# The Regulation of the Localization of the Plasmamembrane Calcium ATPase (PMCA) 2 and 4 isoforms

## Short PhD thesis

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## Introduction

The calcium ion ($Ca^{2+}$) is the most versatile signaling messenger known in nature. The continuous presence of $Ca^{2+}$ throughout the life of a cell is required for a wide array of essential cellular functions such as gene transcription, muscle contraction or neurotransmission. Four-fifths of the cytosolic $Ca^{2+}$ is bound to $Ca^{2+}$-binding proteins or membrane surfaces, while the remaining free cellular $Ca^{2+}$ concentration ranges between 100-200 nM. Balanced operation of cellular calcium signaling requires the generation and maintenance of low intracellular $Ca^{2+}$ concentrations and the Plasma Membrane $Ca^{2+}$ ATPase (PMCA) protein plays a crucial role in this process.

The PMCA pump consists of a transmembrane domain of ten transmembrane helices and cytosolic loops connecting these helices. The conserved A-, N-, and P cytosolic domains make up the catalytic region of the protein. The first cytosolic loop of PMCA contains a region responsible for the binding of acidic phospholipids, whereas the C-terminal of the protein contains several regulatory regions, such as the PDZ-binding sequence allowing protein-protein interactions or the high affinity calmodulin binding region which essential for the regulation of the pump.

According to the current view, the major role of PMCA is the maintenance of the low intracellular $Ca^{2+}$ concentrations by extruding intracellularly accumulated $Ca^{2+}$ ions. Nevertheless, experiments using PMCA knockout mice or different mutant variants of the protein also suggested its involvement in regulating local and cell-type specific $Ca^{2+}$ concentrations, signaling pathways or programmed cell death. Specialized tissue distribution of PMCA2 and unique intracellular localization of its 2w isoform enables the participation of this variant in the process of hearing and lactation. PMCA4 shows a ubiquitous expression pattern; however, recent studies have also proposed its role as a differentiation or a tumor marker.

## Aims

Tissue specific expression of the different PMCA isoforms allow for specialized protein functions, which requires well-defined intracellular localization, compartmentalization and transcriptional regulation as only the correctly targeted and sufficiently expressed protein is able to perform its proper function. Factors regulating the expression and the cellular localization of the different PMCA isoforms have not been fully revealed. In our work, we investigated the *in vitro* intracellular localization, compartmentalization, protein-protein

interactions and regulation of the PMCA2 and PMCA4 isoforms, in order to answer the following questions.

We aimed to:
- investigate the regulation of the localization and protein-protein interactions of PMCA2w/b and 2z/b protein isoforms using confocal microscopy techniques;
- set up and characterize an MDCK cellular model system showing stable overexpression of PMCA2w/b and 2z/b protein isoform which could further be employed to analyze the interaction between the PMCA2w/b isoform and the NHERF2 scaffold protein, and the role of this interaction in the localization of PMCA2;
- investigate the regulation of the expression and localization of PMCA4x/b using C-terminally truncated PMCA4x/b mutants and confocal microscopy and biochemical techniques;
- examine the predicted internalization or retention signals located in the C-terminal region of the PMCA4x/b protein by applying site directed mutagenesis.

**Materials and Methods**

Cell cultures were maintained in supplemented Dulbecco's modified Eagle's medium (DMEM) at 37 ºC in 5% $CO_2$ and humidified atmosphere. Transduced cell lines were cultured in DMEM containing 0.5 mg/ml G418 (Sigma), and G418 was withdrawn under the various treatments. Cell lines used in this study (MDCK II, COS-7, HeLa) were obtained from the ATCC collection (Manassas, VA), while the Phoenix Eco and PG13 cell lines were generous gifts from Dr. Katalin Német (Hungarian National Blood Transfusion Services, Budapest). For cellular transfection, the FuGene HD (Roche) or Lipofectamine 2000 (Invitrogen) transfection reagents were used according to the manufacturers' protocol.

Plasmids encoding the various isoforms and C-terminally truncated variants of PMCA were generous gifts of Drs. J.T. Penniston (Mayo Clinic) and E.E. Strehler (Mayo Clinic). PMCA L>A mutants were generated with the Quickchange II Site-Directed Mutagenesis Kit (Stratagene). Primers were synthesized by the Nucleic Acid Synthesis Laboratory of the Biological Research Centre (Szeged, Hungary).

MDCK cell lines stably expressing the PMCA2 variants were generated by retroviral transduction and the calcium phosphate co-precipitation kit (GIBCO). After transduction single-cell clones were generated and the protein expression level of the transduced PMCA

was verified by Western blot, while localization of the pump was followed by immunohistochemistry and confocal microscopy.

Protein samples were prepared by precipitation with trichloroacetic acid, and protein content of the samples was determined by the modified Lowry method and subsequent Western blot analysis.

Internalization and cell surface abundance of PMCA was measured by biotinylation using the Cell Surface Protein Isolation Kit (Thermo Scientific).

$Ca^{2+}$ transport activity was measured using crude microsomes isolated from COS-7 cells transfected with the PMCA isoforms of interest, in a reaction mixture containing $^{45}CaCl_2$. The amount of intravesicularly accumulated $^{45}Ca^{2+}$ was determined in a Beckman LS Liquid Scintillation Counter 6000. The measured values were weighted with the expression levels of the PMCA protein determined by densitometry analysis of Western blots.

Microscopy studies were carried out 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 to and edited with the Gimp 2.6 (Free Software Foundation Inc, Boston) and Photoshop CS5 (Adobe Systems Inc.) softwares. For data analysis, PRISM 5 (GraphPad Software Inc., San Diego), Microsoft Excel (Microsoft, Seattle), OriginPro 8.5 (OriginLab Corp., Northampton), and ImageJ 1.36b (Rasband, NIH, Maryland) programs were also used.

The ratio of PMCA4 localized in the plasma membrane was determined by analysing microscopy images of HeLa cells expressing PMCA4. Localization of PMCA4 was identified by its colocalization with the plasma membrane or Golgi marker WGA or the ER marker anti-calnexin. In order to measure the apical/basolateral localization ratio of PMCA2, x-z sections of the microscopy images of PMCA2-expressing MDCKII cells were analyzed. The apical or basolateral localization of PMCA2 was identified by its colocalization with the apical marker proteins ezrin and NHERF2 or the basolateral marker protein Na,K-ATPase. Images were analyzed with the Gimp 2.6 software (Free Software Foundation Inc, Boston).

The mobility of the PMCA protein was measured with the Fluorescence Recovery after Photobleaching (FRAP) technique. The kinetics of recovery was determined by exponential fitting of the average data of at least three independent experiments using the non-linear regression algorithm of of the GraphPad Prism 4.0 Software (San Diego, California, USA).

Statistical significance was determined by Student's t-test, applicability of this test was verified by the F-probe. Data were considered as statistically significant if the p-value was

less than 0.05. For the statistical analysis of the intracellular localization of the different PMCA protein variants, two independent investigators uninformed of the experiment's goals scored the confocal images and statistical significance of the image scores was determined using the $\chi^2$test.

**Results**

Our experiments addressing the regulation and the protein-protein interactions of PMCA2 provided the following results:

- We generated and characterized MDCKII cell lines showing stable expression of PMCA2w/b or PMCA2z/b with retroviral transduction, in order to obtain a cellular model system for the subsequent localization studies of these PMCA protein variants.
- Applying these model cells, we determined the subcellular localization and the apical/basolateral ratio of PMCA2w/b and PMCA2z/b with immunocytochemistry, confocal microscopy and biotinylation techniques. We found that in the plasma membrane of the transduced MDCKII cells, localization of PMCA2w/b is predominantly apical, while localization of PMCA2z/b is mostly basolateral.
- Applying polarized MDCKII model cells, we examined the steady-state apical localization ratio of PMCA2w/b using cell surface biotinylation. We showed that no significant internalization of PMCA2w/b occurred in the time scale of our experiments.
- We investigated the role of partner proteins of PMCA2w/b in connecting PMCA2w/b to the cell cytoskeleton and followed the mobility of the PMCA2w/b with FRAP measurement. We demonstrated the the partner protein NHERF2 displays a stabilizing effect on the apical localization of PMCA2w/b.
- In order to examine the connection between PMCA2w/b and the actin cytoskeleton, we treated the PMCA2w/b-expressing MDCKII cells with cytochalasin D. Changes in the localization and internalization of the PMCA2w/b upon cytochalasin D treatment were followed with confocal microscopy and biochemical techinques, respectively. We found that cytochalasin D treatment resulted in disruption of the connection between PMCA2w/b and the actin cytoskeleton; however, under the same conditions internalization ratio of PMCA2w/b was unchanged.
- By generating EGFP-tagged *chimeric* PMCA constructs, we studied the effect of alternative mRNA splicing occurring at the *A-site* on the subcellular localization of PMCA2. The subcellular localization of the PMCA2 protein variants were analyzed by confocal microscopy

and our results support the hypothesis that a 45 amino acid long “w” splice segment contains an apical localization signal, which *per se* provokes apical localization of the protein.

Our studies investigating the regulation of the localization and the expression of PMCA4 yielded the following results:

- Applying transiently transfected cell lines, we examined the impact of confluency on the intracellular localization of PMCA4x/b with confocal microscopy. We found that the increase in cellular confluency positively correlates with the plasma membrane localization ratio of PMCA4x/b.
- Studying various C-terminally truncated PMCA mutants, we examined the role of the C-terminal region in the regulation of the intracellular localization of PMCA4x/b. We found that the C-terminally truncated PMCA mutants showed an altered intracellular localization as compared to the wild type PMCA4x/b. Therefore, we hypothesized that the C-terminal region between amino acids 1157 to 1181 of PMCA4x/b contained one or more internalization or retention signals.
- In order to identify the putative localization signals in the C-terminal region of PMCA4x/b and to analyze their impact on the intracellular localization of PMCA, we generated L>A substituted PMCA4x/b and PMCA4x/b-ct24 mutant variants by site-directed mutagenesis. When examining the protein expression levels and the $Ca^{2+}$-transport activity of these mutant PMCA proteins, we found that all of the mutants were functional and could be activated by the administration of calmodulin.
- Applying transiently transfected cells and confocal microscopy, we examined the role of the dileucine motifs (in position 1147-48 and 1167-69) in the basolateral localization of PMCA4x/b. Our results suggested that basolateral localization of PMCA4x/b was independent of the presence of the examined dileucine motifs.
- Using transiently transfected cell lines, cell surface biotinylation and confocal microscopy, we investigated the effect of the L>A substitution on the plasma membrane localization of PMCA4x/b and PMCA4x/b-ct24 proteins. We found that the 1167-69L>A substitution enhanced the plasma membrane localization in case of all the examined PMCA variants, whereas the impact of the 1147-48L>A substitution was demonstrated only in the case of PMCA4x/b-ct24. Based on these results, we concluded that the 1167-69 dileucine motif is involved in the regulation of the plasma membrane localization of PMCA4x/b.

## Conclusions

In the present work, we examined the regulation of the localization of the PMCA2 and PMCA4 isoforms.

Results obtained from experiments studying the PMCA2 isoform suggest that plasma membrane localization and lateral mobility of PMCA2 are determined by the presence of the apical localization signal formed by a splicing event at the A-splice site and also by the protein-protein interactions mediated by the C-terminal PDZ-binding sequence of the pump.

Our studies concerning PMCA4 revealed the role of the C-terminal dileucine motifs in regulating the plasma membrane localization and distribution of PMCA4x/b. We found that even though the different PMCA4 variants displayed similar $Ca^{2+}$ transport activities, they decreased the intracellular $Ca^{2+}$ concentration with different kinetics as a result of their distinct intracellular localization patterns. Our results thus support the hypothesis that plasma membrane abundance of the PMCA pump is a major determinant of the pattern of the intracellular $Ca^{2+}$ signal and presumably the $Ca^{2+}$ signal-triggered cellular response as well.

## Publications Related to the Subject of the Thesis

Apical scaffolding protein NHERF2 modulates the localization of alternatively spliced plasma membrane Ca2+ pump 2B variants in polarized epithelial cells.
Padányi R, Xiong Y, **Antalffy G**, Lór K, Pászty K, Strehler EE, Enyedi A.
J Biol Chem. 2010 Oct 8;285(41):31704-12. Epub 2010 Jul 27.
PMID: 20663896, Impact factor (2010): 4.773

Apical localization of PMCA2w/b is enhanced in terminally polarized MDCK cells.
**Antalffy G**, Caride AJ, Pászty K, Hegedus L, Padanyi R, Strehler EE, Enyedi A.
Biochem Biophys Res. Commun. 2011 Jul 1;410(2):322-7. Epub 2011 Jun 6.
PMID: 21672522, Impact factor (2011): 2.484

Plasma membrane calcium pump (PMCA) isoform 4 is targeted to the apical membrane by the w-splice insert from PMCA2.
**Antalffy G**, Mauer AS, Pászty K, Hegedus L, Padányi R, Enyedi Á, Strehler EE.
Cell Calcium. 2012 Feb;51(2):171-8. Epub 2012 Jan 16.
PMID: 22252018, Impact factor (2011): 3.766

A C-terminal di-leucine motif controls plasma membrane expression of PMCA4b.

**Antalffy G**, Pászty K, Varga K, Hegedűs L, Enyedi A, Padányi R.

BBA Mol. Cell. Res. 2013 Dec. 1833(12):2561-72. Epub 2013 Jul 2.

PMID: 23830917, Impact factor (2013): 4.808