CALOXINS: NEW CLASS OF PLASMA MEMBRANE Ca$^{2+}$PUMP INHIBITORS
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TITLE: Caloxins: new class of plasma membrane Ca$^{2+}$ pump inhibitors

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ABSTRACT

Caloxin2A1 is a novel peptide that inhibits the activity of Plasma Membrane Calcium ATPase (PMCA). PMCA is known to play a role in homeostasis of cytosolic calcium and cell signaling. There are 4 genes (PMCA1-4) that code for the various isoforms of the calcium pump. Based on hydropathy plots, PMCA proteins have 5 putative extracellular domains. We screened combinatorial peptide phage display library for binding to specific extracellular targets.

Caloxin 2A1 was obtained as a peptide sequence that would bind to the 2\textsuperscript{nd} putative extracellular domain of PMCA 1 isoform. Caloxin2A1 selectively inhibited the Ca\textsuperscript{2+}-Mg\textsuperscript{2+} ATPase activity in human erythrocyte leaky ghosts that express mainly PMCA 4 isoform. It produced 50% inhibition of the pump activity at 0.4 mM. Caloxin2A1 inhibited the formation of the acid stable 140 kDa acylphosphate in the reaction cycle of the calcium pump in the human erythrocyte leaky ghosts. It also produced endothelium dependent relaxation in the pig coronary artery.

The random peptide phage display library was screened again with higher stringency to obtain caloxin with higher affinity in order to be cost effective and with greater therapeutic potential. This time, the targets were the 2\textsuperscript{nd} putative extracellular domain of PMCA 1 and 2\textsuperscript{nd} and 3\textsuperscript{rd} putative domains of PMCA 4. The peptides selected for binding to the 2\textsuperscript{nd} putative extracellular domain of PMCA 4 selectively inhibited the Ca\textsuperscript{2+}-Mg\textsuperscript{2+} ATPase activity in human erythrocyte leaky ghosts but with a similar affinity as Caloxin2A1. The peptide selected for binding to the 3\textsuperscript{rd} putative extracellular domain
of PMCA 4 was hydrophobic and water insoluble. Substitution of its C-terminus amino acid with lysine residue made the peptide water-soluble and it did inhibit the \( \text{Ca}^{2+}\text{-Mg}^{2+} \) ATPase with slightly higher affinity. However, the inhibition was due to hydrophobicity of the peptide as the randomized version of the peptide also produced inhibition.

We have obtained the first selective inhibitor of PMCA and shown that perturbing extracellular targets can affect protein activity even though most of the functional groups of this protein are in the cytosol.
ACKNOWLEDGEMENTS

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<tr>
<td>ANP</td>
<td>atrial natriuretic peptide</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>[Ca$^{2+}$]i</td>
<td>intracellular calcium concentration</td>
</tr>
<tr>
<td>[Ca$^{2+}$]o</td>
<td>extracellular calcium concentration</td>
</tr>
<tr>
<td>CAM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>1,4-dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>(ethylenedinitriilo)-tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid</td>
</tr>
<tr>
<td>ER/SR</td>
<td>endoplasmic/sarcoplastic reticulum</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>Kd</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>K$_i$</td>
<td>inhibition constant</td>
</tr>
<tr>
<td>K$_m$</td>
<td>michaelis mentens constant</td>
</tr>
<tr>
<td>MES</td>
<td>2-([N-Morpholino] ethanesulfonic acid</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-([N- Morpholino] propane sulfonate-NaOH</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide, reduced form</td>
</tr>
<tr>
<td>NCX</td>
<td>sodium-calcium exchanger</td>
</tr>
<tr>
<td>Pi</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PED</td>
<td>putative extracellular domain</td>
</tr>
<tr>
<td>PEP</td>
<td>phospho(enol)pyruvate</td>
</tr>
<tr>
<td>PIP$_2$</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PMCA</td>
<td>plasma membrane calcium ATPase</td>
</tr>
<tr>
<td>ROCC</td>
<td>receptor operated calcium channel</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarco/endoplasmic reticulum calcium ATPase</td>
</tr>
<tr>
<td>SOCC</td>
<td>store operated calcium channel</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TRP</td>
<td>transient receptor potential</td>
</tr>
<tr>
<td>VOCC</td>
<td>voltage operated calcium channels</td>
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1.0 INTRODUCTION

1.1 Calcium Homeostasis

1.1.1 Overview

Ionized calcium (Ca\(^{2+}\)) plays a role in cell signaling when its concentration in the cytosol is elevated. It controls a large number of cellular functions, including modulation of metabolic routes, cell growth, cell cycle, syntheses and release of hormones, muscle and non-muscle motility, multiplicity of membrane-linked processes and apoptosis. Ca\(^{2+}\) regulates gene expression by modulating the activity of various transcription factors by kinases or phosphatases and plays a role in memory storage by altering the activity of enzyme calmodulin kinase II. Since Ca\(^{2+}\) is an important messenger, in a resting cell it is maintained at very low free concentration\(^{1,2,3,4}\). The intracellular calcium concentration \([\text{Ca}^{2+}]_i\) must be tightly regulated in time, space and amplitude since cells extract specific information from these parameters\(^5\). The normal \([\text{Ca}^{2+}]_i\) is around 0.1 \(\mu\text{M}\), which is about 10,000 fold lower than the extracellular Ca\(^{2+}\) concentration \([\text{Ca}^{2+}]_o\) implying a large chemical gradient along with electrical gradient across the membrane\(^3,5,6\). The plasma membrane determines the \([\text{Ca}^{2+}]_i\), over long term, since it is the only ‘organelle’ that can move Ca\(^{2+}\) between cell and the Ca\(^{2+}\) reservoir of the extracellular space. The short-term role of plasma membrane varies from cell to cell, with large cells also relying on endo (sarco) plasmic reticulum (ER/SR) due to low plasma membrane area\(^7\). The ER/SR can store up to 10-15 mM calcium that can be released upon stimulation, thereby transiently
increasing the cytosolic concentration of calcium\(^6\). Thus, the cell has an access to supply of external calcium and more finite internal store sequestered calcium within the ER/SR, which leads to significant increase in its intracellular concentration required for the signaling function. The major mechanisms involved in removal of Ca\(^{2+}\) from the cytosol are outlined here followed by the systems regulating both the entry and release of Ca\(^{2+}\) into the cytosol.

**1.2 Mechanisms of calcium removal from cytosol**

There exist membrane intrinsic proteins in the plasma membrane and membranes of intracellular organelles whose sole function is to maintain low intracellular Ca\(^{2+}\). Multitude of cytosolic proteins in the cell also binds Ca\(^{2+}\) thus buffering intracellular Ca\(^{2+}\) although their primary function involves decoding its information.

**1.2.1 Membrane bound proteins**

The membrane bound proteins are the calcium pumps of the plasma membrane and ER, Na\(^{+}\)- Ca\(^{2+}\) exchanger (NCX) in the plasma membrane and electrophoretic uniporter in mitochondria. The calcium pump of the plasma membrane is an ATPase (PMCA), which represents less than 0.1% of the total intrinsic membrane proteins. It has high calcium affinity and low capacity that allows it to transport Ca\(^{2+}\) from cells even when its concentration is at resting submicromolar level. Quantitatively, it may appear less important in excitable tissues like heart, where NCX predominates, but it likely plays
the role of a fine tuner of cytosolic Ca\(^{2+}\) operating in a concentration range where the low affinity NCX looses efficiency. It is thought to transport calcium with a 1:1 stoichiometry to ATP hydrolyzed. The pump is the product of 4 genes and has a differential tissue distribution. There are many known regulators of the pump such as calmodulin, kinase directed phosphorylation, acidic phospholipids, polyunsaturated fatty acids and oligomerization. General properties, structural organization, regulation and importance of PMCA are dealt with in detail in the following sections.

The calcium pump of the ER/SR is an ATPase (SERCA), which is very abundant. It has molecular weight of 110 kDa with high calcium affinity. It transports calcium with a 2:1 stoichiometry to the ATP hydrolyzed. There are 3 genes coding for the calcium pump in the ER/SR. An acidic proteolipid, phospholamban is a known SERCA regulator. The general features of PMCA and SERCA show similarities as they belong to P-type cation transporters forming a transient phosphorylated intermediate\(^1,6,8,9\). PMCA has longer C-terminal domain with multiple regulatory mechanisms as compared to SERCA.

Besides PMCA, plasma membrane has the NCX as another Ca\(^{2+}\) extruding system. The NCX is a low affinity and high capacity calcium transporting system, which is particularly important in excitable membranes. The turn over number for the NCX is between 2500 – 5000 sec\(^{-1}\). Although the \(K_m\) (Ca\(^{2+}\)) of the NCX varies from 1-10 \(\mu\)M, it is thought that the concentration of Ca\(^{2+}\) in the zone immediately beneath plasma membrane would be significantly higher than bulk cytosol, thus enabling NCX to play a role in Ca\(^{2+}\) homeostasis. As an example, the NCX is abundant in the transverse tubules of sarcolemma in the heart and are thus close to the Ca\(^{2+}\) releasing terminal cisternae of
SR. The NCX extrudes Ca\textsuperscript{2+} against its large electrochemical gradient using the energy provided by the inward movement of Na\textsuperscript{+} down its steep electrochemical gradient and is sustained by sodium pump. The system operates electrogenically, exchanging 3 Na\textsuperscript{+} for 1 Ca\textsuperscript{2+}. It has been determined by balancing one component of thermodynamic driving force, the membrane potential against the other component, the sodium gradient so that no net Ca\textsuperscript{2+} fluxes occurs\textsuperscript{11}. It can contribute to both Ca\textsuperscript{2+} extrusion and influx depending on the membrane potential and the electrochemical gradients of Ca\textsuperscript{2+} and Na\textsuperscript{+}\textsuperscript{1,6,9,10}. The extrusion occurs via consecutive mechanism where charges move in more than one partial step. It is thought that Na\textsuperscript{+} binds to the exchanger at one side of the membrane, is transported across and released on the other side. Only then Ca\textsuperscript{2+} binds and is transported across in the opposite direction\textsuperscript{10}. The NCX has 970 amino acids of which first 32 amino acids are a signal sequence cleaved off in the ER. It has 9 TM domains. The counter transport function of the exchanger is associated with TM segments. Although the NCX does not bind calmodulin, it has a region resembling the calmodulin-binding site in its large intracellular loop. There are three isoforms of NCX that are cloned and show high level of expression in heart, brain and skeletal muscle\textsuperscript{10}.

There occurs transport of the cytosolic calcium into mitochondria by means of a low affinity, high-speed electrophoretic uniporter. The uniporter is coupled to oxidative phosphorylation, and uses the electropotential gradient across the inner mitochondrial membrane to drive Ca\textsuperscript{2+} into mitochondrion\textsuperscript{1,6,9}. The in vitro affinity of the uniporter for Ca\textsuperscript{2+} (K\textsubscript{m}: 10-20 µM) is low in comparison to the cytosolic Ca\textsuperscript{2+} concentration found in vivo during physiological processes (10 nM-2 µM). However, close proximity of
mitochondria to the Ca\(^{2+}\) release sites from the intracellular stores or Ca\(^{2+}\) influx channels in the plasma membrane creates microdomains of high Ca\(^{2+}\) concentration that is transported by mitochondria. Sequestered Ca\(^{2+}\) activates the mitochondrial matrix enzymes of the citric acid cycle producing more adenosine triphosphate (ATP).

The role of nucleus in Ca\(^{2+}\) homeostasis is unclear but since it is interconnected with the SR, it is considered to act as an internal Ca\(^{2+}\) store. A calcium pump activity has been reported to be responsible for transporting cytosolic calcium into the nucleus\(^6\)\(^9\).

1.2.2 Cytosolic proteins

The signaling function of calcium requires low cytosolic concentration of Ca\(^{2+}\). To control calcium levels, evolution has selected reversible complexation by specific proteins, which are soluble or intrinsic to membranes. The high affinity intracellular calcium binding proteins belong to EF-hand group and annexin family of proteins. These proteins also play a role in decoding the information carried by Ca\(^{2+}\) and pass it on to the targets. The proteins of EF-hand family contain repeat units made of two perpendicular \(\alpha\)-helices, interrupted by non-helical loop of 10-12 amino acids where calcium is coordinated to 6-8 oxygen atoms of carboxylic side chains. Two most important members of EF-hand group proteins are calmodulin and troponin C with high calcium buffering capacity. EF-hand proteins change conformation on binding to Ca\(^{2+}\) becoming more hydrophobic on the surface, and collapse around the binding domain of the target. A group of EF-hand proteins called the neuronal calcium sensors are known to play a role in phototransduction and in regulating the release of neurotransmitters, biosynthesis of
polyphosphoinositides, metabolism of cyclic nucleotides and activity of type A K⁺ channels. Annexins are soluble amphipathic proteins with conserved repeats of about 75 amino acids and separated by sequences of variable lengths¹,².

1.3 Plasma Membrane Calcium ATPases (PMCA)

1.3.1 General properties

PMCA belongs to the P₂ subfamily of P-type ion transport ATPases that form a covalent phosphorylated enzyme intermediate, which is intimately linked with the ion translocation process. The γ-phosphate of ATP reacts with the single aspartic acid residue of the enzyme. Such ATPases are inhibited by lanthanum and vanadate, a transition state analog of phosphate. However, although lanthanum decreases the level of the phosphoenzyme in all other pumps, it stimulates the steady state phosphoenzyme level in calcium pump¹⁵,¹⁶. The pump is suggested to have 1:1 stoichiometry with one Ca²⁺ being translocated with hydrolysis of each molecule of ATP and acts as an electrogenic Ca²⁺: H⁺ exchanger¹⁷. The Ca²⁺: H⁺ countertransport and net charge transfer has been demonstrated using PMCA purified from brain and reconstituted in liposomes. Ca²⁺ uptake, H⁺ ejection and electrogeneity were studied by observing the absorption and fluorescence change in arsenazo III, a metallochromic indicator, a fluorescent pH indicator and the transmembrane electrical potential gradient indicator respectively¹⁸. Coexistence of SERCA and PMCA that remove cytosolic Ca²⁺, and other transport channels that allows electrolyte leak in an intact cell along with lack of a specific
inhibitor of PMCA has rendered it difficult to directly characterize the nature of electrogenic Ca\(^{2+}\): H\(^+\) exchanger. Inconsistent results are obtained by studies on inside-out vesicles and on reconstituted proteoliposomes where the various detergents used have different effects on the electrogenic properties of the Ca\(^{2+}\) pump.

Although the exact mechanism of ion translocation in PMCA is not known, it has been studied in detail in SERCA, which also belongs to P-type ATPases. The intermediate steps are formulated in the 4-step scheme: E\(_1\)→E\(_1\)P→E\(_2\)P→E\(_2\)→E\(_1\). In the first step, a high-energy phosphorylated intermediate (E\(_1\)P), with the bound Ca\(^{2+}\) is formed by the reaction of the enzyme with the MgATP. In the step (2), a number of conformational changes take place in the protein leading to translocation of the bound cation across the membrane and conversion of the protein to a low energy intermediate E\(_2\)P. Release of the bound cation takes place concomitant with or following the conversion of the E\(_1\)P to E\(_2\)P. This new conformation E\(_2\)P has low affinity for the cation. In the step (3), phosphate is removed from the enzyme by hydrolysis, followed by the return of the protein to the E\(_1\)P conformation in the last step\(^{16}\).

1.3.2 Structural organisation of PMCA

Proteolytic digestion, hydropathy plots and information obtained from structure of SERCA have been used to understand the organization of PMCA in the plasma membrane. Like the other P-class ATPases, the calcium pump of the plasma membrane has 10 putative transmembrane (TM) spanning helices with about 80% of the pump mass protruding into the cytosol. The TM domains of the pump are connected on the outside of
the membrane with very short loops. The continuation of the polypeptide chain from the TM domains 1-5 projects into the cytosol as ‘stalk’ segments.\textsuperscript{16,17,19}

Three main cytosolic domains protrude from the membrane. The first small cytosolic loop connects the TM domains 2 and 3 and is the ‘A domain’ (actuator region) that couples the translocation of ion to ATP hydrolysis. The second large cytosolic loop between TM domains 4 and 5 has the ‘N domain’ (nucleotide binding site) and the ‘P domain’ (aspartylphosphate formation site) and the third cytosolic region extends from the last TM domain. The small cytosolic loop has \(\beta\)-sheet structure and corresponds to the transducing domain of SERCA that mediates the coupling of ATP hydrolysis to \(\text{Ca}^{2+}\) translocation. It also contains an acid phospholipid responsive region that is rich in basic amino acids. The large cytosolic loop is the catalytic domain that contains the site for aspartyl-phosphate formation and about 140 residues downstream of it is the ATP binding site. The former has a conserved sequence ‘CSDKTGTT’ found in all P-type ATPases. The ATP binding site sequence ‘FSKGAE’ has central three residues conserved in all P-type ATPases. There is also a ‘hinge’ region with sufficient flexibility to bring these two sites close together during the reaction cycle. The last cytosolic unit protruding from the 10th TM domain is unique to PMCA and has sites involved in the multiplicity of regulation of the pump, including the calmodulin (CAM) binding site, and consensus sequences for protein kinases.\textsuperscript{16,17,19} The different isoforms of PMCA show great similarity in their catalytic core region but differ substantially in the downstream regulatory region. The 4 PMCA genes and their splice variants produce this diversity in the regulatory region. Experiments with controlled trypsinization, radioactively labelled
CAM, and CAM coupled to cleavable radioactive photoaffinity cross linker have been
used to identify the calmodulin-binding domain. The calmodulin-binding domain is about
30 residues long and is rich in basic amino acids with a conserved tryptophan in amino
terminal portion. It is flanked by two acidic stretches that have non-catalytic Ca\(^{2+}\) binding
sites\(^{20}\).

1.3.3 Regulation of PMCA

The important property of PMCA that sets it apart from the other members of the
P-type ATPases is the presence of multiple regulatory mechanisms. The catalytic part
of PMCA, which is required for Ca\(^{2+}\) pumping activity, is homologous to SERCA. The
downstream portion of PMCA is the regulatory region that inhibits and controls the
activity of the pump and is unique to PMCA.

1.3.3.1 Regulation by calmodulin

Calmodulin is a Ca\(^{2+}\)-binding protein that directly regulates PMCA. It binds to the
calmodulin-binding domain in the C-terminal end of PMCA and increases both the
affinity of the pump for Ca\(^{2+}\) and the rate of transport of the ion\(^{4,19}\). Studies indicate that
CAM shifts the equilibrium of the enzyme towards the active E\(_1\) conformation and
increases the rate of phosphorylation and dephosphorylation\(^{19}\). The different isoforms of
PMCA have different affinities for CAM. Beyond the affinity of CAM for the pump, its
rate of binding and dissociation are also very important in determining the biological
properties of PMCA. If the binding of CAM to an isoform were slow, the dissociation would be even slower due to the affinity of pump for CAM. Such slow on and off rates for CAM would cause the activation of the pump to respond to fluctuations in Ca\(^{2+}\) levels in an integrative fashion. In the presence of low frequency, low intensity fluctuation in the Ca\(^{2+}\) influx, the slow on rate of CAM would intensify the spikes, allowing cytosolic Ca\(^{2+}\) to increase to higher levels before the pump is activated. In the presence of high frequency, high intensity fluctuations in the Ca\(^{2+}\) influx, the pump would be expected to accentuate the spikes. However, the pump would become more activated with each event of Ca\(^{2+}\) influx, so that the trailing edge of the spike would become steeper and the spike more clearly defined. Thus the different isoforms of PMCA with differential tissue expression would modulate differently the frequency and intensity of Ca\(^{2+}\) spike to meet the needs of the cell\(^7\).

The calmodulin-binding domain has sequences that show resemblance to the IQ motifs (IQXXXRGXXXR) that are well characterized in unconventional myosins and are involved in binding to CAM. Alteration of the IQ motif in the calmodulin binding domain may confer Ca\(^{2+}\) sensitivity to it\(^7\). The calmodulin-binding domain shares several structural features with other CAM binding enzymes including clusters of arginine residues with a preponderance of hydrophobic residues in the first half of the domain\(^20\).

In the absence of CAM, the calmodulin binding domain with other residues further along the enzyme act as an internal repressor of the pump by binding to the A domain and the N domain, thereby limiting substrate access \(^4,8\). The N-terminal half of the calmodulin-binding domain interacts with the large 'N domain' and the C-terminal
half interacts with the small 'A domain'. A key residue within the calmodulin-binding domain is tryptophan that is an essential anchor for autoinhibition and CAM binding\(^{21}\). Although the free concentration of calmodulin in the cell exceeds the amount required to saturate the calcium pump, its binding to the pump is calcium dependent. The modulation of the pump by calmodulin is PMCA isoform dependent and is highly cooperative as the rise from 10-90% of full pump activity occurs by 4 to 5-fold increase in \([\text{Ca}^{2+}]_i\)\(^{22}\). PMCA 2 has abnormally high calmodulin affinity\(^{4,17}\). High affinity of an isoform for calmodulin, leads to its inability to stimulate and it may act as a pseudo-subunit of the pump\(^{4,17}\).

1.3.3.2 Regulation by acidic phospholipids

The acidic phospholipids as phosphatidylserine, phosphatidylinositol, phosphatidic acid and cardiolipin stimulate the pump by increasing the affinity for \(\text{Ca}^{2+}\), the rate of transport and cooperativity for calcium. The concentration of phospholipids in the membrane would be sufficient for half-maximal activation of the pump. They are more effective as activators as compared to calmodulin. Phosphatidylinositol 4,5 bisphosphate (PIP 2) is one of the most potent stimulator of the pump. PIP 2 may be important in keeping PMCA partially active in resting cells, where free \(\text{Ca}^{2+}\) would be low and calmodulin stimulation would be minimal. Its modulation occurs by phosphorylation and dephosphorylation and its phosphorylated derivatives are extremely effective pump activators. The turn over rate of these phospholipids is rapid and their breakdown products are not stimulatory\(^{4,17,19}\). Agonists stimulate phospholipase C (PLC) that degrades PIP 2 into diacylglycerol and 1,4,5-inositol triphosphate, thereby reducing
the activity of the calcium pump. This requires that PLC and PMCA be present in close association. This suggests the localization of PMCA with PLC in caveolae where the PLC can share a common pool of PIP 2 with PMCA. Stimulation of the pump by phospholipids is partly based on their binding to the pump, which may occur through the polar head groups. Polyamines inhibit the stimulatory effect of the acidic phospholipids. Their cationic groups interact with the negatively charged phosphate groups of the phospholipids. The polycation spermine, which is present in many mammalian cells and is highly abundant in the brain, is known to inhibit the Ca\textsuperscript{2+} pump. Studies on the inhibitory effect of spermine have shown that the polycation can also interact with the negatively charged groups of the aminoacid residues on the cytosolic loops of the pump, bridging them and blocking the access of the substrates to the catalytic core.

1.3.3.3 Stimulation by protein kinases

Cyclic AMP dependent protein kinase (PKA): The PKA phosphorylates the serine residue in the sequence KRNSS which appears to fit with the PKA consensus sequence. This sequence is found in PMCA 1b isoform only. This site is present halfway between the C-terminal domain and the calmodulin-binding domain. The phosphorylation decreases the Km (Ca\textsuperscript{2+}) to 2 μM with two-fold increase in V\textsubscript{max} of the pump, perhaps through the modulation of the calmodulin-binding domain. The stimulation of the pump by calmodulin and PKA is non-additive. The difference in the stimulation by the PKA perhaps depends on the pump isoform diversity with varying distances between the phosphorylation site and the calmodulin-binding domain. Cyclic AMP in addition to
phosphorylation may lead to cytosolic alkalinization increasing the calcium extrusion. Studies on parotid acinar cells that lack NCX have shown that agonists that increase the cAMP levels in cells also phosphorylate PMCA and increase its activity. This potentiation is dependent on elevated Ca\(^{2+}\), and is prevented by lanthanum. However, lanthanum is a non-specific inhibitor of ATPases and it cannot be ruled out that reversal of enhanced PMCA activity in the presence of specific inhibitor would be more convincing.

**Cyclic GMP dependent protein kinase (G kinase):** The G-kinase increases the \(V_{\text{max}}\) and \(Ca^{2+}\) affinity of the pump. The exact mechanism of the activation is not known. It may occur directly through phosphorylation of the pump or indirectly through the phosphorylation of another protein that may regulate the \(Ca^{2+}\) pump. In-vivo studies to see the cGMP-dependent regulation of PMCA was carried out using agents as Atrial natriuretic factor that is known to increase the intracellular cGMP levels. Such studies showed an increase in the \([Na^+]_o\)-independent \(Ca^{2+}\) efflux. At 0.1 \(\mu M [Ca^{2+}]_i\), which is the threshold level for contraction, the \(Ca^{2+}\) efflux rate was twice that of the untreated cells.

**Protein kinase C (PKC):** PKC increases the \(V_{\text{max}}\) of the pump. It phosphorylates the pump in the threonine and serine residues in the carboxy terminal of the \(Ca^{2+}\) pump downstream of the calmodulin binding domain, which also has arginine and lysine residues that activate phosphorylation. Smaller amounts of phosphorylation occur at other
sites in the C-terminus; one of these sites is the threonine of calmodulin binding domain\textsuperscript{7}. The phosphorylation relieves the inhibition due to the calmodulin binding domain downstream inhibitory region. It incorporates 1 mole of phosphate per mole of the pump and its phosphorylation is antagonised by calmodulin. The phosphorylation of the isoforms PMCA 2a and PMCA 3a occurs predominantly in the calmodulin binding domain that then prevents the binding of CAM so that the phosphorylated Ca\textsuperscript{2+} pump have constant low activity\textsuperscript{7}. Stimulation of the pump is dependent on the isoenzyme form of the kinase; pump isoform diversity reflected in different calmodulin affinities of the pump isoforms and lipid associated with the pump\textsuperscript{8,17}. The agonists stimulating PLC, which results in PKC signaling pathways, activate the Na\textsuperscript{+}-H\textsuperscript{+} exchanger leading to alkalinisation. This rise in pH increases the affinity of the pump for the calcium\textsuperscript{8}.

\subsection{Effect of proteases}

There occurs controlled degradation of the pump by the intracellular calcium dependent protease calpain that truncates the pump at its COOH terminus. It removes the calmodulin-binding domain in two steps leading to calmodulin insensitivity and permanent activation of the pump. Calmodulin delays the action of calpain. The cytosolic concentration of Ca\textsuperscript{2+} required to activate calpain exceeds 1 µM, which is found in injured cells and calpain activated pump is then acted upon by other proteases completing the degradation process. In such a case, calpain would be part of the cycle leading to cell death due increased cytosolic calcium\textsuperscript{17}. 
1.4 Mechanisms of calcium entry into cytosol

1.4.1 Calcium entry channels

There are two main types of entry channels found in the plasmalemma that are classified on the basis of their regulatory mechanisms; Voltage Operated Calcium Channels (VOCC) and Receptor Operated Calcium Channels (ROCC). VOCC's are found mainly in excitable cells as muscle and neuronal cells, where they are activated by membrane depolarization by K⁺ solutions or agonists. Mammalian VOCC are comprised of five protein subunits. One subunit forms the Ca²⁺ channel and the others serve to regulate the channel gating⁶,⁹,¹²,¹³.

ROCC's are non-specific cation channels with some degree of divalent specificity. They form structurally and functionally diverse channels that are particularly prevalent on the secretory cells and at nerve terminals. They open as a result of binding of an agonist to the receptor. The receptor protein is separate from the channel protein and involves a trimeric G-protein, ER or a mobile intracellular messenger to link the two. The Store Operated Calcium Channels (SOCCs) are a major subfamily of the ROCC's. SOCC's are selective for Ca²⁺ and other ions as Ba²⁺ and Sr²⁺ do not readily enter through this pathway. SOCC's open in response to the depletion of intracellular calcium stores, either by a physiological Ca²⁺ mobilising messenger or pharmacological agents. Light detection by photoreceptor cells in compound eye of Drosophila activates the Ca²⁺ entry channels known as TRP (transient receptor potential) in the photoreceptor cell membrane. Mammalian homologs of TRP have been found and are thought to act as the SOCC's. Of the two prominent schemes for signaling Ca²⁺ influx via SOCC's, one
involves the conformational coupling between the Ca\(^{2+}\) entry channels on the plasma membrane and the Ca\(^{2+}\) release channels on the ER/SR. Direct physical connection of human TRP3 channel (that has been regarded as a candidate for SOCC), with the IP\(_3\)R has been observed by coimmunoprecipitation. The second mechanism may be the release of a diffusible calcium influx factor from the depleted stores that activate Ca\(^{2+}\) entry channels on the plasma membrane. SOCC’s may be one of the most ubiquitous plasma membrane Ca\(^{2+}\) channels\(^{6,9,12,13}\).

Mechanically activated Ca\(^{2+}\) channels that respond to cell deformation are now being found in many cells\(^{13}\).

### 1.4.2 Calcium release channels

There are two types of channels in the ER/SR that are voltage independent. These are the inositol triphosphate receptors (IP\(_3\)Rs) and the ryanodine receptors (RyRs). The IP\(_3\)R is a large structure composed of 4 subunits (total molecular mass ~1200 Kda) and is activated by inositol 1,4,5-triphosphate (IP\(_3\)). In the inositol phosphate calcium signaling pathway, the binding of an agonist to the receptor on the plasma membrane leads to the activation of Phospholipase C which breaks down membrane associated phosphatidylinositol 4,5-bisphosphate into calcium signaling messenger IP\(_3\) and the protein kinase C activator diacylglycerol\(^{6,14}\). The IP\(_3\) is highly mobile in the cytosol and diffuses to encounter the IP\(_3\)R in the ER/SR. The binding of IP\(_3\) to its receptor changes the conformation of IP\(_3\)R to open an integral channel to allow Ca\(^{2+}\) to be released into cytosol. The IP\(_3\)R requires IP\(_3\) to open but their activation is regulated by cytosolic
calcium levels (activation 0.5-1 μM; inhibition >1 μM)$^{6,13}$. IP$_3$ has a lifetime in order of seconds and is metabolized by enzymes. Addition of a phosphate group to IP$_3$ by a Ca$^{2+}$-dependent kinase produces inositol 1,3,4,5-tetrakisphosphate that is known to bind to GTPase-activating protein that modulates Ca$^{2+}$ release$^{13}$.

Ryanodine receptors are sensitive to ryanodine, a neutral plant alkaloid and calcium release is produced by binding of calcium, cyclic ADP ribose and caffeine to the receptor. The RyRs are sensitive to the cytosolic Ca$^{2+}$ concentration (activation at 1-10 μM; inhibition at >10 μM). Caffeine increases the sensitivity of RyRs to Ca$^{2+}$. RyRs are found largely in excitable cell types as muscle and neurons. Their conductance and molecular mass is twice that of IP$_3$R$^{6,13}$.

Another calcium release channel that has recently been found is the sphingolipid Ca$^{2+}$ release mediating protein of the endoplasmic reticulum. It is activated by sphingolipids as sphingosylphosphorylcholine and sphingosine-1-phosphate. It is expressed in many different types of tissues including cardiac muscle, pancreas and liver. It has very small molecular mass—20 kDa$^{6,13}$.

There seems to exist another unknown receptor that is distinct from the IP$_3$R and the RyRs. It is activated by nicotinic acid adenine dinucleotide phosphate$^{13}$.

Mitochondria, which store Ca$^{2+}$ when its level in the cytosol rises, has a sodium calcium exchanger (NCX) that runs at a slow rate and is involved in Ca$^{2+}$ efflux into the cytosol$^{13}$. 
1.5 Importance of PMCA

Calcium efflux across the plasma membrane occurs through the Na\(^+\)-Ca\(^{2+}\) exchanger (NCX) and PMCA. Due to higher affinity of the pump for calcium (Kd- 100 nM), it can extrude significantly more calcium at cytosolic calcium concentration \([\text{Ca}^{2+}]_i\) below 1 \(\mu\text{M}\) than the exchanger (Kd-1000 nM), thus being the primary mechanism responsible for maintaining the low resting levels of calcium in the cells\(^{25,26}\). While NCX removes the bulk of excess calcium following stimulation of the cell, PMCA is responsible for the fine-tuning of the intracellular calcium levels at all times. The extrusion of Ca\(^{2+}\) in exchange for Na\(^+\) depends on the Na\(^+\) gradient that is maintained by the sodium pump. Thus the Na\(^+\) dependent extrusion is limited by the activity of Na\(^+\)-K\(^+\) ATPase. The plasma membrane enriched fraction from the antral smooth muscle has about 4 times higher PMCA activity than Na\(^+\)-K\(^+\) ATPase activity, reflecting the major role of PMCA in calcium extrusion\(^{27}\). Evidence also supports the key role of PMCA in the modulation of muscular tone\(^{28}\).

Studies in smooth muscle have shown that there is higher Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase activity in the plasma membrane than the endoplasmic reticulum, suggesting a relatively higher contribution of PMCA to Ca\(^{2+}\) fluxes\(^6,27\). This however, could also be due to lower density of calcium pumps in the ER than in the plasma membrane\(^6\). The ability of guinea pig ureter smooth muscle cells to reduce the \([\text{Ca}^{2+}]_i\) rapidly following the Ca\(^{2+}\) loading in voltage-gated transients was markedly inhibited if Na\(^+\) concentration gradient was reversed, but was little affected if the Na\(^+\) gradient was decreased by 25 or 50\%. Recovery from Ca\(^{2+}\) load caused by the reversal of Na\(^+\) gradient could occur by the
removal of \([\text{Ca}^{2+}]_o\) in continuing absence of \(\text{Na}^+\) outside, indicating the importance of a \(\text{Na}^+\) independent \(\text{Ca}^{2+}\) removal system\(^{29}\).

Immunocytochemistry revealed that NCX in astrocytes and neuronal somata is confined to plasma membrane microdomains that overlie the underlying sub-plasma membrane junctional ER (jER) that has structural resemblance to the plasma membrane-SR junctions observed in skeletal, cardiac and smooth muscle. PMCA has more uniform distribution in the plasma membrane and is thus involved in the regulation of the bulk cytosolic \(\text{Ca}^{2+}\) while NCX may regulate the \(\text{Ca}^{2+}\) sequestered in the jER\(^{26}\). In the presynaptic nerve terminals only PMCA is clustered at the active zones to maintain low cytosolic \(\text{Ca}^{2+}\) and ‘reprime’ the vesicular release mechanism following the activity\(^{26}\).

It has been suggested that PMCA exchanges a \(\text{H}^+\) for \(\text{Ca}^{2+}\) and thus leads to initial rapid acidification of the cytosol followed by alkalinization after agonist stimulation. This proton influx may play an important role in excitation-response coupling after stimulation\(^{28}\).

However, the distribution of PMCA and NCX varies in different tissues and the lack of a specific inhibitor has been a limitation in understanding the relative contribution of the calcium pump to \(\text{Ca}^{2+}\) homeostasis. Most of the experiments are carried out under conditions that differ from the cellular environment. Some of the parameters in experiments that can affect the result of such studies are: 1) the purification of \(\text{Ca}^{2+}\) pump and its reconstitution in lipids using different detergents in preparation, 2) maintaining experimental temperature at \(37^\circ\text{C}\), 3) purification of pump results in isolation of \(\text{Ca}^{2+}\)
pump from other transporters and channels with which it is associated in the cell microdomains as caveolae, and where it may be functionally coupled to them.

1.6 Inhibitors of PMCA

There are no known specific inhibitors of the calcium pump. In addition to vanadate that inhibits all P-type ATPases and lanthanides, a number of non-specific inhibitors exist that do not have a physiological role\textsuperscript{15}. Eosin, which is commonly used as PMCA inhibitor also inhibits Na\textsuperscript{+}-K\textsuperscript{+}ATPase. Inhibitors of PMCA are required to understand the role of Ca\textsuperscript{2+} in signal transduction and intracellular Ca\textsuperscript{2+} homeostasis. Thapsigargin, a tumor promoting sesquiterpene lactone specifically inhibits SERCA and has hence helped in elucidating its physiological role in maintaining intracellular Ca\textsuperscript{2+} homeostasis and the mechanism of its ion transport capacity\textsuperscript{30}.

Significance of specific inhibitors lies in their power to help understand the structural organization, tissue distribution, mechanism of action, regulation and relevance to the understanding of the pathological processes associated with the dysfunction of the enzyme. This has been well demonstrated by the cardiac glycosides (ouabain, digoxin), specific inhibitors of the Na\textsuperscript{+} pump that bind to it extracellularly\textsuperscript{31}. The Na\textsuperscript{+} pump belongs to the same family of P-type ion ATPases as PMCA and plays an important role in maintaining the gradients of Na\textsuperscript{+} and K\textsuperscript{+} ions across the plasma membrane. It is the only receptor for ouabain that binds to the limited extracellular domain of the \(\alpha\) subunit of the Na\textsuperscript{+} pump which is mainly cytosolic\textsuperscript{31,32}.
The cardiac glycosides are used as therapeutic agents to increase the tone of the failing heart where they bind extracellularly to the Na\(^+\) pump and block it. This increases the intracellular concentration of Na\(^+\), which activates the Na\(^+\)-Ca\(^{2+}\) exchanger in the plasma membrane to increase the cytosolic concentration of Ca\(^{2+}\), which leads to contraction\(^3\). Thus digoxin acts on the Na\(^+\) pump and the chain of events in its action involves raising the cytosolic Ca\(^{2+}\), which could be directly modulated by an inhibitor of PMCA that plays a crucial role in lowering the cytosolic Ca\(^{2+}\) as explained above. Since every cell depends on both Na\(^+\) and Ca\(^{2+}\) pump, thus, obtaining an inhibitor that will selectively modulate the Ca\(^{2+}\) pump, can eventually lead to designing of non-peptide substances that could be used therapeutically as digoxin and angiotensin related peptides\(^{33}\).

1.7 Screening for an inhibitor using Phage display peptide library

Phage display peptide library is unique in having physical linkage between the bacteriophage - surface displayed randomized peptides and the genotype coding for it packaged within the same virion. Combinatorial peptide libraries are developed through site directed mutagenesis using degenerate oligodeoxynucleotides, which are then incorporated into the single stranded circular bacteriophage genome at the N terminus of the gene encoding its coat protein. It has developed as a powerful tool for identifying a novel peptide ligand for peptide or non-peptide targets by process of screening called biopanning. In the simplest form biopanning involves the incubation of the phage with the target, washing away of the non-bound phage followed by elution of the bound phage.
The eluted phage is then amplified and repeated through the same process till the eluted phage is enriched in species binding to the target. Clones are then picked from the enriched phage and analyzed for the sequences coded by it. The Ph.D library has been used in a number of applications including the screening for high affinity novel inhibitors (e.g. for cholesterylester transfer protein), epitope mapping (e.g. in human myeloma proteins in multiple myeloma patient) and studying protein-protein interactions (e.g. streptavidin). The random peptide libraries have either unconstrained peptides that lack any forced, specific constraints in the peptides of different lengths or have constrained peptides with the introduction of covalent constraints e.g. by cysteine residues that can form disulfides.

The Ph.D-12 library, which is based on a combinatorial library of random 12-mer peptide fused to the minor coat protein (p III) of the filamentous coliphage M13 was screened for the ligand to bind to the 2nd putative extracellular domain (PED 2) of PMCA (Fig. 1). The phage that bound PED 2 of PMCA 1b from vascular tissue coded for the peptide that was tested for its inhibitory effect on PMCA and termed caloxin 2A1. Caloxins are defined as substances that bind to one of the 5 putative extracellular domains (PED 1 to 5) of the plasma membrane Ca\(^{2+}\) pump to alter its activity.

1.8 Objectives of study

There are two main objectives of my study. Since caloxin 2A1 was obtained by screening the Ph.D-12 library against the PED 2 of PMCA, its binding to the domain indicates that it would modulate the ATPase activity of the pump by restricting the
movements involved in conformational changes during the reaction cycle. Therefore, in
Aim 1 the focus will be to develop the biochemical assays to characterize caloxin2A1 to
test if it does modulate the activity of PMCA. If it does modulate the activity but with
lower affinity then in Aim II, the random phage display peptide library will be screened
again with higher stringency to obtain higher affinity caloxins.
**Fig. 1. PMCA1 STRUCTURE.** Main model is for rabbit PMCA1b (Khan & Grover, Genbank X59069) and latest model from Dr. Guerrini (Carafoli group). Comparison of PMCA1 to 4 are for human (Swiss protein bank accession #P20020, Q01814, Q16720, P23634). Identical residues are marked as “-”. Transmembrane residues in PMCA4 whose mutation to A cause loss of ATPase activity and acylphosphate formation from ATP: E423, N879, D883, Q971 (Guerini et al (1996) Biochemistry 35:3290-3296). Corresponding residues in PMCA1 sequence are marked with*” Cytoplasmic Sites of Interest: 461-D: Acylphosphate site, 598-IFSKEGASEILK: FITC binding site, 1080-RELRRWQILWFRLNRIQ: calmodulin binding site, 1174-KRNNS: cAMP-dependent phosphorylation site.
2.0 MATERIALS AND METHODS

2.1 Materials

Ph.D-12 phage display library kit was obtained from New England Biolabs, Inc. Agar, trypticase peptone and yeast extract were obtained from Becton Dickinson, (Cockeysville, MD). $^{32}$P-$\gamma$-ATP was from Amersham (Piscataway, NJ). ATP, bovine serum albumin (BSA), imidazole, EGTA, EDTA, ouabain, NADH, phospho(enol)pyruvate (PEP), pyruvate kinase-lactate dehydrogenase, calmodulin (from bovine testes), 3[N-Morpholino]propane sulfonate-NaOH (MOPS), 2-[N-Morpholino]ethanesulfonic acid (MES), keyhole limpet hemocyanin, ovalbumin and tetracycline were obtained from Sigma (St Louis, MO). Acrylamide and agarose were obtained from GibcoBRL (Grand Island, NY). All other chemicals were purchased from standard commercial sources.

2.2 Method of screening Ph.D-12 library for obtaining Caloxin2A1

Phage display peptide library (Ph.D-12), the combinatorial library of random 12-mer peptide fused to the minor coat protein (pIII) of the filamentous coliphage M13 was used for screening for binding to the 2nd putative extracellular domain (PED) of PMCA1b isoform. The PED 2 of PMCA1b in rabbit contains the residues 401-413 (KRPWLAECTPIYI; GenBank accession no X59069). Cysteine in the sequence was replaced by serine, a cysteine added to the COOH terminal end of the sequence and additional flanking amino acid residues were added to synthesize the peptide PMCA398:
WVQKRPWLAESTPIYIQYFVKC. PMCA398 was then conjugated to keyhole limpet hemocyanin (khlh) or ovalbumin. The screening procedure has been previously described\(^\text{10}\). Many clones picked after the final cycle of panning had no inserts but six clones encoded for the peptide VSNSNWPSFPSSGGG-amide, which was synthesized and eventually termed caloxin2A1. Randomization of the residues of caloxin2A1 peptide gave the sequence SWSSFPGSGGVSNPN-amide.

2.3 Synthesis and conjugation of the peptides

The peptides were synthesized by Dalton chemical laboratories (Toronto, Ontario, Canada). The key hole limpet hemocyanin and ovalbumin conjugates of the target peptides were synthesized by Bio-synthesis, Inc (U.S.A.). The peptides and the conjugates were purified by high-pressure liquid chromatography and verified by mass spectroscopy.

2.4 Screening strategy for obtaining high affinity Caloxin in round 1

Ph.D-12 library was screened for binding to the target sequence PMCA398 (WVQKRPWLAESTPIYIQYFVKC) of PMCA 1b isoform.
2.4.1 Panning

The overall screening strategy consisted of first 8 cycles of panning with lower stringency and next 7 cycles of panning with higher stringency. Each cycle of panning for first 8 cycles consisted of following steps:

Day1: the microtiter plate wells were coated with the target peptide, which contained 0.1 mg/ml each of PMCA 398 and PMCA 398-khlh conjugate in sterile phosphate-buffered saline (PBS) with 1 mM sodium azide and left in the fridge overnight covered by saran wrap. PBS contained 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄ and 1.5 mM KH₂PO₄ (pH 7.4).

Day2: the excess target peptide from the wells in the plate was poured off and firmly slapped face down on clean paper towel to remove any residual solution.

- The remaining binding sites in the wells were blocked with blocking buffer containing 5 mg/ml of bovine serum albumin (BSA) in sterile PBS with 1 mM sodium azide for 1h in fridge.
- After discarding the excess blocking solution, the wells were washed 6x with sterile PBS.
- Phage was then added to the wells and incubated at room temperature on the rocker for 1h.
- The unbound phage was poured off and wells were washed 10x with PBS and for 5 min each with blocking solution containing 0.5 mg/ml of each keyhole limpet hemocyanin and ovalbumin and 5% carnation milk in PBS.
The phage was eluted with the low pH elution buffer (0.2M glycine with 1 mg/ml BSA, pH 2.2) for 10 min and the eluate was amplified.

2.4.2 Amplification and precipitation of the eluted phage

The LB-tet (85.6 mM NaCl, 0.5% yeast extract, 1% trypticase peptone, tetracycline10 µg/ml) was inoculated with a colony of XL-1-Blue non-competent cells and allowed to grow at 37°C with shaking for 4h to get an early log culture. The eluted phage was added to the culture and incubated at 37°C with vigorous shaking for 4.5h. The amplified phage was then centrifuged at high speed (18,000 rpm) for 2 min and supernatant transferred to fresh tubes containing 1/3 volume of PEG/NaCl (20% polyethylene glycol–8000, 2.5 M NaCl) to precipitate the phage overnight. The amplified phage was used for the next cycle of panning. After the initial 8 cycles of panning, the eluate of 8th cycle was titered to keep the input plaque-forming unit (pfu) constant.

2.4.3 Phage titering

- The LB-agar (85.6 mM NaCl, 0.5% yeat extract, 1% trypticase peptone, 1.5% agar) plates were prewarmed at 37°C until use.
- An overnight culture started with a single colony of XL-1-Blue non-competent cells in LB-tet was diluted 1:500 in 100ml LB-tet and grown at 37°C with shaking for 4h to density of 0.3-0.6.
• Cells were harvested in cold centrifuge at 18,000 rpm for 2 min and resuspended in LB without tet (25% of original volume).

• The agarose top (0.6% agarose, 85.6 mM NaCl, 0.5% yeat extract, 1% trypticase peptone) was melted and equilibrated in a water bath at 48°C.

• Amplified phage was serially diluted in LB in range of E+2 to E+11.

• The 15 ml sterile tubes were labeled corresponding to the phage dilution, 100 µl cells were dispensed into them, 1 for each dilution, followed by the addition of 100 µl each of the diluted phage.

• One at a time, 4 ml of 0.6% agarose was added to each of the tubes and poured immediately onto the prewarmed LB-agar plates.

• After cooling the plates for 30 min, they were inverted and incubated at 37°C overnight. The plaques were counted next morning and multiplied with the dilution factor to get the titers in pfu/100 µl.

The input pfu was kept constant at E+10 pfu/well for the future cycles of panning. The 9th cycle of panning was carried out as above except that the elution was carried out with specific elution buffer containing PMCA398-ovalbumin conjugate (0.1 mg/ml in PBS, with 10 µg/ml tetracycline and no sodium azide) at different times (0.25, 0.5, 1, 2, 4, 6, 24 and 48h) and then with the low pH buffer. The 48h eluate of 9th panning cycle was amplified, titered and selected again for another round of panning with elution for 24 and 48h followed by the low pH buffer. The unamplified differential time eluates (24 and 48h) of subsequent panning cycles were titered, amplified and titered...
again to add constant pfu of the 48h eluate in the next round of panning. The phage was selected again by the same method for 6 more rounds.

2.4.4 Picking of plaques and amplification

The enriched phage after the final cycle of panning was titered at a dilution to get well-spaced 50-100 plaques per plate. 50 plaques were then picked with sterile wooden sticks and transferred to 700 μl LB containing 15 ml sterile tubes and amplified by incubating with the 300 μl of early log phase culture of XL-1 blue cells. The amplified phage was precipitated overnight in 1/3 volume of PEG/NaCl. The precipitated phage was resuspended in LB and incubated with 10 ml of diluted overnight culture for 4.5h. The cells were centrifuged and the supernatant collected in the fresh tube (~10 ml). Third amplification was carried out by incubating 10 ml of the amplified phage with 1:3 diluted overnight culture started in 3 ml of LB-tet with a single colony of XL-1 blue cells. It was grown for 3.5-h with shaking at 37°C. The cells were then used for the plasmid DNA isolation.

2.4.5 Isolation of plasmid DNA

The plasmid DNA was purified from the XL-1 blue cells after the third amplification cycle by using the Plasmid Midi Purification Kits (QIAGEN, Mississauga, Ontario). Briefly, the XL-1 blue cells were harvested, lysed under alkaline conditions in the presence of RNase A. The lysate was then neutralized, centrifuged at high speed to remove debris. Cleared lysate was then loaded for binding of plasmid DNA to the anion
exchange resin. After washing, the plasmid DNA was eluted, desalted and precipitated in isopropanol. After washing in ethanol, the purified DNA was redissolved in water. The plasmid DNA was then quantified on agarose gels or using a spectrophotometer. The plasmid DNA was then sequenced by MOBIX (McMaster University, Hamilton) using the downstream -96 and -28 primers provided in the phage display peptide kit. The reliability of sequencing was confirmed by using two different downstream primers (-96 and -28) for sequencing the plasmid, which gave the same insert sequence.

2.5 Strategy for obtaining high affinity Caloxin for PED 2 of PMCA 1b isoform

A Ph.D-12 library was screened for binding to the target sequence PMCA398-khlh conjugate (WVQKRPWLAESTPIYIQYFVKC) of the PMCA 1b isoform.

Panning was done similarly to the first round except that the elution was carried out with the specific target PMCA398-ovalbumin conjugate instead of the low pH buffer from first round onwards. For each cycle of panning, the elution was carried out for 24, 48 and 72 h followed with low pH buffer elution. The differential time eluates were titered and amplified. The amplified 72-h eluate was then used for panning in the next cycle at a constant input of 7E+10 pfu/well. Panning was done for 5-6 cycles and then 40 clones were picked, amplified and sequenced as described for round 1.
2.6 Strategy for obtaining high affinity Caloxin for PED 2 and PED 3 of PMCA 4

Two Ph.D-12 libraries were screened for binding to the two different target sequences. One target sequence was the PED 2 of PMCA 4 isoform that contained residues 391-403 (RRPWLPPECTPIYI). Cysteine was substituted by serine, another cysteine was added at the COOH end and additional flanking amino acid residues were added to synthesize the peptide V\text{INRRPWLP}{PESTPIYI}QYFVKC. This target peptide was then conjugated to khlh or ovalbumin. The wells of the microtiter plate were coated with 0.1 mg/ml each of PED 2 and PED 2-khlh conjugate in sterile PBS and 1 mM sodium azide. Elution was carried by specific elution peptide V\text{INRRPWLP}{LAESTPIYI}QYFVKC-ovalbumin conjugate.

The second target sequence was the PED 3 of PMCA 4 isoform with sequence CITQDSPLKA. It was conjugated to khlh and ovalbumin. The wells of the microtiter plate were coated with 0.1 mg/ml each of PED 3 and PED 3-khlh conjugate in sterile PBS and 1 mM sodium azide. Elution was done with ovalbumin conjugate of PED3. The screening was carried out as described above for round 1 and 28 clones were picked for both the target sequences of PED 2 and PED 3 and DNA isolated as described above.

2.6.1 Selectivity assay for clones picked for PED 2 and PED 3

The selectivity assay was based on obtaining more phage upon binding to PED 2-khlh and eluting with PED 2-ovalbumin than with binding to khlh and eluting with ovalbumin. Following is the summary of the individual steps in the assay.
• Coated one well of the microtiter plates with either 0.1 mg/ml each of the PED2 and PED2-khlh conjugate in sterile PBS and 1 mM sodium azide or with 0.1 mg/ml of khlh in sterile PBS and 1 mM sodium azide and incubated overnight at 4°C.

Day2: The excess target peptide and khlh from the wells in the plate was poured off and firmly slapped face down on clean paper towel to remove residual solution.

• The remaining binding sites in both the wells were blocked with blocking buffer containing 5 mg/ml of BSA in sterile PBS with 1 mM sodium azide for 1h in fridge.

• After discarding the excess blocking solution, the wells were washed 6x with sterile PBS.

• Amplified clone at concentration of E+08 pfu/μl was then added to both the wells and incubated at room temperature on the rocker for 1h. Also added same concentration of phage library.

• The unbound phage was poured off and wells were washed 10x with PBS and for 5 min each with blocking solution containing 0.5 mg/ml of each keyhole limpet hemocyanin and ovalbumin and 5% carnation milk in PBS.

• The bound phage was eluted with the PED2-ovalbumin-tet (0.1 mg/ml in PBS, with 10 μg/ml tetracycline and no sodium azide) in the first well and ovalbumin (0.1 mg/ml in PBS, with 10 μg/ml tetracycline and no sodium azide) in the other well at different times (24, 48 and 72 h).

• The eluted phage from both the wells were titered as described above.
In parallel, panning was done with the E+08 pfu/μl of the non-selected Ph.D-12 library for the binding to PED 2-khlh and khlh targets respectively.

2.7 Preparation of human erythrocyte leaky ghosts

Human erythrocyte leaky ghosts were prepared as previously described. Briefly, 25-30 ml of blood in acid citrate dextrose was obtained and centrifuged at 6,000 rpm for 5 min. The clear buffy layer containing platelets and plasma were siphoned off. The lower layer of erythrocytes was then transferred to four 250 ml centrifuge bottles and mixed with 10 volumes of 172 mM Tris-HCl (pH 7.6 at 4°C). The tubes were centrifuged at 5,000 rpm for 5 min and clear supernatant was removed. The erythrocytes were washed in 172 mM Tris-HCl for 4 times. The red blood cells were then lysed in 14 volumes of chilled distilled water and centrifuged at 12,000 rpm for 10 min. The red supernatant was removed, leaving behind a tight and loose pellet. The pellets were then washed in 14 volumes of imidazole-EDTA (10 mM imidazole-HCL, 1 mM EDTA, pH 7 at 23°C) by centrifugation at 12,000 rpm for 10 min. This washing was repeated about 8 times and then the pale loose pellet was transferred to new 250 ml tubes leaving behind the tight red pellet. This loose pellet was then washed in 14 volumes of imidazole-HCl (40 mM imidazole-HCl, pH 7 at 23°C). The loose pellets were then pooled, centrifuged at 18,000 rpm for 5 min and made into 500 μl aliquots, which were then stored at −80°C until use. An aliquot was used for protein estimation.
2.8 **Protein estimation**

Protein estimation was done with Bradford reagent (Bio-Rad, Hercules, CA) using bovine serum albumin to make the standard curve. Absorbance was measured at 595 nm. The standard curve was fitted and linear regression was performed using the Lotus 1-2-3 computer programme.

2.9 **Coupled enzyme assay**

This ATP regenerating system measures continuously the disappearance of the reduced form of nicotinamide adenine dinucleotide (NADH) in the reaction medium using a fluorometer (excitation at 340 nm and emission at 460 nm) at 37°C as previously described\(^3^9,^4^1\). Erythrocyte ghosts were incubated with or without caloxin for 30 min on ice before the assay. Basal Mg\(^{2+}\) ATPase activity was measured first as slope for the disappearance of NADH fluorescence in the 135 µl reaction mixture in the cuvette which contained 0.2-0.4 mg/ml ghost protein, 0.1 mM ouabain, 100 mM NaCl, 20 mM KCl, 6 mM MgCl\(_2\), 30 mM imidazole-HCl (pH 7.0), 0.5 mM EDTA, 0.2 mM NADH, 1 mM phosphoenol pyruvate, excess pyruvate kinase-lactate dehydrogenase, 0.5 mM ATP, 0.5 mM EGTA, and 4 µg/ml calmodulin. After about 7-8 min, 0.55 mM CaCl\(_2\) was added to the cuvette and disappearance of NADH monitored for another 15-17 min. The slope after the addition of calcium gave the measure of total ATPase activity. (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase activity was obtained by subtracting the basal Mg\(^{2+}\) ATPase activity from the total ATPase activity. Na\(^+\)-K\(^+\) ATPase activity was measured in the same solution used to
measure basal Mg\(^{2+}\) ATPase activity, except that ouabain, a sodium pump inhibitor, calcium and calmodulin were not added.

### 2.10 Acylphosphate assays

The formation of the acid stable Ca\(^{2+}\)-dependent 140 kDa acylphosphate was determined with sodium dodecyl sulphate polyacrylamide gels at pH 4.0 as previously described\(^42\). The ghosts were incubated for 30 min at 0°C with either caloxin or random peptide (RP) obtained by the randomization of the amino acid composition of caloxin 2A1. The ghosts were then added to the reaction mixture. The reaction mixture had 100 mM KCl, 30 mM imidazole-HCl (pH 6.8 at 20-23°C), 0.05 mM CaCl\(_2\), 4 μg/ml calmodulin, 0.4-0.7 mg/ml membrane protein and 0.005 mM ATP with trace amounts of \(^{32}\)P-γ-ATP in volume of 0.2 ml. The reaction was run for 60 sec’s and then quenched with 0.25 ml of ice-cold stopping solution (TCAP) containing 10% tricholoroacetic acid, 50 mM phosphoric acid and 0.5 mM unlabelled ATP. The proteins were precipitated after centrifugation at 4°C, the supernatant discarded and the pellet washed again with TCAP. The proteins were then resuspended in MEDS buffer pH 5.5 (10 mM MOPS, 1 mM EDTA, 10% sucrose, 3% SDS, 10mM DTT and 0.01% methyl green) and electrophoresed using sodium dodecyl sulphate polyacrylamide gels at pH 4.0. The acylphosphate was quantified using Phosphor Imager and Image Quaint software to determine the intensity of each band after subtracting the background intensity.
2.11 Data Analysis

Values given are mean ± SEM. Where applicable student's t-test was used and values of p<0.05 were considered to be statistically significant. Non-linear regression was used to determine the inhibition constant using the software FigP (Biosoft Corporation, Ancaster, Ontario).
3.0 RESULTS

Caloxin2A1 is a specific peptide sequence coded by a phage that bound to the second putative extracellular domain of PMCA1b isoform, during an in vitro screening of the phage display peptide library. The present study attempted to develop the biochemical assay to test if caloxin 2A1 modulates the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase activity of PMCA (AIM I) and to screen the phage display peptide library for obtaining high affinity caloxins (AIM II).

3.1 AIM I: Developing biochemical assay

The calcium pump has Ca\(^{2+}\) stimulated Mg\(^{2+}\) ATPase activity where the energy of hydrolysis of ATP is used to pump Ca\(^{2+}\) ions. The activity of PMCA can be studied experimentally by measuring Ca\(^{2+}\) fluxes, by determining the \(^{32}\)P incorporation in acylphosphate formation in the reaction cycle of the pump or by assaying the Ca\(^{2+}\) stimulated Mg\(^{2+}\) ATPase hydrolysis of ATP. Ca\(^{2+}\) flux studies are limited by the membrane sidedness of the vesicles requiring the rightside out sealed membrane vesicle preparations. Although caloxin2A1 is expected to bind on the extracellular site of the Ca\(^{2+}\) pump, its other substrates as Ca\(^{2+}\) and ATP, and pump modulator calmodulin are known to bind intracellularly. This would require membrane vesicles to be leaky and thus unable to carry out the Ca\(^{2+}\) flux.

The Ca\(^{2+}\) stimulated Mg\(^{2+}\) ATPase hydrolysis of ATP to release inorganic orthophosphate P\(_i\) can be assayed spectrophotometrically or with the coupled enzyme
assay. Spectrophotometric measurement of Pi can give over estimation due to non-
enzymatic hydrolysis of ADP, AMP and pyrophosphate in the reaction solution during the
time of assay. In the coupled enzyme assay used in this study, the hydrolysis of ATP is
coupled to the oxidation of NADH using pyruvate kinase and lactate dehydrogenase. This
procedure has the advantage of continuous measurement of the ATP hydrolysis and the
buildup of inhibitory ADP is prevented.

Human erythrocyte leaky ghosts provide an optimal membrane system to test the
Ca\(^{2+}\) stimulated Mg\(^{2+}\) ATPase activity. The erythrocyte ghosts have low basal
Mg\(^{2+}\) ATPase and ecto Ca\(^{2+}\) ATPase activity. The lack of intracellular organelles prevents
interference due to ATPase activities of endoplasmic reticulum and nucleus. The ghosts
can be made leaky thereby providing access to the substrates and activator calmodulin
that bind to the calcium pump intracellularly. Leaky ghosts allow for the binding of the
caloxin2A1 to the extracellular side and equilibration of the other reaction components on
both sides of the membranes facilitating a continuous measurement of the Ca\(^{2+}\)-Mg\(^{2+}\)
ATPase activity of the ghosts. Erythrocyte ghosts can be prepared and stored at -80°C for
a month without any decrease in the activity.

The coupled enzyme assay was optimized for the protein and NADH
concentrations to determine the initial reaction velocity. Different concentrations of
ghosts were tested to get linear reaction so that only fraction of NADH was used.
Including ouabain in reaction solution, the specific inhibitor of the Na\(^{+}\) pump, the Na\(^{+}\)-K\(^{+}\)
ATPase activity of the ghosts was inhibited. Adding the ion chelator, EGTA, optimized
the Mg\(^{2+}\) and free Ca\(^{2+}\) concentration for the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase activity. Background
fluorescence in the absence of NADH was measured to confirm no effect on the measurement of Ca\(^{2+}\)-Mg\(^{2+}\) ATPase activity.

3.1.1 *Caloxin2A1 modulates PMCA activity*

Caloxin2A1 was obtained by binding to the PED 2 of PMCA 1b isoform. PED 2 connects the transmembrane domains (TM) 3 and 4. Mutagenesis has shown that TM 4 is involved in the formation of channel for the translocation of ion\(^{56}\). It was hypothesized that since caloxin2A1 was encoded by the phage that bound to the PED 2, caloxin2A1 will bind and perturb the extracellular domain of the calcium pump and modulate PMCA activity. Fig. 2 shows the tracing of the ATPase activity of the ghosts measured by the disappearance of NADH with a fluorometer (excitation 340 nm, emission 460 nm). The basal Mg\(^{2+}\) ATPase activity was determined in the reaction solution in the absence of Ca\(^{2+}\). After running the experiment for about 6-min, 8 mM CaCl\(_2\) was added to the same reaction solution to get the total ATPase activity of the ghosts seen as the steep slope in the Fig. 2. The Ca\(^{2+}\)-Mg\(^{2+}\) ATPase is obtained by the difference in the slopes of the total ATPase and basal Mg\(^{2+}\) ATPase calculated in absence of Ca\(^{2+}\). The inhibition of the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase activity of ghosts in the presence of caloxin2A1 results in a decrease in the slope after addition of CaCl\(_2\).
Fig. 2: Ca$^{2+}$-Mg$^{2+}$ ATPase activity of ghosts measured with a fluorometer. The Ca$^{2+}$-Mg$^{2+}$ ATPase activity is measured by the disappearance of NADH (excitation 340 nm, emission 460 nm) with time. The difference in the slopes before and after the addition of CaCl$_2$ gives the measure of Ca$^{2+}$-Mg$^{2+}$ ATPase activity. The inhibition due to 0.9 mM caloxin2A1 is seen as the decrease in the slope of the activity of the ghosts.
3.1.2 *Caloxin2A1 affinity for PMCA*

To observe the kinetics of inhibition, the effect of different concentrations of caloxin2A1 on the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase activity of PMCA was tested. Fig. 3 shows the inhibition of Ca\(^{2+}\)-Mg\(^{2+}\) ATPase activity of PMCA with different concentrations of caloxin2A1. Effect of 0, 0.23, 0.47, 0.93 and 1.87 mM caloxin2A1 were tested. For each experiment, the values of Ca\(^{2+}\)-Mg\(^{2+}\) ATPase activity obtained in presence of caloxin2A1 were expressed as a percentage of the control mean of that day. Data from several experiments performed on different days were pooled. Using these data points and assuming 100% inhibition, caloxin2A1 produced 50% inhibition at 0.4 ± 0.1 mM.

3.1.3 *Selectivity of caloxin2A1*

Caloxin2A1 was obtained from phage that bound the PED 2 of the PMCA 1b isoform and these short extracellular loops are not known to be homologous to the extracellular loops of other members of P-type ATPases. It was hypothesized that caloxin2A1 would bind and inhibit specifically the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase activity of PMCA. Fig. 4 shows that 0.9 mM caloxin2A1 inhibits only the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase activity of ghosts and has no effect on the Mg\(^{2+}\) ATPase activity calculated in same reaction solution in the absence of Ca\(^{2+}\). Caloxin2A1 did not inhibit the activity of Na\(^{+}\)-K\(^{+}\) ATPase of the ghosts, which was tested in the same reaction solution but in the absence of ouabain (specific inhibitor of sodium pump), Ca\(^{2+}\) and calmodulin. Caloxin2A1 also did not inhibit the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase activity of the sarcoplasmic reticulum. The sequence of caloxin2A1
VSNSNWPSFPSGGG was randomized to obtain random peptide (RP: SWSSFPGSGGVSNPN) to show that the amino acid sequence and not just the composition of caloxin2A1 is required for the inhibition of the Ca$^{2+}$-Mg$^{2+}$ ATPase activity of ghosts. Fig. 4 shows no change in Ca$^{2+}$-Mg$^{2+}$ ATPase activity of the ghosts in the presence of the random peptide (RP). Student t-test was used and only significant (P < 0.05) inhibition was with caloxin2A1 on the Ca$^{2+}$-Mg$^{2+}$ ATPase activity of PMCA in ghosts. These results show that caloxin2A1 is specific for the Ca$^{2+}$-Mg$^{2+}$ ATPase activity of PMCA in ghosts. Although, caloxin2A1 was obtained for binding to PMCA 1b isoform, it produced an inhibition of the Ca$^{2+}$-Mg$^{2+}$ ATPase activity of the PMCA in ghosts that express mainly PMCA 4 isoform.
Fig. 3: Caloxin2A1 concentration dependence of inhibition of plasma membrane Ca\(^{2+}\)-Mg\(^{2+}\)ATPase. The graph shown is from a total of 42 data points pooled from experiments on 7 different days using different preparations of the ghosts. On each day, the data were normalized, with the mean value of the Ca\(^{2+}\)-Mg\(^{2+}\)ATPase activity in the absence of caloxin2A1 taken as 100%. SE values are shown as error bars. The data fit best with the IC\(_{50}\) value of 0.4 ± 0.1 mM. (Modified from Fig. 1A in reference 39).
Fig. 4: Caloxin2A1 (VSNSNWPSFPSSGGG-amide) inhibits plasma membrane $\text{Ca}^{2+}$-$\text{Mg}^{2+}$ATPase selectively. Concentration of caloxin2A1 or RP, random peptide (SWSSFPGSGGVSNPN-amide) used is 0.9 mM. The types of activity monitored are shown as $\text{Ca}^{2+}$-$\text{Mg}^{2+}$ATPase, $\text{Mg}^{2+}$ATPase or $\text{Na}^+$$\text{K}^+$ATPase. G, ghosts; S, skeletal muscle sarcoplasmic reticulum. The values are expressed as the percent inhibition of the control mean ± sem. The only significant ($P < 0.05$) inhibition by caloxin2A1 was of the $\text{Ca}^{2+}$-$\text{Mg}^{2+}$ATPase activity of the ghosts. (Modified from Fig. 1B in reference 39).
3.1.4 Effect of caloxin2A1 on Ca\textsuperscript{2+} dependent acylphosphate formation

In the forward step of reaction cycle of PMCA activity it forms a Ca\textsuperscript{2+} dependent acid stable, alkali labile 140-kDa phosphorylated intermediate from ATP. The terminal phosphate of ATP on hydrolysis forms an acylphosphate with a conserved aspartate residue in the second cytosolic loop of the calcium pump. This acylphosphate then undergoes rapid hydrolysis followed by conformational change of the pump to start another reaction cycle. Caloxin2A1 could inhibit the pump activity by affecting any of these steps of the reaction cycle. Using \textsuperscript{32}P-\gamma-ATP in the reaction with erythrocyte ghosts can help follow the formation of the acylphosphate intermediate in the PMCA activity cycle. Since 0.4 mM caloxin2A1 produced 50% inhibition, 3.4 mM caloxin2A1 will cause greater than 90% inhibition of the Ca\textsuperscript{2+}-Mg\textsuperscript{2+} ATPase activity of the ghosts. Thus this concentration of caloxin2A1 was used to see its effect on the phosphorylated intermediate of the pump. Fig. 5 shows that at given concentration of 3.4 mM, caloxin2A1 produced nearly complete inhibition of acylphosphate formation (lane 3). In Fig. 5, lane 1 shows the Ca\textsuperscript{2+} dependence of the formation of acylphosphate as the Ca\textsuperscript{2+} chelator EGTA inhibits the formation of the acylphosphate. The same concentration of random peptide had no effect on the formation of phosphorylated intermediate in the presence of Ca\textsuperscript{2+}(lane 4).
Fig. 5: A Phosphor Image of a gel showing the effect of caloxin2A1 on the 140-kDa acylphosphate intermediate of PMCA. The erythrocyte ghosts were treated with 3.4 mM caloxin2A1 or random peptide (RP). The acylphosphate formation was carried out using $^{32}$P-$\gamma$-ATP and the samples were analyzed by acid gel electrophoresis. The lane one contained 1 mM EGTA. The position of the bands was compared to SERCA 2b pump acylphosphate (110-kDa) which migrated slightly faster than PMCA acylphosphate (140-kDa). (Modified from Fig. 2 in reference 39)
3.2 AIM II: Screening Ph.D-12 library for high affinity caloxin

Caloxin2A1 inhibited the $\text{Ca}^{2+}$- $\text{Mg}^{2+}$ ATPase activity of the ghosts with $K_i \approx 0.4$ mM. The cost of high-pressure liquid chromatography purified peptides makes this low affinity peptide expensive to use in experiments for further characterization. Caloxin2A1 was selected by screening the Ph.D-12 library for binding to PED 2 of PMCA 1b isoform. However, it inhibited the $\text{Ca}^{2+}$- $\text{Mg}^{2+}$ ATPase activity of the ghosts, which express mainly PMCA 4 isoform. High affinity caloxin could be more isoform selective. High affinity caloxin is also an essential requirement in paving way for the future therapeutic design of a non-peptide caloxin. Therefore, the Ph.D-12 library was screened again with higher stringency to select for high affinity caloxin.

3.2.1 Screening Ph.D-12 library for binding to PED2 of PMCA1b isoform

The sequence of the 2nd putative extracellular domain of PMCA 1b was used as the target peptide (WVQKRPLAESTPIYIQYFVKC) in round 1. Screening the Ph.D-12 library in the first 8 cycles of panning involved elution with low pH glycine buffer (pH 2.2) which is a strong elution buffer that would disrupt the linkage of all the bound phage still left in the well after number of wash steps. The phage pool would be comparatively enriched after 8 rounds of panning. The amplified phage from the 8th cycle was then titered to keep the input phage constant and the elution thereafter was carried out with a specific elution buffer, PED 2-ovalbumin conjugate.
Fig. 6: Changes in the output pfu/ul with differential time elution in the 9th cycle of panning. The target coating peptide was mix of PMCA 398 (WVQKRPWLAESEPIIQYFVKC) and PMCA398-khlh (WVQKRPWLAESEPIIQYFVKC-khlh) conjugate. The elution was carried out with PMCA398-ovalbumin (WVQKRPWLAESEPIIQYFVKC-ovalbumin) conjugate. The 48 h eluate was amplified and used for panning in the subsequent cycles at constant input concentration of E+10 pfu/well.
(WVQKRPWLAESTPIYIQYFVKC-ovalbumin) in an effort to elute phage bound specifically to the target PED 2. Fig. 6 shows the titers of the unamplified differential time eluates of the 9th cycle of panning. The output pfu decreases with increase in the elution time. The phage from the 48-h eluate was expected to be of higher affinity with lower dissociation constant (Kd) requiring longer time to elute. The 48-h eluate was amplified and used in the subsequent cycles of panning at constant input of E+10 pfu/well. Putting constant pfu in each cycle of panning enabled to observe the change in the output phage, which is expected to increase with each panning cycle due to the enrichment in the phage pool binding selectively to the target PED 2 sequence. The elution was carried with specific elution buffer which was the ovalbumin conjugate of PMCA 398 (WVQKRPWLAESTPIYIQYFVKC) for 24 and 48 h with a wash in PBS after each elution. Any bound phage after 48-h was eluted with the low pH glycine elution buffer. Table 1 shows the change in the titers of the output phage eluted for 24 and 48-h with the panning cycles. The output pfu/μl increases for initial rounds before stabilizing.

3.2.1.1 Selected clones and encoded peptide sequence

50 clones were picked after the stabilization of the output pfu, amplified and plasmid DNA purified from each of them. Sequencing of the 50 plasmid DNA with −28 and −96 sequencing primers and inverting the sequence obtained by extension of primers gave the same DNA insert. The sequence of DNA insert and the peptide encoded by it is given in Table 2.
Table. 1: Change in the output pfu/μl with panning for PED 2 of PMCA 1b. The input pfu for each panning round was kept constant at E+08 pfu/μl and the elution was carried out for 24 and 48h with specific elution buffer followed with the low pH glycine buffer (pH 2.2). The target sequence was PMCA 398 (WVQKRPMWLAESTPIYIQYFVKC) and keyhole limpet hemocyanin conjugate of PMCA398 (WVQKRPMWLAESTPIYIQYFVKC- khlh). The elution of the phage for 24 and 48 h was carried out with the specific elution peptide (WVQKRPMWLAESTPIYIQYFVKC- ovalbumin).
Table 2: Sequences obtained upon screening for peptides binding to PED 2 of PMCA 1b. 50 clones were picked after the final panning cycle for the target sequence PMCA 398 (WVQKRPWLAESTPIYIQYFVKC) and its keyhole limpet hemocyanin conjugate. All 50 clones had the same DNA insert.

<table>
<thead>
<tr>
<th>No. of clones</th>
<th>Sequence of DNA insert</th>
<th>Sequence of peptide coded by insert</th>
</tr>
</thead>
</table>
| 1-50          | gat gtg tct tca gga tta gta gat  
                 |    tcc tcc tgg tgg agg ttc ggc  
                 |    cct ttc tat tct cac tct ccg cgg  
                 |    cct agg cct ccg act atg ctg  
                 |    gct cct acg | DVSSGLVDSSWWRFGPF  
                 |                     | YSHSPRPRPPTMLAPT |

3.2.2 Screening Ph.D-12 library for binding to PED 2 of PMCA 1b isoform

The sequence of the 2nd putative extracellular domain of PMCA 1b and its khlh conjugate was used as the target peptide (WVQKRPWLAESTPIYIQYFVKC) in round 2. The Ph.D-12 library was screened as described in Material and Methods. With the hypothesis that the specific elution buffer (WVQKRPWLAESTPIYIQYFVKC-ovalbumin) will require longer time to elute the phage with higher binding affinity to the target, the elution time was increased to 72 h. The input phage was kept constant at
7E+10 pfu/well. Table 3 shows the change in the output pfu with differential time elution in different panning cycles.

3.2.2.1 Selected clones and encoded peptide sequence

After final round of panning, 40 clones were picked, amplified and sequenced. Sequencing of the 40 plasmid DNA with –28 and –96 sequencing primers and inverting the sequence obtained by extension of primers gave the same DNA insert. The sequence of DNA insert and the peptide encoded by it is given in Table 4.

3.2.2.1.1 Effect of peptide QWPSVYPTPSSH on PMCA activity

The peptide QWPSVYPTPSSH selected was then tested for its effect on the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase activity of the ghosts. The peptide did not produce inhibition of Ca\(^{2+}\)-Mg\(^{2+}\) ATPase activity of ghosts. Since this peptide was obtained by screening under more stringent conditions, it could be of high affinity and thus also isoform specific or may be a non-modulatory high affinity peptide.
Table 3: Change in the output pfu/μl with panning for PED 2 of PMCA 1b. The input pfu for each panning round was kept constant at 7E+08 pfu/μl and the elution was carried out for 24, 48 and 72-h and with the low pH glycine (pH 2.2). The target sequence was PED 2 sequence of PMCA 1b and its keyhole limpet hemocyanin conjugate (WVQKRPWLAEStPIYIQYFVKC). The elution of the phage for 24, 48 and 72-h was carried out with the elution peptide (WVQKRPWLAEStPIYIQYFVKC- ovalbumin) followed by elution with low pH glycine buffer (pH 2.2).

<table>
<thead>
<tr>
<th>Panning cycle</th>
<th>Titer of 24-h eluate (pfu/μl)</th>
<th>Titer of 48-h eluate (pfu/μl)</th>
<th>Titer of 72-h eluate (pfu/μl)</th>
<th>Titer of pH eluate (pfu/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&gt;12000</td>
<td>&gt;660</td>
<td>&gt;700</td>
<td>&gt;50</td>
</tr>
<tr>
<td>2</td>
<td>&gt;65000</td>
<td>&gt;3000</td>
<td>&gt;300</td>
<td>~180</td>
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<tr>
<td>3</td>
<td>&gt;88000</td>
<td>&gt;3700</td>
<td>&gt;1500</td>
<td>&gt;6000</td>
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<td>&gt;30000</td>
<td>&gt;7000</td>
<td>&gt;43000</td>
</tr>
<tr>
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<td>&gt;700000</td>
<td>~69000</td>
<td>&gt;18000</td>
<td>&gt;19000</td>
</tr>
</tbody>
</table>
Table 4: Sequences obtained upon screening for peptides binding to PED 2 of PMCA 1b. 50 clones were picked after the final panning cycle for the target sequence PMCA 398 (WVQKRPLAESTPIQYFVKC) and its keyhole limpet hemocyanin conjugate. All 50 clones had the same insert DNA sequence.

<table>
<thead>
<tr>
<th>No. of clones</th>
<th>Sequence of the insert</th>
<th>Sequence of the peptide encoded by DNA insert</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- 40</td>
<td>cag tgg cct tcg gtg tat ccg act cct agt tct cat</td>
<td>QWPSVYPTPSSH</td>
</tr>
</tbody>
</table>

3.2.3 Screening Ph.D-12 library for binding to PED 2 of PMCA 4 isoform

Human erythrocyte ghosts that mainly express PMCA 4 isoform have been successfully used to measure the Ca$^{2+}$-Mg$^{2+}$ ATPase activity of PMCA. Screening the Ph.D-12 library for binding to the PED 2 of PMCA 4 isoform would facilitate the easier testing of the peptide for its effect on the Ca$^{2+}$-Mg$^{2+}$ ATPase activity. The sequence of the 2nd putative extracellular domain of PMCA 4 and its khlh conjugate was used as the target peptide (VINRRPWLPSTPIQYFVKC). The Ph.D-12 library was screened as described in Material and Methods. VINRRPWLPSTPIQYFVKC-ovalbumin was used as specific elution buffer for differential time elution of the bound phage. The input phage was kept constant at 3E+11 pfu/well. Table 5 shows the change in the output pfu of the differential time elution in different panning cycles.
3.2.3.1 Selected clones and encoded peptide sequence

After the final round of panning, 27 clones were picked, amplified and sequenced. Table 6 shows the DNA sequence coded by the clones. 27 clones yielded 6 different DNA sequences. Each of the 6 different types of the clones was then tested for its selectivity for binding to the target PED2 sequence. The output pfu obtained with the clone panned against the target sequence khlh was expressed as ratio of the output pfu obtained by panning the clone against the PED2 and its khlh conjugate target which was taken as 100%. Ph.D-12 library was also panned simultaneously against the PED2 and its khlh conjugate target and khlh target and the output pfu’s were similarly expressed as ratios. Fig. 7 shows the result of selectivity of one of the clones. It can be seen that clone J1 bound to the target PED2 sequence with much higher selectivity. Two of the 6 clones, J1 and J6 gave higher selectivity ratios and were thus selected for synthesis.
Table 5: Change in the output pfu/μl with panning for PED 2 of PMCA 4. The input pfu for each panning round was kept constant at 3E+09 pfu/μl and elution was carried out for 24, 48 and 72-h and with the low pH glycine buffer (pH 2.2). The target peptide was PED 2 sequence of PMCA4 and its keyhole limpet hemocyanin conjugate (VINRRPWLPESTPIYIQYFVKC). The elution of the phage at 24, 48 and 72-h was carried out with the elution peptide (VINRRPWLPESTPIYIQYFVKC – ovalbumin)

<table>
<thead>
<tr>
<th>Panning cycle</th>
<th>Titer of 24-h eluate (pfu/μl)</th>
<th>Titer of 48-h eluate (pfu/μl)</th>
<th>Titer of 72-h eluate (pfu/μl)</th>
<th>Titer of pH eluate (pfu/μl)</th>
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</thead>
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<tr>
<td>1</td>
<td>&gt;7000</td>
<td>&gt;700</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<td>&gt;3800</td>
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<td>&gt;46000</td>
<td>&gt;6600</td>
</tr>
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<td>&gt;25000</td>
<td>&gt;5700</td>
<td>&gt;1000</td>
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<td>&gt;380000</td>
<td>&gt;5800</td>
<td>&gt;2500</td>
<td>&gt;330</td>
</tr>
</tbody>
</table>
### Table 6: Sequences obtained upon screening for peptides binding to PED 2 of PMCA 4.

27 clones were picked after the final panning cycle for the target sequence PED 2 of PMCA4 (VINRRPWLPESTPIYIQYFVKC) and its keyhole limpet hemocyanin conjugate.
Fig. 7: Selectivity assay of clones binding to PED 2 of PMCA 4. The 2nd putative extracellular domain and its keyhole limpet hemocyanin conjugate and the keyhole limpet hemocyanin alone were used as the two target sequences for panning with constant input pfu of the selected sequence ASTNVFARPMYL (J1) and the phage display Ph.D peptide library. It shows the comparison of the output plaque forming units for each panning. The pfu obtained with the selected sequence (J1) is taken as 100%.
3.2.3.1.1 *Effect of peptide J1 (ASTNVFARPMYL) and J6 (HVTYLNNPQGPS) on PMCA activity*

The peptides J1 and J6 were tested for their effect on the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase activity of the ghosts. Both the peptides inhibited the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase activity of the ghosts. Fig. 8 shows that the two peptides J1 and J6 did not have affinity higher than caloxin2A1.

![Graph showing effect of peptides on PMCA activity](image)

**Fig. 8: Effect of the peptides J1 and J6 on PMCA.** The effect of 0.9 mM each of the peptides J1 (ASTNVFARPMYLGGG-amide) and J6 (HVTYLNNPQGPSGGG-amide) on the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase activity of the ghosts was compared with that of 0.9 mM caloxin2A1. The values are expressed as percent inhibition of the control mean ± sem.
3.2.4 Screening Ph.D-12 library for binding to PED 3 of PMCA 4 isoform

The 3rd putative extracellular domain is conserved in four different PMCA isoforms and was used for screening the Ph.D-12 library in an attempt to obtain a high affinity caloxin. The sequence of the 3rd putative extracellular domain of PMCA 4 and its khlh conjugate was used as the target peptide (CITQDSPLKA -khlh). The Ph.D-12 library was screened as described in Material and Methods. CITQDSPLKA -ovalbumin was used as the specific elution buffer for the differential time elution time of the bound phage. The input phage was kept constant at 3E+11 pfu/well. Table 7 shows the change in the output pfu of the differential time elution in different panning cycles.

3.2.4.1 Selected clones and encoded peptide sequence

After the final round of panning, 27 clones were picked, amplified and sequenced. Of the 27 clones only 2 clones had insert. Table 8 shows the DNA sequence coded by the two clones. Each of the 2 different types of the clones was then tested for its selectivity for binding to the target PED 2 sequence. The output pfu obtained with the clone panned against the khlh target sequence was expressed as ratio of the output pfu obtained by panning the clone against the PED 2 target, which was taken as 100%. Ph.D-12 library was also panned simultaneously against the PED 2 target and khlh target and the output pfu's were similarly expressed as the ratios. Due to high selectivity ratio, C5 peptide was synthesized.
<table>
<thead>
<tr>
<th>Panning cycle</th>
<th>Titer of 24 h eluate (pfu/μl)</th>
<th>Titer of 48 h eluate (pfu/μl)</th>
<th>Titer of 72 h eluate (pfu/μl)</th>
<th>Titer of pH eluate (pfu/μl)</th>
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</table>

Table 7: Change in the output pfu/μl with panning for PED 3 of PMCA 4. The input pfu for each panning round was kept constant at 3E+09 pfu/μl and elution carried out at 24, 48 and 72 h and with pH glycine (pH 2.2). The target sequence was PED 3 sequence of PMCA 4 and its keyhole limpet hemocyanin conjugate (CITQDSPLKAC). The elution of the bound phage for 24, 48 and 72-h was carried out with the specific elution peptide (CITQDSPLKAC – ovalbumin) followed with pH glycine elution buffer.
<table>
<thead>
<tr>
<th>No. of clones</th>
<th>Sequence of DNA insert</th>
<th>Sequence of the peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 out of 27</td>
<td>agt gtg tgg tcg gct act ttt ctg tct tct ccg</td>
<td>SVWSATFLSSS (C5)</td>
</tr>
<tr>
<td>1 out of 27</td>
<td>tcg tgg ttg acg ccg tct cct cct att agg ctg cat</td>
<td>SWLTPSSPIRLH</td>
</tr>
</tbody>
</table>

Table 8: Sequences obtained upon screening for peptides binding to PED 3 of PMCA 4. 27 clones were picked after the final panning cycle for the target sequence PED 2 of PMCA 4 (CITQDSPLKAC) and its keyhole limpet hemocyanin conjugate.

3.2.4.1.1 Effect of peptide C5 (SVWSATFLSSSP) on PMCA activity

The peptide C5 was tested for its effect on the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase activity of the ghosts. The peptide C5 was hydrophobic and did not dissolve in the buffer containing the ghosts. The detergent triton (final concentration 0.01 %) and DMSO (final concentration of 1 %) were used to dissolve the peptide C5 that did not dissolve completely. Fig. 9 shows the effect of C5 peptide on the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase activity of the ghosts. The peptide was then made hydrophilic by substituting the COOH-terminal glycine with a lysine (C5K peptide). The peptide C5K produced high inhibition of the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase activity of ghosts. However, on synthesizing a randomized version of C5K peptide (SSLSFSAVSTPWGGK), the random peptide also produced inhibition of the
Ca\(^{2+}\)- Mg\(^{2+}\) ATPase activity of the ghosts suggesting that it was the detergent like properties of the peptide C5K that was producing the inhibitory effect (Fig. 10).

![Bar graph showing inhibition of PMCA by CS peptide with caloxin2A1.](image)

**Fig. 9: Comparison of inhibition of PMCA by C5 peptide with caloxin2A1.** The figure shows the amount of inhibition produced by 0.9 mM each of caloxin2A1 and the C5 peptide. The Ca\(^{2+}\)-Mg\(^{2+}\) ATPase activity of the ghosts was measured using the coupled enzyme assay. It is from the total of 9 data points from experiments performed on different days. The values are expressed as the percent inhibition of the control mean ± SEM.
Fig. 10: Inhibition of Ca$^{2+}$-Mg$^{2+}$ATPase activity by C5K and C5K-RP. The figure shows the amount of inhibition produced by 0.23 mM each of caloxin2A1, C5 peptide (SVWSATFLSSSGGK) and C5K random peptide (SSLSFSAVSTPWGGK). The Ca$^{2+}$-Mg$^{2+}$ATPase activity of the ghosts was measured using the coupled enzyme assay. Values are expressed as the percent inhibition of the control mean ± SEM.
4.0 DISCUSSION

Caloxin2A1 was obtained by screening a phage display peptide library against the 2nd putative extracellular domain of the PMCA 1b isoform. It selectively inhibited the Ca$^{2+}$-Mg$^{2+}$ ATPase activity of the human red blood cell ghosts that express mainly PMCA 4 isoform. In addition, caloxin2A1 interferes with the formation of the acid stable, alkali labile 140-kDa acylphosphate from ATP in the partial forward reaction of ATPase cycle. Discussion focuses on the comparison of these results with other known inhibitors of the Ca$^{2+}$ pump. Aim II involved the screening of the phage display peptide library to obtain a high affinity caloxin. However, screening of the Ph.D-12 library did not yield peptides with affinity higher than caloxin2A1. The reasons for this will also be discussed.

4.1 Specificity of caloxin2A1

Results have shown that caloxin2A1 is the specific inhibitor of PMCA that inhibits the Ca$^{2+}$-Mg$^{2+}$ ATPase activity of the calcium pump in red blood cell ghosts producing 50% inhibition (Ki) at 0.4 ± 0.1 mM. Randomization of the sequence of caloxin2A1 did not produce any inhibition of the Ca$^{2+}$-Mg$^{2+}$ ATPase activity of the Ca$^{2+}$ pump.

There are no known extracellularly acting specific inhibitors of PMCA as more than 80% of the pump is cytosolic with five short putative extracellular domains. These extracellular domains are regarded as peptide segments connecting the 10 transmembrane domains(TM) without any other known function. Another difficulty in obtaining specific
inhibitors is the similarity between PMCA and other ion pumps. PMCA belongs to the type II class of P-type ATPases that include all enzymes that transport the abundant ions of basic cellular metabolism: Ca$^{2+}$, Na$^+$, K$^+$. The other well-studied members of this class include SERCA and Na$^+$-K$^+$ ATPase. All P-type ATPases have sequence similarities while sharing the key features of ATP hydrolysis and central core of transmembrane helices. The crystal structure of SERCA, the first reported structure of an active transport protein has recently been determined at 2.6\AA resolution$^{43}$. The alignment of the homologous regions of the various P-type ATPases with SERCA sequence and their mapping onto SERCA structure has revealed extensive similarity of SERCA with PMCA and Na$^+$-K$^+$ ATPase$^{43}$. The study showed that all of ‘P domain’ (acylphosphate forming site), most of ‘N domain’ (nucleotide binding site) and portion of ‘A domain’ (the first cytosolic loop) of PMCA is homologous with SERCA. There are conserved amino acid residues in the transmembrane domains of P-type ATPases that are proposed to mediate ion translocation. PMCA lacks two of these conserved residues in TM 5 and TM 8. Recombinant PMCA with mutation of single amino acid residue in TM 5 to the conserved residue found in other P-type ATPases acquired properties of SERCA$^{44}$. These studies show the extent of similarity between members of P-type ATPases due to which most of the inhibitors of PMCA are not selective. Caloxin2A1 does not inhibit the Mg$^{2+}$ ATPase and the Na$^+$-K$^+$ ATPase activity of the red blood cell ghosts. It also did not inhibit the Ca$^{2+}$-Mg$^{2+}$ ATPase activity of SERCA. Of the other known inhibitors of the pump, Lanthanum inhibits SERCA$^{45}$. Vanadate, a phosphate analog that inhibits PMCA, also inhibits Na$^+$-K$^+$ ATPase and SERCA$^{46}$. Eosin inhibits both PMCA and Na$^+$-K$^+$
ATPase\textsuperscript{47}. In a study to determine the role of plasma membrane Ca\textsuperscript{2+} efflux transporters in the generation of agonist independent [Ca\textsuperscript{2+}]\textsubscript{i} oscillations, both eosin and caloxin were used to inhibit PMCA. Caloxin2A1 transiently elevated [Ca\textsuperscript{2+}]\textsubscript{i} that returned to basal levels with complete blocking of the [Ca\textsuperscript{2+}]\textsubscript{i} oscillations. Eosin elevated the basal level of cytosolic Ca\textsuperscript{2+} before blocking the [Ca\textsuperscript{2+}]\textsubscript{i} oscillations. An effect similar to that of eosin was also shown by the inhibitor of the sodium calcium exchanger that elevated the basal [Ca\textsuperscript{2+}]\textsubscript{i} before blocking the [Ca\textsuperscript{2+}]\textsubscript{i} oscillations. Since the effect of eosin and inhibitor of sodium calcium exchanger was similar, eosin could also be modulating the functioning the exchanger\textsuperscript{62}. Fluoroaluminate complex, AlF\textsubscript{4}\textsuperscript{−} causes a complete inhibition of the Na\textsuperscript{+}-K\textsuperscript{+} ATPase and PMCA activity and a partial inhibition of SERCA\textsuperscript{48}.

Caloxin2A1 was obtained by screening the Ph.D –12 library against the PED 2 of PMCA 1b isoform. However, it inhibited the Ca\textsuperscript{2+}-Mg\textsuperscript{2+} ATPase activity of PMCA 4, which is the main isoform expressed in red blood cell ghosts. This can be explained by the similarity between the PED 2 of the two isoforms. Of the 13 amino acid residues only two are different between the two isoforms. One of the amino acid substitutions involves lysine→ arginine where the positive charge is conserved. Ouabain, the specific extracellular inhibitor of the sodium pump has been shown to have same affinity for all the α-subunit isoforms of the pump. Heart expresses all 3 isoforms of α-subunit of Na\textsuperscript{+}-K\textsuperscript{+} ATPase, which are the receptors for the cardiac glycosides used in treatment of congestive heart failure. The observed differences in the ouabain sensitivity are due to the decrease in the pump abundance with age and heart failure and not due to the difference in the affinities of the various isoforms\textsuperscript{49}. However, it cannot be ruled out that
caloxin2A1 may have higher affinity for the PMCA 1b isoform which cannot be tested at present due to the difficulties in overexpressing this isoform.

4.2 **Sidedness of Caloxin2A1 action**

Since caloxin2A1 was obtained by screening a phage display peptide library to bind the 2nd PED of PMCA 1b isoform, it is the only known inhibitor that may bind extracellularly to the pump surface and modulate its activity. Ouabain, a specific inhibitor of Na pumps has its binding site on the extracellular side of the protein\(^43\). It binds to the site formed by the small extracellular loops connecting the transmembrane domains TM 1-TM 2, TM 3-TM 4, TM 5-TM 6 and TM 7-TM 8. This has been shown by ouabain resistant mutations, studies using photoaffinity derivative of ouabain, homology studies and solid-state NMR studies\(^43, 50\). Lanthanum acts by arresting the protein in the phosphorylated form and has been shown to produce an inhibition from both the inside and outside of the protein in the pump. It is suggested that if its binding site is the same, then lanthanum could be binding at or near the calcium binding sites that alternately face both the inside and the outside of the cell\(^51\). Vanadate is present in the tissues and can rapidly equilibrate across the plasma membrane via an anion exchange system. It binds the intracellular domain of the ATPases to inhibit their activity\(^52\). Eosin an inhibitor of PMCA, partitions across the plasma membrane and acts from the intracellular side of the Ca\(^{2+}\) pump at a site different from ATP binding site\(^47\). AlF\(_4\) with a structure similar to phosphate ion acts from the cytosolic side of Ca\(^{2+}\) pump of the plasma membrane by interacting with the phosphate-binding site on the pump\(^48\).
4.3 Mechanism of caloxin2A1

Caloxin2A1 inhibits the $\text{Ca}^{2+}$-$\text{Mg}^{2+}$ ATPase activity of the PMCA with $K_i$ of 0.4 ± 0.1 mM. Inhibition of the activity is paralleled with decrease in the $\text{Ca}^{2+}$ dependent formation of acid stable, alkali labile 140-kDa acylphosphate intermediate from ATP in the forward reaction cycle. The effect of caloxin2A1 on the partial reactions of the activity cycle of PMCA is shown in Fig. 11 based on these results and a study by Holmes et al. The E1-E2 reaction cycle of activity is based on the model for SERCA. Although caloxin2A1 inhibits the formation of acylphosphate in the reaction starting with ATP, it does not affect the formation of the phosphoenzyme from inorganic phosphate $\text{Pi}$.

![Diagram](image)

**Fig. 11: Mechanism of caloxin2A1.** Caloxin2A1 inhibits the formation of 140-kDa acylphosphate starting from ATP and not $\text{Pi}$. 

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The probable explanation for the inhibitory effect of caloxin2A1 can be summarized as follows:

i) Caloxin2A1 can bind the E1 state of the Ca\(^{2+}\) pump, which has high affinity for Ca\(^{2+}\) and reacts with ATP. Upon binding to PMCA, caloxin2A1 can bring about a conformational change. Since, caloxin2A1 binds extracellularly and the substrates of PMCA as ATP and Ca\(^{2+}\) bind intracellularly, it is not expected to compete with the other substrates of the plasma membrane Ca\(^{2+}\) pump. This has been shown in a study on its mechanism of action\(^{53}\). As caloxin2A1 does not compete with ATP for its binding site, its action could be mediated by an allostERIC effect that prevents the binding of ATP to its site in the cytosolic domain of the Ca\(^{2+}\) pump.

Caloxin2A1 binds the 2\(^{nd}\) PED of PMCA that connects the TM3-TM4. The TM 3 is the continuation of the C-terminal of the cytosolic actuator domain (domain A) into the membrane. The TM 4 extends into the cytosol to form the N-terminal part of large cytosolic loop that has a site for the binding of ATP (domain N) and the formation of acylphosphate (domain P). X-ray diffraction studies of SERCA at 2.6\(^{°}\)A resolution suggest that the phosphorylation site is about 25\(^{°}\)A away from the bound nucleotide and involves large domain movements to bring the two close together during ATP hydrolysis\(^{54}\). It is regarded that the domain A works as an anchor for the movement of domain N. Domain A is connected to the TM helices and is released on binding of Ca\(^{2+}\) to its high affinity sites. Domain A can then facilitate the large movements of the cytosolic domains during ATP hydrolysis\(^{54}\). Mutation of the conserved residues in TM 4 has also shown that it has a Ca\(^{2+}\) binding site\(^{55}\). Thus, binding of caloxin2A1 to PED 2
could restrict the TM domains 3 and 4, further affecting the free movements of the cytosolic domains linked to these TM helices. Vanadate, a non-specific inhibitor of PMCA is known to exert negative interactions in affinity between its binding site and regulatory binding site of ATP\textsuperscript{46}.

ii) Caloxin2A1 may allow the binding of ATP to the N domain of PMCA but prevent its hydrolysis or block the phosphorylation reaction to decrease the formation of phosphoenzyme. Residues that are critical for ATP hydrolysis surround the aspartate residue that accepts the terminal phosphate group of ATP\textsuperscript{54}. Restricted movements in presence of caloxin2A1 could prevent their accessibility to the N domain to facilitate ATP hydrolysis.

iii) decrease in the level of the phosphoenzyme in the presence of caloxin2A1 can also result by inhibition of the other partial reactions of the ATPase cycle. Caloxin2A1 could arrest the pump in the E2 conformation thereby preventing its conversion to the ATP sensitive, high Ca\textsuperscript{2+} affinity E1 conformation. Vanadate also decreases the ATPase activity by blocking the conversion of E2→E1 following the hydrolysis of the phosphoenzyme. Vanadate forms a stable complex with the E2 conformation decreasing the availability of E1 conformation to be phosphorylated by ATP\textsuperscript{56}.

4.4 Screening using the Ph.D-12 mer library.

Screening of the phage display peptide library for binding to the target PED 2 and PED 3 was carried out under stringent conditions. The elution time was increased with the hypothesis that the phage that remains bound for longer time after the washes to
remove the weakly bound phage, should have lower dissociation constant. However, the peptides that were obtained from screening did not yield peptides of affinity higher than caloxin2A1. One limitation in the screening is the presentation of the target. The conformation of the target sequence may be different from the one that exists in the native membrane. Therefore, though the phage-displayed peptide may bind tightly to the target in the well of a microtiter plate, the binding to the pump in the membrane may not be as effective. Screening has been carried out successfully with the Ph.D library to obtain ligands for the target overexpressed in cells, maintaining its structural complexity in the natural environment.

The 33 amino acid peptide selected during the first round of screening of the Ph.D-12 library to bind to the PED 2 of PMCA 1b isoform resulted from the non-specific binding due to its larger size. The second screening for the PED 2 of PMCA 1b isoform gave the sequence “QWPSVYPTSSH” in all 50 clones and did not produce higher inhibition of Ca\(^{2+}\)-Mg\(^{2+}\) ATPase activity of PMCA in red blood cell ghosts. The screening of Ph.D-12 library for PED 2 of PMCA 4 isoform also did not give high consensus peptide motifs and the ones tested did not inhibit with higher affinity as compared to caloxin2A1. The Ph.D-12 library used for screening has 12-mer random peptide sequence fused at the N-terminus of the minor coat protein, pIII and there are 5 copies of the peptide per phage particle. Lower affinity peptide variants gain an advantage in the selection due to the multiple-point attachment of the phage to the multiple copies of the target immobilized on the solid surface leading to their slow dissociation rates and consequent distortion of the intrinsic affinities. Cwirla et al failed
to obtain high affinity peptides for binding to a monoclonal antibody that is specific for
the N-terminus of β-endorphin. They suggested that the multivalent interaction between
the low affinity phage carrying 5 copies of the peptide leads to high avidity and tenacious
adherence of the phage during the washing. To reduce the effects of avidity,
monovalent phage display systems are constructed where the number of fusion peptides
per virion is reduced to as few as one. Monovalent display allows for one to one binding
affinity in the phage selections of the fusion peptides. This technique has been used
successfully to find a receptor-specific variant of atrial natriuretic peptide (ANP) from the
library of filamentous phage particles displaying single copies of random ANP mutants
fused to gene III protein. The identified mutations were combined to produce an
efficiently expressed ANP analog that is as potent as wild-type ANP in stimulating
natriuretic peptide receptor A- guanylyl cyclase activity. Advantages of both polyvalent
peptide display that can give low affinity binding ligands and monovalent peptide display
for a more direct affinity based selection have been used to obtain high affinity ligands.
The polyvalent peptide library was screened first to obtain a ligand to bind the interleukin
receptor with $K_m$ of 150 µM and affinity selection with the variants of this ligand gave a
peptide that bound to the interleukin receptor with higher affinity and acted as a cytokine
antagonist at this receptor site.

Another limitation to obtaining a tight binding ligand is the size of the library that
is limited by the number of transfectants that can be obtained with reasonable number of
E.coli cells. The practical limitation for the number of combinations of the residues
within a given peptide is $10^{11}$ and the size of a peptide that can be completely randomized with 99% confidence that all the variants will be recovered is limited to 6-838.

Affinity selection process can also pose a problem with the adsorption of the wild phage that has the advantage of rapid propagation due to lack of any insert and selection of the plastic binding phage particles. Adey et al (1995) characterised the phage that binds to the different types of plastic and can be selected during the screening of the Ph.D library for a ligand to bind the immobilised target.$^{61}$

The sequence obtained for binding to the PED 3 of PMCA 4 isoform was highly hydrophobic. On substituting the terminal glycine residue to lysine made the peptide soluble and it did produce high inhibition of the $\text{Ca}^{2+}-\text{Mg}^{2+}$ ATPase activity of PMCA. However, the randomised version of the peptide produced same extent of inhibition suggesting that the hydrophobic peptide had acquired detergent like properties.

4.5 Studies using caloxin2A1

Since the recent publication of the caloxin2A1 as a novel specific inhibitor of PMCA in 2001$^{39}$, other laboratories have used it successfully in their experiments validating its importance. Kawano et al used caloxin2A1 to specifically inhibit PMCA to determine the contribution of $\text{Ca}^{2+}$ efflux pathways towards the generation of $\text{Ca}^{2+}$ oscillations in the human bone marrow derived mesenchymal stem cells$^{62}$. Their study is important in that not much is understood about the $\text{Ca}^{2+}$ efflux extrusion mechanisms in non-excitable cells or their link to the $\text{Ca}^{2+}$ oscillations$^{62}$. In another study to observe the role of PMCA in the stimulation of the extracellular $\text{Ca}^{2+}$ sensing receptor, caloxin2A1
was used as a specific inhibitor of the Ca$^{2+}$ pump. Using caloxin2A1 the study reported a novel autocrine and paracrine mechanism for the stimulation of extracellular Ca$^{2+}$ sensing receptor that leads to the generation of more Ca$^{2+}$ spikes$^{63}$. This revealed a gap junction independent mechanism of cell-cell communication by Ca$^{2+}$. Further information on the mechanism of action of caloxin2A1 has been published stating that the inhibition is non-competitive with respect to the substrates of the Ca$^{2+}$ pump. Since caloxin2A1 binds to the extracellular domain of the pump, it does not compete with the binding of Ca$^{2+}$ and ATP to their respective binding sites in the Ca$^{2+}$ pump. Calmodulin, the known modulator of PMCA also binds to the C-terminus of the pump and thus does not compete with caloxin2A1$^{53}$.

4.6 Conclusion

Caloxin2A1 is a novel specific inhibitor of PMCA. It binds to the pump extracellularly and inhibits the Ca$^{2+}$-Mg$^{2+}$ ATPase activity of PMCA with a $K_i$ of 0.4 ± 0.1 mM. It also decreases the formation of the acid stable 140-kDa acylphosphate in the partial reaction of the ATPase cycle. It has also provided evidence that the putative extracellular loops connecting the transmembrane domains of the Ca$^{2+}$ pump plays a role in the pump activity. Caloxin2A1 does not inhibit the basal Mg$^{2+}$ ATPase or the Na$^+$-K$^+$ ATPase activity of ghosts and has no effect on the Ca$^{2+}$-Mg$^{2+}$ ATPase activity of SERCA. The amino acid sequence of caloxin2A1 is essential for its inhibitory effect, as the randomized sequence of caloxin2A1 does not produce any inhibition.
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