Published in final edited form as:

Cell Calcium. 2012 February ; 51(2): 171–178. doi:10.1016/j.ceca.2011.12.010.

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 PMCA 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 dileucine 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.

Keywords

Alternative splice; apical membrane targeting; MDCK cells; NHERF2; Plasma membrane Ca^{2+} ATPase; Polarized cells

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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 (PMCA) 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 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 pMM₂-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 *AflIII* 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', *AflIII* site underlined). The w-insert from pMM₂-PMCA2w/b was amplified by PCR with the pMM₂-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 pMM₂-PMCA2w-AflIII reverse primer (PMCA2wAflIIR 5'-gct gcc ccg tcc tgt tgc tta agt ttg ctc tgg ctg gcg-3', *AflIII* site underlined). The constructs were double digested using *PvuI* and *AflIII* 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 pMM₂-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 PMCA4wnAflIIF 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 PMCA. Cell lysates were prepared 48 h after transfection by washing the cells on ice with Ca²⁺/Mg²⁺-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 3,000 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 0.45µm PVDF (Millipore) membrane on ice at 60V for 1 h. Blots were blocked for 1 h at room temperature using 5% non-fat dried milk (NFDM) in TBST (20mM Tris-HCl, pH 7.4, 150mM 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 five-minute 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 five-minute washes with TBST the blots were developed using the ECL Plus Western Detection 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) diluted in blocking buffer. After washing with DPBS, the cells were incubated for 1 h at room temperature with the appropriate Alexa Fluor conjugated secondary antibodies diluted 1:250 in blocking buffer. After repeated washes, samples were studied with an Olympus IX-81/FV500 laser scanning confocal microscope, using an Olympus PLAPO 60× (1.4 NA) oil immersion objective (Olympus Europa GmbH). Images were imported and edited using Adobe Photoshop CS5. Apical or basolateral localization of GFP-tagged PMCA was detected by GFP fluorescence and was identified by its co-localization with the marker proteins ezrin and Na,K-ATPase, respectively. Localization analysis was performed using Fluoview 4.3 software (Olympus Corporation). Images of x-z sections of 25–50 cells from three independent experiments were collected and the ratios of mean PMCA fluorescence intensities of equal regions of interest in apical versus middle sections of the cells were determined by using the Analysis tool of Fluoview 4.3 software. Immunostaining of specific markers, i.e., Na,K-ATPase and ezrin/NHERF2 was used to determine the position of the lateral and apical sections, respectively. The data were displayed in a bar graph showing the average ratio numbers of the mean fluorescence intensities for each of the PMCA transfections. Means and standard errors were calculated using GraphPad Prism 5 software (GraphPad Software Inc.) as described [13].

2.5 Ca²⁺ signal measurements

MDCK cells were cultured on 8-well Nunc Lab-Tek Chambered Coverglass in DMEM supplemented with 10% FBS. After 24 h cells were co-transfected with the appropriate PMCA construct and the genetically encoded calcium indicator GCaMP2 [17]. 48 h after transfection, the medium was replaced by Phenol Red free DMEM supplemented with 10% FBS and 10 mM HEPES (pH 7.4). Single cell calcium signal measurements were carried out in Ca²⁺-free Hanks' Balanced Salt Solution supplemented with 20 mM Tris, 0.9 mM MgCl₂, 100 µM CaCl₂ and 100 µM EGTA (pH 7.4). The reaction was initiated by the addition of 100 µM ATP as indicated. Cells were studied with an Olympus IX-81/FV500 laser scanning confocal microscope using an Olympus PLAPO 60× (1.4 NA) oil immersion objective. For GCaMP2 imaging cells were excited with the 488 nm laser line and emission was collected between 505 and 535 nm. Time lapse sequences were recorded and images were analyzed

with Fluoview Tiempo (v4.3) time course software. The experimental data were normalized to baseline fluorescence (F/F_0) and fitted using GraphPad Prism software (<http://www.graphpad.com>). 20–30 cells were measured in a single experiment. The area under the curves of the Ca^{2+} signal of 40–50 cells was determined from three independent determinations. Data are expressed as mean $\pm$ standard error (SE).

3. Results

3.1. The w-splice insert from PMCA2w confers apical targeting to PMCA4

To test if the w-insert from PMCA2 is capable of altering the targeting of PMCA4x/b from its normally strictly basolateral localization, we engineered the 45 amino acid sequence from 2w precisely into the corresponding position of PMCA4, generating a chimeric PMCA4(2w)/b (Fig. 1A). Previous studies showed that the presence of an N-terminal GFP-tag does not affect the targeting of PMCA isoforms in MDCK cells [11]. The PMCA4(2w)/b and all additional constructs were therefore tagged by EGFP to facilitate detection of the recombinant proteins in transfected cells. Western blotting of transiently transfected HeLa cells confirmed that the recombinant proteins were expressed as full-length proteins at comparable levels (Fig. 1B). Fig. 2 shows confocal fluorescence images of MDCK cells transfected with GFP-PMCA4(2w)/b and GFP-PMCA4x/b. The cells were co-stained with antibodies against the apical marker protein ezrin and the basolateral protein Na,K-ATPase. As previously reported [11], PMCA4x/b was strictly basolateral (Fig. 2, left panels). Strikingly, however, PMCA4(2w)/b showed a similar distribution to that of PMCA2w/b, which is targeted to the apical membrane in addition to basolateral localization [11, 13]. As shown in Fig. 2 (right panels), the chimeric pump was prominently targeted to the apical domain in addition to basolateral localization. Because PMCA4(2w)/b is identical to PMCA4x/b except for the 2w insert at splice site A, these data show that the w-insert is capable of re-localizing the normally basolateral PMCA4 pump to the apical membrane.

3.2. NHERF2 greatly enhances apical localization of PMCA4(2w)/b with a C-terminal Val to Leu mutation

The C-tail of PMCA2b ends with a Leu residue (Fig. 1A) and conforms to the canonical PDZ ligand sequence for interaction with the apical scaffolding protein NHERF (Na^+/H^+ exchanger regulatory factor); thus when PMCA2w/b is co-expressed with NHERF2 its apical localization is greatly enhanced [13, 19]. In contrast, the C-terminal residue of PMCA4b is Val, shifting the PDZ domain preference of this pump towards membrane-associated guanylate kinase (MAGUK) proteins such as SAP97, PSD95 and CASK [20–22] commonly found at the lateral membrane of polarized epithelial cells [23, 24]. To test if the apical localization of the chimeric PMCA4(2w)/b could be enhanced if its C-terminal sequence corresponded to the consensus NHERF binding motif, we mutated the terminal Val residue to Leu and studied the localization of the resulting construct in MDCK cells co-transfected with NHERF2. Fig. 3 shows a comparison of the localization of GFP-PMCA4(2w)/b-V>L and GFP-PMCA4x/b-V>L in cells co-stained for (apical) NHERF2 and (basolateral) Na,K-ATPase. The data reveal an enhanced apical localization of PMCA4(2w)/b-V>L in the presence of NHERF2 (compare Fig. 3, left panels with Fig. 2, right panels, and see quantification in Fig. 4). In contrast, PMCA4x/b-V>L, which lacks the 2w-insert, was still exclusively basolateral even when co-expressed with NHERF2 (Fig. 3, right panels; Fig. 4). Thus, while the ability to interact with NHERF2 is not sufficient to target the PMCA to the apical membrane, this interaction enhances the amount of pump directed to the apical membrane due to the 2w insert.

3.3. The di-leucine motif in the C-tail of PMCA4 is not essential for basolateral targeting

A previous study suggested that a conserved Leu-Ile motif in the C-tail of PMCA2x/b (and PMCA1x/b) is necessary for basolateral targeting [12]. In PMCA4x/b, the corresponding residues are Leu(1147)-Leu(1148) thus representing a similar di-hydrophobic motif (Fig. 1A). To test if this motif contains important basolateral targeting information, we mutated both Leu residues to Ala, generating GFP-PMCA4x/b-LL>AA and GFP-PMCA4(2w)/b-LL>AA (Fig. 1). We predicted that this mutation would enhance the apical localization of PMCA4(2w)/b-LL>AA by removing a potentially conflicting signal to the apical targeting information provided by the 2w-insert. Similarly, PMCA4x/b-LL>AA may no longer be exclusively basolateral but display both lateral and apical localization, as has been reported for the analogous L(1153)>A and I(1154)>A mutations in PMCA2x/b [12]. Surprisingly, however, PMCA4(2w)/b-LL>AA did not show enhanced apical localization compared to PMCA4(2w)/b, with both pumps being equally distributed to the apical and lateral membrane of MDCK cells (Fig. 4A). The LL>AA mutation also had no effect on the localization of PMCA4x/b: PMCA4x/b-LL>AA was still strictly basolateral (Fig. 4). These results demonstrate that, unlike in PMCA2b (and 1b), the di-Leu motif at positions 1147–1148 is not required for basolateral targeting of PMCA4b in polarized MDCK cells.

3.4. PMCA isoforms retain their isoform-specific activity regardless of membrane targeting

PMCA isoforms differ significantly in their functional properties, with PMCA4b being a “slow” pump showing low basal activity and strong auto-inhibition, and PMCA2 being “fast” with high basal activity and rapid activation by Ca^{2+} /calmodulin [25, 26]. To investigate if the 2w insert-mediated re-direction of PMCA4b from its normally basolateral location to the apical membrane might alter its functional Ca^{2+} handling characteristics, we performed single-cell Ca^{2+} imaging experiments using the genetically encoded Ca^{2+} sensor protein GCaMP2 in MDCK cells expressing PMCA2x/b, PMCA2w/b, PMCA4x/b or the chimeric PMCA4(2w)/b. Single Ca^{2+} spikes were elicited in cells kept in nominally Ca^{2+} free medium by adding ATP (100 μM) to release Ca^{2+} from intracellular stores, and the change in $[\text{Ca}^{2+}]_i$ was recorded as GCaMP2 fluorescence change over time. Fig. 5 shows that the PMCA isoforms handle such evoked Ca^{2+} signals in an isoform-specific manner that is independent of the pump's localization in the basolateral or apical membrane: Cells expressing the (apical) PMCA4(2w)/b displayed virtually identical Ca^{2+} signals to cells expressing the strictly basolateral PMCA4x/b (Fig. 5A, left), and these signals were distinctly different from those recorded from cells expressing (apical) PMCA2w/b or (basolateral) PMCA2x/b (Fig. 5A, right). Both the amplitude and the duration of the Ca^{2+} signal were greater in cells expressing the PMCA4 pumps than in cells expressing the PMCA2 variants, confirming the slower activation and different function of PMCA4 compared to the fast and highly active PMCA2. This is supported by the data in Fig. 5B showing quantification of the Ca^{2+} signal by calculating the area under the curves. Importantly, Fig. 5C shows that all PMCA variants were expressed at comparable levels in these cells, confirming that the two different Ca^{2+} signal patterns are not due to differences in PMCA expression. Taken together, our data demonstrate that the 2w splice insert functions solely as (apical) targeting element in the PMCA and does not affect the intrinsic isoform-specific functional properties of the pump.

4. Discussion

This study shows that the 45 amino acid w-splice insert from PMCA2 acts as independent apical targeting signal in the PMCA. When the 2w sequence was inserted precisely into the A-splice position of the PMCA4 isoform, the chimeric pump was similarly distributed to the apical membrane of MDCK cells as the original PMCA2w. These results suggest that the w-insert functions independently of the isoform sequence upstream and downstream of the

insertion, at least when present at the same location in the first cytosolic loop of the pump. This raises the question whether the w-insert could act as an autonomous apical targeting signal if inserted elsewhere in the pump sequence, or when appended to an unrelated (membrane) protein? Although this has not yet been tested, we think it is unlikely for several reasons. (i) A previous study showed that the primary sequence of the w-insert is not essential for apical targeting: when the 20 amino acids in the middle of the insert were replaced by an unrelated sequence of equal length the resulting “scrambled” PMCA2w was still targeted to the apical membrane in hair cells [12]. (ii) The same study also indicated that the size of the insert was crucial: only when it encompassed ≥ 31 residues was the PMCA targeted to the apical membrane. This suggests that the w insert functions as a conformational targeting element, rather than having a defined sequence motif recognized by apical sorting proteins. (iii) A yeast two-hybrid search using the w-insert together with its flanking sequence as bait to screen a brain cDNA expression library did not yield any specific interaction partners (J. Rockwood and E.E.S., unpublished observation). (iv) Sequence analysis using bioinformatics tools such as PSIPRED and DomPred (<http://bioinf.cs.ucl.ac.uk/>) shows no significant similarity to any known protein domain for the 2w insert. Moreover, structural analysis using available software including DISOPRED, IUPRED (<http://iupred.enzim.hu/>), and PONDR (<http://www.pondr.com>) predicts that most of the w-insert is intrinsically unstructured. A prominent feature of intrinsically disordered protein domains is their dynamic flexibility, which appears to be important for their function [27, 28]. The w-insert may form a flexible loop embedded in the first cytosolic loop of the PMCA capable of interacting with or shielding other regions of the pump. This may result, e.g., in “masking” of a default basolateral targeting signal widely separated in the primary sequence.

Among such targeting motifs the di-leucine motif in the C-tail of PMCA4b (at positions 1147–1148) is an excellent candidate because the corresponding Leu-Ile sequence in PMCA2x/b (and PMCA1x/b) has previously been shown to be a strong basolateral targeting signal [12]. However, our alanine mutagenesis results do not support the hypothesis that this di-leucine motif is required for basolateral targeting of PMCA4b. This is remarkable because the sequence surrounding the di-hydrophobic motif is highly conserved in PMCA4b (P-L-L-D-E) and PMCA1b/2b (P-L-I-D-D). On the other hand, the overall sequence similarity between the C-tails of PMCA4b and 1b/2b is rather modest (~40% identical over the last 100 residues), suggesting that the location and arrangement of sequence motifs responsible for basolateral targeting of PMCA4b are distinct from those in PMCA1b/2b.

The presence of a strong ligand for the PDZ domains of NHERF2 can influence the membrane distribution of the PMCA [13]. We found that replacing the C-terminal Val residue of PMCA4b by Leu (changing the PDZ ligand sequence from ...E-T-S-V to ...E-T-S-L) enabled NHERF2 to recruit the pump to the apical membrane and to enhance its apical-to-basolateral ratio. However, the ability to interact with NHERF is not sufficient for the PMCA to be localized in the apical domain. The presence of an apical targeting signal such as the 2w-insert is required to send the PMCA to the apical membrane; only then can the pump be “captured” by the scaffolding protein NHERF2 to enhance its apical distribution. This is clearly seen when NHERF2 is overexpressed in MDCK cells transfected with the GFP-PMCA constructs (Fig. 3) but can also be seen with the endogenous NHERF2 present in these cells [29]. Here we showed that the apical distribution of PMCA4(2w)/b-V>L was similar to that of “wild type” PMCA2w/b and enhanced compared to that of PMCA4(2w)/b unable to interact with NHERF (Fig. 4A). In contrast PMCA4x/b-V>L, which has the proper PDZ ligand sequence to interact with NHERF remained basolateral because it lacks the (w-insert) apical targeting signal.

The w splice option is only observed in PMCA2 transcripts and does not exist in the other PMCA genes. PMCA2 is mainly expressed in excitable tissues such as brain and muscle, but significant levels of PMCA2 are also found in pancreatic beta cells and in lactating mammary epithelial cells [3]. Although the relative abundance and distribution of the different A-splice variants 2z, 2x, and 2w remains to be determined in many of these tissues, it is remarkable that PMCA2w is only found in highly polarized cells. In hippocampal neurons, 2w is enriched in dendritic spines where it localizes close to the postsynaptic density [30]. In sensorineural epithelial cells (e.g., cochlear hair cells) and in lactating mammary epithelial cells, 2w is exclusively present in the apical membrane where it is essential for the vectorial export of Ca^{2+} into the endolymph and duct lumen, respectively [7, 31, 32]. Thus the PMCA2w splice variant appears to be expressed specifically in polarized cells requiring efficient local Ca^{2+} export from a defined membrane compartment. The w-splice may have evolved specifically in PMCA2 to facilitate the efficient targeting to such a desired compartment. Although other PMCA isoforms including PMCA1 and 4 may also be localized in apical membrane domains (e.g., in parotid acinar cells [33]), the targeting of these pumps must follow a different mechanism from that of PMCA2w.

We show here that “redirecting” the normally strictly basolateral PMCA4b to the apical membrane in polarized MDCK cells does not alter its functional characteristics. When global changes in $[\text{Ca}^{2+}]_i$ were measured using the genetically encoded sensor GCaMP2, the amplitude and half-time of decay of an ATP-evoked Ca^{2+} spike were indistinguishable in cells expressing the basolateral PMCA4x/b or the mostly apical PMCA4(2w)/b. These results also address the question whether introducing the 2w-insert in PMCA4b affects the pump's functional properties. Consistent with earlier work indicating that the splice site A configuration (z, x, w) has minimal impact on the functional properties of PMCA2 splice variants [26, 34, 35], our data show no effect of the w-insert on PMCA4b Ca^{2+} handling characteristics. Thus, the PMCAs retain their isoform-specific functional properties regardless of the cellular localization in the basolateral or apical membrane. This is also supported by the data (Fig. 5) showing identical handling of an ATP-evoked Ca^{2+} signal by the basolateral PMCA2x/b and the apical PMCA2w/b. Taken together, these results indicate a clear “separation of labor” between the targeting information specified by the splice site A w-insert and the functional information (basal activity, kinetics of activation, maximal turnover) residing in other parts of the pump. Understanding the mechanism by which the w-insert acts as a “dominant” signal to affect the membrane targeting of the PMCA will be an important focus of future work.

Acknowledgments

We thank Krisztina Lor for the excellent technical assistance. This work was supported in part by NIH Grant R01-NS51769 to E.E.S and by Hungarian Academy of Sciences Grants OTKA CK 80283 and ETT 215/2009 to A.E.

References

- [1]. Bootman MD, Lipp P, Berridge MJ. The organisation and functions of local Ca^{2+} signals. *J. Cell Sci.* 2001; 114:2213–2222. [PubMed: 11493661]
- [2]. Kiselyov K, Wang X, Shin DM, Zang W, Muallem S. Calcium signaling complexes in microdomains of polarized secretory cells. *Cell Calcium.* 2006; 40:451–459. [PubMed: 17034849]
- [3]. Strehler EE, Caride AJ, Filoteo AG, Xiong Y, Penniston JT, Enyedi Á. Plasma membrane Ca^{2+} ATPases as dynamic regulators of cellular calcium handling. *Ann. N. Y. Acad. Sci.* 2007; 1099:226–236. [PubMed: 17446463]
- [4]. Brini M, Carafoli E. Calcium pumps in health and disease. *Physiol. Rev.* 2009; 89:1341–1378. [PubMed: 19789383]

- [5]. Strehler EE, Zacharias DA. Role of alternative splicing in generating isoform diversity among plasma membrane calcium pumps. *Physiol. Rev.* 2001; 81:21–50. [PubMed: 11152753]
- [6]. Penniston JT, Enyedi A. Modulation of the plasma membrane Ca^{2+} pump. *J. Membr. Biol.* 1998; 165:101–109. [PubMed: 9744998]
- [7]. Hill JK, Williams DE, LeMasurier DE, Dumont RA, Strehler EE, Gillespie PG. Splice-site A choice targets plasma-membrane Ca^{2+} -ATPase isoform 2 to hair bundles. *J. Neurosci.* 2006; 26:6172–6180. [PubMed: 16763025]
- [8]. VanHouten JN, Neville MC, Wysolmerski JJ. The calcium-sensing receptor regulates plasma membrane calcium adenosine triphosphatase isoform 2 activity in mammary epithelial cells: a mechanism for calcium-regulated calcium transport into milk. *Endocrinol.* 2007; 148:5943–5954.
- [9]. Borke JL, Minami J, Verma A, Penniston JT, Kumar R. Monoclonal antibodies to human erythrocyte membrane $\text{Ca}^{++}\text{-Mg}^{++}$ adenosine triphosphatase pump recognize an epitope in the basolateral membrane of human kidney distal tubule cells. *J. Clin. Invest.* 1987; 80:1225–1231. [PubMed: 2445778]
- [10]. Magyar CE, White KE, Rojas R, Apodaca G, Friedman PA. Plasma membrane Ca^{2+} -ATPase and NCX1 $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger expression in distal convoluted tubule cells. *Am. J. Physiol.* 2002; 283:F29–F40.
- [11]. Chicka MC, Strehler EE. Alternative splicing of the first intracellular loop of plasma membrane Ca^{2+} -ATPase isoform 2 alters its membrane targeting. *J. Biol. Chem.* 2003; 278:18464–18470. [PubMed: 12624087]
- [12]. Grati M, Aggarwal N, Strehler EE, Wenthold RJ. Molecular determinants for differential membrane trafficking of PMCA1 and PMCA2 in mammalian hair cells. *J. Cell Sci.* 2006; 119:2995–3007. [PubMed: 16803870]
- [13]. Padányi R, Xiong Y, Antalffy G, Lőr K, Pászty K, Strehler EE, Enyedi A. Apical scaffolding protein NHERF2 modulates the localization of alternatively spliced plasma membrane Ca^{2+} pump 2b variants in polarized epithelial cells. *J. Biol. Chem.* 2010; 285:31704–31712. [PubMed: 20663896]
- [14]. DeMarco SJ, Chicka MC, Strehler EE. Plasma membrane Ca^{2+} ATPase isoform 2b interacts preferentially with $\text{Na}^{+}/\text{H}^{+}$ exchanger regulatory factor 2 in apical plasma membranes. *J. Biol. Chem.* 2002; 277:10506–10511. [PubMed: 11786550]
- [15]. Hall RA, Premont RT, Chow CW, Blitzer JT, Pitcher JA, Claing A, Stoffel RH, Barak LS, Shenolikar S, Weinman EJ, Grinstein S, Lefkowitz RJ. The b_2 -adrenergic receptor interacts with the $\text{Na}^{+}/\text{H}^{+}$ -exchanger regulatory factor to control $\text{Na}^{+}/\text{H}^{+}$ exchange. *Nature.* 1998; 392:626–630. [PubMed: 9560162]
- [16]. Hall RA, Ostedgaard LS, Premont RT, Blitzer JT, Rahman N, Welsh MJ, Lefkowitz RJ. A C-terminal motif found in the b_2 -adrenergic receptor, P2Y1 receptor and cystic fibrosis transmembrane conductance regulator determines binding to the $\text{Na}^{+}/\text{H}^{+}$ exchanger regulatory factor family of PDZ proteins. *Proc. Natl. Acad. Sci. USA.* 1998; 95:8496–8501. [PubMed: 9671706]
- [17]. Nakai J, Ohkura M, Imoto K. A high signal-to-noise Ca^{2+} probe composed of a single green fluorescent protein. *Nature Biotechnol.* 2001; 19:137–141. [PubMed: 11175727]
- [18]. Ujhelly O, Özvegy C, Várady G, Cervenak J, Homolya L, Grez M, Scheffer G, Roos D, Bates SE, Váradi A, Sarkadi B, Németh K. Application of a human multidrug transporter (ABCG2) variant as selectable marker in gene transfer to progenitor cells. *Hum. Gene Therapy.* 2003; 14:403–412.
- [19]. Enyedi A, Strehler EE. Regulation of apical membrane enrichment and retention of plasma membrane Ca^{2+} ATPase splice variants by the PDZ-domain protein NHERF2. *Communic. & Integr. Biol.* 2011; 4:340–343.
- [20]. Kim E, DeMarco SJ, Marfatia SM, Chishti AH, Sheng M, Strehler EE. Plasma membrane Ca^{2+} ATPase isoform 4b binds to membrane-associated guanylate kinase (MAGUK) proteins via their PDZ (PSD-95/Dlg/ZO-1) domains. *J. Biol. Chem.* 1998; 273:1591–1595. [PubMed: 9430700]
- [21]. Schuh K, Uldrijan S, Gambaryan S, Roethlein N, Neyses L. Interaction of the plasma membrane Ca^{2+} pump 4b/CI with the Ca^{2+} /calmodulin-dependent membrane-associated kinase CASK. *J. Biol. Chem.* 2003; 278:9778–9883. [PubMed: 12511555]

- [22]. Padányi R, Pászty K, Strehler EE, Enyedi A. PSD-95 mediates membrane clustering of the human plasma membrane Ca^{2+} pump isoform 4b. *Biochim. Biophys. Acta.* 2009; 1793:1023–1032. [PubMed: 19073225]
- [23]. Wu H, Reuver SM, Kuhlendahl S, Chung WJ, Garner CC. Subcellular targeting and cytoskeletal attachment of SAP97 to the epithelial lateral membrane. *J. Cell Sci.* 1998; 111:2365–2376. [PubMed: 9683631]
- [24]. Nix SL, Chishti AH, Anderson JM, Walther Z. hCASK and hDlg associate in epithelia, and their src homology 3 and guanylate kinase domains participate in both intramolecular and intermolecular interactions. *J. Biol. Chem.* 2000; 275:41192–41200. [PubMed: 10993877]
- [25]. Caride AJ, Filoteo AG, Penheiter AR, Pászty K, Enyedi Á, Penniston JT. Delayed activation of the plasma membrane calcium pump by a sudden increase in Ca^{2+} : fast pumps reside in fast cells. *Cell Calcium.* 2001; 30:49–57. [PubMed: 11396987]
- [26]. Brini M, Coletto L, Pierobon N, Kraev N, Guerini D, Carafoli E. A comparative functional analysis of plasma membrane Ca^{2+} pump isoforms in intact cells. *J. Biol. Chem.* 2003; 278:24500–24508. [PubMed: 12716903]
- [27]. Tompa P, Fuxreiter M. Fuzzy complexes: polymorphism and structural disorder in protein-protein interactions. *Trends Biochem. Sci.* 2008; 33:2–8. [PubMed: 18054235]
- [28]. Schlessinger A, Schaefer C, Vicedo E, Schmidberger M, Punta M, Rost B. Protein disorder - a breakthrough invention of evolution? *Curr. Op. Struct. Biol.* 2011; 21:412–418.
- [29]. Antalffy G, Caride AJ, Pászty K, Hegedus L, Padányi R, Strehler EE, Enyedi Á. Apical localization of PMCA2w/b is enhanced in terminally polarized MDCK cells. *Biochem. Biophys. Res. Commun.* 2011; 410:322–327. [PubMed: 21672522]
- [30]. Burette AC, Strehler EE, Weinberg RJ. A plasma membrane Ca^{2+} ATPase isoform at the postsynaptic density. *Neurosci.* 2010; 169:987–993.
- [31]. Wood JD, Muchinsky SJ, Filoteo AG, Penniston JT, Tempel BL. Low endolymph calcium concentrations in deafwaddler^{2J} mice suggest that PMCA2 contributes to endolymph calcium maintenance. *J. Assoc. Res. Otolaryngol.* 2004; 5:99–110. [PubMed: 15357414]
- [32]. Reinhardt TA, Lippolis JD, Shull GE, Horst RL. Null mutation in the gene encoding plasma membrane Ca^{2+} -ATPase isoform 2 impairs calcium transport into milk. *J. Biol. Chem.* 2004; 279:42369–42373. [PubMed: 15302868]
- [33]. Baggaley E, McLarnon S, Demeter I, Varga G, Bruce JIE. Differential regulation of the apical plasma membrane Ca^{2+} -ATPase by protein kinase A in parotid acinar cells. *J. Biol. Chem.* 2007; 282:37678–37693. [PubMed: 17938178]
- [34]. Hilfiker H, Guerini D, Carafoli E. Cloning and expression of isoform 2 of the human membrane Ca^{2+} ATPase. *J. Biol. Chem.* 1994; 269:26178–26103. [PubMed: 7929331]
- [35]. Ficarella R, Di Leva F, Bortolozzi M, Ortolano S, Donaudy F, Petrillo M, Melchionda S, Lelli A, Domi T, Fedrizzi L, Lim D, Shull GE, Gasparini P, Brini M, Mammano F, Carafoli E. A functional study of plasma-membrane calcium-pump isoform 2 mutants causing digenic deafness. *Proc. Nat. Acad. Sci. U.S.A.* 2007; 104:1516–1521.

Fig. 1.

PMCA constructs and mutants studied in this work. (A) A scheme of the PMCA with its 10 membrane-spanning segments (numbered 1–10) is shown on the top. The alternative splice site A is indicated, and the x- and w-splice inserts are shown as boxes corresponding to the included exon(s). The position of a di-leucine (LL) motif in the C-tail is also indicated, and the PDZ-binding sequence (PDZ-BD) is represented by a black box. The amino acid sequences corresponding to the A-splice site in PMCA4x, PMCA2x, PMCA4(2w) and PMCA2w, as well as partial sequences of the C-tail including the di-leucine and PDZ-binding motifs of PMCA4x/b, PMCA2x/b, PMCA4x/b-V>L, PMCA4(2w)/b-V>L, and PMCA4(2w)/b-LL>AA are shown below. Amino acid residue numbers are given for the first and last residue in each sequence. The A-splice insert, the di-leucine motif, and the C-terminal residue are in bold print. (B) Expression of the recombinant GFP-tagged PMCA4 constructs. Aliquots of lysate from HeLa cells transfected with the constructs indicated on top of each lane were separated by SDS-PAGE and probed by Western blotting with an anti-GFP antibody to detect the recombinant GFP-PMCA protein (top panel) or with an antibody against GAPDH as loading control (bottom panel). All GFP-PMCA constructs are expressed at comparable levels as full-length proteins of the expected size.

Fig. 2.

The 2w-insert confers apical targeting to PMCA4b in polarized MDCK cells. MDCK cells grown for 72 h on Lab-Tek II Chambered Coverglass were transfected with cDNAs encoding the indicated GFP-tagged PMCA variants. 48 h post-transfection, cells were fixed and stained with the apical marker anti-ezrin (indicated in red) and the basolateral marker anti-Na,KATPase (indicated in blue), and analyzed by confocal microscopy. Co-localization of the PMCA (detected by its GFP fluorescence) with ezrin is indicated in yellow in the merged images. Representative apical and middle (lateral) x-y scans are shown in the first and second rows of each panel. In the third row of each panel, x-z projections of the x-y scans are presented. Note the prominent apical localization of the chimeric PMCA4(2w)/b, while PMCA4x/b remains strictly basolateral. Data shown are representative of at least three independent experiments. Scale bars represent 10 μ m.

Fig. 3.

NHERF2 enhances the apical localization of PMCA4(2w)/b with a C-terminal Val to Leu mutation. MDCK cells were transiently co-transfected with NHERF2 and GFP-PMCA4(2w)/b-V>L or GFP-PMCA4x/b-V>L as indicated. Fixed cells were stained for NHERF2 (red) and Na,K-ATPase (blue) and observed by confocal fluorescence microscopy as described in the legend for Fig. 2. The expressed PMCAs were detected by the GFP fluorescence of their tag. NHERF2 localized to the apical membrane where it markedly co-localized with PMCA4(2w)b-V>L (left panels) but was clearly separated from PMCA4x/b-V>L (right panels), which remained strictly basolateral. Scale bar = 10 μ m.

Fig. 4.

Quantitative evaluation of the localization of PMCA constructs in MDCK cells and effect of NHERF2 on the apical/basolateral distribution of the PMCAs. MDCK cells were transfected with the indicated constructs and processed as described in the legend for Fig. 2.

Localization of the GFP-tagged PMCAs was evaluated by their co-localization with the apical marker ezrin and the basolateral marker Na,K-ATPase. Quantification was performed as described in section 2.4. (A) Ratio of apical to basal/lateral distribution of different PMCA variants in the absence and presence of NHERF2. PMCA4x/b, 4x/b-LL>AA and 4x/b-V>L are strictly basolateral and insensitive to the presence of NHERF2. The 2w splice insert promotes apical localization of PMCA4(2w)/b, 4(2w)/b-LL>AA and 4(2w)/b-V>L. Note the significantly enhanced apical to basolateral ratio for PMCA4(2w)/b-V>L comparable to that of PMCA2w/b. A C-terminal leucine residue permits protein-protein interactions between the PMCA and NHERF2. Therefore, over-expression of NHERF2 further enhances the apical localization of PMCA2w/b and PMCA4(2w)/b-V>L, but not of PMCA4(2w)/b without the C-terminal leucine residue. Also note that mutation of a dileucine putative basolateral targeting sequence did not enhance apical localization of PMCA4(2w)/b-LL>AA. Values represent the mean $\pm$ SEM of calculations from 25 to 50 cells of three independent experiments. Significance by t-test: ** $p < 0.002$; *** $p < 0.0001$.

(B) Summary of the localization of different PMCA constructs in polarized MDCK cells. The 2w-insert functions as primary apical targeting signal; NHERF2 can enhance the apical distribution of the PMCA but only if the pump is targeted to the apical membrane (via the 2w insert) AND contains an optimal NHERF2-interacting C-terminal sequence.

Fig. 5.

PMCA^s handle global Ca²⁺-signals in an isoform-specific manner regardless of their localization. **(A)** MDCK cells were cultured on Nunc Lab-Tek Chambered Coverglass and co-transfected with the indicated PMCA construct and the genetically encoded Ca²⁺ indicator GCaMP2. Single-cell Ca²⁺ signal measurements were carried out in Ca²⁺ free HBSS. Ca²⁺ signals were elicited by addition of 100 μ M ATP (arrows). 20–30 cells were measured in a single experiment, time-lapse sequences were recorded, images were analyzed and the experimental data were fitted as described in section 2.5. *Left panel:* average fluorescence profile of Ca²⁺ signals in cells expressing PMCA4(2w)/b (black) and PMCA4x/b (grey). *Right panel:* average fluorescence profile of Ca²⁺ signals in cells expressing PMCA2w/b (black) and PMCA2x/b (grey). Stippled lines indicate the standard deviation (SD) of each average curve. **(B)** Quantification of Ca²⁺ signal measurements shown in (A). Data were obtained by calculating the area under the curves of Ca²⁺ signal time-lapse sequences of 40–50 cells from three independent determinations. Data are expressed as mean $\pm$ standard error (SE). There was no significant difference in Ca²⁺ handling by the x- and w-variants of either PMCA2 or PMCA4, however, note the distinct difference in Ca²⁺ handling between the slow PMCA4 and the fast PMCA2 isoforms. **(C)** Confocal images of representative populations of MDCK cells expressing the various PMCA protein variants applying the same detector gain, zoom, amplitude offset and gain, transmission percent, pinhole size, and scan speed for each image. The data indicate that the constructs were equally expressed in MDCK cells.