Allosteric inhibitors of plasma membrane Ca\(^{2+}\) pumps: Invention and applications of caloxins

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INTRODUCTION

This review focuses on the concepts behind the invention of a novel class of plasma membrane (PM) Ca\(^{2+}\) pump (PMCA)-specific inhibitors called caloxins. PMCA is a high affinity Ca\(^{2+}\) removal system found in all mammalian cells. Regulation of Ca\(^{2+}\) concentration in the cytosol (Ca\(^{2+}\)\(_i\)) is essential for cell survival and signal transduction. In this context, we provide an overview of membrane Ca\(^{2+}\) transport proteins responsible for control of Ca\(^{2+}\)\(_i\) and the need for PMCA-specific inhibitors to delineate its role in Ca\(^{2+}\)\(_i\) homeostasis and signaling. We introduce the extracellular domains (exdoms) of PMCA as allosteric targets to obtain prototype caloxins that have been used to study PMCA physiology. More recently, the importance of genetic diversity of PMCA isoforms is becoming obvious. Therefore, we have started to develop isoform-selective caloxins. We briefly outline the importance of various PMCA isoforms in health and disease and describe the isoform-selective caloxins ob-
tained to date. We conclude with the potential of caloxins as research and therapeutic tools in the field of PMCA physiology and pathophysiology.

MAJOR PLAYERS OF CALCIUM DYNAMICS

Ca$^{2+}$ is a key intracellular signaling molecule which controls different cellular processes in various cells. In a resting cell, Ca$^{2+}$ is kept low at around 0.1 μmol/L, which is 10,000-fold lower than its concentration outside the cell. Since the membrane potential is negative inside the cell, the large electrochemical gradient allows Ca$^{2+}$ entry through pathways in the PM that open during cell excitation [1-3]. The Ca$^{2+}$ entry upon cell stimulation involves a number of Ca$^{2+}$ channels and reverse mode Na$^+$-Ca$^{2+}$ exchanger (NCX) in the PM. Ca$^{2+}$ is also stored at high concentration in the sarco/endoplasmic reticulum from where it can be released by Ca$^{2+}$ channels. In addition, other organelles, like mitochondria and Golgi bodies, may also store and release Ca$^{2+}$ into the cytosol. Following the completion of the signaling event, Ca$^{2+}$ has to be lowered to the resting levels. The lowering of Ca$^{2+}$ in the cytosol occurs mainly by its sequestration into the sarco/endoplasmic reticulum or its extrusion from the cell. Ca$^{2+}$ sequestration into the sarco/endoplasmic reticulum is carried out by the sarco/endoplasmic reticulum Ca$^{2+}$ pumps (SERCA). Ca$^{2+}$ extrusion from the cells can be carried out by PMCA and forward mode NCX. NCX uses the electrochemical gradient of Na$^+$ to extrude Ca$^{2+}$ from the cells with a low affinity (Km for Ca$^{2+}$ = 1-10 μmol/L). PMCA uses the energy derived from ATP hydrolysis to expel Ca$^{2+}$ from cells with a high affinity (Km for Ca$^{2+}$ = 0.2-0.5 μmol/L). Thus, PMCA is the only high affinity Ca$^{2+}$ extrusion mechanism found in eukaryotic cells which may play a key role in long-term regulation of Ca$^{2+}$-i. PMCA act as dynamic regulators of Ca$^{2+}$-i involved in both short-term signaling events as well as long-term cellular processes like cell growth and differentiation. PMCA are crucial to cell survival and signaling. However, it is difficult to study the exact role of PMCA in a cell due to the presence of other Ca$^{2+}$-i lowering systems like NCX and SERCA. Specific inhibitors are needed to understand the role of PMCA in various cell types in health and disease.

CALOXINS - A NOVEL CLASS OF ALLOSTERIC INHIBITORS OF PMCA

Allosteric sites have been exploited as pharmacological targets because of their greater specificity than the active sites of enzymes and receptors. Digoxin and ouabain are allosteric inhibitors of Na$^+$-pump which have proven useful as research and therapeutic tools. Similarly, SERCA pump inhibitors, such as thapsigargin, have led to the exploration of the role of this protein in signal transduction. In contrast, inhibitors based on regulatory and active sites with consensus sequences conserved among various proteins may not be specific. For example, vanadate and eosin are currently used as inhibitors to study PMCA[12-15]. Vanadate is a phosphate analog that competes for ATP binding in the catalytic domain of all ATPases. It has much higher affinity for the Na$^+$-pump than for PMCA. Decavanadate also inhibits a variety of ATPases and other proteins[16]. Similarly, eosin is also not PMCA specific as it acts by interfering with the binding of ATP to a conserved site found in all ATPases.

Therefore, PMCA specific inhibitors are required to understand the role of PMCA in Ca$^{2+}$ homeostasis and cell signaling.

We pioneered the concept of caloxins: short peptides that specifically inhibit PMCA by binding to the allosteric sites on the protein. The exdoms, which are short loops connecting the transmembrane helices of PMCA on the extracellular surface, were chosen as allosteric targets to obtain caloxins. PMCA have 5 exdoms and the regulatory and active sites of PMCA are all cytosolic. The first reason for choosing exdoms was that the exdom sequences of PMCA do not have significant homology with other proteins[2,17,18]. The second reason was that thapsigargin inhibits SERCA by binding to its luminal loops which have been shown to undergo conformational changes during its reaction cycle and the exdoms of PMCA would be similar in function to luminal loops of SERCA. Also, exdom 1 of Na$^+$-K$^+$-ATPase is the allosteric site involved in its inhibition by ouabain[19]. Hence, exdoms of PMCA were chosen as the allosteric sites to which caloxins would bind and specifically inhibit PMCA when added extracellularly.

INVENTION OF CALOXIN 2A1

The first caloxin to be invented was caloxin 2a1[20]. A phage library displaying 12-amino acid random peptides fused to its coat protein was screened by a process called panning for binding to a target[21]. The target was a synthetic peptide corresponding to the exdom 2 sequence (Figure 1) of PMCA1 conjugated to carrier protein keyhole limpet hemocyanin or ovalbumin. The target peptide conjugated to keyhole limpet hemocyanin was immobilized by passive adsorption to the wells of a plastic microtiter plate. The phage library in solution was allowed to bind to the target in the well. The wells were washed extensively to remove the non-specific phage. This was followed by elution of the target-bound phage with a solution of target peptide conjugated to ovalbumin. The eluted phage was amplified and the above screening process repeated for 8 cycles. Following the last round of phage screening, the consensus peptide sequence VSNSNWPSSFSS was selected. This peptide was chemically synthesized with addition of GGG sequence at its C-terminus to obtain caloxin 2a1 (VSNSNWPSSFGG-amide). GGG sequence is found as a short spacer between the variable peptide sequence and the coat pro-
tein of the phage. Caloxin 2a1, at a concentration of 0.4 ± 0.1 mmol/L, produced 50% inhibition of Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase activity of PMCA in the human erythrocyte ghosts. It also inhibited the formation of Ca\(^{2+}\)-dependent acylphosphate in a partial reaction of PMCA catalytic cycle. Caloxin 2a1 did not inhibit any other ATPases tested. Consistent with being an allosteric inhibitor, caloxin 2a1 inhibited the PMCA ATPase in a manner non-competitive with respect to Ca\(^{2+}\), ATP and calmodulin (Figure 2)[22]. Screening protocol similar to that used for caloxin 2a1 resulted in affinity selection of caloxins 1a1 and 3a1 for binding to synthetic exdom 1 and 3 respectively[23,24]. The caloxins invented by screening phage display libraries using synthetic exdoms as targets were termed series A caloxins (see Figure 1 for nomenclature).

**APPLICATIONS OF CALOXIN 2A1**

Caloxin 2a1 has been used to answer a number of questions concerning PMCA action. It was used to investigate a long standing hypothesis about the Ca\(^{2+}\) transport mechanism of PMCA. PMCA had been suggested to exchange Ca\(^{2+}\) for proton(s) so as to remain electroneutral[18,23]. Increase in Ca\(^{2+}\) in cultured mouse cerebellar granule cells induced by phytotoxin (Palytoxin) or glutamate receptor activation was shown to be accompanied by intracellular acidification. This acidification was blocked with caloxin 2a1, suggesting that it was related to proton influx that accompanied Ca\(^{2+}\) removal by PMCA[12,13]. The synchronous activation of CA1 pyramidal neurons of hippocampus was associated with a rapid increase in extracellular pH. The pH transients at the extracellular surface were measured using a single neuron or neuronal population. Caloxin 2a1 inhibited the extrasynaptic alkaline transient observed in single neurons or neuronal population. This provided direct evidence that the countertransport of proton(s) by PMCA generates extrasynaptic alkaline shifts observed upon synchronous activation of a neuronal population[24].

Caloxin 2a1 has helped in determining the role of PMCA in Ca\(^{2+}\) oscillations which are a mode of signaling in both excitable and non-excitable cells. The extracellular Ca\(^{2+}\) sensing receptor translates the changes in extracellular Ca\(^{2+}\) to the cell interior via oscillatory Ca\(^{2+}\) changes. HEK293 cells expressing the extracellular Ca\(^{2+}\) sensing receptor elicited Ca\(^{2+}\) oscillations upon receptor activation. PMCA-mediated Ca\(^{2+}\) extrusion was essential in reinforcing the receptor stimulation to maintain and modulate the periodicity of Ca\(^{2+}\) oscillations which were eliminated in the presence of caloxin 2a1[27]. The role of PMCA in generation of spontaneous Ca\(^{2+}\) oscillations was also shown in human bone marrow-derived mesenchymal stem cells. Caloxin 2a1 inhibited the oscillations as did the non-selective inhibitor, carboxyeosin. However, caloxin 2a1 induced a Ca\(^{2+}\) transient followed by the return of Ca\(^{2+}\) to basal levels. In contrast, carboxyeosin markedly increased the basal Ca\(^{2+}\) before stopping the oscillations. The difference in the action of the two agents is consistent with PMCA-specific inhibition by caloxin 2a1 leading to an initial increase in Ca\(^{2+}\) which is then lowered by other Ca\(^{2+}\)-removing systems. In contrast, the increase in basal Ca\(^{2+}\) by carboxyeosin can be explained by its non-specific effects on sodium pump or SERCA, that in turn can affect Ca\(^{2+}\) influx or uptake into the intracellular Ca\(^{2+}\) pool[19]. Similar differences in the effects of caloxin 2a1 and carboxyeosin have been observed in other cell types like mouse embryonic stem cells[28].
Caloxin 2a1 has also been used to study the effect of PMCA inhibition in vascular tissues. Consistent with the inhibition of PMCA in vascular endothelium, caloxin 2a1 produced an endothelium-dependent relaxation that was reversed by N(G)-nitro-L-arginine methyl ester[^21]. Thus caloxin 2a1 is the first known PMCA selective inhibitor. Despite its low affinity for PMCA, it has been used to study PMCA physiology in various tissues and cell types.

**GENETIC DIVERSITY IN PMCA**

PMCA are encoded by four genes: PMCA 1-4. Alternative splicing of the primary gene transcripts results in a large number of PMCA variants which differ in their regulatory and kinetic properties, as reviewed elsewhere[^32-35]. Here, we focus on the diversity observed in the expression of the PMCA isoforms 1-4 in various tissues and cell types and the differences in their subcellular localization. PMCA1 and 4 are most widely expressed while PMCA2 and 3 have tissue-specific distribution.

PMCA isoform expression varies in a cell-type dependent manner in various tissues, e.g. in coronary arteries, the endothelial cells express mainly PMCA1 whereas the smooth muscle cells express more PMCA1 than PMCA4[^33] (unpublished). Similarly, various regions of the brain differ in their expression of the four PMCA genes[^34,35]. The frontal cortex expresses high levels of all four PMCA genes; the hippocampus expresses PMCA genes 1, 3 and 4 and the cerebellum has higher levels of PMCA2 and 3[^4,5]. Cell-type specific expression of PMCA isoforms has also been observed in different classes of retinal neurons that differ in their signaling. The neurons can signal with graded potentials involving sustained elevation of Ca^{2+} (photoreceptors, horizontal and bipolar cells), action potentials involving Ca^{2+} transients (ganglion cells) or both (amacrine cells). PMCA1 is expressed in photoreceptors, horizontal cells and cone bipolar cells. PMCA2 is found in rod bipolar cells and it is coexpressed with PMCA3 in amacrine and ganglion cells. In stratified corneal epithelium, there are differences in the PMCA isoforms expressed in the different layers of cells[^17,38]. Thus, the PMCA isoforms exhibit tissue and cell-specific expression that may reflect differences in their Ca^{2+} handling requirements.

The PMCA isoforms may exhibit differential PM localization within a single cell type. The spatially distinct demands of Ca^{2+} influx and efflux observed especially in polarized cells like epithelial and neuronal cells may determine the differential distribution of PMCA isoforms in the PM. Duodenal mucosa cells express PMCA1, which is localized in the basolateral membrane. This allows the transcellular transport of luminally absorbed Ca^{2+}, which is pumped out of the basolateral membrane into the interstitial space[^39]. In some instances, PMCA may be localized in lipid rafts which are cholesterol/spingolipid-rich microdomains of PM or in a specialized subset of lipid rafts called caveolae[^40,41]. Caveolae are small (50-100 nm) invaginations of PM that are enriched in the protein caveolin. They are rich in receptors, channels, signal transducers, effectors and structural proteins and may act as Ca^{2+} signaling microdomains[^42]. It is not known if the PMCA isoforms localized in these specialized PM domains are different from those present in non-caveolar PM. PMCA4b has been shown to localize in caveolae where it can interact with neuronal NO synthase and regulate its activity[^43]. The functional significance of differences in the distribution of PMCA isoforms observed at tissue, cell or subcellular levels needs to be determined and would require the isoform specific inhibitors of PMCA.

**PMCA ISOFORMS AND DISEASE**

Changes in the levels of expression or activity of various PMCA isoforms have been associated with several pathologies including heart disease, hypertension, carcinogenesis, cataract formation, diabetes and neurodegenerative diseases[^44-47]. Gene targeting studies have shown that PMCA1 null mice are embryolethal suggesting an essential housekeeping role for PMCA1[^44,45]. However, mice with heterologous PMCA1 ablation are normal. Studies on oral squamous cell carcinoma have suggested an epigenetic inactivation of PMCA1 gene as a frequent and early event during oral carcinogenesis[^46]. Altered PMCA1 expression has also been observed in breast cancer[^47]. In vascular smooth muscle cells, the regulation of PMCA1 expression by the transcription factor c-Myb may control cell proliferation[^48]. An increase in the level of PMCA1 expression is associated with the loss of Ca^{2+} homeostasis observed in human cataract lenses[^49,50]. Studies using cultured human lens epithelial cell line have shown that oxidative stress, a major contributor to cataract development, also induces changes to the level of expression of PMCA1.

The changes in the levels of PMCA2 have been reported in several diseases including hearing defects, multiple sclerosis, spinal cord injury, cancer and cataract. PMCA2 null mice exhibit deafness and ataxia, whereas the heterozygous mice are predisposed to age or noise related hearing loss[^51]. The role of PMCA2 in hearing loss has also been verified in humans. The hearing loss in a family, caused by homozygous mutations in cadherin 23, was exacerbated in individuals heterozygous for a mutation in the PMCA2 gene[^45]. Besides being a regulator of Ca^{2+} homeostasis, PMCA2 has been shown to be essential for Ca^{2+} secretion in milk[^46]. An increase in levels of PMCA2 is seen during lactation, which decreases again upon weaning. An increase in levels of PMCA2 is also observed in breast cancer cell lines[^50,52-54]. Its inhibitory interaction with calcineurin, and therefore of calcineurin-mediated apoptosis, suggests a regulatory role for PMCA2 in breast tumorigenesis. PMCA2 expression is also altered in cataract lenses as compared to age-matched clear lenses[^55].

PMCA3 is expressed at high levels in choroid plexus and may thus play a role in regulating ionic composition...
of the cerebrospinal fluid which is essential in brain function and development\cite{51}. PMCA3 mRNA levels in the placenta correlate with neonatal bone mineral content, bone area, placental weight and birth weight suggesting that it may be crucial in calcium absorption by fetal bones\cite{52}. Lack of availability of PMCA3 knockout animals has limited studies on PMCA3 physiology.

Despite the ubiquitous distribution of PMCA4, the major phenotype observed in null mice is male infertility due to loss of sperm hypermotility\cite{44}. The role of PMCA in sperm motility has also been confirmed by carboxy-echinin-mediated inhibition of PMCA in wild type mice\cite{53}. Although PMCA is thought to play a less important role than NCX in excitable cells, a loss of PMCA4 impaired phasic contractions and caused apoptosis in the portal vein smooth muscle studied \textit{in vitro} from some strains of mice\cite{49}. PMCA4 may also play a more direct role as modulator of Ca\textsuperscript{2+} signaling pathways. PMCA4b overexpression in mice gave unexpected increase in arterial reactivity and increased blood pressure\cite{58,59}. In cardiomyocytes, the overexpression attenuated the \beta-adrenergic inotropic response\cite{60}. The observed results were due to a direct interaction of PMCA4 with the neuronal NO synthase leading to impairment of its NO synthase activity. PMCA4 may also regulate hypertrophy and heart failure. In human failing hearts, PMCA4 protein expression decreased by 60% as compared to the normal hearts. PMCA4 was also downregulated by 25% in hypertrophic mouse hearts following transverse aortic constriction\cite{62}. Gene targeting of PMCA4 increased the susceptibility of hearts to hypertrophy, whereas its cardiac-specific inducible expression rendered it anti-hypertrophic in response to pressure overload. Association of PMCA4 with calcineurin and alteration of calcineurin-mediated hypertrophic response may be the underlying mechanism of PMCA4 regulated hypertrophy. Abnormal platelet Ca\textsuperscript{2+} homeostasis in diabetes mellitus is also associated with increase in the level of PMCA4 expression\cite{63}. PMCA4 expression levels may change during cancerogenesis. Differentiation of HT-29 colon cancer cells was associated with an upregulation of PMCA4, whereas breast cancer cell lines show decrease in levels of PMCA4\cite{47,54,63}.

**PMCA ISOFORM SELECTIVE CALOXINS: NEEDS AND CHALLENGES**

The genetic diversity that exists in PMCA emphasizes the need for isoform-selective caloxins to understand PMCA physiology and pathophysiology. The invention of isoform-specific inhibitors, however, is an unprecedented task. The challenges in the invention of isoform-selective caloxins are discussed below.

The first challenge in the invention of isoform-selective caloxins is the identification of allosteric target sites in PMCA where ligand binding can produce inhibition and which are sufficiently diverse among the four PMCA gene products to confer isoform selectivity to the ligand. Our initial work shows that exdoms 1, 2 or 3 can be used as targets to obtain caloxins\cite{21,22,23,24}. Of these, only the amino acid sequence of exdom 1 differs significantly among the proteins encoded by the four PMCA genes (Figure 1). Therefore, exdom 1 was chosen as a target to invent isoform-selective caloxins\cite{53}. In addition, the alternative splicing of the four primary gene transcripts does not affect the exdom 1 sequence in the splice variants. Therefore, exdom 1 based isoform-selective caloxin would be expected to inhibit all the splice variants of the gene. However, exdom 1 is very long and has a cysteine residue in the middle. It is not known if the cysteine participates in any disulfide bonding affecting the conformation of the exdom. Therefore, exdom 1 has been arbitrarily divided into exdom 1X and 1Y around the cysteine. Synthetic peptides corresponding to each half are used as targets to screen for isoform-selective caloxins.

The second challenge is the development of appropriate protocols to screen the phage display random peptide libraries for binding to the target\cite{45,59}. Series A caloxins have been obtained using only the synthetic exdom peptides as targets. In nature, the conformation of the exdom 1 in PMCA protein may be different from that in the synthetic peptides and hence the resulting caloxins would have low affinities and may not distinguish between different isoforms. This problem may be overcome by screening phage libraries using purified PMCA as the target. However, a major disadvantage of this method is the loss of specificity due to exdom recognition. Therefore, a two-step screening procedure was developed to affinity select phage-encoded peptides that retain exdom specificity and can bind the exdom in its native conformation in PMCA with high affinity. In the first step, synthetic exdom peptide is used as a target to screen the phage library by biopanning for 3-4 rounds to obtain a sub-library of phage clones showing some preference for binding to the synthetic exdom. In the second step, the sub-library is screened for binding to PMCA protein by affinity chromatography. This takes advantage of the ability of PMCA to bind to calmodulin only in the presence of Ca\textsuperscript{2+}. The phage is allowed to bind to PMCA immobilized on a calmodulin resin. PMCA-phage complex is then eluted in a solution containing Ca\textsuperscript{2+} chelator. The method can be further refined by introducing negative chromatography to eliminate selection of non-specific phage. The phage pool is pre-adsorbed with calmodulin resin alone or with immobilized PMCA that differs in its isoform type as compared to the target before use in screening by affinity chromatography. Phage copy number bias after screening can arise from methods used in library construction or by preferential amplification of certain phage clones in between the screening rounds. Therefore, following the two-step screening, the enriched phage pool was subjected to competitive screening by affinity chromatography. In competitive screening, equal plaque forming units of each type of phage clone in the enriched pool was allowed to compete for binding to PMCA to select a dominant clone based on its
affinity for the target. The resulting caloxins are termed series B caloxins and exhibit higher affinity with PMCA-isofrom preference as compared to series A caloxins. The affinity and isoform selectivity of series B caloxins can be improved further by limited mutagenesis of series B caloxin to create a library and then screen it by affinity chromatography to obtain series C caloxins. The next major concept is to take advantage of the information that exdom 1 has been arbitrarily divided into exdom 1X and 1Y to be used as targets for screening as shown in Figure 1. The series C caloxins directed against exdom 1X and 1Y can be optimally linked to obtain series D bidentate caloxin. The bidentate caloxin is expected to have affinity and PMCA-isofrom selectivity much higher than the either of the two partner caloxins.

The third challenge is to obtain PM source that is relatively rich in only one of the four PMCA isoforms. Human erythrocyte PM expresses mainly PMCA4 and pure PM can be obtained as erythrocyte ghosts. Several tissues were tested to discover that PM of rabbit duodenal mucosa is rich in PMCA1 (unpublished). We have not yet tested tissues to identify a rich source for PMCA2 or 3. However, microsomes prepared from the insect cells overexpressing these isoforms have been used in biochemical assays for measuring the activity of PMCA2 and 3.

PMCA4 SELECTIVE CALOXINS

Caloxin 1b1 is a series B caloxin that was obtained by two-step screening using synthetic exdom 1X of PMCA4 and PMCA protein purified from erythrocyte ghosts as a target. It inhibited the Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase activity of PMCA in leaky erythrocyte ghosts that express mainly PMCA4 isoform with a Ki (inhibition constant) value of 46 ± 5 μmol/L - an affinity which was 10 × higher than that of the first reported series A caloxin 2a1. It was selective for PMCA4 as it inhibited the other PMCA isoforms with lower affinity: PMCA1 (105 ± 11 μmol/L), PMCA2 (167 ± 67 μmol/L), PMCA3 (274 ± 40 μmol/L). It did not inhibit any other ATPases tested. It increased the force of contraction produced by a submaximum concentration of phenylephrine in de-endothelialized rat aortic rings. In cells cultured from pig coronary artery, it caused a greater increase in Ca\(^{2+}\) in the arterial smooth muscle cells (expressing PMCA4 and PMCA1) than in endothelial cells (expressing mainly PMCA1).

Caloxin 1c2 is a series C caloxin that was obtained by limited mutagenesis of caloxin 1b1. Caloxin 1c2 inhibited the PMCA activity in erythrocyte ghosts with a Ki value of 2.5 μmol/L. This affinity is 10-20x higher than that of the parent caloxin 1b1, and 100-200x higher than that of caloxin 2a1. The structure activity relationship of various residues in caloxin 1c2 is illustrated in Table 1. Caloxin 1c2 has the 1c2 specific sequence TAWSEVLHR and the conserved domain GGGSK. A study of the mutants of 1b1 that were selected for the selection of 1c2 showed that the residue W was crucial. Substitution of W with benzoylphenylalanine decreased the affinity while substitution of K with the same residue had very little effect. Based on mutagenesis and the modification studies, the moiety WSEV/L/V was key to this inhibition. Caloxin 1c2 was PMCA4 selective in that it had greater than 10-fold higher affinity for PMCA4 than for PMCA1, 2 or 3.

Caloxin 1c2 increased the basal tone of the de-endothelialized arteries and increased the Ca\(^{2+}\) sensitivity of the tissue to produce greater force of contraction at low extracellular Ca\(^{2+}\) when NCX and SERCA were inhibited. In another study, the effect of caloxin 1c2 on smooth muscle contractility was examined in intestine of caveolin-1 knockout mice. Immunohistochemistry and immunoblot studies suggested caveolar localization of PMCA4 and its role in Ca\(^{2+}\) extrusion from a privileged cytosolic space formed by close spatial arrangement between caveolae and sarcoplasmic reticulum. Thus, caloxin 1c2 is being used in studies to provide insight into the physiological function of PMCA4 in tissues.

We have also obtained caloxin 1b2 based on exdom 1Y of PMCA4 (unpublished). In a preliminary experiment, caloxins 1c2 and 1b2 were linked to obtain a bidentate caloxin which inhibited PMCA activity in erythrocyte ghosts with a Ki of 500 nmol/L, indicating higher affinity compared to either caloxin 1c2 or 1b2. This preliminary experiment shows the feasibility of linking the exdom 1X- and 1Y-based caloxins to obtain the series D bidentate caloxins with nanomolar affinity and very high selectivity for PMCA4.

PMCA1 SELECTIVE CALOXINS

Our interest in coronary artery endothelium led us to identify PMCA1 as the major isoform expressed in this tissue. Hence, a PMCA1-specific caloxin is needed to elucidate its role in coronary artery function. We carried out a two-step screening of the phage library using synthetic exdom 1X of PMCA1 and PMCA protein purified from plasma membrane Ca\(^{2+}\) pump (PMCA) ATPase in the human erythrocyte ghosts, which contain mostly PMCA4. B: Benzoylphenylalanine.

| Caloxin | Peptide sequence | Ki (μmol/L) |
|--------|-----------------|-------------|
| 1b1    | TAWSEVLHR GGGSK | 45 ± 4      |
| 1c1    | TTVEVHR GGGSK   | 20 ± 3      |
| 1c3    | ASWSEVLHR GGGSK | 18 ± 3      |
| 1c2    | TAWSEVLHR GGGSK | 23 ± 0.3    |
| 3B1c2biotin | TBSEVLDLRR GGGSK(biotin)-amide | 50 ± 6 |
| 16B1c2biotin | TAWSEVLDLRR GGGSK(biotin)-amide | 5.1 ± 0.8 |

Caloxins 1c1, 1c2 and 1c3 were obtained by random limited mutagenesis of caloxin 1b1. The values of inhibition constants (Ki) are for plasma membrane Ca\(^{2+}\) pump (PMCA) ATPase in the human erythrocyte ghosts, which contain mostly PMCA4. B: Benzoylphenylalanine.

FUTURE OF CALOXIN RESEARCH

The existence of gene diversity and differential tissue and cell-specific distribution of PMCA has turned the initial concept of developing PMCA inhibitors into a more daunting challenge of obtaining isoform-selective inhibitors. Significant progress has been made in this area with the invention of prototypes of PMCA4 and PMCA1 selective caloxins (unpublished). Caloxin research may unfold in several directions: study of PMCA-isoform specific physiology and pathophysiology, developing PMCA2 and 3 selective caloxins, designing targets based on PMCA protein structure with higher modulatory potential, tissue targeted expression of caloxins, obtaining non-peptide inhibitors by caloxin displacement.

In the invention of isoform-selective caloxins, exdom 1 has been divided arbitrarily into 1X and 1Y, due to the presence of a cysteine residue in the middle (Figure 1). The proximity of exdomain 1X and 1Y can allow for the development of a bidentate caloxin. However, optimization of the orientation of exdomain 1X and 1Y based caloxins and the length of the linker between them is essential in obtaining a high affinity bidentate caloxin. Synthetic peptide based on the complete sequence of exdomain 1 was not used as a target due to the presence of cysteine whose participation in disulfide bond is not known at present. Examination of the primary structure of PMCA protein shows the presence of a cysteine residue in the middle of the exdomain 2 and exdomain 5. There is a surface cysteine in transmembrane loop 5 of PMCA. Taken together, these cysteines may be involved in disulfide bridge formation. In homology model based on X-ray structure of SERCA, exdomain 1 is in proximity to exdomain 5. Therefore designing a target where synthetic peptides based on exdomain 1 and 5 are joined by a disulfide bridge may result in higher affinity caloxins. A layer of selectivity may also be added by targeted expression of secretable caloxins. Regardless of these approaches to obtain high selectivity of inhibition by caloxins, the problem will remain that peptides will be susceptible to proteolytic attack and hence short-lived. In this context, one can select non-peptide inhibitors by high-throughput screening.

Thus, there are several ways to improve the affinity and isoform-selectivity of current caloxins with the potential to improve their bioavailability.

A few examples are provided where caloxins may also aid in understanding the basis of various disorders and become clinically useful. Hypertension is associated with defects in PMCA. Current antihypertensive therapies involve decreasing arterial excitability with Ca2+ channel blockers or angiotensin converting enzyme inhibitors. Caloxins will help us explain how the increased artery tone in hypertension can result from PMCA inhibition in smooth muscle. PMCA selective caloxins may be preferentially used to increase endothelial NO production, resulting in a decrease in artery tone. PMCA4 is required for sperm motility. Thus, PMCA4 selective caloxins may form a new class of contraceptive agents. The reduction in sperm motility may also aid in storing sperm for future use. Ca2+ regulation plays a key role in cell cycle and changes in PMCA expression have been reported in several forms of cancer. The effects of caloxins on cancer are being realized and isoform-specific caloxins may aid in developing therapies for cancer. PMCA isoforms play specific roles in neuronal signaling that may change in various neuronal pathologies. PMCA isoform-selective caloxins will aid in delineating the underlying mechanisms. Isoform specific caloxins may help in understanding the basis of retinopathies as expression and distribution of the PMCA isoforms in retina and lens may alter during disease. We anticipate that isoform-selective caloxins will become useful tools in understanding and/or managing some of the disorders discussed above.

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