seen as yellow in the merged panel (top), confirming that GFP-PMCA2b is predominantly localized in the apical membrane of the MDCK cells. Bar = 5 μm. C, MDCK cells were co-transfected with GFP-PMCA2b and HA-NHERF2 constructs. GFP-PMCA2b (green) and HA-NHERF2 (red) are expressed in the apical domain of transfected cells and show a significant amount of colocalization (yellow in merged panels). Bar = 20 μm in x-y sections, and 5 μm in x-z sections.

Pull-down experiments using GST fusion proteins of the PMCA2b and PMCA4b COOH termini revealed a high degree of specificity in the PMCA/NHERF2 interaction. The COOH-terminal tail of PMCA4b did not interact with either NHERF2 or the related NHERF1 in these experiments. The same result was obtained in communoprecipitations of full-length proteins from transiently transfected COS cells. This finding is in agreement with previous studies demonstrating the importance of a terminal leucine residue for high affinity peptide interaction with NHERF (27, 42). The recent solution of the crystal structure of the NHERF1 PDZ1 domain complexed with its own -D-E-Q-L* COOH-terminal sequence or the -Q-D-T-R-L* sequence from the cystic fibrosis transmembrane conductance regulator (CFTR) has provided a structural explanation for the importance of the terminal leucine residue (38, 43); the side chain and carboxylate moiety of Leu enter a deep cavity formed by specific residues of the PDZ domain, and extensive hydrophobic contacts and hydrogen bonds stabilize the interaction. Importantly, the isobutyl side chain of Leu fits tightly into this hydrophobic cavity, providing a likely explanation for the requirement of a terminal Leu (rather than, e.g., Val or Ala). This satisfactorily explains the difference between PMCA2b and 4b in their ability to bind NHERF2. Because PMCA1b and 3b end in the identical sequence -E-T-S-L* found in PMCA2b, we predict that these isoforms are also able to interact with NHERF. On the other hand, the distinct preference of PMCA2b for NHERF2 compared with NHERF1 is more difficult to rationalize. The PDZ domains of NHERF1 and NHERF2 are highly similar (~70% identity in pairwise comparisons), and the residues directly involved in contacting the terminal Leu and the Thr at position -2 are conserved in the two NHERFs (43). PMCA2b contains a Ser at position −1 and a Glu at position −3, whereas other high affinity NHERF-interacting proteins such as the CFTR, β2-adrenergic receptor, and PDGF receptor contain a different residue at position −1 (Arg, Leu, and Phe, respectively) and an Asp at position −3 (27). Because the penultimate residue may influence the binding affinity of a ligand for the NHERF PDZ domains (27, 43), it is possible that Ser at −1 in PMCA2b contributes to the pump’s preference of NHERF2 over NHERF1. Similarly, the Glu at position −3 in PMCA2b may favor the PDZ domains of NHERF2 over those of NHERF1, although this appears doubtful based on gel overlay experiments indicating that replacing Asp at −3 by Glu decreases the binding affinity of a peptide ligand for PDZ1 of both, NHERF1 and NHERF2 (27). Given the high conservation among NHERF1 and NHERF2 PDZ domains in the binding pockets recognizing the last four residues of their ligands, it is thus more likely that residues upstream of position −3 in PMCA2b are involved in determining its binding preference for NHERF2 over NHERF1.

The colocalization of GFP-PMCA2b and NHERF2 at the apical membrane of MDCK epithelial cells is an interesting finding. Information on the subcellular targeting of the PMCA isotypes has only recently become available for some neuronal and epithelial cell types (39, 44–46). Mammalian epithelia contain primarily PMCA isoforms 1b and/or 4b (9), and Ca2+ transport studies have indicated that the PMCA is concentrated on the basolateral membrane of gut and distal kidney epithelia (47, 48). More recent reports suggest that in certain epithelia with high Ca2+ transporting requirements (e.g. lactating breast (Ref. 37)), PMCA2b is found at the apical membrane, providing the means for high affinity Ca2+ extrusion into the luminal space of these cell types. When transfected into MDCK epithelial cells (which do not express significant amounts of endogenous PMCA2b), the exogenously expressed GFP-PMCA2b was targeted to the apical membrane. Apical targeting of the pump occurred in the absence of exogenously co-expressed NHERF2, suggesting that an interaction with NHERF2 is not required for membrane targeting (Fig. 4, A and B). In support of this, we have recently found that a COOH-terminally truncated GFP-PMCA2b construct unable to bind PDZ domains is still targeted to the apical domain in MDCK cells.2 The apical sorting signal in the GFP-PMCA2b must therefore be located upstream from the PDZ domain-interacting COOH-terminal tail. Independent support for this hypothesis stems from a recent study on the distribution of PMCA2 in the cochlea; using splice variant-specific antibodies, Dumont et al. (39) demonstrated that the major PMCA isoform in the apical stereociliary membrane of cochlear outer hair cells corresponds to PMCA2a. This confirms that the apical membrane localization of PMCA2 does not require a PDZ domain binding COOH-terminal tail (which lacks in the a splice form). The situation for PMCA2 is thus different from that for the CFTR; in the CFTR, an intact PDZ domain-binding sequence appears to be required for proper apical membrane targeting and functional expression (41, 42).

NHERF associates with several other membrane transporters and receptors, including the apical Na+/H+ exchanger NHE3 (33), the β2-adrenergic receptor (26), the mammalian Trp4/5 calcium channels (49), and the apical type II Na/Pi cotransporter (50). Although NHERFs may play a direct role in membrane targeting of proteins such as the CFTR, other functions have also been proposed for the NHERFs. It is thought that these modular PDZ proteins provide a scaffolding mechanism, bringing together transmembrane proteins, signaling molecules, and the actin cytoskeleton, in a regulated fashion.

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2 S. J. DeMarco and E. E. Strehler, unpublished observation.
Plasma Membrane Ca\(^{2+}\) Pump 2b Binds NHERF2

(51). The association of NHERFs with the ezrin-radixin-moesin family of proteins provides a linkage to the cortical actin cytoskeleton (for reviews, see Refs. 51 and 52), implicating NHERFs in the stabilization and membrane retention of their interacting partners. Related roles include the regulation of receptor internalization and endocytic sorting (53), mediating cAMP-dependent transporter regulation (e.g. via recruitment of cAMP-dependent protein kinase) (35, 54), and facilitating transporter activation via dimerization (55). Any of these roles could potentially apply to the interaction between NHERF2 and PMCA2b. The interaction between apically targeted PMCA2b and NHERF2 may provide an indirect link between the Ca\(^{2+}\) pump and the actin cytoskeletal network, potentially stabilizing the pump in a particular membrane microdomain and allowing its regulation by co-assembled cAMP-dependent protein kinase. Alternatively, NHERF2-mediated co-clustering of multiple PMCA2b molecules may facilitate their oligomerization, which has been shown to lead to calmodulin-independent pump activation (56). Previous reports have implicated NHERFs in the regulation of H\(^+\) and Cl\(^{-}\} conductance through association with NHE3 and CFTR, respectively. The recent report of Tang et al. (49) on the TrpR45 Ca\(^{2+}\) channels, and now our data on PMCA2b, indicate an involvement of NHERF2 in the regulation of Ca\(^{2+}\) transport as well. Future experiments will attempt to determine the precise role of this exciting new and specific PMCA2b-NHERF2 interaction for localized Ca\(^{2+}\) handling in vivo.

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