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Plasma Membrane Calcium ATPases as Novel Candidates for Therapeutic Agent Development

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Abstract

Plasma membrane Ca^{2+} ATPases (PMCAs) are highly regulated transporters responsible for Ca^{2+} extrusion from all eukaryotic cells. Different PMCA isoforms are implicated in various tasks of Ca^{2+} regulation including bulk Ca^{2+} transport and localized Ca^{2+} signaling in specific membrane microdomains. Accumulating evidence shows that loss, mutation or inappropriate expression of different PMCAs is associated with pathologies ranging from hypertension, low bone density and male infertility to hearing loss and cerebellar ataxia. Compared to Ca^{2+} influx channels, PMCAs have lagged far behind as targets for drug development, mainly due to the lack of detailed understanding of their structure and specific function. This is rapidly changing thanks to integrated efforts combining biochemical, structural, cellular and physiological studies suggesting that selective modulation of PMCA isoforms may be of therapeutic value in the management of different and complex diseases. Both structurally informed rational design and high-throughput small molecule library screenings are promising strategies that are expected to lead to specific and isoform-selective modulators of PMCA function. This short review will provide an overview of the diverse roles played by PMCA isoforms in different cells and tissues and their emerging involvement in pathophysiological processes, summarize recent progress in obtaining structural information on the PMCAs, and discuss current and future strategies to develop specific PMCA inhibitors and activators for potential therapeutic applications.

INTRODUCTION

The plasma membrane calcium ATPases (PMCAs) are members of the large family of P-type ion pumps characterized by the formation of a phosphorylated intermediate (hence their name) during the reaction cycle [1]. P-type ion pumps are integral membrane proteins found in the plasmalemma or organellar membrane of all cells, where they are responsible for the generation and maintenance of steep ion gradients (e.g., Ca^{2+}, Na^{+}/K^{+} or H^{+} pumps) or the removal of toxic heavy metal ions (e.g., Cd^{2+}, Co^{2+}, Pb^{2+}, Zn^{2+}), among other functions [2, 3]. The PMCAs belong to the type IIB subfamily [4] or family 2 [5] (see www.tcdb.org) of P-type ATPases and represent the main high-affinity system responsible for the expulsion of Ca^{2+} across the cell membrane. Mammalian PMCA isoforms 1-4 are encoded by separate genes on chromosomes 12q21.3, 3p25.3, Xq28, and 1q32.1 in humans, and on chromosomes 10C3, 6E3, XA7.3, and 1E4 in mice, respectively. Each of the PMCA genes (human genome database nomenclature ATP2B1-ATP2B4) shows a complex intron-exon structure including multiple alternatively spliced exons. Alternative RNA splicing at two main “hotspots” referred to as sites A and C leads to the production of multiple splice variants for each PMCA isoform [6]. Over 30 different PMCA splice forms can thus be generated in

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mammalian organisms. A schematic overview of the main splice options and the nomenclature for labeling the PMCA splice variants is given in Figure 1.

PMCAs are single polypeptides of about 1,100 to 1,250 amino acid residues with a molecular mass of 125 to 140 kDa. They contain 10 membrane-spanning segments and their N- and C-termini are both on the cytosolic side, as is the bulk of their mass including the two major intracellular loops making up most of the A (actuator) and N/P (nucleotide-binding/catalytic phosphorylation) domains [7]. In animal PMCAs, the C-terminal tail is crucial for pump regulation: it contains the auto-inhibitory sequence that interacts with the major cytosolic loops to keep the PMCA in a relatively inactive state at low $[Ca^{2+}]_i$, as well as the calmodulin-binding domain (which partially overlaps with the auto-inhibitory domain) and sites of regulatory phosphorylation and protein-protein interactions [7-11]. A generic scheme of the PMCA illustrating its main domain architecture is shown in the top panel of Figure 1.

The importance of the PMCA for the maintenance of intracellular Ca^{2+} homeostasis was recognized soon after the existence of such a dedicated system for expelling Ca^{2+} against a large concentration gradient had been demonstrated [12, 13]. However, because the function of the PMCA was thought to be essentially that of a generic “sump pump” responsible for restoring and maintaining the low global resting $[Ca^{2+}]_i$ level following a temporary increase, there was only modest interest in pursuing the PMCA as a drug target in the absence of more specific information on tissue expression and functional specialization. As will be discussed below, formidable additional challenges have also hampered earlier attempts to develop PMCA modulators. On the other hand, the advent of molecular cloning, whole-genome and transcriptome sequencing revealed the presence of a large family of different PMCA isoforms, and work over the past 10-15 years has shown that many PMCAs fulfill specialized roles and are expressed in cells and tissues in a spatially and temporally restricted manner. Together with rapidly emerging evidence that these pumps play diverse roles in different diseases, these recent developments have dramatically increased the interest in targeting the PMCAs for therapeutic agent development.

PMCA DIVERSITY SUGGESTS FUNCTIONAL SPECIALIZATION

The presence of a large family of PMCA isoforms and splice variants immediately begs the question of the functional significance of this diversity. Attempts to address this question have included determining the expression pattern of the PMCAs during development and in different organs and tissues, studying their subcellular localization in specialized cell types, and their co-localization with proteins that may be functionally connected. In parallel, biochemical studies on isolated PMCAs either purified from a suitable tissue source (mainly human erythrocyte ghosts) or expressed as recombinant protein have been instrumental in determining enzymatic, regulatory, and structural differences among isoforms and splice variants. Combined with *in vitro* mutagenesis studies, Ca^{2+} signaling assays in cultured cells, and physiological experiments on tissues and in animal models, these approaches have clearly shown that PMCA isoforms and splice variants perform very different functions ranging from vectorial transport of bulk Ca^{2+} to fine-tuning local Ca^{2+} signaling events in localized membrane microdomains.

Evidence from Expression and Localization

Studies at the mRNA and the protein level indicate that PMCA1 is ubiquitously expressed in all tissues [14-17] and can be detected by *in situ* hybridization from the earliest time points (9.5 dpc) in mouse embryonic development [18]. Consequently, PMCA1 has been referred to as a “housekeeping” PMCA, although this terminology is misleading and should be abandoned in the face of evidence showing that different PMCA1 splice variants are

uniquely expressed in defined cell types (e.g., fast muscles and specific neurons) where they probably perform specialized functions [15, 16, 19-23]. PMCA4 is similarly detected in most adult tissues although it appears later in mouse development (around 12.5 dpc) and at much lower levels [18]. PMCA4x/b is the major pump isoform found in human erythrocytes, whereas PMCA4z/a and 4x/a are relatively abundant in the heart and smooth muscles [24]. In contrast to PMCA1 and PMCA4, PMCA2 and PMCA3 show a much more restricted tissue distribution, with abundant expression in specific areas of the brain and in skeletal muscle [24]. The full complexity of PMCA isoform expression becomes evident when the dynamic patterns of splice variant distribution are taken into account. Thus, even within the same tissue or cell type, multiple PMCAs are frequently expressed simultaneously, but at different levels and with distinct subcellular localization. This is especially notable in highly polarized cells such as in retinal photoreceptor cells where PMCA1b is present on the inner segment and synaptic terminal membrane and PMCA4b is restricted to the synaptic terminals [25], or in cochlear hair cells where PMCA2w/a is exclusively present on the apical stereocilia and PMCA1x/b on the basolateral membrane [26].

It is therefore not surprising that the lack or altered activity of specific PMCAs results in very different phenotypes and pathological outcomes, supporting the notion of functional specialization of these pumps.

Evidence from Interacting Proteins

Evidence for the functional differentiation among PMCAs has also come from molecular, biochemical, and cellular studies that have identified a growing number of proteins that specifically interact with one or several PMCA isoforms or splice forms. The binding of calmodulin as the major regulator of the PMCAs has already been mentioned. Although all PMCAs can bind calmodulin, different isoforms and the C-terminal splice variants “a” and “b” of each isoform differ significantly in their affinity for calmodulin and more importantly, in the kinetics of activation/inactivation by calmodulin [27-30]. Yeast two-hybrid and other protein interaction search strategies have uncovered a surprisingly large and diverse number of proteins that interact with different domains of the pumps [24, 31, 32]. A schematic summary of these interactions is shown in Figure 2. The C-terminal sequence of the b- (and most c- and d-) splice variants of all PMCAs conforms to the consensus binding sequence for PDZ domain-containing proteins. Accordingly, many PDZ proteins have been shown to interact promiscuously with multiple PMCA isoforms or more selectively with specific PMCAs via their C-terminal tail. These include membrane-associated guanylate kinase (MAGUK) family members such as PSD95, SAP97, and CASK [33-35] as well as diverse scaffolding, signaling and regulatory proteins including CLP36, NOS-1, Homer, NHERF and PISP/AIPP1, among others [36-40]. Additional proteins were shown to bind to the N-terminal tail (14-3-3ε; [41]) or distinct regions of the intracellular loops of some PMCAs (RASSF-1, calcineurin A, α1-syntrophin) [42-44].

Interactions with these proteins serve to target PMCAs to distinct membrane domains, recruit them into multi-protein signaling complexes or to modulate their activity and regulation [11, 32, 45-48]. These dynamic protein-protein interactions further augment the functional diversity of different PMCA isoforms.

Evidence from *In Vitro* Biochemical Studies

Early biochemical studies on the enzymatic, regulatory and Ca^{2+} transport properties of the PMCA were performed on material isolated from human erythrocytes [49-52], which was found to correspond mainly to isoform PMCA4b [53]. The advent of molecular cloning and expression of recombinant proteins in different eukaryotic systems allowed subsequent

studies on specific PMCA isoforms and splice variants overexpressed in mammalian cells (COS, CHO) as well as in the baculovirus insect cell and in yeast expression systems [28, 30, 54-57]. These studies unequivocally showed that PMCA isoforms differ significantly in their basal Ca^{2+} ATPase activity, apparent Ca^{2+} affinity and regulation by Ca^{2+}/calmodulin, activation or inhibition by protein kinase C, and phosphorylation by other kinases (e.g., PKA, tyrosine kinase src) [24]. This is also true for the major C-terminal alternative splice variants (a and b) of the same PMCA isoform, a finding that is not surprising given that the splice directly affects the calmodulin-binding domain (see Fig. 2). Perhaps most significantly, detailed kinetics studies showed that a major distinctive feature of different isoforms and C-terminal splice variants (at least of PMCA2a and 2b, PMCA4a and 4b) concerns their calmodulin association and dissociation rates, which in turn determines the timing of their activation and inactivation [27, 29, 58, 59]. This feature is largely responsible for the finding that there are “fast” and “slow” PMCA isoforms and for what has been called the “memory effect” of the PMCAs, i.e., the concept that PMCAs with slow off-rates for calmodulin dissociation react more rapidly to successive Ca^{2+} spikes after an initial Ca^{2+} transient due to calmodulin that remains bound to the pump during the intermittent decrease in $[Ca^{2+}]_i$ [28, 58]. Different PMCAs thus have different “memories” for past activation, resulting in significant differences in how they handle different Ca^{2+} loads.

Evidence from Calcium Signaling Studies

To show that PMCA isoforms differ in how they handle intracellular Ca^{2+} loads, Ca^{2+} imaging studies have been performed in intact cells (over-) expressing specific PMCAs. Most studies have either used global Ca^{2+} imaging with (ratiometric) fluorescent Ca^{2+} indicators (e.g., Fura-2) or global or organelle-specific imaging using the aequorin/coelenterazine luminescence system. This work demonstrated differences between PMCA isoforms in their efficiency of returning single evoked Ca^{2+} spikes of similar amplitude back to the resting level, as indicated by characteristically different $t_{½}$ values (half-times of the decay of a given Ca^{2+} spike). In addition, PMCA isoforms also showed different capacity to reduce the amplitude of Ca^{2+} signals elicited by IP3-mediated release from the endoplasmic reticulum [60, 61]. In a more recent study, the genetically engineered Ca^{2+} sensor GCaMP2 was used to demonstrate the very different, isoform-specific capacity of PMCA2 and PMCA4 in handling ATP-induced Ca^{2+} loads in MDCK cells [62]. Evidence for isoform-specific function *in vivo* has also come from studies in which different PMCAs have been selectively down-regulated by RNA knock-down strategies. In an interesting recent study in MDA-MB-231 breast cancer cells, which express both PMCA1b and 4b, Curry et al. found that silencing of PMCA1 resulted in a very different cellular response to ATP-evoked Ca^{2+} signals than silencing of PMCA4, and that the outcome on cell survival and apoptosis was also distinct [63]. Although the above studies represent only a small fraction of the many reports that have investigated the role of different PMCAs in cellular Ca^{2+} handling, they serve to illustrate the non-equivalence of the PMCA isoforms and their functional specialization to fulfill different tasks in cell regulation. This, in turn, suggests that targeting specific PMCAs holds promise in manipulating distinct cellular signaling pathways for potential therapeutic benefit.

PMCA INVOLVEMENT IN DISEASE

Genetic Evidence from Mutations, Gene Deletion, and Genome-wide Association Studies

The most compelling evidence for the functional specificity of different PMCAs stems from both natural gene mutations and targeted gene manipulations. Targeted deletion of PMCA1, 2, and 4 (Atp2b1, Atp2b2, Atp2b3) in mice showed vastly different phenotypes with homozygous loss of PMCA1 being embryonic lethal, whereas loss of PMCA2 results in deafness, imbalance and ataxia, and loss of PMCA4 results in male infertility with otherwise

few overt consequences [64-67]. The early embryonic lethality of PMCA1 knockout mice during the pre-implantation stage supports the essential role of this ubiquitous isoform. While this is not surprising, the phenotypes of Atp2b1+/-heterozygotes are perhaps more interesting; studies in these animals showed an important contribution of PMCA1 to (bladder) smooth muscle contractility and cellular susceptibility to apoptosis [65, 68, 69]. From a human disease standpoint, the most exciting recent developments concern findings showing a causal link between PMCA1 genetic variants and hypertension and cardiovascular disease risk [70, 71]. Studying mice with vascular smooth muscle cell-specific Atp2b1 deletion, Kobayashi and co-workers found that PMCA1 knockout resulted in elevated basal [Ca^{2+}] as well as increased phenylephrine-stimulated [Ca^{2+}] in cultured vascular smooth muscle cells [72]. Importantly, phenylephrine-induced vasoconstriction in arterial rings was higher in the absence of PMCA1, and these mice exhibited elevated systolic blood pressure under resting conditions. Reduced PMCA1 expression and function in the vasculature are therefore a risk factor for hypertension.

The importance of PMCA2 in hearing and balance is obvious from the severe phenotype of engineered Atp2b2-/-mice [64] as well as from numerous spontaneous or chemically induced Atp2b2 mutants whose names (deafwaddler, wriggle, joggle, oblivion, etc.) reflect some of the most notable deficiencies in the affected animals [73-76] (for review, see [77]). The expression, proper targeting (e.g., to the stereocilia of cochlear outer hair cells) and function of PMCA2 are essential for hearing as well as other specific physiological processes such as calcium transport into milk [78], motor neuron coordination and cerebellar synaptic plasticity [79-82]. Specific missense mutations in the ATP2B2 gene have also been identified in at least two separate human pedigrees with congenital sensorineural hearing loss, providing unequivocal evidence for the involvement of this PMCA isoform in specific disease pathology [77, 83, 84].

Evidence for the involvement of PMCA3 in disease is currently sparse. However, exome sequencing recently revealed a missense mutation in the ATP2B3 gene of a human family afflicted with congenital X-linked cerebellar ataxia [85]. As mentioned earlier, PMCA3 is expressed in a highly tissue-specific manner mainly in excitable cells, including at cerebellar parallel fiber-Purkinje neuron synapses [23, 86]. Thus, deficiencies in PMCA3 function may well underlie or contribute to a variety of neuronal communication disorders.

The male infertility of Atp2b4 (PMCA4) knockout mice has already been mentioned. Proper function of the PMCA4 isoform is essential for dynamic Ca^{2+} handling to allow hyperactivated sperm motility [65, 66]. Although it is not yet clear if PMCA4 is the major PMCA isoform in human sperm, targeting the PMCA with specific inhibitors has already been proposed as a potential male contraceptive strategy [87]. What has become clear, however, is that PMCA4 - although expressed widely in most tissues - is not so much a “generic” pump for bulk Ca^{2+} export but rather a signaling molecule involved in highly cell-specific local Ca^{2+} signaling [24, 32]. Genetic manipulation of PMCA4 expression has demonstrated the importance of this pump isoform in cardiovascular biology [32, 88]. Overexpression of PMCA4b specifically in the heart of transgenic rats and mice showed a role for this pump in the agonist-mediated hypertrophic response of cardiomyocytes, an effect mediated in part by the signaling function of PMCA4b via its physical interaction with nitric oxide synthase (nNOS) [89]. The PMCA4b/nNOS-syntrophin interaction also influences the β-adrenergic contractile response in cardiomyocytes; overexpression of PMCA4b resulted in a decreased contractile response by affecting NOS-dependent cGMP/cAMP signaling [90, 91]. Inducible overexpression of PMCA4b in the mouse heart also antagonized pressure overload-induced cardiac hypertrophy due to the interaction of PMCA4b with calcineurin [43] and the resulting decrease in NFAT signaling [92]. Accordingly, adult Atp2b4-/-(PMCA4 knockout) mice showed a slight increase in heart

weight and pressure overload-dependent cardiac hypertrophy [92]. On the other hand, overexpression of PMCA4b in vascular (arterial) smooth muscles of transgenic mice resulted in increased blood pressure related to the PMCA4-mediated inhibition of NOS function [93, 94]. Further evidence for the importance of PMCA4 in (human) disease stems from a recent genome-wide association study (GWAS) comparing severe malaria patients and controls from large African population cohorts. This study found a strong association of multiple SNPs (single nucleotide polymorphisms) within the ATP2B4 gene with severe malaria [95], suggesting that alteration of PMCA4 expression or structure/function contributes to the susceptibility and resistance to severe malaria. Importantly, PMCA4 is the major PMCA isoform of human erythrocytes, contributing over 70% of the total PMCA in this tissue [53].

Indirect Causes: Overexpression, Down-Regulation, Altered Localization and Dysregulation

There is now a large literature on the correlation of altered expression, localization, regulation and function of different PMCA isoforms with pathophysiological processes. While correlation should not be equated with causality, the strong association of the change in a specific PMCA with a distinct phenotypic outcome suggests at least an indirect contribution of altered pump function to the observed outcome. The studies using knockdown approaches (using antisense oligonucleotides, siRNAs) or overexpression (by transient and stable transfection of cells in culture) are too numerous to describe individually but they have provided unequivocal evidence for the selective role(s) that different PMCA isoforms and splice variants play in processes such as cell differentiation, cell proliferation, protection against excitotoxicity and apoptosis, or regulation of amplitude and frequency of agonist-evoked Ca^{2+} signals in different cell types [47, 61, 96-101]. A recent study nicely demonstrated the different roles of PMCA1 and PMCA4 in bulk Ca^{2+} handling and the regulation of cell death pathways in the same MDA-MB-231 breast cancer cell line [63]. Studies on tissues and cells reflecting different diseases including diabetes, osteoporosis, hypercalciuria, hypertension, cancer, and neurodegenerative disease suggest that different PMCA isoforms play central roles in the pathological outcomes, although in most cases their altered expression and regulation is probably a consequence, rather than the cause, of the underlying disease [7, 102-105]. Nevertheless, the specific link of different PMCAs with defined signaling outcomes suggests that pharmacological targeting of these pumps should be of therapeutic interest [104, 106]. For example, robust changes in the expression of PMCA1, 2, and 4 have been reported in several cancers, e.g., breast and colon, leading to remodeling of cellular Ca^{2+} handling and subsequent changes in gene transcription, cell differentiation and proliferation [107-111]. Because silencing of PMCA1 and PMCA4 has different consequences on cell viability and apoptotic response to toxic stress [63], targeting the individual PMCA isoforms may be useful as a tailored approach in therapeutic applications.

PMCA ISOFORMS AS THERAPEUTIC TARGETS

Challenges for the Development of Specific PMCA Inhibitors

The PMCAs are integral membrane proteins like many receptors and ion channels that have long been recognized as a major class of drug targets. Given the overwhelming evidence for the specific involvement of PMCAs in disease, why is there not a single drug on the market targeting these proteins specifically? To answer this question, it is useful to consider some of the peculiar features of the PMCAs. Unlike traditional ligand-dependent receptors or ligand-gated channels, the PMCAs have no specific peptide or small molecule extracellular ligand and hence there is no obvious targetable binding site for agonist or antagonist development from the outside of the cell. Most of the mass of the PMCAs is intracellular (see, e.g., Fig.

2), with only short extracellular loops connecting adjacent transmembrane domains. Thus, there is only limited access to the protein from the outside for possible drug targeting. The generally low abundance of the PMCAs in natural cell membranes, together with the difficulty of purifying or enriching them in an intact form, and the fact that most cells express more than one isoform have further hampered attempts to develop specific PMCA modulators. Although pump function can be relatively easily assayed (e.g., as enzymatic Ca^{2+}-dependent ATPase activity or $^{45}Ca^{2+}$ uptake into microsomal vesicles), the low amount of PMCA in the membrane raises signal-to-noise issues due to the presence of contaminating ATPases. The large diversity of isoforms and splice variants presents a further challenge to the development of selective PMCA modulators. In this context, the lack of high-resolution structural information on much of the PMCA molecule has been a further serious impediment to the rational design of modulators of pump function (but see below for recent advances and new opportunities).

Generic PMCA Inhibitors Lack Specificity

Several small molecule inhibitors of PMCA function have been in use for over 30 years and have been extensively applied to block the pumps in applications ranging from *in vitro* Ca^{2+} uptake studies to physiological studies evaluating the contribution of the PMCAs to cellular Ca^{2+} homeostasis and specific Ca^{2+}-dependent events such as neurotransmitter release or epithelial Ca^{2+} transport [13, 112]. The most frequently used strategies to block PMCA function are La^{3+}, orthovanadate, fluorescein analogs (e.g., (carboxy-)eosin), extracellular alkalization, and antagonizing calmodulin action (trifluoperazine, calmidazolium). These agents can be grouped into the 4 major categories of inorganic ions (La^{3+}, VO_3^-), molecules interacting with amino acid side chains (eosin), calmodulin antagonists, and thermodynamics-based blockade of counterion transport (reduced availability of protons due to alkalization). These modulators obviously work by very different mechanisms: La^{3+} blocks the conformational transition of the phosphorylated pump from the E1-P to the E2-P state [113]; orthovanadate acts as a transition state analog of phosphate and blocks the E2 to E1 step of the pump cycle [114]; eosin and other fluorescein analogs interact with the ATP binding site [115]; and calmidazolium (and other calmodulin antagonists) prevent calmodulin-stimulation of the PMCA but may also inhibit by direct interaction with the pump [116, 117]. Although all of these inhibitors are small, easy to use in most cellular applications, and relatively inexpensive, they are all non-specific with significant “off-target” effects (e.g., calmodulin inhibitors!) and most importantly, they are not isoform-selective.

Development of Antibodies and Inhibitory Peptides with Isoform Specificity

Both polyclonal and monoclonal antibodies have been raised against different PMCA isoforms [17, 118-123] and have found widespread use in applications ranging from mapping of structural domains to tissue expression and cellular localization studies. When the epitopes for several of the monoclonal antibodies were delineated [122, 124-126], only two antibodies recognized extracellular peptide sequences [119, 122] and very few affected pump function, either modestly stimulating or partially inhibiting Ca^{2+} ATPase activity [119, 120, 124]. Because of the very limited accessibility of the PMCAs from the extracellular side, raising antibodies recognizing the PMCAs on intact cells has been challenging. Perhaps more unexpected is the apparent difficulty of generating high-affinity antibodies that inhibit PMCA function. Antibodies thus appear to be of limited usefulness as pump modulators.

A more promising strategy to develop PMCA-specific inhibitors was introduced by Ashok Grover, whose laboratory focused on isolating short (~10-15 residues) PMCA-binding peptides by panning of random peptide phage display libraries with specific PMCA

sequences corresponding to the pump's extracellular loop regions [127]. These so-called caloxins (for extracellular plasma membrane Ca^{2+} pump inhibitors, in analogy to the Na^+/K^+ pump inhibitor digoxin) act as allosteric modulators of the PMCA presumably by interfering with important conformational transitions that occur during normal pump function [128]. Caloxins against the first, second and third extracellular loop of the PMCA (see Fig. 1) have been developed and accordingly were named caloxins 1, 2, and 3, respectively (see [127] for explanation of the caloxin nomenclature). While first generation caloxins are not very isoform-specific and have relatively high K_i values on the order of 0.5 mM (e.g., caloxin 2A1 [129]), the more recently developed caloxins (e.g., caloxin 1B1) show considerable isoform-selectivity and improved (lower) K_i values (~50 μM) when added to the medium of intact cells [130]. Improved affinity and isoform selectivity were achieved by increasing the stringency of screening (using the full-length PMCA in addition to the synthetic PMCA peptides as target) and introducing selective amino acid changes to further optimize the caloxins. This has resulted in the B- and C-series caloxins, respectively, e.g., caloxins 1B1 and 1C1 [130, 131]. Considerable isoform-selectivity has been reported for some of these newer caloxins, with caloxin 1B1 showing about 2-fold higher efficacy against PMCA4 than PMCA1 [130] and caloxin 1B3 being more effective against PMCA1 (K_i ~ 17 μM) than against PMCA4 (K_i ~45 μM) [132]. The value of these more selective caloxins for whole-cell physiological studies of PMCA isoform function has been demonstrated in several recent publications, e.g., showing the importance of PMCA1 in endothelial cell Ca^{2+} regulation [132] and of PMCA4 in aortic smooth muscle contraction [130, 131]. Caloxins with K_i values in the low micromolar range are now available [127] and it is realistic to expect that further improvements in selection strategies and/or chemical modification will lead to the development of new generations of caloxins with even higher affinity and selectivity for specific PMCA isoforms.

High-throughput Chemical Library Screening

The need for relatively large amounts of highly purified and functional PMCA has been a major hurdle in the development of high-throughput assays. This is especially true for isoform- or splice variant-specific screenings because there is no natural tissue source that expresses only a single PMCA, and over-expression and purification of recombinant PMCAs in eukaryotic systems are not trivial. However, the minimization of assay volumes (in nanodrops or sub-nanoliter compartments) and increasingly sensitive detection methods for assay output (e.g., fluorescence-based enzymatic assay for product release) should help to mitigate this problem. Indeed, the idea of performing high-throughput screens for PMCA inhibitors has already been proposed as a desirable and viable strategy to identify specific inhibitors of PMCA4 in the treatment of cardiovascular disease [88] and in male fertility control [87]. The first proof-of-principle study describing an assay that may be suitable for the high-throughput screening of large libraries of small compounds as modulators of PMCA4 function is reported in this Special Issue of the Journal of Pharmacy and Pharmaceutical Sciences [133], showing the promise of this approach for the discovery of novel and specific PMCA modulators. Importantly, such assays can (and should) be geared towards identifying both inhibitors and activators of specific PMCAs because different pathological conditions will require antagonists to suppress the activity of a specific PMCA isoform (e.g., PMCA4 in sperm) or agonists to selectively increase a pump's activity (e.g., PMCA2 in hearing).

FUTURE PROSPECTS

The evidence for the involvement of specific PMCA isoforms and splice variants in diverse human pathologies is overwhelming, reflecting their tissue-, developmental-, and context-specific contribution to normal Ca^{2+} handling and their integration in complex Ca^{2+} signaling events. With our increased understanding of where, when, and why a specific

PMCA isoform is required in normal cell function and how this may change in disease, it is now clear that the PMCAs should be considered as novel targets for therapeutic intervention. Both, inhibitors and activators are needed to modulate the activity of specific PMCAs. While it is generally simpler and more straightforward to identify and develop inhibitors of an enzyme, the unique regulation of the PMCAs opens equally promising avenues for the development of activators. This is so because the PMCAs are intrinsically auto-inhibited, i.e., they are normally in a low-activity basal state and need to be activated (by calmodulin, phosphorylation, acidic phospholipids, oligomerization) to increase their Ca^{2+} extrusion rate. In addition, the specific association of different PMCAs with scaffolding, regulatory and signaling proteins via direct protein-protein interactions [24, 45, 134] opens the door to targeting these interactions as a means to modulate PMCA function. The delineation of specific protein-binding domains in the PMCAs is facilitating random screenings of high-throughput small molecule libraries but is also helping with the development of rationally designed compounds because atomic resolution structures are more easily obtained for defined soluble protein-binding subdomains than of the entire PMCA membrane protein. The regulatory C-tail of the PMCAs is of particular interest because it contains multiple protein-binding domains including the calmodulin-binding and PDZ protein-interacting domains as well as domain(s) involved in the auto-inhibitory interaction with the cytosolic loops of the pump. The NMR solution structure of the calmodulin-binding domain of PMCA4b bound to calmodulin has been reported in 2010 [135] and an X-ray crystal structure of the *A. thaliana* PMCA regulatory domain in complex with two calmodulin molecules has been determined even more recently [136]. Similarly, structures of the complex of specific PDZ domains with the PDZ protein-interacting C-terminal tail of different PMCA isoforms should soon become available, further demonstrating the feasibility of high-resolution structural information on specific domains of the PMCAs. Moreover, the structural homology of the PMCAs to the SERCA (sarco/endoplasmic reticulum Ca^{2+} ATPase) pumps, for which multiple X-ray crystal structures have been obtained in different conformational states [137, 138], is further assisting with structural studies of the PMCAs. Combined with increasingly sophisticated molecular modeling and biophysical and functional data from mutagenesis experiments, this is resulting in structural models (Figure 3) that may serve as a framework for further studies focusing on the development of modulators targeting specific domains of the PMCA for functional intervention.

While the PMCAs remain challenging targets for the development of modulating agents, the discovery of isoform-specific protein-protein interactions involving defined PMCA domains, combined with increasing structural information on these domains and their protein complexes, will undoubtedly lead to increased efforts to identify selective inhibitors and activators of these important membrane transporters. It is attractive to envision the not-too-distant future where PMCA modulators are part of a physician's arsenal of pharmaceutical tools in the treatment of diseases ranging from cardiovascular and neurological disorders to cancer and osteoporosis.

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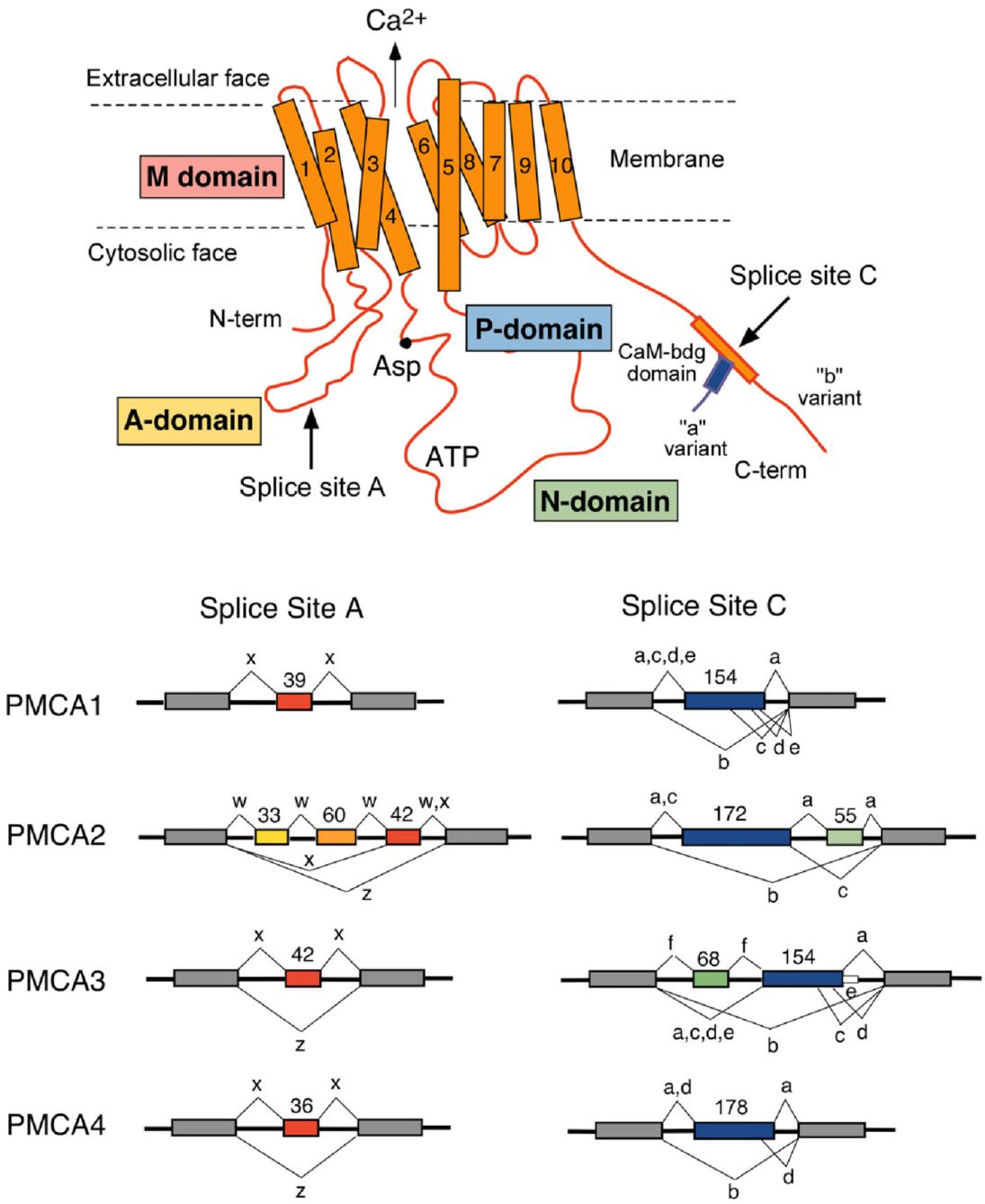

Figure 1.

Scheme of the PMCA (top) and of the alternative splice options generated from the 4 mammalian PMCA genes (bottom). The major domain organization of the PMCA is shown in the scheme on top. The ten membrane-spanning regions are numbered and shown as cylinders forming the M domain. The amino- (N-term) and carboxy-terminal ends (C-term), the conserved aspartate (Asp) residue undergoing phosphorylation during the reaction cycle, and the ATP binding site (ATP) are labeled. The direction of Ca^{2+} transport is indicated by an arrow. The three main cytosolic domains are labeled A (actuator), P (phosphorylation), and N (nucleotide-binding). Splice sites A and C are indicated by arrows. Splicing at site C affects the calmodulin-binding (CaM-bdg) domain and results in major splice variants “a”

and “b”, which differ in their C-terminal amino acid sequence due to a shift in reading frame (indicated as separate lines). Bottom: Exon structure of the regions involved in alternative splicing in the four human PMCA genes. Constitutively spliced exons are shown as gray boxes. The sizes of alternatively spliced exons (colored) are given in nucleotides, the alternative splicing options are indicated by connecting lines, and the resulting splice products are labeled by their lowercase symbol. Combinatorial use of the splice options at sites A and C yields over 30 possible PMCA splice variants.

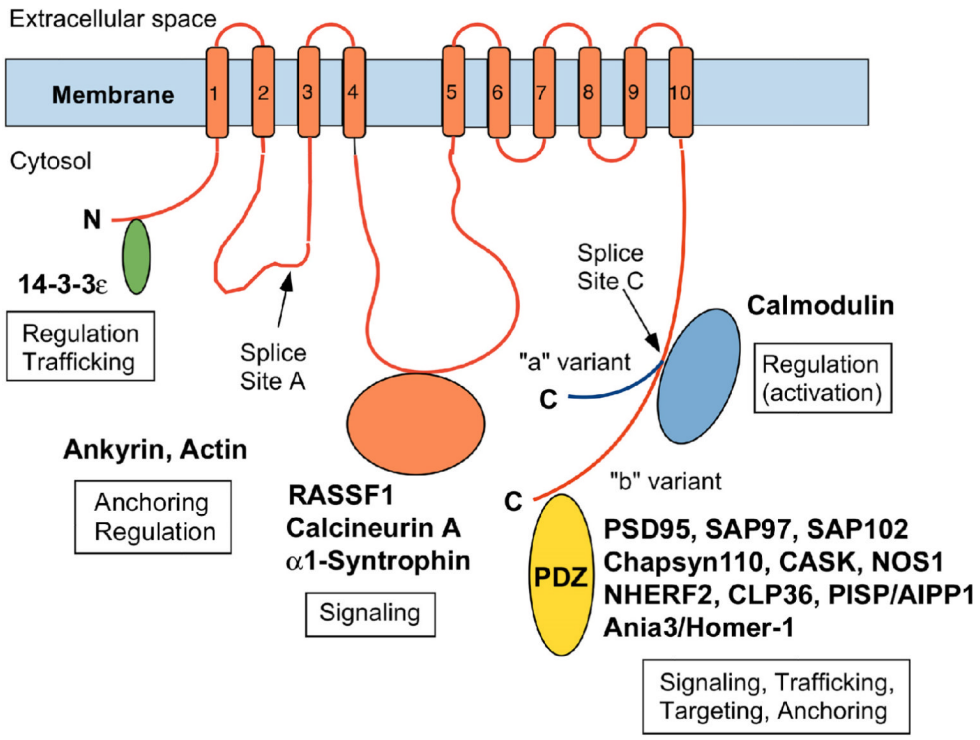

Figure 2.

Interacting protein partners of the PMCAs and their roles in PMCA regulation and function. PMCA-binding proteins are schematically shown as colored ovals near the domain of the PMCA where they interact. Known or suspected roles of these proteins in PMCA function, regulation, and signaling are indicated in boxes beneath the listed proteins. The two major sites of alternative splicing are also indicated, and the main C-terminal splice variants “a” and “b” are shown with separate C-tails to indicate their sequence divergence. Note that PDZ domain-containing proteins (PDZ) only bind to “b-splice” variants of the PMCA, and that not all PMCA isoforms interact with all of the listed proteins. Ankyrin and actin also interact with the PMCA although the site of interaction has not yet been established.

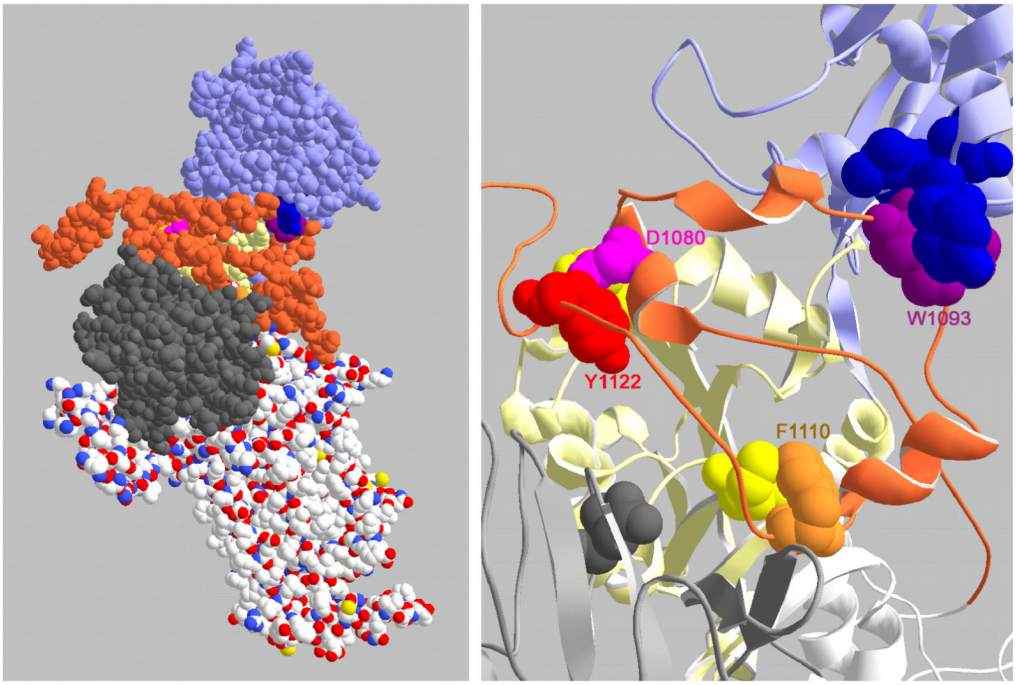

Figure 3.

Model of PMCA4b in the E1-Ca^{2+} state highlighting intramolecular autoinhibitory interactions. A structural model was built for the PMCA4b based on its homology to SERCA, and taking into account experimental constraints derived from mutagenesis and crosslinking data. A space-filling representation of the model is on the left, and it shows how the C-tail (orange-brown) fits in the bottom of a cleft, lying on the surface of the pale yellow P domain, between the grey A domain and the light blue N domain. The stalk and transmembrane portions of the molecule are shown in CPK colors. Note that the cytosolic side is on top and the extracellular side on the bottom in this model. The right-hand panel shows specific interactions of the C-tail with the core; it is enlarged, but has the same orientation and colors as the left panel. This is a ribbon representation with the interacting residues shown as space-filling for emphasis. The interacting residues in the C-tail are highlighted in color (D_{1080}, magenta; W_{1093}, purple; F_{1110}, pale orange; Y_{1122}, red); the core residues they interact with are shown as space-filling in the same colors as the domain they are part of, but their colors are more intense. The left panel shows that the appropriate parts of C-tail are partly buried in the E1-Ca^{2+} state. Figure courtesy of Dr. John T. Penniston.