Multifaceted plasma membrane Ca^{2+} pumps: from structure to intracellular Ca^{2+} handling and cancer.

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Abbreviations: CaM, calmodulin; CBS, calmodulin-binding sequence; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; HDAC, histone deacetylase; HUVEC, human umbilical vein endothelial cells; IP_3, inositol-1,4,5-trisphosphate; MLEC, mouse lung endothelial cells; NCX, Na^+/Ca^{2+}-exchanger; NFAT, nuclear factor of activated T-cells; NHERF2, Na^+/H^+ exchanger regulatory factor 2; nNOS, neuronal nitric oxide synthase; $PI(4,5)P_2$, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; PMCA, plasma membrane Ca^{2+} ATPase; RANKL, nuclear factor κB ligand; RASSF, Ras association domain family; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; SOCE, store operated Ca^{2+} entry; SPCA, secretory pathway Ca^{2+} ATPase; STIM1, stromal interaction molecule 1; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cell;

1. Abstract

Plasma membrane Ca^{2+} ATPases (PMCAs) are intimately involved in the control of intracellular Ca^{2+} concentration. They reduce Ca^{2+} in the cytosol not only by direct ejection, but also by controlling the formation of inositol-1,4,5-trisphosphate and decreasing Ca^{2+} release from the endoplasmic reticulum Ca^{2+} pool. In mammals four genes (PMCA1-4) are expressed, and alternative RNA splicing generates more than twenty variants. The variants differ in their regulatory characteristics. They localize into highly specialized membrane compartments and respond to the incoming Ca^{2+} with distinct temporal resolution. The expression pattern of variants depends on cell type; a change in this pattern can result in perturbed Ca^{2+} homeostasis and thus altered cell function. Indeed, PMCAs undergo remarkable changes in their expression pattern during tumorigenesis that might significantly contribute to the unbalanced Ca^{2+} homeostasis of cancer cells.

Keywords

Plasma membrane Ca^{2+} ATPase, Ca^{2+}-calmodulin, phosphatidylinositol-4,5-bisphosphate binding, Ca^{2+} signaling, differentiation, tumor progression

2. Introduction

Among the P-type ATPases, plasma membrane Ca^{2+} ATPases comprise a separate sub-group of Ca^{2+} pumps that is called ATP2B (name of the gene) or PMCA (name of the protein). The three types of Ca^{2+} pumps of the P-type ATPase family - SERCA, SPCA and PMCA - share essential basic properties, including membrane topology and reaction mechanism but they also display significant differences, particularly in the areas related to regulation. Fundamental properties of the PMCA have been discussed in numerous excellent reviews [1-3].

PMCAs are encoded by 4 different genes, all are located on different chromosomes; PMCA1 on 12q21–23, PMCA2 on 3p25.3, PMCA3 on Xq28 and PMCA4 on 1q25–q32 (see refs [1] and [4] for review). Alternative splicing of the primary transcripts at two different sites produces over 20 different variants. Splicing at site A changes the length of the first intracellular loop (A domain) that affects targeting while splicing at site C alters the length of the C-terminal tail that gives different regulatory and trafficking characteristics to the variants (*Figure 1*).

A unique property of the PMCA proteins is their complex regulatory feature. The C-terminus of PMCAs contains a high affinity calmodulin-binding site that also serves as an auto-inhibitor. Within the C-terminal region there are also sites for phosphorylation with protein kinases, proteolytic sites for caspase-3 and calpain, motifs that control trafficking of the pumps and a C-terminal PDZ-binding motif present only in the b splice forms. PMCAs are also known to bind to and being activated by acidic phospholipids such as the signaling molecule, phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$).

The basic function of the PMCA is to maintain intracellular Ca^{2+} homeostasis. When Ca^{2+} increases in the cytosol, it binds to calmodulin (CaM), and then the Ca^{2+}-CaM complex binds to several different target proteins to activate down-stream events. Binding of Ca^{2+}-CaM to the PMCA releases auto-inhibition of the pump and consequently it becomes active and removes Ca^{2+} from the cytosol. This negative feedback mechanism eventually terminates the signal. Thus, the task is relatively simple yet mammalian development resulted in many - over 20 - distinct PMCA variants.

PMCAs are expressed in tissue and cell-type specific pattern manner. PMCA1 is the house-keeping PMCA form present in all cell types. PMCA2 and 3 are typically present in excitable cells, in the brain, hair cells of the inner ear or skeletal muscle. The b splice variant of PMCA4 is found in relatively large amounts in the heart, endothelial cells, pancreas and in the epithelium. The principal piece of the sperm tail expresses specifically the a splice form of PMCA4.

The particular needs of the distinct cell types - being as diverse as neurons in the brain on the one hand and cells of the epithelium on the other - are satisfied by the unique targeting/trafficking and regulatory characteristics of the individual pump variants. Individual targeting features and response-time allow PMCAs to modulate effectively the complex spatial-temporal dynamics of Ca^{2+} signaling events of polarized cells. These unique features of PMCAs are complemented with their highly differentiation-specific expression pattern that can be significantly altered in less differentiated cancer cells.

In the present review we will concentrate on recent developments of the PMCA field including specific localization and regulatory characteristics of the pumps. We will highlight how distinct regulatory features of PMCAs are translated into distinct Ca^{2+} signaling patterns

in the living cells, we will elucidate the dual function of PMCAs in PI(4,5)P_2 signaling, and finally we will discuss PMCA function in cancer.

3. Structural elements and regulatory regions of the PMCA

Structural elements of PMCAs. Sequence homology between P-type ATPases predicted 10 transmembrane helices, a corresponding set of stalks on the cytoplasmic side and three large cytoplasmic domains in the PMCAs (a schematic representation of PMCA structure is shown in *Figure 1*) [3, 5]. The three cytoplasmic domains are 1/ the N domain which binds ATP, 2/ the P domain containing an aspartic acid residue that is phosphorylated by ATP during the reaction cycle and 3/ the A domain which coordinates the movements of the other domains. The transmembrane helices provide the Ca^{2+}-coordinating residues, which can bind one Ca^{2+} ion per reaction cycle [6]. In addition, homology modeling of the PMCA has detected a nearly continuous positively charged ring in the membrane-proximal stalk region that is missing from the SERCA structure [7]. Thus, PMCAs follow the positive-inside rule of plasma membrane proteins like the recently crystalized Zn transporting P-type ATPase [8]. Being a transmembrane protein, PMCA interacts with lipids and the presence of lipids is needed for activity. Reconstitution of the PMCA into a neutral phospholipid bilayer enhances the enzyme turnover rate [9] but more acidic phospholipids are needed for a fully functional pump [10].

Conformational changes during the enzyme cycle. Similarly to other P-type ATPases, the PMCA has been proposed to undergo a series of structural and chemical transitions during its reaction cycle. Conventionally, the cycle is initiated by binding of Ca^{2+} to the high Ca^{2+} affinity E1 state followed by ATP binding and phosphoenzyme (EP) formation. The cycle is completed by translocation of Ca^{2+} through the membrane, dephosphorylation and regaining the E1 conformation from the low Ca^{2+} affinity E2 state that allows the enzyme to begin a new cycle. Crystal structures at different conformational states [11] and kinetic analysis [12] of the SERCA pump have suggested that SERCA probably binds ATP first (E2.ATP) followed by Ca^{2+} binding (ATP.E1.2Ca^{2+}) and phosphoenzyme formation (ADP.E1P. 2Ca^{2+}). Recently, conformational states of the reaction cycle of PMCA have been determined using specific photoactivatable lipid probes [13]. These lipid probes have allowed the measurement of conformational changes between the inhibited and Ca^{2+}- calmodulin activated states of the pump [14]. From these data, equilibrium constants for various ligand binding, regulation of Ca^{2+} transport in the presence of ATP or vanadate and conformational movements of functional domains [15] have been determined. These authors determined the functional cycle

of ATP binding and hydrolysis by PMCA and found that in resting condition the pump exists in two intermediate states, E1ATP and E2ATP, to which Ca^{2+} could bind and initiate ATP driven Ca^{2+} transport.

Regulatory regions. PMCA is a bigger molecule than the other P-type ATPases; two of the extra regions contain extra regulatory functions including regulation of activity and changes in localization. Localization of the PMCAs is mainly controlled by alternative RNA splicing at site A in the first intracellular loop while regulation of activity has been related mostly to the C-tail. Importantly, alternative RNA splicing affects both regulatory regions, generating multiple versions of the PMCA with distinct regulatory and targeting functions, according to the needs of the particular cell type.

Regulatory regions - the first intracellular loop. Alternative splicing at site A occurs in the region encoding the first intracellular loop (*Figure 1*) where a single exon is either included or excluded in the mature transcripts, producing the x and z splice variants of PMCAs. In mammalian PMCA2, one or two additional exons can be inserted at the A splice site, producing further splice variants y and w [3]. The w PMCA splice variant has the longest insert (45 amino acid residues) in its first intracellular loop; this insert targets PMCA2 to the apical membrane of polarized cells [16].

Regulatory regions - the C-tail. Alternative splicing at site C affects the cytosolic C-terminal tail and produces splice variants, a, b, c, d, e; among those the most frequently occurring ones are the a and b forms (*Figure 1*). The C-tails of the b splice variants of the PMCA1-3 isoforms have relatively high sequence homology but the PMCA4b isoform is quite different. The C-tail has been known as the main regulatory unit of the PMCA. Regulatory functions of proteins are often carried out by intrinsically disordered regions; such regions provide a large interaction surface exposing short linear motifs that allow interactions with different protein partners [17, 18]. Indeed, the C-tail of PMCAs shows an enrichment of both polar and charged residues typical characteristics of such regions. Analysis by different bio-informatics tools (IUPred (http://iupred.enzim.hu/) [19], PONDR (http://www.pondr.com/) [20] and PREDISORDER (http://sysbio.rnet.missouri.edu/) [21] predicts that the C-tail of PMCA contains both ordered and disordered regions. *Figure 2* shows the prediction of disordered regions in the C-tail of PMCA4b from Phe^{1045} to the C-terminal Val^{1205} and the complex pattern of different binding and regulatory sequences within the C-tail. In addition to the flexible hinges, C-tail contains an about 30-40-residue-long region (from Asp^{1080} to Leu^{1114})

that are predicted to be structurally well organized (*Figure 2B* dashed lines). This region overlaps the calmodulin-binding sequence (CBS), which is important for both CaM binding and autoinhibitory interactions with the core of PMCA (see below). Another program ANCHOR (http://anchor.enzim.hu/) [22] identifies binding sites, which are likely to gain stabilizing energy by interacting with globular protein partners. ANCHOR found two peaks (*Figure2B*, blue stripes) within the CBS with relatively high probability to serve as binding sites. This agrees with the existence of two anchor sites being involved in the interaction between the two lobes of Ca^{2+}-calmodulin (*see below*). Interestingly, ANCHOR indicates strong binding sites at both the previously identified downstream di-leucine-like motif [23] and the PDZ-binding tail [24]. Between CBS and the di-leucine-like motif another peak suggest additional binding sites within a region known to contain PKC phosphorylation and calpain cleavage sites [25-27]. Similar sites in the C-tail are also predicted by the DISOPRED3 program (http://bioinf.cs.ucl.ac.uk/psipred/) [28]. The CBS and the PDZ binding tail (b variants only) share some sequence homology between the PMCA variants, while other motifs (di-leucine-like, phosphorylation and calpain cleavage sites) are present only in certain variants. This complex arrangement of sites shows the finely-tuned regulatory potential of the C-terminal region.

The Calmodulin-binding sequence. As discussed above the C-tail of the PMCA contains sequence motifs involved in regulation. One of these motifs is the ~28-residue long calmodulin-binding sequence (CBS). It was possible to study the interaction of this sequence with calmodulin by using the synthetic peptides shown in Table 1.

Free Ca^{2+}-CaM has an extended dumbbell structure in which two globular domains are connected by a helix. Activation of the pump requires binding of Ca^{2+}-CaM to the CBS. The details of the conformational changes of CaM and peptides representing the CBS were first studied by small angle X-ray scattering, which showed changes in the radius of gyration of CaM and its complexes indicating that the binding of C20 to CaM did not alter the extended structure of CaM, but that use of C24 caused the complex to collapse into a globular structure [29]. An NMR structural study of the interaction between CaM and C20 confirmed that CaM did not collapse when C20 was used [30]. A more recent NMR study was done using a longer peptide (C28); it found that calmodulin was collapsed and wrapped around the C28 peptide utilizing two anchors from the peptide that were 18 residues apart, with Trp^{1093} as the first anchor and Phe^{1110} the second [31].

Autoinhibitory interactions. An important characteristic of the PMCA isoforms is their basal activity, which is their activity without the activator Ca^{2+}-calmodulin. The basal activity of the pumps depends on the degree of interaction between the C-tail and the catalytic core. If the interaction is strong the pump has low basal activity (PMCA4b and PMCA4a) whereas in the case of a weak interaction the basal activity is high (PMCA2 and 3 variants). The basal activity is determined by sequences of both the C-tail and the core regions to which the C-tail binds. The differences between the structures of the C-tails resulted in a higher basal activity of PMCA4a as compared to that of PMCA4b [32]. On the other hand, the high basal activity of PMCA2b was attributed to the differences in the core regions rather than to the C-tail [33]. Importantly, any change made in the C-tail of PMCA4b that increased the basal activity also increased the rate of activation by CaM [33-35], demonstrating that autoinhibitory interactions also affect CaM binding.

The autoinhibition of PMCA is removed by Ca^{2+}-calmodulin-binding or by proteolytic removal of the whole C-tail. Interestingly, a caspase-3 cleavage site (Asp^{1080}) is located 5 residues upstream of the CaM-binding sequence between the inhibitory region and the catalytic core of PMCA4b. When caspase-3 is activated during the course of apoptosis, it cuts immediately after Asp^{1080} and removes the complete inhibitory region [36-38]. Moreover, mutation of Asp^{1080} activated PMCA4b suggesting that this site functions as an anchor between the autoinhibitor and the catalytic core [34]. Another anchor point is Trp^{1093} which is located in the calmodulin-binding sequence and involved in CaM binding (see above) [35]. Intramolecular fluorescence resonance energy transfer between the C- and N-terminal segments of the PMCA showed that the N-terminal tail is also involved in the autoinhibition [39] and Glu^{99} was determined as a critical anchor point within this region [40].

These complex structural features of PMCAs are manifested in functions specific to each variant that allow cells regulate delicately their Ca^{2+} homeostasis and relevant downstream effectors.

4. Ca^{2+}-calmodulin activation kinetics.

An important feature of the PMCAs is the tight regulation of their activity. Among other less characterized modulators, such as acidic phospholipids and phosphorylation with protein kinases, the Ca^{2+}-calmodulin complex (Ca^{2+}-CaM) is the primary regulator of the PMCA. The regulatory properties of the PMCA isoforms have been determined by studying cells in which

one of the isoforms is dominant. The first studies determined the dissociation constants for Ca^{2+}-CaM binding to PMCA4b - the major PMCA form present in red cell membrane - under steady state conditions using either red cell membrane vesicles or isolated pump. Later overexpressed cell systems were suited better to compare steady-state, as well as kinetic characteristics of the isoforms. The first steady-state studies revealed pumps with high and low affinity or more precisely pumps with low and high dissociation constants (K_d) for Ca^{2+}-CaM, respectively. As an example PMCA4b binds Ca^{2+}-CaM with high affinity while the alternatively spliced variant PMCA4a has low affinity [41]. Most of the other variants tested in this respect bind Ca^{2+}-CaM with high affinity [27].

Measuring kinetics of calmodulin binding and PMCA activation sheds light on further details of the regulatory properties of the variants. Kinetic constants of Ca^{2+}-CaM binding/dissociation and PMCA activation/inactivation rates at different Ca^{2+} concentrations were determined for the most common variants of PMCA2, 3 and 4 (kinetic characteristics of the isoforms are summarized in *Figure 3A*). These experiments showed that PMCA4b binds Ca^{2+}-CaM slowly (low k_{on}) and it remains bound to the pump for couple of minutes (low k_{off}) [42, 43]. Surprisingly, however, PMCA4a binds Ca^{2+}-CaM quickly (high k_{on}) but then - unlike from PMCA4b - Ca^{2+}-CaM dissociates from this pump also quickly (high k_{off}), which is the reason why PMCA4a has low affinity for Ca^{2+}-CaM ($K_d=k_{off}/k_{on}$) in spite of its fast activation rate [32]. Because of the rapid inactivation, PMCA4a should have no memory of earlier Ca^{2+} spikes. The PMCA2 and PMCA3 variants are quickly activated and stay active for a long time [44, 45]. This property gives them long lasting memories of earlier Ca^{2+} spikes. Based on the activation rates of the pumps, PMCA4b is referred to as the slow pump while the other forms are fast pumps.

Unfortunately, we know very little of the kinetic characteristics of PMCA1. Although PMCA1 is ubiquitous, we do not have access to cells in which it is dominant or expressed in appreciable amounts to perform kinetic studies. PMCA4b has been extensively studied because it is the dominant Ca^{2+} transporter of human red cells. The other forms have been made dominant by overexpression, but this has been difficult for PMCA1. In an earlier study overexpressed PMCA1 was reported to be highly sensitive to proteolytic degradation in Sf9 cells [46]. In another study, successful overexpression of functional PMCA1a in rat aortic endothelial cells led to altered expression of other elements (SERCA, IP3R, CRAC pathway) of the calcium signaling toolkit [47], making it difficult to study its characteristic features separately.

5. Shaping the Ca^{2+} signal in live cells.

The shape of the cytoplasmic calcium signal is determined by the rate of calcium influx (through plasma membrane or endomembrane calcium channels) and the rate of subsequent calcium removal from the cytosol (by calcium pumps and the Na^+/Ca^{2+} exchanger; this latter is present only in certain types of cells) [48, 49]. The calcium signal may be oscillatory or a single calcium wave, and the latter could be a transient spike or a sustained signal [50, 51]. Surprisingly, among the many factors that are known to shape the Ca^{2+} signal the plasma membrane calcium pumps have not been extensively examined. Most of the existing studies discuss the role of PMCAs as part of the extrusion systems but little is known about the specific function of the individual isoforms.

One of the main difficulties of these studies has been the lack of PMCA-specific inhibitors. La^{3+} is an effective PMCA inhibitor [52] but it also inhibits (although with lower affinity) Ca^{2+} channels and other Ca^{2+} transporters [53]. Carboxyeosine is a potent PMCA inhibitor [54]; however, it is a fluorescent compound that may interfere with fluorescent Ca^{2+} sensors used to detect the Ca^{2+} signal in the living cells. More recently a small molecule inhibitor, aurintricarboxylic acid (ATA) was identified by high-throughput screening [55]. While ATA was reported to inhibit PMCA4 function specifically at a relatively low concentration, at higher concentrations it can affect several vital cellular functions, and therefore, this compound should be used with caution in live cells. Caloxins are a family of short peptide inhibitors that by now also include isoform-specific versions [56-59]; however, their affinity is quite low and they have not been thoroughly tested for specificity in live cells, but they may become very useful in the future.

Alterations in plasma membrane abundance and function. One aspect of the pumps' impact on Ca^{2+} signaling is when the expression is either reduced or up-regulated. The lack or the over-production of the PMCAs, with concomitant modulation of the expression levels of other key components of the Ca^{2+} signaling toolkit, result in increased Ca^{2+} influx or efflux and/or altered Ca^{2+} sequestration thus, remodeling calcium signaling of the cells.

PMCA1, the house-keeping isoform, is ubiquitously expressed in all kinds of cells, most often together with one or more other PMCA isoforms. Not surprisingly, the decrease of PMCA1 expression level and/or activity in cases where it is the obligate or dominant isoform leads to abnormal $[Ca^{2+}]_i$ handling. In pig coronary artery endothelial cells the PMCA1 activity was

reduced by the selective inhibitor, caloxin1b3, resulting in elevated cytosolic calcium concentration [58]. In the vascular smooth muscle cells (VSMCs) of VSMC-specific PMCA1 KO mice the cytosolic Ca^{2+} level was also raised resulting higher systolic blood pressure in the animals [60]. PMCA1 was suggested to play an essential role in gastric epithelial surface repair using a PMCA1+/- model [61]. These authors showed that PMCA1 was responsible for the increased extracellular Ca^{2+} concentration near the damaged cells that was required for the repair. On the other hand, there are several data emphasizing the importance of PMCA1 in transcellular Ca^{2+} transport. Intestinal Ca^{2+} uptake is known to be stimulated by 1,25(OH)2D3 that induces a gene network involved in the regulation of transcellular Ca^{2+} uptake. Ribiczey and others showed that 1,25(OH)2D3 controls the expression of PMCA1 in enterocytes thus, it is particularly involved in the transcellular Ca^{2+} transport of these cells [62, 63]. Transcellular Ca^{2+} transport is also typical of kidney cells. Although, various kidney cells express a diversity of PMCA isoforms [64] it seems that it is the PMCA4 isoform that contributes to renal transepithelial Ca^{2+} flux rather than PMCA1 [65].

The substantial alteration of the protein expression pattern is a characteristic feature of certain tumors and differentiating cells. There are emerging data regarding the modulation of PMCA expression and consequent alteration of the Ca^{2+} signal during differentiation. Indeed, increased PMCA4 expression and thus, enhanced Ca^{2+} clearance was reported during differentiation of breast cancer, gastric and colon carcinoma cell lines [66-68]. The downregulation of PMCA4 contributes to tumorigenesis by reorganization of the calcium signal that influences proliferative pathways in these cells.

In tissues and cells of neural origin the expression of the fast PMCA isoforms (PMCA2 and/or PMCA3) is pronounced. Differentiation of a human neuroblastoma cell line IMR-32 upregulated the expression of PMCA2, PMCA3 and PMCA4 isoforms that increased Ca^{2+}-efflux from the cells after depolarization [69]. When PMCA2 and PMCA3 levels were reduced in PC12 cell lines, even though several compensatory mechanisms (up-regulation of PMCA1, PMCA4 and SERCAs), these cells had decreased capacity to remove calcium [70]. This led to altered differentiation abilities of the cells with longer neurites, retracted growth cones and more varicosities than in control PC12 cells. Moreover, decreased PMCA2 expression led to increased susceptibility to apoptosis. Similarly, in an aging brain model, where suppressed expression of PMCA2 in embryonic rat brain neurons and SH-SY5Y neuroblastoma cells showed disruptions of normal Ca^{2+} signaling, enhanced cell death to toxic stimuli was observed [71].

PMCA2 is also abundant in lactating mammary gland along with decreased PMCA4 expression [72] and in some breast cancer cells the PMCA composition was also reported to be changed. Increased intracellular calcium levels were observed in PMCA2 deficient mammary epithelial cells that led to increased apoptosis, whereas overexpression of PMCA2b in T47D breast cancer cells reduced intracellular calcium and consequently reduced apoptosis in response to ionomycin treatment [73]. Resistance to apoptosis indicated by PMCA2 overexpression might contribute to cancer progression and refer to poor prognosis in breast cancer.

A shift in PMCA level in itself does not necessarily correlate with modified Ca^{2+} extrusion as the pump may not localize properly to the plasma membrane. In subconfluent cultures PMCA4 was shown to be localized predominantly in intracellular compartments [23, 74]. Increasing confluence enhanced plasma membrane localization of either transiently or endogenously expressed PMCA4b, and consequently augmented the Ca^{2+} extrusion capacity of the cells [23].

There is growing evidence that several kinds of disease (e.g. deafness, cerebellar ataxia, familial spastic paraplegia, etc.) are accompanied by mutations in PMCA genes. Almost all of these disorders result in disturbances of the Ca^{2+} handling of the cells reducing the extrusion efficiency and increasing the basal cytosolic Ca^{2+} level (PMCA2: [75]; PMCA3: [76, 77]; PMCA4: [78].

Interactions with scaffold and signal molecules. There are several lines of evidence that the interactions of PMCAs with different scaffold and signal molecules and/or localization of the pump might also influence the local and/or global Ca^{2+} signaling of the cells. In these interactions the activity of either the PMCA and/or its partner is altered. These findings have shed light on novel functions of PMCA other than simply extruding excess Ca^{2+} from cells. Through these interactions PMCAs become components of various signaling pathways and are able to modify downstream signaling directly. One example is the role of PMCAs during T cell activation. When T cells are stimulated an elevated global Ca^{2+} signal is observed that enhances the translocation of NFAT to the nucleus [79]. Soboloff et al. found that both STIM1 and PMCA4 are upregulated during lectinphytohemagglutinin (PHA)-induced activation of T cells and the subsequent interaction of PMCA with STIM1 reduces PMCA-mediated $[Ca^{2+}]_i$ clearance [80]. Further investigations revealed that the ER resident scaffold protein POST also interacts with PMCAs, STIM1 and other components of the Ca^{2+} signaling

complex [81]. In store-depleted Jurkat cells the STIM1-POST complex inhibited PMCA activity resulting in a higher cytosolic Ca^{2+} level. Presumably, POST might act as an adaptor of STIM1-mediated PMCA inhibition during T cell activation. T cell activation was also shown to result the re-organization of PMCA to the close proximity of mitochondria at the immunological synapse [82]. Mitochondria by rapidly taking up the inflowing Ca^{2+} reduce local Ca^{2+}-dependent PMCA activity thus, contributing to the elevated global Ca^{2+} signal. Another example where PMCA activity is influenced by the interplay with other signal molecules is the constitutive interaction of G protein-coupled estrogen receptor 1 (GPER/GPR30) and PMCA4b via their PDZ-binding motifs (through PSD-95) [83]. Via this interaction each molecule affects the other's function, i.e. PMCA is inhibited while GPER/GPR30 is activated, resulting in a prolonged Ca^{2+} signal, thus affecting the activity of downstream Ca^{2+} dependent elements.

Direct comparison of the isoforms. Most the above mentioned studies investigated the effects of PMCAs on the overall Ca^{2+} handling of the cells. In an earlier study the four main PMCA isoforms and their splice variants were expressed in Chinese hamster ovary (CHO) cells and their Ca^{2+} extrusion capacity was compared [84] in response to a G-protein coupled receptor agonist. In a more recent paper, the effects of PMCA isoforms were compared in a simplified model, where the shape of the Ca^{2+} signal relied mainly on the interplay between plasma membrane channels and PMCAs [85]. In this model three kinetically well-characterized PMCA isoforms (PMCA4b, 4a and 2b, respectively) were overexpressed in HeLa and HEK-293 cells and the down-regulation of store operated Ca^{2+} entry (SOCE) was compared. Surprisingly, the different activation and/or inactivation rates of these isoforms produced three distinct Ca^{2+} signal types (oscillation, transient spike or spike with sustained plateau) (*Figure 3B-C*). Mathematical modeling revealed that delayed activation of the slow PMCA4b was required for the formation of the oscillation pattern. It is interesting to note, that similar time delayed feedback mechanisms often induce oscillation in physiological systems. Indeed, the fast pumps PMCA2b and PMCA4a, which are activated quickly with no delay, could not generate such oscillation pattern. In this case a single calcium transient is produced but because of the distinct memories of these pumps – PMCA4a gets inactivated quickly while PMCA2b remains active for a long time – PMCA4a allows an elevated $[Ca^{2+}]_i$ after the spike while PMCA2b quickly reduces $[Ca^{2+}]_i$ to its basal level.

An earlier study also showed that plasma membrane Ca^{2+} pumps contribute to sustained intracellular Ca^{2+} oscillations in human bone marrow–derived mesenchymal stem cells [86].

However, in this case Ca^{2+} oscillations were generated by IP_3 induced Ca^{2+} release from ER stores and yet blocking of PMCA activity (that was shown to be PMCA4) completely eliminated oscillation. Similarly, in airway smooth muscle cells inhibition of PMCA1 and 4 abolished UTP-induced Ca^{2+}oscillations [87]. Interestingly, in a different system in HEK293 cells, that express preferably the PMCA1 isoform [85], neither Ca^{2+} entry nor Ca^{2+} extrusion by PMCA was necessary for sustained Ca^{2+} oscillation [88].

The main message of the above studies is that PMCAs being regulated by calmodulin, as well as by several scaffold and signal molecules in a complex way can effectively shape Ca^{2+} signals. Moreover, PMCAs themselves can transform Ca^{2+} transients into distinct patterns: oscillation, single spike or a spike with a plateau. The fact that PMCA variants can have such a great impact on the Ca^{2+} signal suggests that loss-of-function mutations or an aberrant expression and/or localization pattern could indeed contribute to the unbalanced Ca^{2+} signaling reported in a variety of disease states.

6. Downstream of Ca^{2+} signaling – the NFAT pathway

A change in the pattern of Ca^{2+} signaling might be coupled to changes in a variety of downstream signaling pathways [50, 89]. The role of PMCA as a regulator of downstream intracellular Ca^{2+} signaling has long been suggested. In this respect the nuclear factor of activated T-cells (NFAT) - a family of transcription factors regulated by calcineurin [90, 91] - has been the best-characterized pathway. A direct interaction was described between the calcium/calmodulin dependent phosphatase calcineurin and the catalytic core of both PMCA2 and PMCA4 resulting in a decreased activation of NFAT [92, 93]. These authors suggested that the inhibition of the calcineurin/NFAT pathway was a result of the suppressed activity of calcineurin due to its low Ca^{2+} microenvironment provided by the PMCA. In several breast cancer cell lines the same authors showed that by disruption of the interaction between calcineurin and PMCA2, the NFAT activity could be restored [94]. Another study suggested that PMCA4b interacted through its catalytic domain with the tumor suppressor RASSF1 protein and this interaction decreased the activation of ERK after epidermal growth factor stimulation of the cells [95]. These results suggested that PMCAs can inhibit the NFAT/AP-1 transcriptional activity in these model systems.

Distinct roles of PMCAs were proposed during the early and late phases of osteoclast differentiation [96]. In undifferentiated osteoclasts receptor activator of nuclear factor κB

ligand (RANKL) induces Ca^{2+} oscillations that trigger the activation of NFATc1, which regulates the transcription of osteoclastogenic genes. NFATc1 also binds to the promoter region of PMCA1 and 4 and increases their expression. The upregulation of PMCAs has a major anti-apoptotic effect in mature osteoclasts. In good accordance with these data knockdown of PMCA1 and 4 enhanced Ca^{2+} oscillation and osteoclastogenesis in bone-marrow derived macrophages and increased apoptosis in mature osteoclasts, while overexpression of PMCA1 or 4 strongly decreased RANKL induced Ca^{2+} oscillations and NFATc1 activity reducing osteoclastogenetic potential. These findings revealed the importance of PMCA as a core component of an autoregulatory loop between the NFATc1 and Ca^{2+} signaling pathways, and PMCA adjusting the course of osteoclastogenesis.

Endothelial cells provide another model system where the inhibitory effect of PMCA4 on NFAT activation has been described. *Baggott et al.* showed that overexpression of PMCA4b in human umbilical vein endothelial cells (HUVEC) decreased NFAT activity after vascular endothelial growth factor (VEGF) activation [97]. Furthermore, PMCA4 deficient mouse lung endothelial cells (MLEC) produced increased NFAT activation after stimulation. In addition, the expression of NFAT target genes COX2 and RCAN1.4, which are involved in angiogenesis, were inhibited by PMCA4 overexpression and were increased by PMCA4 silencing. These authors also found that PMCA4b overexpression decreased the migratory ability of HUVECs while PMCA4 deficient MLECs showed increased migration. It is important to note that PMCA4 did not affect cell proliferation and ERK phosphorylation in these models. The ability of the cells to form endothelial tubes was also investigated both in vitro and in vivo, and as a physiological consequence, PMCA4 decreased blood vessel formation and VEGF-induced angiogenesis.

An opposite effect of PMCA4 variants on NFAT activity was found in vascular smooth muscles cells (VSMC) [98]. In VSMCs from PMCA4b knock-out mice the expression of NFATc3 was decreased by the increase of two proteins, Decorin (G1 arrest marker) and regulator of G-protein signaling-16 (Rgs-16; MAPK inhibitor). This effect could be reversed by the overexpression of either PMCA4a or PMCA4b. Decorin and Rgs-16 have been shown to inhibit the calcineurin/NFAT pathway; hence PMCA4 by regulating signal molecules of the NFAT signaling network other than calcineurin could increase NFAT activity in certain cell system.

An inhibitory effect on NFAT signaling by PMCAs was also shown in neuroendocrine cells [99]. In PC12 cells, PMCA2 and PMCA3 are present in addition to the ubiquitous PMCA1 and 4. Downregulation of PMCA2 or PMCA3 in these cells caused a permanent increase in the intracellular Ca^{2+} level in resting cells and an increased Ca^{2+} influx after stimulation. This led to an elevated activation of NFAT 1 (NFATc2) and 3 (NFATc4) and consequently a reduction of dopamine secretion of the cells. These authors also showed that inhibition of NFAT with 11R-VIVIT changed splicing of the PMCA isoforms [100] suggesting that NFAT could control the production of certain PMCA2 and 3 splice variants in PC12 cells.

All these results highlight the complex relationship of PMCA and NFAT signaling that depends strongly on cell- and tissue types and intracellular localization of the pumps.

7. Targeting PMCAs to the appropriate membrane compartment

PMCAs are capable of completing their tasks efficiently when they are correctly placed in the right plasma membrane compartment. Localization of the PMCA to these compartments is a highly regulated process depending on both the properties of the PMCA variants, interaction partners and cell type.

Controlling plasma membrane abundance of PMCA4b – the C-terminal di-leucine-like motif. One way to regulate the function of plasma membrane proteins is to control their concentration in the plasma membrane; which is often accomplished by tyrosine- or di-leucine-based sorting signals [101, 102]. Recent experiments showed that a large portion of PMCA4b could reach the plasma membrane only in confluent cell cultures [23]. It was suggested that a di-leucine-based sorting signal at the C-tail of PMCA4b controls plasma membrane abundance of this pump. Mutation of this motif changed dramatically the subcellular distribution of the pump and increased its abundance in the plasma membrane compartment. Human PMCA4b has two independent di-leucine-based motifs at its C-tail of which the upstream motif (^{1147}LL/LI) is conserved in the b splice variants of all PMCAs (PMCA1-4) while the downstream motif (^{1167}LLL), which appears to be the main sorting signal, is present exclusively in PMCA4b. The upstream di-leucine motif was identified as a basolateral sorting motif in PMCA1b and PMCA2b [103] but not in PMCA4b [23].

In some cases the di-leucine-based sorting motif is masked by other molecules that prevent the protein containing the motif from internalization (E-cadherin, GABAB receptor) [104, 105]. In case of PMCA4b no such sorting-motif masking partner has been found, however,

interactions with distinct scaffolding proteins can alter localization of the pump in certain membrane compartments as discussed below in more details.

Cellular distribution of the PMCAs in polarized cells – the role of the A splice site. Establishment of cell polarity (in neurons, epithelial and migrating cells,) generating distinct plasma membrane compartments requires an even more complex regulation of PMCA distribution. Splicing at site A can change distribution of PMCAs in polarized cells (see also Section 3). The x and z splice variants of PMCA 1 and 4 localize mostly to the basolateral membrane compartment of polarized epithelia of lung, intestine and kidney where they can facilitate transcellular Ca^{2+} fluxes [65, 106]. The w insert at the A splice site is the longest and acts as a strong apical signal. One example where the w insert concentrates PMCA in the apical compartment is the stereocilia at the apical pole of mammalian sensory hair cells of the inner ear where PMCA2w/a is required for normal hearing [107-109]. Interestingly, the size rather than the particular sequence of the w insert was critical for apical targeting of PMCA2w/a [103]. Another example is the lactating mammary gland where the role of PMCA2w/b is to provide milk calcium as demonstrated in PMCA2 knock-out mice [110]. Apical membrane localization of PMCA2 has also been reported in salivary glands, where Ca^{2+} is secreted into the saliva by PMCA2 as a part of a transcellular Ca^{2+} transport system, including CaT-Like Ca^{2+} channels, calbindin-2 and SERCAs [111].

Cellular distribution of the PMCAs – the role of PDZ interactions. The different sequences generated by alternative splicing at the C splice site also influence subcellular localization of the PMCAs. At the C-termini of all b variants is a PDZ domain-binding sequence by which they can interact with various scaffold proteins [112, 113]. The PDZ-binding sequence in the PMCA1b, 2b and 3b variants are identical (-ETSL) while the last amino acid residue is different in PMCA4b (-ETSV). This minor change in the binding sequence results in different binding partners with varying affinities for the different PMCA isoforms. As a result the Na^{+}/H^{+} exchanger regulatory factor 2 (NHERF2) scaffold protein interacts specifically with PMCAs having the –ETSL binding sequence but not with that of the PMCA4b variant [114]. It was shown that in polarized MDCK cells PMCA2w/b was anchored to the apical cytoskeleton via a PDZ-domain mediated interaction with NHERF2 stabilizing and thus further increasing the amount of PMCA in the apical plasma membrane compartment [115]. PMCA1b trafficking to the membrane was enhanced in response to muscarinic G-protein coupled receptor activation and PDZ interaction with NHERF2 played a key role in maintaining the pump at the cell surface [116].

A distinct localization pattern could be generated by the interaction between the PDZ-binding sequence of the PMCA4x/b variant and its binding partner PSD-95 [117]. PSD-95 is a member of the membrane-associated guanylate kinase (MAGUK) scaffold family containing multiple protein-protein interaction modules that allow clustering of trans-membrane proteins. Co-expression of PMCA4x/b and PSD-95 in COS-7 and Neuro-2A cells increased the plasma membrane expression of PMCA4x/b and redistributed it into clusters within the plasma membrane. The PMCA4b/PSD-95 clusters were fenced by the underlying actin cytoskeleton resulting in reduced lateral mobility of the pump. Thus, distinct binding partners can alter local abundance of the pump via different type of contacts between PMCAs and the actin cytoskeleton, i.e. anchorage with NHERF2 or fence with PSD-95. Dean et al. also found that while in resting platelets little PMCA (PMCA1b and PMCA4b) was associated with the actin cytoskeleton, PMCA redistributed dramatically to the actin cytoskeleton upon activation with thrombin via PDZ mediated processes [118]. Interestingly, G-actin directly interacts with and stimulates the activity of the purified erythrocyte Ca^{2+} pump independent of the PDZ interactions [119].

PDZ-interactions can recruit PMCAs into distinct microdomains where they can specifically alter the activity of their interacting partners. In cardiomyocytes, where PMCA4b binds to neuronal nitric oxide synthase (nNOS) through its C-terminal PDZ-binding sequence, PMCA has a major structural function, rather than the global Ca^{2+} removal, by sustaining the integrity of the nNOS signaling complex in a plasma membrane microdomain. In PMCA4 knock-out animals more than one third of the nNOS showed cytoplasmic localization eventually resulting in increased cardiomyocyte contractility [120, 121].

PMCA localization in neurons. In neurons PMCA isoforms also show functionally distinct distribution pattern. In many neuronal cell types (hippocampal neurons, Purkinje cells, interneurons) the different PMCAs are concentrated in different microdomains of dendritic spines [24, 122, 123] or the presynaptic terminals [124, 125]; and several lines of evidence suggest that they play crucial role in the regulation of Ca^{2+} signaling at the synapse [124, 126, 127]. Many studies show that PDZ interactions with scaffolding proteins (e.g. PSD-95) recruit PMCA into the appropriate supramolecular complexes in neurons [128, 129]. Homer1 also interact with PMCA via its PDZ-binding motif [130]. This interaction was suggested to affect both PMCA activity and its recruitment to certain membrane microdomains. Homer2 also regulates PMCA4 expression and activity in non-excitable cells, however, not via the PDZ

binding motif but rather through a putative Homer-binding sequence located in the N-terminus of PMCA4 [131].

Garside et al. studied the localization of PMCA2 within a synapse enriched preparation in cerebellum and showed that PMCA2b interacts with PSD95 and the NMDA glutamate receptor subunits NR1 and NR2a at post-synaptic sites and with syntaxin-1A at the pre-synaptic sites [129]. At the presynaptic plasma membrane of the photoreceptor the localization of the PMCA is regulated by a unique protein complex including PSD-95 and another scaffolding protein membrane palmitoylated protein 4 (MPP4) [132].

In the aspiny dendrites of interneurons nicotinic acetylcholine receptors are functionally linked to PMCA2b via PSD-95 and the activity of the PMCA2b regulates the number of receptor clusters [133]. In different central nervous system (CNS) areas (spinal cord, brainstem, and retina) there is a compartmentalized interaction between the neuronal glycine transporter 2 (GlyT2), the Na^+/Ca^{2+}-exchanger1 (NCX1) and the PMCA2 and PMCA3 isoforms, that regulates GlyT2 activity. The GlyT2•PMCA2,3•NCX1 complex is found in lipid raft domains and PMCA and NCX activities are necessary for GlyT2 activity [134].

Studies on PMCA2 knock-out mice revealed that besides regulation of the presynaptic Ca^{2+} handling PMCA can also influence the morphology of neurons. The lack of PMCA2 dramatically altered the morphology of the Purkinje neurons in cerebellum [135]. Similarly, reduced size of the Purkinje cell dendritic tree was observed when PMCA activity was inhibited chronically [136].

Association of PMCA and lipid rafts. Localization of PMCA also depends on the composition of the microdomains (lipid rafts) in the plasma membrane. Sepulveda et al. found that in pig cerebellum only the PMCA4 isoform associates with cholesterol/sphingomyelin-rich lipid rafts while other PMCA variants are found in the detergent-soluble fractions [137]. In MDCK cells PMCA2w/b variant partitioned into low-density membranes associated with lipid rafts in the apical membrane. Depletion of membrane cholesterol resulted in reduced lipid raft association and loss of PMCA2w/b from the apical membrane [138]. Recently, Jiang et al. described that all PMCA isoforms can be located in lipid rafts in rat brain synaptosomal membranes. They found that the raft PMCA pool showed the highest specific activity and that activity decreased progressively with age [139]. Another study found that both PMCA and calmodulin are localized within lipid rafts in primary cultured neurons and this relationship

influences the function of the pump. The depletion of cholesterol strongly inhibited the raft-associated PMCA activity [140].

PMCA localization in migrating cells.

Ca^{2+} gradients have been recognized between the front and back of different types of migrating cells. Recent experiments demonstrated that a front-to-rear polarity of PMCA expression resulted in low cytosolic Ca^{2+} in the front of migrating human endothelial cells and suggested that this specific distribution of PMCA was at least partially responsible for the typical front-to-rear Ca^{2+} concentration gradient essential for directed and collective migration of endothelial cells [141]. Knocking-down or inhibition of PMCA activity abolished the Ca^{2+} gradient and impaired directed cell migration underlying the importance of PMCA in endothelial cell migration.

All these examples indicate that PMCA has highly specialized roles at distinct membrane regions such as cell junctions, post or pre-synaptic densities or lamellipodia. To recruit the PMCA to these sites, it is necessary to establish the appropriate protein-protein interaction with scaffolding proteins, actin cytoskeleton or regulator partner. These processes are determined by the localization signals of PMCAs which are highly various due to the A and C-splice sites. Both the C-tail and other regions of the pump variants may have additional, not yet recognized, trafficking/localization motifs that may further define their highly specialized tasks and shed light on the role of the less acknowledged forms of this large Ca^{2+}ATPase family.

8. A novel role of PMCAs in PI(4,5)P_2 signaling

PI(4,5)P_2 is a key signaling phospholipid molecule of the inner leaflet of the plasma membrane involved in cell motility, adhesion, exocytosis, endocytosis, and in many other vital cellular processes [142]. It regulates various channels and pumps, forms clusters with a variety of PI(4,5)P_2 -binding proteins and controls their function. PI(4,5)P_2 binding may occur via electrostatic interactions with polybasic regions and/or through binding to specific binding pockets of proteins.

PI(4,5)P_2 has long been known to activate PMCA [143]. Two acidic phospholipid-binding sites have been identified: one is the so called AL region near the A splice site close to transmembrane segment M3 [144, 145] and the other is the positively charged calmodulin

binding sequence at the C-tail (*Figure 1*). More recently, in addition to the previously determined positively charged regions, homology models of PMCA4b showed the presence of a ring of basic residues (the blue collar) on the cytoplasmic side, in the stalk region next to the membrane [146]. Placement of $PI(4,5)P_2$ molecules at random positions near the stalk disclosed 4 probable binding pockets in the collar of the PMCA4b molecule. Since almost all basic residues of the pockets are conserved among PMCAs similar $PI(4,5)P_2$ binding pockets are expected to be formed in nearly all PMCA isoforms.

In regions of the membrane which have a high concentration of PMCA, there will be an equilibrium between free and bound $PI(4,5)P_2$. The relation between free and bound $PI(4,5)P_2$ and its probable impact on cellular Ca^{2+} signaling is shown schematically in *Figure 4.* $PI(4,5)P_2$ molecules which bind to PMCA are protected from hydrolysis to inositol-triphosphate (IP_3) and diacylglycerol [146]. Since IP_3 triggers release of Ca^{2+} from intracellular stores [147], binding to PMCA will limit release of Ca^{2+}. Recently Pena et al showed that by binding $PI(4,5)P_2$ the myristoylated alanine-rich C-kinase substrate (MARCKS) also could control intracellular calcium mobilization during acrosomal exocytosis [148]. However, in addition to sequestering $PI(4,5)P_2$, PMCAs can remove Ca^{2+} from the vicinity of $PI(4,5)P_2$ molecules protecting them from Ca^{2+}-dependent phospholipase C mediated hydrolysis resulting in less IP_3 release throughout the agonist stimulation phase.

The above findings suggest that PMCAs are not simply Ca^{2+} gatekeepers but they offer double-protection for the cells during Ca^{2+} signaling by means of removing excess Ca^{2+} from the cytosol and controlling intracellular Ca^{2+} store depletion in the same time.

9. PMCAs in cancer cells and tumors.

The expression of PMCAs 2-4 is cell/tissue-type and differentiation-stage specific while the housekeeping form PMCA1 is expressed in the early embryonic state and does not change drastically during development. However, a specific splice shift from PMCA1b to PMCA1a occurs during the development of neurons [123, 149]. Tumorigenesis is generally associated with inhibition of cell differentiation. Numerous observations indicate that remodeling of intracellular Ca^{2+} homeostasis is an important step of tumorigenesis and metastasis formation [150-156]. Several types of Ca^{2+} entry channels – STIM/ORAI, secretory pathway calcium ATPase 2 (SPCA2)/ORAI1 and TRP channels - have been reported to be upregulated in cancer cells that may lead to an overall rise in intracellular Ca^{2+} concentration and

consequently to increased tumor progression. In addition, Ca^{2+} channels were shown to promote migration and invasion [157, 158]. In line with these observations, an altered expression of the PMCAs has been also reported in a variety of cancer cell types that was accompanied by a significantly altered Ca^{2+} signaling pattern. PMCA1 and PMCA2 mRNA expression levels were found to be upregulated, while PMCA4 expression was downregulated in breast cancer cell lines compared to non-tumorigenic breast epithelial cells [159, 160]. In addition, pronounced PMCA4b expression in normal, mature ductal breast epithelium has also been demonstrated [68]. Furthermore, downregulation of PMCA4 expression in colon carcinoma has been reported while a comparatively high level of PMCA4 mRNA level was detected in normal colon tissue [74]. Interestingly, specific downregulation of the PMCA4 protein expression level was observed in high-grade colon adenoma, colon cancer and lymph node metastasis [161]. These studies suggest that the abundance of PMCA4 is highly sensitive to the degree of differentiation of cells and that the expression of PMCA4 (PMCA4b in particular) is downregulated in tumors. In contrast to the frequent downregulation of PMCA4b expression in tumors, changes in the expression of PMCA1 do not show a clear enough picture to accurately predict its role in tumorigenesis. While a modest upregulation of PMCA1 has been described in breast cancer cell lines, downregulation of its expression was seen in SV40 transformed skin and lung fibroblast [162] as well as in oral cancers where PMCA1 was found to be epigenetically inactivated [163].

Upregulation of PMCA4b in differentiating cancer cell models. Epigenetic downregulation of the expression of differentiation-associated genes by deregulation of DNA methylation and histone acetylation are typical characteristics of cancer cells. Histone deacetylase (HDAC) inhibitors such as short chain fatty acids, suberoylanilide hydroxamic acid (Vorinostat) or valproic acid are known to induce differentiation and apoptosis, and block proliferation of tumor cells; hence they have been used in clinical trials as anticancer drugs [164-166]. While decreased PMCA expression was found in poorly differentiated tumor cells, differentiation inducing HDAC inhibitors were shown to upregulate the expression of distinct PMCA isoforms in the same type of cells. In MCF-7 breast carcinoma cells induction of cell differentiation by HDAC inhibitors or phorbol ester led to the selective enhancement of PMCA4b expression [68]. Moreover, it has been shown that induction of cell differentiation of colon and gastric carcinoma cell lines also leads to the upregulation of PMCA4b protein levels [67, 167]. It is important to note, that no changes in the expression of either PMCA1 or PMCA2 were described in either of the above differentiation models of colon or breast cancer

cell lines. In these model systems differentiation-induced increase in PMCA4b expression resulted in enhanced Ca^{2+} clearance from cells suggesting that PMCA4b could, in principle, inhibit Ca^{2+} entry-mediated tumor progression.

Correlation of Ca^{2+} pump expression profiles in cancer cells. PMCA4b upregulation is often accompanied by the differentiation-specific induction of the SERCA3 protein in cells of epithelial origin such as colon, lung and breast cancer cells [68, 152, 168, 169]. Also, downregulation of SERCA3 expression in tumors has been demonstrated [170, 171]. Interestingly, SERCA3 is controlled by Ca^{2+} itself; it gets activated only at relatively high Ca^{2+} concentrations that might be attained mostly locally near the opening of Ca^{2+} channels [172]. In that sense, SERCA3 may also allow larger Ca^{2+} signals to develop similarly to the PMCA4b pump with its delayed response to the same signal. Thus, simultaneous upregulation of SERCA3 and PMCA4b may be particularly well suited to the specific Ca^{2+} signaling character of the differentiated epithelial cells.

Little is known about the potential involvement of the third family of Ca^{2+}-ATPases, the secretory pathway calcium ATPases (SPCA), in cancer. SPCA1 was reported to have cell type specific effects: SPCA1 haploinsufficiency increased the incidence of squamous cell tumors of the skin and esophagus [173], whereas SPCA1 levels were significantly elevated in clinical samples of breast cancer of the basal subtype [174]. Alterations in SPCA1 levels had no substantial effect on global cytosolic calcium signaling. Rather, it affected the processing of proteins important in tumor progression. On the other hand, besides its conventional Ca^{2+} transporting role, a novel function was attributed to SPCA2, that is triggering store independent Ca^{2+} entry by direct interaction with plasma membrane Ca^{2+} channels [151]. Accordingly, high levels of SPCA2 in cancer cells result in sustained elevation of cytoplasmic Ca^{2+} concentration. Considering that cancerous cells often have reduced PMCA expression (thus reduced Ca^{2+} extrusion capacity), the effect of increased SPCA2 levels is synergistic with the decreased PMCA levels in tumorigenesis.

Conclusions

Since the early discovery of the erythrocyte Ca^{2+} pump [175] research progressed first on understanding the basic properties of PMCAs using biochemical and later molecular biological tools, isolated pumps or membrane preparations. The discovery of an increasing number of pump variants with distinct characteristics has raised further interests on their

biological function. From the major phenotypes of PMCA knockout mice we learned that PMCA1 is the house-keeping form; PMCA2 is essential for hearing and for providing proper amount of milk Ca^{2+}; and PMCA4 for male fertility. From the many studies both in situ and in vivo we also learned that the functional diversity of the pump isoforms probably arises from a not yet clearly understood regulation of their gene expression and the structural divergence between the regulatory regions of the variants. Accordingly, 1/ PMCA expression is development-, cell- and tissue-type specific; 2/ Subcellular localization, abundance and activity of individual PMCA isoforms can be altered by alternative splicing and interactions with specific adaptor, regulator and/or scaffold proteins; and 3/ Expression of pumps with distinct regulatory properties can have significant impact on the Ca^{2+} signal and can generate typical patterns such as oscillation, single spike or a spike with a plateau. These distinct types of signals then could lead to diverse downstream events altering cellular function and eventually cell fate.

Conventionally, the function of PMCAs is to maintain cellular Ca^{2+} homeostasis by removing excess Ca^{2+} from the cytosol. In addition to this vitally important task, a recently recognized new role of the PMCA is to control the abundance of free $PI(4,5)P_2$ molecules in the plasma membrane and the formation of IP_3, and thereby agonist induced discharge of the Ca^{2+} pool. The great diversity and cellular specificity of PMCAs imply that disturbances in any of the above PMCA function (altered expression, activity and/or cellular localization) may result in disease causing properties. Mutations in the ATP2B2 and ATP2B3 genes can cause deafness, or cerebellar ataxia, respectively. Reduced expression of PMCA1 has been associated with altered bone mineralization and gastric epithelial wound healing among others. Reduced expression of PMCA4, on the other hand, often coincides with tumorigenesis or may be linked to male infertility.

In summary, although we have learned a great deal about the structural basis of PMCAs' function, we still do not have a 3-D crystal structure of the molecule to understand structural details of auto-inhibition, interactions with regulator proteins and lipids nor their reaction steps. Although, we understand quite well why so many PMCA isoforms are needed to satisfy cellular needs, we still do not have isoform specific and high affinity small molecule inhibitors to ease studying the role of individual PMCA variants in cell function. During the last years it also became apparent that altered levels of PMCA abundance might be related to tumor progression, but the exact mechanisms that could control PMCA expression are not

very well understood. Thus, future research aiming to answer these questions may help to find new ways to prevent PMCA-associated diseases and reduce the risk of cancer.

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Figure legends

Figure 1: Model of plasma membrane Ca^{2+}ATPase

Schematic representation of membrane topology of the PMCA is shown on top. Catalytic phosphorylation site (P), ATP-binding site (ATP) , Acidic lipid (AL) binding region and calmodulin-binding sequence (CBS) are indicated. Among the alternative splice variants, only the A splice variants of PMCA2 isoform and C splice variants of PMCA4 isoform are shown.

Figure 2: C-terminal tail of PMCA4b

A, Schematic representation illustrating the composition of the binding sites and other motifs in the C-terminal tail of PMCA4b. Abbreviations of previously determined binding sites: CBS – calmodulin-binding sequence, LLL – di-leucine-like motif starting at ^{1167}Leu [23], PDZB – PDZ-binding sequence [24], PKC – protein kinase C phosphorylation sites [27], pp60src – pp60src phosphorylation site (^{1176}Tyr) [176]. The red lines show protease cleavage sites.
B, Disordered probability of the C-tail. The continuous and dotted lines show the disorder probability of the C-tail calculated by IUPRED (red), PREDISORDER (blue) and PONDR-VSL2 (green) predictors. Regions predicted to be disordered (scores are higher than 0.5) are marked with continuous lines. The blue stripes in the background indicate disordered binding regions predicted by ANCHOR program.

Figure 3: Ca^{2+} signal patterns formed by different PMCA variants

A, Different kinetic properties of the PMCA variants. The points refer to the activation and inactivation character (slow or fast) of the individual PMCA variants according to the parameters described in [42-45].

B-C, PMCA specific SOCE mediated Ca^{2+} signal patterns in epithelial cells. Simulations were performed using the kinetic parameters of the model from Paszty et al. [85], with permission from AAAS. *B,* Low expression level of PMCA; *C1- C3*, High expression levels of PMCA4b, PMCA2b and PMCA4a variants, respectively.

Figure 4: Binding of $PI(4,5)P_2$ to PMCA decreases the intracellular Ca^{2+} level by two mechanisms.

This scheme shows dynamic equilibrium between free and PMCA-bound $PI(4,5)P_2$. At a relatively high PMCA abundance $PI(4,5)P_2$ will bind to the PMCA, depleting the pool of free $PI(4,5)P_2$. In this case, less free $PI(4,5)P_2$ will be available for hydrolysis to IP_3. Less IP_3 will then release less Ca^{2+} from the intracellular stores, diminishing the Ca^{2+} signal in response to extracellular stimuli. At the same time, by binding $PI(4,5)P_2$ PMCA becomes more active hence removing Ca^{2+} from the cell more rapidly than in its unbound state.

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