Published in final edited form as:
Biochem Biophys Res Commun. 2011 July 1; 410(2): 322–327. doi:10.1016/j.bbrc.2011.05.147.

Apical localization of PMCA2w/b is enhanced in terminally polarized MDCK cells

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Abstract

The “w” splice forms of PMCA2 localize to distinct membrane compartments such as the apical membrane of the lactating mammary epithelium, the stereocilia of inner ear hair cells or the post-synaptic density of hippocampal neurons. Previous studies indicated that PMCA2w/b was not fully targeted to the apical domain of MDCK cells but distributed more evenly to the lateral and apical membrane compartments. Overexpression of the apical scaffold protein NHERF2, however, greatly increased the amount of the pump in the apical membrane of these epithelial cells. We generated a stable MDCK cell line expressing non-tagged, full-length PMCA2w/b to further study the localization and function of this protein. Here we demonstrate that PMCA2w/b is highly active and shows enhanced apical localization in terminally polarized MDCK cells grown on semi-permeable filters. Reversible surface biotinylation combined with confocal microscopy of fully polarized cells show that the pump is stabilized in the apical membrane via the apical membrane cytoskeleton with the help of endogenous NHERF2 and ezrin. Disruption of the actin cytoskeleton removed the pump from the apical actin patches without provoking its internalization. Our data suggest that full polarization is a prerequisite for proper positioning of the PMCA2w variants in the apical membrane domain of polarized cells.

Keywords

apical membrane; membrane cytoskeleton; membrane targeting; NHERF2; PMCA; polarized cells

Introduction

Plasma membrane Ca^{2+} pumps (PMCAs) are essential elements of Ca^{2+} signaling because in many cell types these pumps are the primary mechanisms for restoring and maintaining low intracellular Ca^{2+} levels. More than twenty PMCA variants exist, and many of them

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have highly specific roles. The specificity of different PMCAs partly arises from their differential localization, protein-protein interaction partners and/or Ca^{2+} sensitivity, which are affected by alternative splicing [1]. The different splice forms of PMCA2 illustrate this ability especially well: alternative splicing at site A determines the final destination of the pump, whereas alternative splicing at site C determines interaction with specific protein partners. Thus, the "w" splice (site A) sends this pump to the apical membrane domain of polarized cells [2] while the "b" splice form (site C) has the ability to interact tightly with the apical scaffold protein NHERF2 [3]. The splice-dependent localization has specific physiological implications: PMCAs that are located in the basolateral membrane contribute to Ca^{2+} clearance and transcellular Ca^{2+} transport whereas PMCAs located on the apical side also modulate the Ca^{2+} concentration of the extracellular fluid, as in the case of PMCA2w/a in cochlear hair cells modulating the Ca^{2+} concentration of the endolymph [4, 5] and PMCA2w/b in mammary cells contributing to Ca^{2+} secretion in milk [6].

Recent experiments demonstrated that the PMCA2w/b variant shows prominent apical localization, however, with a relatively large amount of pump staying in the lateral membrane of confluent MDCK cell monolayers [7]. Overexpression of NHERF2 greatly enhanced the apical localization and stabilized this pump in the apical compartment by anchoring it to the apical cytoskeleton. These experiments suggested an interplay between the alternative splice options at sites A and C in determining the unique localization pattern of the PMCA2 splice variants. However, a limiting factor in these studies was that transient expression of the pump does not allow for longer culture times, which are necessary to ensure full polarization of MDCK epithelial cells [8]. Here, by using stably transfected cells we show that the apical localization of PMCA2w/b is greatly enhanced in filter grown, fully polarized PMCA2w/b-MDCK cell cultures. Our results also suggest that the pump is stabilized in the apical membrane by interaction with the apical actin cytoskeleton via endogenous NHERF2.

Materials and methods

Reagents and antibodies

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. TRITC-phalloidin (used at 0.1 μg/ml) was obtained from Sigma. 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. Rabbit polyclonal antibodies against PMCA2 (NR2) and NHERF2 (Ab720) have been described [3, 9] and were used at 1:1000 and 1:2000, respectively. The genetically encoded calcium indicator GCaMP2 was a generous gift from Junichi Nakai (RIKEN Brain Science Institute, Saitama, Japan) [10]. FuGene HD Transfection Reagent was obtained from Roche Applied Science. All other chemicals used were of reagent grade.

Generation of stably transfected cells and Western blotting

Stably transfected MDCK cells expressing PMCA2w/b and PMCA2z/b were generated following a protocol used previously as described [11, 12]. Cells were grown at 37°C in a 5% CO_2 humidified incubator in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics (1% penicillin and 1% streptomycin, 500 μg/ml G418). The level of hPMCA2 and NHERF2 protein expression was tested by Western blot analysis. Equal amounts of protein samples were resolved on 7.5% acrylamide gels following the procedure of Laemmli with some modifications [11]. Proteins were subsequently transferred onto PVDF membranes, and the blots were immunostained with the anti-PMCA2 antibody NR2 or the anti-NHERF2 antibody Ab720, followed by

horseradish peroxidase-coupled goat anti-rabbit secondary IgG (GE Amersham, 1:2000) and detection by the ECL system according to the manufacturer's protocol (GE Amersham).

Image acquisition

MDCK cells were seeded into eight-well Nunc Lab-Tek II chambered coverglass (Nalge Nunc International) or Transwell dishes (Corning Inc.) at 5×10^4 cells/well or 1×10^5 cells/well. Cells were cultured 8 to 12 days prior to the experiments to ensure complete polarization, and grown for 48 h after transfection when needed. To perform immunostaining for localization studies, cells were gently washed with Dulbecco's modified phosphate-buffered saline (DPBS), fixed with 4% paraformaldehyde (PFA) in DPBS for 10 min, washed with DPBS and permeabilized in 0.2% PFA and 0.2 % Triton-X 100 for 2 min at room temperature. The samples were 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 [7]. The cells were then incubated for 1 h at room temperature with primary antibody diluted in blocking buffer. After washing with DPBS, the cells were incubated for 1 h at room temperature with Alexa Fluor conjugated secondary antibody 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 60x (1.4 NA) oil immersion objective (Olympus Europa GmbH). Where indicated, cytochalasin D was added in media without serum at a final concentration of 2 μM and incubated at 0°C for 5 min before staining.

Surface biotinylation and internalization

Surface biotinylation was performed on MDCK cells stably expressing PMCA2w/b or PMCA2z/b. Cells were cultured for 8 to 12 days in Transwell permeable supports (Corning Inc.) prior to the experiments to ensure complete polarization. The cells were incubated twice for 30 min in PBS buffer containing 1 mg/ml of Sulfo-NHS-SS-Biotin (Thermo Scientific) at 4°C. After biotinylation, cells were washed twice with PBS and incubated with 50 mM glycine in PBS for 15 min at 4°C to quench the excess biotinylating reagent. Cells were then washed twice with PBS and returned to the 37°C incubator. After the indicated times at 37°C (to allow surface protein internalization), biotin attached to protein that was not internalized was cleaved by incubating the cells (2 × 30 min) with 200 mM MESNA (mercaptoethane sulfonic acid) in PBS at 4°C. After 2 washes in PBS, cells were lysed in lysis buffer (PBS containing 1% Nonidet-P40, 1% sodium deoxycholate, 1 mM EDTA, 0.1 mM PMSF, 1 mM $NaVO_3$, 0.5 mM NaF, 2 μg/ml aprotinin and 4 μg/ml leupeptin). Cell lysates were incubated overnight with 5% Streptavidin agarose beads (Thermo Scientific). The beads were washed 3x with lysis buffer and subsequently resuspended in 100 μl electrophoresis sample buffer. After 10 min at 37°C, beads were pelleted and supernatants were subjected to SDS-PAGE and Western blotting for PMCA2 (using antibody NR2) as described [9].

Ca^{2+} signal measurements

Control or PMCA2w/b expressing MDCK cell clones were cultured on 8-well Nunc Lab-Tek Chambered Coverglass in DMEM supplemented with 10% fetal bovine serum. After 48 hours, cells were transfected with the genetically encoded calcium indicator GCaMP2 [10] using the FuGENE HD (Roche) transfection reagent. Before confocal imaging cells were kept in Phenol Red free DMEM, 10% FBS, 10 mM HEPES. Calcium signal measurements were carried out in Hanks' Balanced Salt Solution supplemented with 20 mM Hepes (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 60x (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. GraphPad Prism4 software (http://www.graphpad.com) was used to fit the experimental data.

Results

Characterization of MDCK cells stably expressing PMCA2w/b

Previous experiments suggested that the N-terminal GFP-tag interferes with PMCA2w targeting in the hair-bundle membrane of cochlear and vestibular hair cells [13]. To avoid interference of the GFP-tag with apical localization we generated a stable MDCK cell line that expresses a non-tagged version of PMCA2w/b. Western blot analysis (Fig. 1A) shows a high level of expression of the PMCA2w/b isoform in the stably transduced MDCK cells compared to empty vector transduced control cells. The level of expression of PMCA2w/b did not change over an extended cell culture period of more than 10 days (Fig. 1B). However, confocal imaging showed that similarly to the GFP-tagged protein [7], the non-tagged PMCA2w/b pump was targeted nearly equally to the apical and lateral membrane when the stably expressing MDCK cells were grown on glass (Fig. 1C). We next tested the functionality of the PMCA2w/b expressed in these MDCK cells, using the genetically encoded Ca^{2+} indicator GCaMP2 [10] to follow Ca^{2+} transients. 48 hours after transfection with the GCaMP2 plasmid, Ca^{2+} transients were evoked by the addition of 100 μM ATP. Fig. 1D shows the time-course of Ca^{2+} transients and demonstrates that expression of PMCA2w/b dramatically shortens the duration of the Ca^{2+} signal compared to control cells that express mainly PMCA4b [14]. In agreement with previous findings *in vitro* [15] and *in vivo* [16, 17], our data demonstrate that PMCA2w/b is a very powerful pump that clears Ca^{2+} rapidly from the cell.

Increased apical localization of PMCA2w/b in fully polarized, filter-grown MDCK cells

Previous studies using transient transfection were done on less than fully polarized MDCK cell cultures. Stable expression now made it possible to study the localization of the PMCA2w/b protein during long-term culturing; i.e. when MDCK cells reach full confluence and polarity. The Western blots in Fig. 2A (see also Fig. 1B) show that the expression of PMCA2w/b was stable during an extended culture period (up to 2 weeks) and did not change when the cells were grown on glass or a semi-permeable filter support. Importantly, we also detected endogenous NHERF2 expression in the PMCA2w/b expressing cells using an isoform-specific anti-NHERF2 antibody (bottom panel in Fig. 2A). The right panel of Fig. 2A shows x-z sections of confocal images of cells cultured for 9 days on a filter support. The picture demonstrates that in fully polarized, filter-grown cells the apical domain is highly enriched in PMCA2w/b with much less pronounced lateral staining for the pump (Figs. 2A and 2B). It is also important to emphasize that the cells do not show apoptotic features; i.e. they grow tall and the middle sections of the images show cobblestone-like structures typical of healthy, polarized epithelial cells (Fig. 2B). To determine the steady-state plasma membrane distribution of PMCA2w/b, filter-grown MDCK cells were surface-biotinylated from the apical or the basolateral side followed by streptavidin precipitation and immunoblotting (Fig. 2C). In these studies stably transduced PMCA2z/b expressing MDCK cells were used as control. In accordance with the confocal images, substantial accumulation of the biotinylated PMCA2w/b was detected in the apical domain compared to the basolateral domain, while PMCA2z/b appeared mostly in the basolateral membrane of the cells. Thus, both surface labeling (Fig. 2C) and confocal imaging (Figs. 2A and 2B) suggest that the PMCA2w/b isoform distribution is mostly apical in highly polarized cells.

PMCA2w/b in polarized MDCK cells is linked to the apical actin cytoskeleton and has limited membrane mobility

When polarized cells were co-stained with anti-PMCA2 and anti-ezrin antibodies, a substantial overlap of the fluorescence signals was observed on the apical side (Fig. 3A). This suggests that the apical pump is tethered to the apical cytoskeleton through a PDZ mediated interaction with the endogenous NHERF2 and ezrin, as proposed earlier [7]. To test if PMCA2w/b is stabilized in the apical membrane of fully polarized cells, we performed reversible surface biotinylation followed by streptavidin precipitation and immunoblotting. Figure 3B illustrates that MESNA treatment (which strips surface-bound biotin) removed a large fraction of biotin from the PMCA even after 40 minutes of incubation at 37°C, indicating that only a relatively small fraction (20–30% of the total) of the pump was internalized. These results suggest that PMCA2w/b is immobilized in the apical membrane resulting in slow endocytic trafficking and an increased overall membrane residence time of the pump.

To investigate the link of PMCA2w/b with the underlying apical actin cytoskeleton, we treated fully polarized MDCK cells with cytochalasin D to disrupt the actin filament network, and then studied the localization and membrane residence time of the pump. The confocal images in Fig. 4A show a substantial overlap of the PMCA2w/b and the TRITC-phalloidin (actin labeling) fluorescence signals at the apical side of polarized cells. Cytochalasin D treatment disorganized the actin filaments and detached the pump (and ezrin) from the actin patches although the pump remained apical. Indeed, reversible surface biotinylation (Fig. 4B) showed that only a small fraction of the PMCA2w/b was internalized even after cytochalasin D treatment, indicating that the pump was retained in the apical membrane.

Discussion

Our data suggest that full polarity is required for proper apical localization of PMCA2w/b. Previous experiments using FRAP and surface biotinylation assays demonstrated that interaction with exogenously expressed NHERF2 can stabilize GFP-PMCA2w/b in the apical membrane of confluent MDCK cell cultures [7]. Here we suggest that in filter-grown, fully polarized cells PMCA2w/b is anchored to the apical actin cytoskeleton by the endogenously expressed NHERF2 (Fig. 2). In good accordance with these findings, reversible surface biotinylation using filter-grown MDCK cells showed that only a small fraction of PMCA2w/b was internalized while a larger fraction of the pump stayed in the apical membrane for extended times. Interestingly, disruption of the cytoskeleton did not induce enhanced internalization of the PMCA2w/b suggesting that it is part of a membrane-associated larger molecular complex (see also [7]) that stays in the membrane even in cytochalasin D treated cells.

It is important to emphasize that an N-terminal GFP-tag does not interfere with the targeting of PMCA2w/b in MDCK cells [2] although it may reduce apical targeting in cochlear hair cells [13]. The distribution of the PMCA, on the other hand, is greatly affected by the polarization of the cells: when the cells were grown on a glass support, the PMCA (tagged or not) was almost equally distributed to the apical and the lateral membrane domain (Fig. 1C). In contrast, when the cells were grown on a filter support the apical localization of the PMCA2w/b was greatly enhanced (Figs. 2 and 3A).

Recently, it has been reported that PMCA2w/b overexpression in BRIN-BD11 cells depletes cytosolic, ER and mitochondrial Ca^{2+} stores and induces apoptosis via the mitochondrial pathway [18]. Our stable PMCA2w/b-expressing MDCK cell clones formed highly polarized monolayers during the relatively long culture period indicating that over-

expression of functional (Fig. 1D) PMCA2w/b did not interfere with normal epithelial growth and maturation. We also note that no increase in cells with apoptotic/necrotic morphology could be detected in the PMCA2w/b compared to the empty vector expressing MDCK cell cultures (not shown). In fact, the PMCA2w/b expressing cells grew as fast as the control cells suggesting that over-expression of this pump does not negatively affect proliferation. Although PMCA2w/b expression itself did not induce apoptosis in these cells, further experiments will be needed to determine if the PMCA2w/b expressing MDCK cell clones are more vulnerable to mitochondrial or receptor mediated cell death, as was the case with the insulin-secreting β-cells.

PMCA2w/a is abundantly expressed in the apical stereocilia of fully developed and polarized hair cells [13, 19, 20]. In contrast to PMCA2w/b, the 2w/a splice form lacks a PDZ domain-interacting C-terminal sequence and is therefore unlikely to be recruited to the apical membrane domain by NHERF2. This indicates that the PDZ-binding tail is not essential for apical targeting of the PMCA2w forms. Instead, it is the splice configuration at site A that acts as primary signal for apical PMCA2 delivery [20]. Without the PDZ-binding tail, however, the PMCA2w/a is quite mobile in the membrane bilayer, even if it has a relatively long membrane residence time in the stereocilia membrane [21]. Our data demonstrate that PMCA2w/b, which can interact with NHERF2 and may thus be actively recruited to the apical domain, is concentrated in the apical membrane of MDCK cells only when they are fully polarized. This suggests that complete separation of lateral and apical membranes is required to achieve the final stable, asymmetric distribution of PMCA2w/b. It is apparent that the “w” splice insert is essential for apical delivery of the PMCA2, but the mechanism that sends the pump to the apical compartment remains to be determined. In the case of PMCA2w/b, the interaction with scaffolding PDZ proteins such as NHERF helps to keep the pump in the apical domain. This likely serves to maintain the high Ca^{2+} extrusion capacity required on the apical side of many secreting epithelia including those of the lactating mammary gland.

Acknowledgments

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

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Highlights

- MDCK cell clones stably expressing PMCA2w/b were characterized
- PMCA2w/b in MDCK cells efficiently removes Ca^{2+} following a transient Ca^{2+} spike
- Apical localization of PMCA2w/b is enhanced in fully polarized, filter-grown MDCK cells
- Endogenous NHERF2 promotes apical enrichment of PMCA2w/b via the membrane cytoskeleton

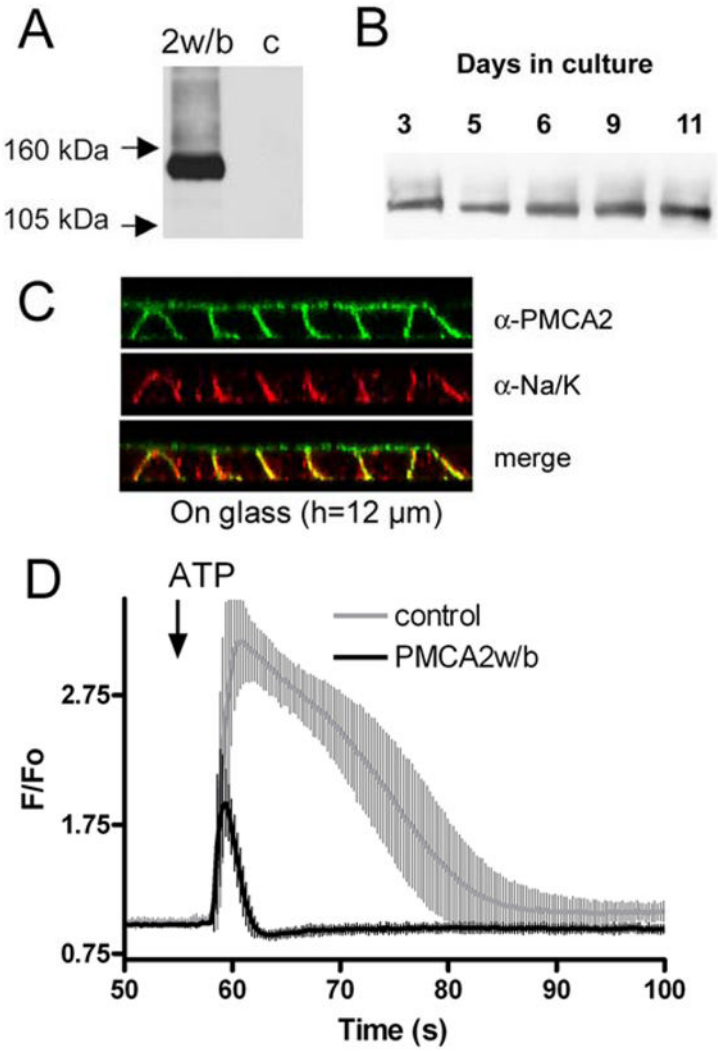

Fig. 1. Stable expression and function of PMCA2w/b in MDCK cells

(A, B) SDS-PAGE/immunoblot analysis of stably transfected MDCK cells grown on glass. PMCA2 was detected with specific antibody NR2. Equal amounts of protein from stably transfected MDCK cells expressing empty vector were used as a control (c) for antibody specificity (**A**). The level of PMCA2w/b expression shows little change over 11 days of cell culturing (**B**). (**C)** Cell surface localization of PMCA2 and Na^+/K^+-ATPase in stably transfected MDCK cells grown on glass (height = 12 μm). The anti-PMCA2 and anti-Na^+/K^+-ATPase stained cells were analyzed by confocal laser scanning microscopy with vertical z-scans. PMCA2w/b is localized nearly equally to the apical and lateral membrane. (**D**) Empty vector control or PMCA2w/b expressing MDCK cells were cultured on 8-well Nunc Lab-Tek Chambered Coverglass and transfected with GCaMP2. Single cell calcium signal measurements were carried out as described in Materials and methods. Arrows indicate administration of 100 μM ATP. 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 Materials and methods.

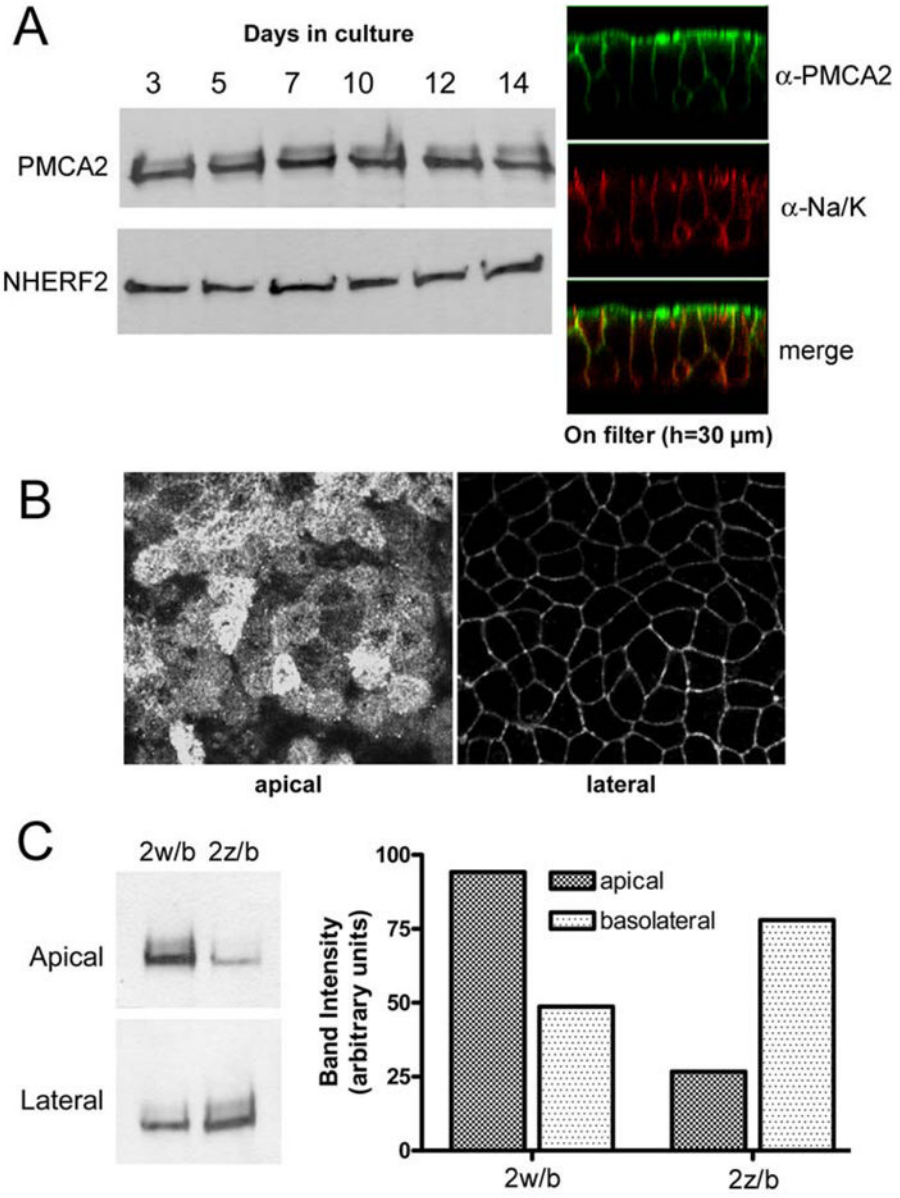

Fig. 2. Characterization of PMCA2w/b in MDCK cells grown on a filter support

(**A)** Stably transfected MDCK cells expressing PMCA2w/b were grown on Transwell permeable supports for 3–14 days. SDS-PAGE/immunoblot analysis of PMCA2w/b (upper panel) and NHERF2 (lower panel) expression is shown on the left. The images on the right show 9 day old cultures stained for PMCA2 (top) and Na^+/K^+-ATPase (middle) analyzed by confocal laser scanning microscopy with vertical z-scans (merged image on bottom). Note that these fully polarized cells grew to a height of 30 μm. (**B**) The anti-PMCA2 stained cells were analyzed by confocal laser scanning microscopy with horizontal x-y scans. In highly polarized, filter-grown cells the apical domain is enriched for PMCA2w/b (*left*) with less pronounced staining of the lateral membrane (*right*). (**C)** Amount of PMCA 2w/b and 2z/b biotinylated from the apical or the basolateral side. MDCK cells stably expressing PMCA 2w/b or 2z/b were grown in Transwell permeable supports and labeled with Sulfo-NHS-SS-biotin, washed, and prepared for immunodetection of PMCA2 as described in Materials and methods. Quantification of the Western blot is shown on the right.

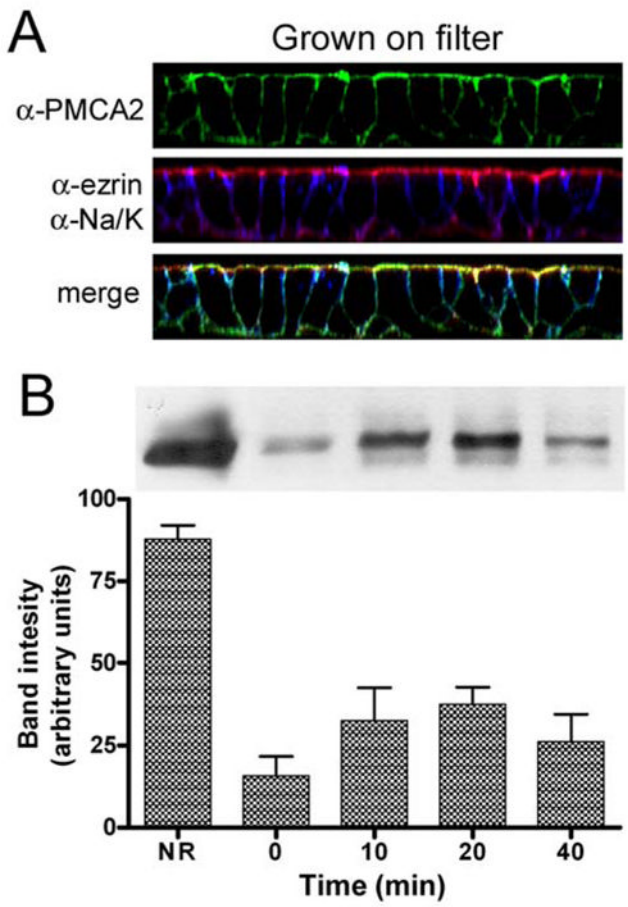

Fig. 3. PMCA2w/b is stabilized in the apical membrane of highly polarized MDCK cells

(A) MDCK cells stably expressing PMCA2w/b were grown on filter support for 9 days. When co-stained with antibodies against PMCA2 (green), Na^+/K^+-ATPase (blue) and ezrin (red), a substantial overlap of the fluorescence signals for PMCA2 and ezrin was observed on the apical side (merged image on bottom). Cells were analyzed by confocal laser scanning microscopy with vertical z-scans. (**B**) Internalization of PMCA2w/b as a function of time. MDCK cells stably expressing PMCA2w/b were grown on Transwell supports and labeled from the apical side with Sulfo-NHS-SS-biotin, washed and returned to the incubator. At the indicated times the remaining surface biotin was removed and the cells were lysed and prepared for immunodetection of PMCA2 as described in Materials and methods. NR corresponds to a non-reduced sample and represents the total amount of apical biotinylated PMCA2. The upper panel shows the result of a typical experiment and the lower panel shows the quantitative evaluation from 3 experiments (± SE).

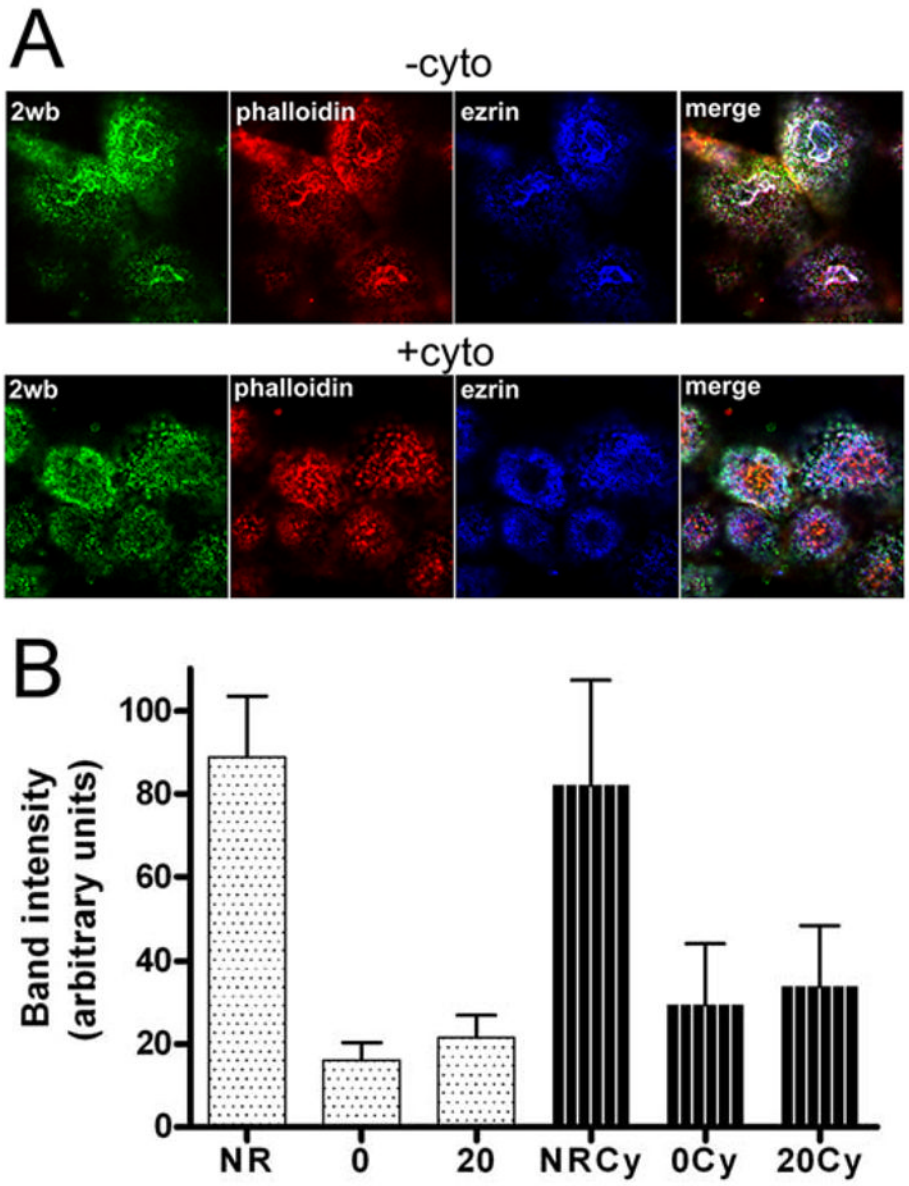

Fig. 4. Cytochalasin D treatment disrupts the actin cytoskeleton-PMCA2w/b co-localization but does not affect internalization of the pump

(A) Co-localization analysis of PMCA2w/b with the apical marker ezrin and the actin cytoskeleton in the absence (−cyto) and presence (+cyto) of cytochalasin D added at the apical side of fully polarized MDCK cells. Cells were co-stained for PMCA2 (green), actin (TRITC-phalloidin (red)) and ezrin (blue), and fluorescence images of the apical side were acquired using confocal laser scanning microscopy with horizontal x-y scans. Cytochalasin D was added in media without serum at a final concentration of 2 μM and incubated at 0 °C for 5 min (+cyto) before staining. (**B)** Internalization of PMCA2w/b as a function of time in the absence and presence (Cy) of cytochalasin D. MDCK cells stably expressing PMCA2w/b were grown on Transwell permeable supports, labeled from the apical side with Sulfo-NHS-SS-biotin, and treated as described for Fig. 3B. The bars represent data from 3 separate experiments (± SE).