Chapter 1
The Plasma Membrane Calcium ATPase: Historical Appraisal and Some New Concepts

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Abstract The plasma membrane calcium ATPase (PMCA pump) was discovered nearly 50 years ago. Among its functional properties, the wealth of regulatory mechanisms singles it out from all other members of the P-type ion pumps superfamily. The cytosolic C-terminal tail of the protein contains a binding domain for calmodulin, which binds to sites near the active site and maintains the enzyme autoinhibited in the resting state. Calmodulin removes the C-terminal domain from these docking sites, relieving the inhibition. Other pump regulators are the acidic phospholipids of the inner leaflet of the membrane, which are in principle sufficient for 50 % of maximal pump activity. The activation by acidic phospholipids could perhaps also be involved in the process of apoptosis, which is known to transfer the activatory phosphatidylserine to the outer leaflet of the membrane bilayer: the decreased Ca^{2+} ejection activity of the pump could amplify the cytosolic Ca^{2+} overload frequently involved in apoptosis. Another novel concept on the PMCA pump is the conclusion that its Ca^{2+} ejection activity is less important to the total regulation of cytosolic Ca^{2+} than that of the SERCA pump and the plasma membrane Na/Ca exchanger. The main role of the PMCA pump is instead the regulation of Ca^{2+} in restricted cytosolic domains in which it interacts with numerous important enzymes. The local regulation of Ca^{2+} necessarily confers to the activation by calmodulin an oscillatory character: as Ca^{2+} decreases in the local pump environment, calmodulin will leave the pump, terminating its activation.

Keywords Calcium signaling • Calcium ATPases • Calcium pumps • Calmodulin • Acidic phospholipids • Local calcium homeostasis

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1 Introduction

The plasma membrane Ca^{2+} ATPase (PMCA pump) was discovered nearly 50 years ago [1]. Studies in the following years have characterized it as the most complex member of the superfamily of P-type ion pumps [2]. One of its properties should be mentioned at the outset, as it has seriously complicated the study of the pump: it is its great propensity to aggregate in the purified state. The pump dimerizes through its C-terminal calmodulin-binding domain [3], but in the isolated state, aggregates are invariably formed which are much larger. They have so far defeated all attempts to monomerize the pump in a functionally active state. Perhaps the most important structural property that sets the PMCA pump apart from all other members of the superfamily is the presence of a long cytosolic C-terminal tail, which has an essential role in the regulation of the activity of the enzyme: it is the locus of interaction of regulatory partners, chief among them calmodulin, and is the structure responsible for the mechanism of autoinhibition, which is a distinctive properties of the PMCA pump. Another distinctive property is the wealth of regulatory mechanisms, which act with different and still incompletely understood mechanisms. Traditionally, the most important among them have been calmodulin and acidic phospholipids, but others have recently emerged, e.g., protein partners that may interact with the pump in a spatially confined cell environment.

2 General Properties of the Plasma Membrane Ca^{2+} Pump: A Succinct Summary

The tertiary structure of the PMCA pump is not available, but molecular modeling work on the SERCA pump template (Fig. 1.1) shows a topology of ten transmembrane domains and three main cytosolic protrusions that correspond to the A, N, and P cytosolic domains of the SERCA pump. It also shows that, as in the case of the SERCA pump, a large conformational change occurs when the pump binds Ca^{2+}: the change affects both the transmembrane helices and the cytosolic portion, but is most evident in the latter, which becomes far more "open" in the presence of Ca^{2+}. The PMCA pump has long been known to have a unique, unstructured C-terminal cytosolic tail of about 150 residues that contains a canonical calmodulin-binding domain (a second, lower-affinity domain that binds calmodulin and has recently been identified downstream of the first in some splicing variants of the pump [4]). The C-terminal tail also contains consensus sites for two activatory kinases: that for PKA is isoform specific and is located downstream of the calmodulin-binding domain [5], whereas PKC has two target sites that are not isoform specific [6], a Thr within the calmodulin-binding domain and a Ser further downstream. One early finding on the PMCA pump which was made before its purification is its activation by acidic phospholipids [7]. The finding was extended to the purified pump [8], and it was calculated that the amount of acidic phospholipids in the environment of the

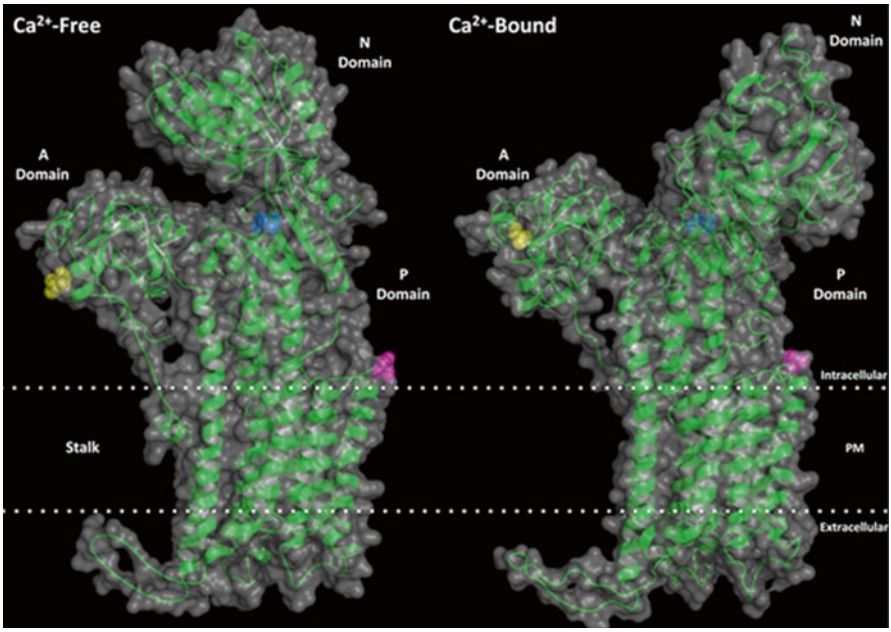

Fig. 1.1 Structure of the PMCA pump (isoform 3): Ca^{2+}-induced structural changes). Cartoon representation (*green*), superimposed on a space filling representation (*gray*) of the Ca^{2+}-free (*left*) and Ca^{2+}-bound (*right*) structures of the pump, were built on the basis of the respective SERCA structures (PDB 3W5B and 1SU4, respectively). The *blue dots* represent the catalytic aspartate. The N-terminus and the C-terminus of the pump are shown as *yellow* and *magenta dots*, respectively

erythrocyte membrane was in principle sufficient for about 50 % of maximal pump activity [9]. The mechanism of the activation by acidic phospholipids is still not understood, but it has been found that they reduce the K_m Ca^{2+} to even lower levels than calmodulin does [10] and that they interact with the calmodulin-binding domain and the first loop that protrudes into the cytosol between transmembrane domains 2 and 3 [11].

As all P-type pumps, the PMCA pump conserves temporarily the energy of the ATP that is hydrolyzed during the reaction cycle in the form of an aspartyl-phosphate. Interestingly, the PMCA pump transfers one Ca^{2+} across the membrane for each hydrolyzed ATP, instead of the two Ca^{2+} per ATP transferred by the SERCA pump: thus, it has only one Ca^{2+}-binding site, which corresponds to site two of the SERCA pump; this is so because one conserved acidic residue in transmembrane domain 5, which is essential for the formation of Ca^{2+}-binding site 1 in the SERCA pump, is absent in the PMCA pump—its insertion by mutagenesis in transmembrane domain 5 evidently completes the missing binding site for Ca^{2+}, restoring the Ca^{2+}/ATP stoichiometry of the PMCA pump to 2 [12].

The PMCA pump was cloned in 1988 [13, 14]. At the time of the cloning, some of its important domains had been already recognized and sequenced, e.g., the

calmodulin-binding domain [5]. Other domains were identified after the cloning, including the sites that interact with acidic phospholipids [11] and the sites that are responsible for the process of autoinhibition [15, 16], in which the C-terminal tail of the pump folds over under resting conditions of low Ca^{2+} and binds with its calmodulin-binding domain to two sites in the main body of the pump. One is close to the active site in the main cytosolic protruding unit, and the other is located in the large cytosolic unit that protrudes between transmembrane domains 2 and 3. When Ca^{2+} in the environment increases, calmodulin becomes associated with its binding domain, somehow removing it from the main body of the pump and restoring activity.

Soon after its successful cloning, it was found that four separate mammalian genes produce four distinct basic isoforms of the pump [see [17] for a comprehensive review]. The four basic pumps have a sequence identity of about 80 %: the differences do not concern the catalytic core of the pumps, but the regions that are involved in the regulation of their activity [18]. Two of the basic pumps are expressed ubiquitously (PMCA1 and 4), and two (PMCA2 and 3) are restricted to some tissues, with preference for the brain. Until a few years ago it was assumed that both ubiquitous isoforms were housekeeping pumps, but recent work has instead shown that isoform 4 has specific roles in some tissues, e.g., the testis [19]. As expected, the catalytic properties of the four pumps are the same, but the rate of activation by Ca^{2+} (and also the rate of inactivation by its removal) is slower in the two ubiquitous isoforms, which are therefore characterized as "slow" pumps: their role correlates with the type of Ca^{2+} signals in tissues, which could be slow or fast [20]. A striking difference between the ubiquitous and the tissue restricted isoforms if the affinity for calmodulin, which is significantly higher in the latter pumps. Concerning calmodulin, one striking property of PMCA2 which is not yet molecularly understood is its peculiar ability to express high activity in the absence of calmodulin [21, 22]. PMCA2, therefore, continuously pumps Ca^{2+} very effectively irrespective of the presence of its most important activator: this property may respond to particular Ca^{2+} homeostasis demands of specialized cell types, e.g., the outer hair cells of the inner ear [23]. The sustained Ca^{2+} ejection ability of the PMCA2 pump in the absence of calmodulin does not reflect differences in the C-terminal calmodulin-binding domain, the sequence of which is the same in the four basic pump variants in the portion. It evidently reflects differences in the domain(s) of the main body of the PMCA2 molecule that mediate(s) the autoinhibition process. In addition to the four basic isoforms, a large number of additional pump variants are produced by the alternative splicing of the primary transcripts of the four genes. The splicing inserts, naturally, do not involve the catalytic portion of the pump. They occur at site A in the cytosolic loop protruding between transmembrane domains 2 and 3 and at site C within the C-terminal calmodulin-binding domain. The number of the inserted exons, at both site A and site C, varies with the pump and the tissues: it confers to the variants properties that satisfy the functional Ca^{2+} homeostasis requirements of given cell types, and may be responsible for their targeting to particular domains of the plasma membrane [24]. The alternative spicing process is peculiarly complex in the PMCA2 pump, adding to it another property that singles it out from the other three basic pumps.

3 Emerging New Concepts on the Regulation and Function of PMCA Pumps

3.1 Local or General Action of the PMCA Pumps

The correct functioning of PMCA pumps is essential to all animal cells: this is clearly underlined by the increasing number of pathologies of numerous tissues that recognize the malfunction of PMCA pumps as the causative factor. Naturally, the mechanistic function of PMCA pumps is to eject Ca^{2+} from cells: however, in most cells this function of the PMCA pumps is quantitatively overshadowed by that of more powerful systems, i.e., the SERCACa^{2+} pump of the endo(sarco)plasmic reticulum and—in excitable cells—the Na^+/Ca^{2+} exchanger of the plasma membrane (cells like the erythrocytes are obviously exceptions). Prima facie, then, considering the quantitative aspects of the cytosolic homeostasis of Ca^{2+}, the coexistence of PMCA pumps alongside the other systems does not have a logical rationale. The simplest way out of the conundrum would be by proposing that the *raison d'être* of PMCA pumps would not be the bulk homeostasis of cytosolic Ca^{2+}, but its selective regulation in the microdomains surrounding the cytosolic portion of the pump. The concept, in essence, proposes that the role of the PMCA pump in the total extrusion of cell Ca^{2+} in many—perhaps most—cell types could be minor or even quantitatively irrelevant: its main function would be the locally restricted regulation of cytosolic Ca^{2+}. Ca^{2+} can still be used as a signaling agent even in cells that experience large physiological oscillations in its concentration because the pools controlled by the activity of the PMCA pumps would be spatially restricted: for instance, in the cardiomyocytes the general Ca^{2+} pool to be used for contractility is separated from the pool used for signaling purposes [25]. Mohamed et al. [26] have nicely shown that the PMCA 4 pump is indeed only involved in heart Ca^{2+} signaling, not in its general the beat-to-beat control in the contraction/relaxation process. A necessary prerequisite of such signaling role of the PMCA pump would be its localization to spatially restricted microdomains of the plasma membrane in which the pump would preferentially interact with enzyme partners. That the PMCA pump is specifically localized to caveolae has indeed been shown more than 20 years ago [27]. Others have confirmed the finding [28] and have extended it to show that in the subplasma membrane domain of caveolae, which are important sites of cell signaling [29], the PMCA pump indeed interacts specifically with important signaling partners [30]. In the heart, the interaction with neuronal nitric oxide synthase (nNOS) regulates indirectly cardiac contractility by acting on a spatially confined cyclic nucleotide microdomain [26].

3.2 Acidic Phospholipids

The preferred localization of the PMCA pump to the caveolae, or to similar domains like rafts and dendritic spines, brings back the matter of the regulation of the pump by phospholipids. These specialized plasma membrane domains have particular

phospholipid composition and fluidity and could modulate the activity of the PMCA pumps by their lateral mobility [31–33]. It has also been shown that the association of the PMCA pumps with caveolae or rafts, and thus its regulation, could be isoform specific [34]. But the regulation of the activity of the PMCA pumps by acidic phospholipids could come into play in a condition of wider general significance: the apoptotic death of cells [35]. As is well known, an early event of apoptosis is the externalization of phosphatidylserine by scramblases (one of which is Ca^{2+} dependent) to make cells recognizable by phagocytes [36, 37]. Phosphatidylserine is mostly localized to the inner leaflet of the plasma membrane bilayer, where its concentration around the PMCA pump (see above) has been calculated to activate it to about 50 % of maximal [9]. An interesting mechanism could thus be proposed in which scramblases would become activated in apoptotic cells in response to the increase of cytosolic Ca^{2+}. They would transfer phosphatidyl serine to the external lipid monolayer, depriving the PMCA pump of an important activating factor and amplifying the cytosolic Ca^{2+} overload that would eventually execute the apoptotic death of the cell.

The matter of the activation of the PMCA pump by phospholipids deserves some additional discussion: the (acidic) phospholipids of the inner leaflet of the plasma membrane bilayer are supposed to be physically separated from the catalytic core of the pump that protrudes into the cytosol. Therefore, their direct involvement in the operation of the reaction cycle of the pump cannot be easily envisaged, as it would demand that the cytosolic portions of the pump somehow establish contact with the surface of the lipid bilayer. The activatory effects of acidic phospholipids could instead be linked to something structural, i.e., to the modulation of the access of Ca^{2+} to its binding site within the membrane. Surprisingly, however, a report has appeared in which acidic phospholipids have been shown to act on the reaction cycle proper, as they accelerate the dephosphorylation of the pump [38].

Another issue in the matter of the activation by phospholipids is the time scale of their effects: changes of the phospholipid environment of the pump are unlikely to be as rapid as those, for instance, of calmodulin, suggesting that phospholipids could be longer range modulators of pump activity. One possible exception would be the doubly phosphorylated product of phosphatidylinositol (PIP2), which is the only phospholipid that changes rapidly concentration in response to plasma membrane agonists. A reversible, rapid activation of the PMCA pump by PIP2 has actually been proposed [39].

One last point on acidic phospholipids deserves some discussion: its functional interplay with the activation by protein kinases. Both PKA and PKC (see above) activate the PMCA pump (the effect of PKC has aspects that are somewhat controversial). The activity of PKC requires diacylglycerol (DAG), which comes from the hydrolysis of PIP2, and the most important adenylyl cyclase is activated by Ca^{2+}, the increase of which in the cytosol is promoted by InsP3, which also comes from the hydrolysis of PIP2. So, here we have a seemingly paradoxical situation: the activation of the PMCA pump by the protein kinases demands the disappearance of PIP2, which is the most powerful pump activatory phospholipid: possibly, as suggested elsewhere [35] one should consider temporal, or even spatial, factors in the effect of the kinases with respect to the suppression of the effect of PIP2.

3.3 The Autoinhibition of the Pump

The first indication that the PMCA pump at rest was in an autoinhibited state mediated by the C-terminal CaM-binding domain came from experiments in which the proteolytic removal of the C-terminal tail of the pump, including its CaM-binding domain by calpain, irreversibly removed the autoinhibition [5, 9]. The indication was directly verified by experiments in which the autoinhibited state was induced by adding synthetic peptides corresponding to the CaM-binding domain to the pump that had been made fully active by C-terminal truncation [15]. The proposal that the C-terminal tail of the pump would "close" the access route to the active site was provided by experiments in which blue and green fluorescent proteins were fused to the N- and C-termini of the pump [40]. The distance between the two fluorophores was about 45 A in the autoinhibited state and became greater when the pump was activated by CaM (or acidic phospholipids). Other pump regions distant from the CaM-binding domain and from the C-terminal tail could also have a role in the autoinhibition process, i.e., the "stalk" region, in which Asp-170 could help in the stabilization of the autoinhibited state [40, 41]. The "stalk" region has been proposed to form basic pockets that would be suitable for the binding of the head groups of PIP2, which would be protected from the degradation by phospholipase C.

3.4 The Regulation of the PMCA Pump by Calmodulin

The built-in stop mechanism in the activation of the PMCA pump discussed for the effect of the protein kinases has more general significance and can be extended to the case of CaM. Once Ca^{2+} increases in the microenvironment of the autoinhibited pump, CaM becomes bound by the pump and activates the ejection of Ca^{2+}. However, as this occurs, Ca^{2+} decreases in the microenvironment of the pump, leading to the detachment of CaM and to the restoration of the autoinhibited state. Thus, by definition, the activation of the pump by CaM cannot be permanent, but must necessarily follow an oscillatory pattern, in which bursts of activation are followed by periods of relative inactivity. Only conditions of sustained cytosolic Ca^{2+} overload, as frequently occurring in pathology, can produce permanent activations of the pump. These conditions would probably also activate calpains, which would cleave out the C-terminal tail of the pump, contributing to its permanent state of maximal activation and to the amplification of the Ca^{2+} overload condition.

4 Conclusions

The PMCA pumps stand out from all other pumps of the P-type superfamily because of the wealth and complexity of their regulatory mechanisms. They have been discussed in this contribution, which has tried to put them in their respective roles and functional perspectives. The discussion has emphasized aspects of the regulation of

the pump which have not been considered so far: the most interesting concept that has emerged is that the pump, in the physiological conditions prevailing in the cell cytosol, can only be activated by its most important partner, calmodulin, in temporarily limited bursts. The contribution has also underlined the concept that the main role of the PMCA pumps is not to contribute a quantitatively important fraction of the total Ca^{2+} ejection activity of cells: it is the regulation of Ca^{2+}, and of the partners of the pumps sensitive to it, in selected microdomains within the cells.

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