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Plasma membrane calcium pump (PMCA) isoform 4 is targeted to the apical membrane by the w-splice insert from PMCA2

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ABSTRACT

Local Ca^{2+} signaling requires proper targeting of the Ca^{2+} signaling toolkit to specific cellular locales. Different isoforms of the plasma membrane Ca^{2+} pump (PMCA) are responsible for Ca^{2+} extrusion at the apical and basolateral membrane of polarized epithelial cells, but the mechanisms and signals for differential targeting of the PMCAs are not well understood. Recent work demonstrated that the alternatively spliced w-insert in PMCA2 directs this pump to the apical membrane. We now show that inserting the w-insert into the corresponding location of the PMCA4 isoform confers apical targeting to this normally basolateral pump. Mutation of a di-leucine motif in the C-tail thought to be important for basolateral targeting did not enhance apical localization of the chimeric PMCA4(2w)/b. In contrast, replacing the C-terminal Val residue by Leu to optimize the PDZ ligand site for interaction with the scaffolding protein NHERF2 enhanced the apical localization of PMCA4(2w)/b, but not of PMCA4x/b. Functional studies showed that both apical PMCA4(2w)/b and basolateral PMCA4x/b handled ATP-induced Ca^{2+} signals with similar kinetics, suggesting that isoform-specific functional characteristics are retained irrespective of membrane targeting. Our results demonstrate that the alternatively spliced w-insert provides autonomous apical targeting information in the PMCA without altering its functional characteristics.

1. Introduction

Intracellular Ca^{2+} concentrations must be controlled with high spatial and temporal precision to allow proper cell function. This requires the presence of the different components of the Ca^{2+} handling toolkit at the proper subcellular locale such as at pre-synaptic boutons of neurons or the apical domain of secreting epithelial cells [1,2]. How the appropriate Ca^{2+} influx, buffering and efflux mechanisms are assembled and targeted to specific subcellular domains is, however, still poorly understood.

The plasma membrane Ca^{2+} pumps (PMCAs) are responsible for the expulsion of Ca^{2+} from all eukaryotic cells. They help re-set and maintain the global resting free Ca^{2+} levels, as well as participate in dynamic and localized Ca^{2+} signaling [3,4]. Mammals express four different PMCA isoforms (PMCA1-4) but the total number of PMCA variants is much larger due to alternative splicing affecting two major sites (called sites A and C) located in the first intracellular loop and the C-terminal tail of the pump, respectively [5]. PMCA isoforms differ in the kinetics of activation and regulation by Ca^{2+}-calmodulin, with some variants such as PMCA4b being "slow" and others such as PMCA2b being "fast" and showing high basal activity [6]. In agreement with their functional specialization several PMCA isoforms are highly concentrated in specific cellular domains, e.g., PMCA2w/a in the apical stereocilia of cochlear hair cells [7], PMCA2w/b in the apical membrane of lactating mammary epithelial cells [8] or PMCA4x/b in the basolateral membrane of kidney epithelial cells [9,10].

The mechanisms and signals that target different PMCA isoforms to specific membrane compartments of polarized cells are not well understood. Recent work has shown that alternative splicing at site A affects the targeting of PMCA2 variants in inner ear hair cells and in MDCK kidney epithelial cells [11,12]. Inclusion of the w-splice insert of 45 "extra" amino acids in the first intracellular loop of the pump resulted in apical localization of PMCA2w/a and PMCA2w/b in MDCK cells and hair cells, while the x- and z-splice

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variants (containing 13 and 0 spliced-in residues, respectively, in the first loop) were almost exclusively targeted to the basal and lateral membrane [7,11–13]. This raises the possibility that the w-insert from PMCA2 acts as a dominant apical targeting element in the pump. To test this hypothesis, we transplanted the PMCA2 w-insert into the equivalent position of PMCA4b and determined the localization of the chimeric PMCA4(2w)/b in polarized MDCK cells. We also analyzed the effect of mutation of a putative basolateral (di-leucine) targeting motif in the C-terminal tail of the pump and of the C-terminal residue involved in PDZ domain recognition of the apical scaffolding protein NHERF2. Our results show that the w-insert from PMCA2 functions as an autonomous apical targeting element in the PMCA, and that apical localization of the pump is significantly enhanced by interaction with NHERF but not by mutation of the di-leucine motif in the C-tail.

2. Materials and methods

2.1. Reagents and antibodies

FuGene HD Transfection Reagent was obtained from Roche Applied Science, and Lipofectamine™ was from Invitrogen. DMEM and Opti-MEM were obtained from Invitrogen. Affinity-purified rabbit polyclonal anti-NHERF2 antibody 720 [14] was used at a dilution of 1:500. Mouse monoclonal anti-ezrin antibody was from BD Biosciences and used at a dilution of 1:100. Chicken polyclonal anti-Na^+/K^+-ATPase antibody was from Chemicon International and used at a dilution of 1:250. Mouse monoclonal anti-GFP antibody JL-8 was from Clontech and used at 1:2000, and mouse monoclonal anti-GAPDH was from Research Diagnostic Inc. and used at 1:4000. Alexa Fluor 488-, 594-, and 633-conjugated goat anti-mouse and anti-rabbit IgGs and Alexa Fluor 594-conjugated goat anti-chicken IgG were obtained from Invitrogen. All other chemicals were of reagent grade.

2.2. Plasmid constructs

The plasmid for *EGFP-hPMCA4(2w)/b* was generated by inserting the w-splice fragment of pMM_2-PMCA2w/b [11] into pEGFP-PMCA4x/b [11] using the Quickchange® II XL Site-Directed Mutagenesis Kit (Stratagene). Primers were constructed to insert a *PvuI* restriction site at the beginning of the x-splice insert in EGFP-PMCA4x/b (EGFP-PMCA4xb:PMCA4xPvuIF 5′-ggg gag aaa aag cga tcg ggt aaa aaa caa gga gtc ctt-3′, *PvuI* site underlined). Primers were then constructed to insert an *AflII* site at the end of the x-insert in EGFP-PMCA4x/b (EGFP-PMCA4xb:PMCA4xAflIIF: 5′-aat cgc aac aaa ctt aag acc caa gac gga gtg gcc ctg-3′, *AflII* site underlined). The w-insert from pMM_2-PMCA2w/b was amplified by PCR with the pMM_2-PMCA2w-PvuI forward primer (PMCA2wmPvuIF: 5′-gag aag aaa gac cga tcg ggt gtg aag aag ggg gat ggc-3′, *PvuI* site underlined) and the pMM_2-PMCA2w-AflII reverse primer (PMCA2wAflIIR 5′-gct gcc ccg tcc tgt tgc tta agt ttg ctc tgg ctg gcg-3′, *AflII* site underlined). The constructs were double digested using *PvuI* and *AflII* restriction enzymes (New England Biolabs). The digested EGFP-PMCA4x/b vector was shrimp-alkaline-phosphatase (SAP) treated (Roche) and the double digested PCR amplified w-fragment from pMM_2-PMCA2w/b was ligated into the EGFP-PMCA4b construct. Primers were then constructed to back-mutate the restriction sites to the original sequence in the newly created EGFP-hPMCA4(2w)/b (PMCA4wnPvuIF 5′-ggg gag aaa aag aag aaa ggt gtg aag aag ggg gat ggc-3′ and PMCA4wnAflIF 5′-agc cag acg aaa gca aag acc caa gac gga gtg gcc ctg-3′, insertion of original sequences underlined). *EGFP-hPMCA4(2w)/b-V>L* was generated from EGFP-hPMCA4(2w)/b by mutating the C-terminal valine to leucine using the primer PMCA4bV2LF 5′-cag agc cta gag aca tca ctt tga ctc gag ctc aag ctt-3′ (nucleotide change resulting in V to L mutation underlined). *EGFP-hPMCA4(2w)/b-LL>AA* and *EGFP-hPMCA4x/b-LL>AA* were generated from EGFP-hPMCA4(2w)/b and EGFP-hPMCA4x/b, respectively, by mutating the di-leucine starting at position 1147 in PMCA4x/b to di-alanine using the primer PMCA4b4789AAF 5′-gag ttg cca cga aca cca gcc gcg gat gag gaa gag gag-3′ (nucleotide changes resulting in LL to AA mutations are underlined). Plasmids EGFP-PMCA4x/b, EGFP-PMCA2w/b and EGFP-PMCA2x/b for expression of human EGFP-tagged PMCA isoforms in mammalian cells have been described [11]. The mammalian expression construct for NHERF2 was a kind from gift from Dr. Randy Hall (Emory University, Atlanta) and has been described previously [15,16]. The genetically encoded calcium indicator GCaMP2 was a generous gift from Dr. Junichi Nakai (RIKEN Brain Science Institute, Saitama, Japan) [17].

2.3. Cell culture, transfection and Western blotting

MDCKII cells were seeded into eight-well Nunc Lab-Tek II chambered coverglass (Nalge Nunc International, No.: 155411) at 5×10^4/well cell density, and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine for 5 days after seeding. MDCK cells were transfected with the GFP-tagged PMCA constructs as described previously [13]. The constructs were also transfected into HeLa cells to check the expression of the recombinant PMCAs. Cell lysates were prepared 48 h after transfection by washing the cells on ice with Ca^{2+}/Mg^{2+}-free DPBS (Invitrogen), followed by lysis in 50 mM HEPES, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate (pH 7.4) containing a protease inhibitor cocktail for 10 min on ice. Cells were then scraped and frozen at −20 °C until use. After spinning for 10 min at 3000 rpm, total protein concentration in the lysates was determined using the BCA Protein Assay Kit (Pierce) according to the manufacturer's protocol. Samples for electrophoresis were prepared in NuPage LDS Sample Buffer (Invitrogen) supplemented with DTT (final concentration 100 mM) and urea (125 mg/ml) and incubated for 10 min at 37 °C. 40 μg of protein were loaded per lane on an Invitrogen 8% Bis-Tris mini gel and run according to the manufacturer's protocol using MOPS/SDS running buffer, then blotted onto a 0.45 μm PVDF (Millipore) membrane on ice at 60 V for 1 h. Blots were blocked for 1 h at room temperature using 5% non-fat dried milk (NFDM) in TBST (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20), and then incubated in primary antibody (monoclonal anti-GFP, 1:2000 or monoclonal anti-GAPDH, 1:4000) overnight at 4 °C in 5% NFDM in TBST. After three 5-min washes with TBST at room temperature, blots were placed in the appropriate secondary antibody (HRP conjugated goat anti-mouse IgG, Santa Cruz cat # sc-2055) diluted 1:3000 in 5% NFDM in TBST and incubated for 1.5 h at room temperature. After 4 5-min washes with TBST the blots were developed using the ECL Plus Western Dectection System (GE Healthcare/Amersham) according to the manufacturer's protocol. Blots were exposed to Hyblot CL film (Denville cat # E3018) for 5 min prior to developing.

2.4. Confocal fluorescence microscopy, image acquisition and quantification

To perform immunostaining for localization studies, transfected cells were gently washed with Dulbecco's modified phosphate-buffered saline (DPBS), fixed with 4% paraformaldehyde in DPBS for 10 min, washed with DPBS and permeabilized with 0.2% Triton X-100 in DPBS for 5 min at room temperature. The samples were then blocked for 1 h at room temperature in DPBS containing 2 mg/ml BSA, 1% fish gelatin, 0.1% Triton X-100 and 5% goat serum [18] and then incubated for 1 h at room temperature with the appropriate primary antibody (as indicated in the figure legends)