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A novel plasma membrane Ca^{2+}-pump inhibitor: caloxin 1A1

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Abstract

The plasma membrane Ca^{2+}–Mg^{2+}-ATPase is a Ca^{2+}-pump that expels Ca^{2+} from cells. Here we report caloxin 1A1–a novel peptide inhibitor (Ki=100 μM) of plasma membrane Ca^{2+}-pump–obtained by screening a cysteine bridge-constrained random peptide library for binding to the first extracellular domain of plasma membrane Ca^{2+}-pump. Dithiothreitol removed the inhibition indicating that the constraint imposed by the cysteine bridge is required for the inhibition. Caloxin 1A1 also inhibited the fast twitch sarcoplasmic reticulum Ca^{2+}–Mg^{2+}-ATPase although weakly. Glutathione dimers (containing a cysteine bridge) inhibited the Ca^{2+}–Mg^{2+}-ATPase activity of sarcoplasmic reticulum Ca^{2+}–Mg^{2+}-ATPase, but not that of plasma membrane Ca^{2+}-pump. Caloxin 1A1 stabilised Ca^{2+}-dependent formation of the acid stable 140-kDa acylphosphate which is a partial reaction of this enzyme. Thus caloxin 1A1 inhibits the plasma membrane Ca^{2+}-pump by perturbing the first extracellular domain indicating that the transmembrane domains 1 and 2 play a role in its reaction cycle. This finding is consistent with rearrangements that occur in transmembrane helices 1 and 2 during reaction cycle of sarcoplasmic reticulum Ca^{2+}-pump. Caloxin 1A1 caused an increase in cytosolic Ca^{2+} concentration in endothelial cells.
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1. Introduction

Ca^{2+}-pumps are transmembranous Ca^{2+}–Mg^{2+}-ATPases which hydrolyse ATP and use the energy of hydrolysis to expel Ca^{2+} from the cell. Ca^{2+}-pumps in the plasma membrane are the only high affinity Ca^{2+} transporting systems present in all mammalian cells (Carafoli, 1992; Grover and Khan, 1992). Unlike the Ca^{2+}-pumps in the sarco/endoplasmic reticulum, plasma membrane Ca^{2+}-pump has a broad pH profile, lower Km values for Mg^{2+}-ATP, and do not show any co-operativity for Ca^{2+}(Grover and Samson, 1986). The pump activity can be modified by calmodulin, and in some plasma membrane Ca^{2+}-pump isoforms by cyclic AMP-dependent protein kinase (Carafoli, 1992; Grover and Khan, 1992). We have earlier suggested that plasma membrane Ca^{2+}-pump may be more suitable than sarco/endoplasmic reticulum Ca^{2+}-pump in maintenance of low cytosolic Ca^{2+} in resting cells but one can not rule out their role in lowering cytosolic Ca^{2+} following cell excitation. Cells also contain Na^{+}–Ca^{2+}-exchangers that may remove cytosolic Ca^{2+} (Fliegel and Dyck, 1995; Yamanaka et al., 2003). The three systems are expressed at different levels in various tissues and also regulated by diverse mechanisms. Hence, specific inhibitors of the plasma membrane Ca^{2+}-pumps are necessary in order to understand the contribution of the pump in maintenance of calcium homeostasis in various tissues. Recently we reported that caloxin 2A1 inhibited the Ca^{2+}–Mg^{2+}-ATPase in erythrocyte ghosts (Chaudhary et al., 2001). It was selective in that it did not inhibit Mg^{2+}-ATPase or Na^{+}–K^{+}-ATPase activities in erythrocyte ghosts, nor the sarcoplasmic reticulum Ca^{2+}–Mg^{2+}-ATPase in the skeletal muscle sarcoplasmic reticulum.

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Plasma membrane Ca^{2+}-pumps are encoded by transcripts of 4 plasma membrane Ca^{2+}-pump genes (Carafoli, 1992). Based on available hydropathy plots and other biochemical data, the plasma membrane Ca^{2+}-pump is a protein with 10 transmembrane domains, 5 putative extracellular domains and 3 major cytosolic domains. Known functions of the pump, such as high-affinity Ca^{2+}-binding, binding of ATP, acylphosphate formation and hydrolysis, and calmodulin activation occur in the cytosolic domains of the protein. Consistently inhibition by caloxin 2A1 was not competitive with the substrates Ca^{2+} and ATP or the activator calmodulin (Holmes et al., 2003). Mutagenesis of key transmembrane residues in the plasma membrane Ca^{2+}-pump protein suggest a role for the putative extracellular domains in the pump activity (Guerini et al., 1996). A role for the extracellular domains is also suggested from X-ray diffraction studies on the fast twitch sarcoplasmic reticulum pump (Toyoshima et al., 2000). Yet in the literature, putative extracellular domains are the least studied domains. Caloxin 2A1 was selected for binding to putative extracellular domain 2 of plasma membrane Ca^{2+}-pump isoform 1b (Chaudhary et al., 2001). The role of the putative transmembrane domain 1 in the plasma membrane Ca^{2+}-pump is not known. Here we report a peptide sequence obtained by screening a phage display library for binding to putative extracellular domain 1 of plasma membrane Ca^{2+}-pump isoform 4.

2. Materials and methods

2.1. Screening of the phage display library

The putative extracellular domain 1 of plasma membrane Ca^{2+}-pump 4 in humans consists of residues 116–147 with the residue 132 being a cysteine (Genbank accession number NM_001684). We synthesized the peptide plasma membrane Ca^{2+}-pump 133 which contained residues 133–147 and an added C-terminal cysteine (GQVATTPEDENEAQAC)to link the peptide to different proteins. Plasma membrane Ca^{2+}-pump 133 was conjugated to keyhole limpet haemocyanin or ovalbumin through the terminal cysteine by Biosynthesis Inc. (USA). We panned an M13 phage display library expressing random peptides flanked by two cysteines that form a cross-bridge (PhDc7c, New England Biolabs, USA). For panning, the wells of a microtitre plate were coated with a mixture of 10 μg each of plasma membrane Ca^{2+}-pump 133 peptide and plasma membrane Ca^{2+}-pump 133-keyhole limpet haemocyanin conjugate dissolved in a total volume of 100 μl of the coating buffer (phosphate-buffered saline (PBS) containing in mM: 137 NaCl, 2.7 KCl, 8 Na_2HPO_4 and 1.5 KH_2PO_4, pH 7.4 with 1 mM NaN_3) overnight at 4 °C. The following day, the wells were blocked with 200 μl of blocking buffer (5 mg/ml bovine serum albumin in sterile PBS, 1 mM NaN_3) and kept in the fridge for an hour. The wells were then washed with 6×250 μl of PBS. A solution containing 5 mg/ml of bovine serum albumin and 0.5 mg/ml each of ovalbumin and keyhole limpet haemocyanin in PBS was prepared and the phage were diluted in it to obtain 10^{11} pfu/100 μl and then added to each well. After 60 min of mixing at 22–24 °C on a rocker, the wells were washed with 100 μl of the solution containing bovine serum albumin, ovalbumin, and keyhole limpet haemocyanin, 1×250 μl PBS, 250 μl 5% carnation skim milk in PBS, and then again 10×250 μl PBS. 100 μl of plasma membrane Ca^{2+}-pump 133-ovalbumin (0.1 mg/ml in PBS) was then added for 24 h after which the eluates were removed. A 0.2 M glycine–HCl buffer pH 2.2 was then added to elute the remainder of the phage and then pooled in neutralization buffer (1 M Tris–HCl, pH 9.1). Titers of the eluates were determined. The eluate from 24 h was amplified and used for the next round of panning. The panning was repeated for 6 rounds after which phage from individual plaques were picked, amplified, and used for isolating plasmid DNA. DNA was sequenced at the MOBIX facility at McMaster University, using primers provided in the phage display kit. The primer was 96 bp downstream of the random library site.

2.2. Biochemical assays

Ca^{2+}–Mg^{2+}-ATPase assays were typically performed by following the hydrolysis of [^{33}P]-γ-ATP using a procedure previously described (Narayanan et al., 1991). Human erythrocyte leaky ghosts were incubated on ice for 60 min with different concentrations of the caloxin 1A1 in Ca^{2+}-containing or Ca^{2+}-free (1 mM ethylene glycol-*O*,*O*′-bis-[2-amino-ethyl]-*N*,*N*,*N*′,*N*′,-tetraacetic acid) solutions and then used in a 55 μl reaction mixture containing the following: 30 mM imidazole–HCl pH 7.1, 0.4 mM ethylenediamine tetraacetic acid, 0.33 mM Ethylene glycol-*O*,*O*′-bis-[2-amino-ethyl]-*N*,*N*,*N*′,*N*′,-tetraacetic acid, 3 mM $MgCl_2$, 0.2 mM ATP with trace amounts of [^{33}P]-γ-ATP, 2 μg ghost protein, and 0 or 0.5 mM $CaCl_2$. The samples were incubated in a shaking water bath for 60 min at 37 °C and then placed on ice. To each tube 10 μl of a chilled solution containing bovine serum albumin (1 mg/ml), and 100 μl of trichloroacetic acid-ATP (10% trichloroacetic acid, 50 mM ATP) were added. The samples were mixed on a vortex mixer and then centrifuged for 10 min at 10,000×*g* at 4 °C. Supernatant (100 μl) was removed from each tube and mixed with 200 μl of a solution containing ammonium molybdate (1% w/v) and 3.2% sulfuric acid (v/v). Then 800 μl of butyl acetate was added to each tube, the contents were mixed and centrifuged again at 14,000×*g* for 2 min. Supernatant (200 μl/tube) was placed in a scintillation vial and mixed with 5 ml of a scintillation cocktail (Ready safe, Beckman). The samples were placed in a scintillation counter and counts obtained after 3–4 h were used as measure of radioactivity. Ca^{2+}–Mg^{2+}-ATPase activity was determined

as the difference in the activity in the presence and absence of Ca^{2+}. Results on Ca^{2+}–Mg^{2+}-ATPase inhibition were also verified using an ATP regenerating system in a coupled enzyme assay, by continuously monitoring the disappearance of fluorescence of NADH (excitation at 340 nm and emission at 460 nm) at 37 °C as previously described (Chaudhary et al., 2001). Skeletal muscle sarcoplasmic reticulum was a gift from Dr. N. Narayanan (University of Western Ontario, Canada). The Ca^{2+}-dependent formation of the 140-kDa acid stable acylphosphate intermediate was determined from [^{33}P]-γ-ATP as described previously (Chaudhary et al., 2001). The acylphosphates were quantified using a Phosphorimager.

2.3. $[Ca^{2+}]_i$ measurement

Endothelial cells from pig coronary artery were cultured on cover slips as described earlier (Grover and Samson, 1997). Phenotypic characteristics of the cells used here have been reported previously (Grover and Samson, 1997). In Western blots, the cell lysates react positively to anti-von Willebrand factor and anti-endothelial nitric oxide synthase but negatively to anti-smooth muscle α-actin. The cells were removed from the culture medium and rinsed with a solution containing (in mM): 115 NaCl, 5.8 KCl, 2 $CaCl_2$, 0.6 $MgCl_2$, 12 glucose, 25 HEPES–Na, pH 7.4 at 22–24 °C. The cells, while still attached to the cover slips, were loaded with FLUO 3/AM and probenecid and then used for $[Ca^{2+}]_i$ measurement at 37 °C as previously described (Grover and Samson, 1997).

3. Results

3.1. Phage display

Based on hydropathy plots, the peptide sequence (ISLVLSFYRPAGEENELCGQVATTPEDENEAQA) corresponding to residues in plasma membrane Ca^{2+}-pump 4b connects transmembrane helices 1 and 2 of plasma membrane Ca^{2+}-pump thereby forming its first and the largest extracellular domain. The extracellular nature of this domain has been confirmed using an antibody. Since this domain contains a cysteine in the middle and because it is too large, we used only the domain 133–147 (GQVATTPEDENEAQA) as the target peptide. We used this target to screen a library in which each phage displays random 7 amino acids flanked by cysteines that formed a disulfide bridge. This configuration puts more conformational constraints on the peptides expressed. Screening gave the major random sequence–PWWSPHA–based on which we synthesized caloxin 1A1 (ACPWWSPHACGGG). A second sequence obtained was PIWQPHY, which was also inhibitory, but much lower yields were obtained during its synthesis and hence only caloxin 1A1 was characterized further.

3.2. Inhibition of plasma membrane Ca^{2+}-pump Ca^{2+}–Mg^{2+}-ATPase

Fig. 1 shows that caloxin 1A1 inhibited the plasma membrane Ca^{2+}–Mg^{2+}-ATPase in erythrocyte leaky ghosts with a K*i* value of 86±12 μM using the [^{33}P]-γ-ATP hydrolysis assay. K*i* values of approximately 100 μM were obtained by replicating this experiment. For Fig. 2, the Ca^{2+}–Mg^{2+}-ATPase assay was carried out using a coupled enzyme assay and a similar inhibition pattern was obtained.

Since the peptide contained a disulfide bridge, the effect of thiol reagent dithiothreitol was examined (Fig. 2). Including 1 mM dithiothreitol in the assay mixtures increased the K*i* value of caloxin 1A1 from 86±12 μM to 1939±246 μM, indicating that the presence of the disulfide bridge was needed for the inhibition.

Since a cysteine residue is present in the extracellular domain of plasma membrane Ca^{2+}-pump, we tested the possibility that the disulfide bridge itself was the mechanism by which caloxin 1A1 inhibited plasma membrane Ca^{2+}–Mg^{2+}-ATPase (Fig. 3). Glutathione dimer was used at a concentration of 1 mM which was approximately 10× the K*i* value of caloxin 1A1. However, no inhibition was observed. This strongly argues against the disulfide bridge being sufficient for the inhibition.

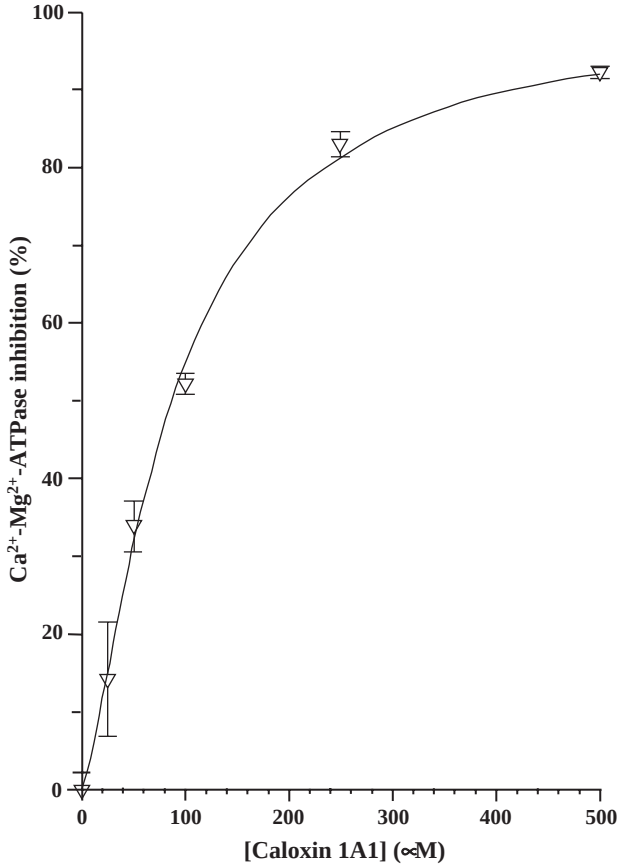

Fig. 1. Inhibition of plasma membrane Ca^{2+}–Mg^{2+}-ATPase by Caloxin 1A1. Values are mean±S.E.M. of replicates.

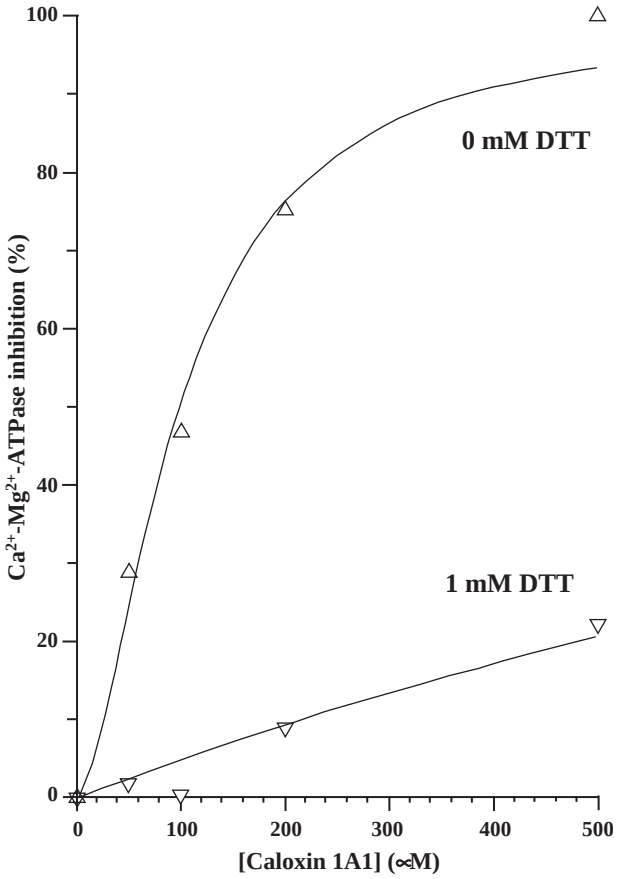

Fig. 2. Effect of dithiothreitol (DTT) on plasma membrane Ca^{2+}–Mg^{2+}-ATPase inhibition by Caloxin 1A1.

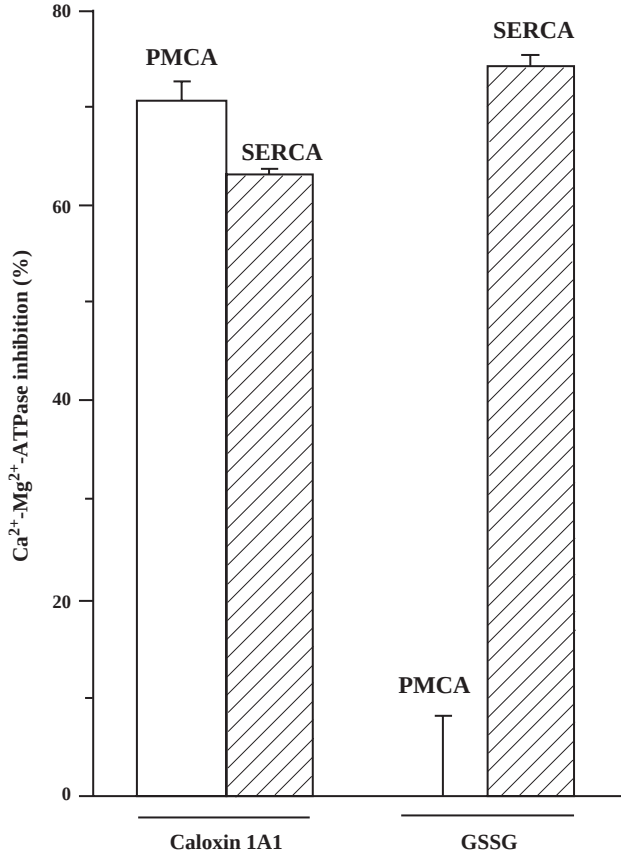

Fig. 3. Effects of 200 μM Caloxin 1A1 and 1 mM glutathione dimer on Ca^{2+}–Mg^{2+}-ATPase of plasma membrane and sarcoplasmic reticulum Ca^{2+}-pumps.

3.3. Inhibition of sarcoplasmic reticulum Ca^{2+}–Mg^{2+}-ATPase

We compared the effects of 200 μM caloxin 1A1 on Ca^{2+}–Mg^{2+}-ATPase in plasma membrane and fast skeletal muscle sarcoplasmic reticulum (Fig. 3). Caloxin 1A1 inhibited the Ca^{2+}–Mg^{2+}-ATPase in plasma membrane more strongly than that in sarcoplasmic reticulum. Glutathione dimer at a concentration of 1 mM strongly inhibited (74±2%) the sarcoplasmic reticulum Ca^{2+}–Mg^{2+}-ATPase. Therefore, we conclude that the disulfide bridge in caloxin 1A1 may have been sufficient to inhibit sarcoplasmic reticulum Ca^{2+}–Mg^{2+}-ATPase. The greater susceptibility of sarcoplasmic reticulum Ca^{2+}-pump to glutathione dimer is consistent with previous observations that sarcoplasmic reticulum Ca^{2+}-pumps are more susceptible to loss of activity due to sulfhydryl group oxidation than the plasma membrane Ca^{2+}-pumps (Grover and Samson, 1988). However, caloxin 1A1 inhibits plasma membrane Ca^{2+}-pump by the more selective pathway involving the extracellular domain 1. Thus, in broken cell preparations, caloxin 1A1 could inhibit Ca^{2+}-pumps in plasma membranes and in sarco/endoplasmic reticulum. However, in studies using cells, it could still be used selectively for the plasma membrane Ca^{2+}-pump since it is unlikely to enter the cell and affect the sarco/endoplasmic reticulum Ca^{2+}-pump.

3.4. Effects of caloxin 1A1 on acylphosphate formation

Acylphosphate intermediate formation from [^{33}P]-γ-ATP in erythrocyte ghosts gave only one major band at 140 kDa (Fig. 4). The band was not observed in presence of excess ethylene glycol-*O*,*O*′-bis-[2-amino-ethyl]-*N*,*N*,*N*′,*N*′,-tetra-acetic acid. Preincubation of the ghosts with caloxin 1A1 prior to the acylphosphate formation reaction increased the intensity of the band by 150 ± 5% (P<0.05).

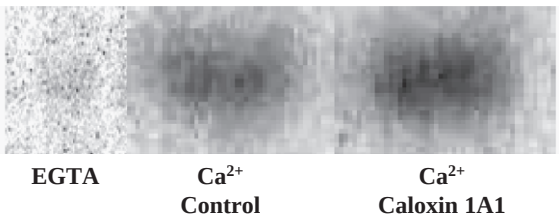

Fig. 4. Effect of 1 mM Caloxin 1A1 on acylphosphate formation. The intensity of the caloxin 1A1 band in 4 gels was 150±5% of that of the control band.

3.5. Effects of caloxin 1A1 on $[Ca^{2+}]_i$ in pig coronary artery endothelial cells

We next tested the effects of PMCA inhibition by caloxin 1A1 on $[Ca^{2+}]_i$ in pig coronary artery cultured endothelial cells. We first determined how different concentrations of the Ca^{2+} ionophore (4-bromo-A23187) would affect $[Ca^{2+}]_i$ in these cells. As expected, the Ca^{2+} ionophore increased $[Ca^{2+}]_i$ in a concentration dependent manner (not shown). 100 nM ionophore caused a large transient increase in $[Ca^{2+}]_i$ following which there was a small steady increase in $[Ca^{2+}]_i$ over the basal level. We added 10 μM cyclopiazonic acid (to inhibit the sarco/endoplasmic reticulum Ca^{2+}-pump) plus the ionophore to the cells. This resulted in a rapid increase in $[Ca^{2+}]_i$ that lasted 2–3 min (Fig. 5). However, adding caloxin 1A1 after this decline in $[Ca^{2+}]_i$ produced a further increase in $[Ca^{2+}]_i$ (Fig. 5), consistent with the decreased ability of the endothelial cells to expel Ca^{2+} upon plasma membrane Ca^{2+}-pump inhibition.

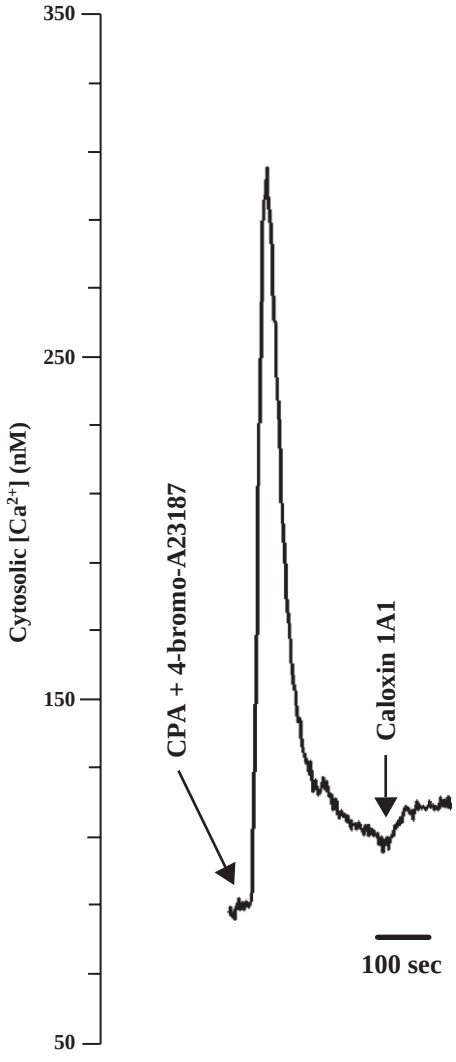

Fig. 5. Effect of caloxin 1A1 on $[Ca^{2+}]_i$ in pig coronary artery cultured endothelial cells. Cyclopiazonic acid (CPA) plus 100 nM 4-bromo-A23187 was added at the time indicated by the first arrow, and 0.37 mM caloxin 1A1 was added at the time indicated by the second arrow.

The results show that caloxin 1A1, a peptide obtained by screening for binding to PED1 sequence 133–147 of the plasma membrane, Ca^{2+}-pump protein inhibits this pump. The peptide also increases cytosolic Ca^{2+} in endothelial cells. However, caloxin 1A1 also causes inhibition of the sarco/endoplasmic reticulum Ca^{2+}-pump.

4. Discussion

Discussion will focus on comparison of caloxin 1A1 with caloxin 2A1 reported earlier (Chaudhary et al., 2001) and biochemical implications of the observations made in this work.

Caloxin 2A1 was the first selective inhibitor of plasma membrane Ca^{2+}-pump but it has a very low affinity (Chaudhary et al., 2001). It was obtained by screening a linear randon 12 amino acid peptide phage display library. Caloxin 1A1 was obtained by screening a disulfide bridge constrained 7 random amino acid peptide phage display library. The latter screening gave a tighter binding peptide. However, thiol dimers inhibited the sarco/endoplasmic reticulum Ca^{2+}-pump leading to a lower selectivity in broken cell preparations. Caloxin 1A1 inhibited the expulsion of Ca^{2+} from cells in which the sarco/endoplasmic reticulum Ca^{2+}-pump had already been inhibited. This inhibition confirms that this peptide acts when applied to extracellular surface of the cells. Since the peptide would not cross the membranes, it can be used as a selective inhibitor in cellular and tissue preparations.

Preincubation of the ghosts with caloxin 1A1 increased the amount of acylphosphate formed. This is in contrast to the decrease in the acylphosphate formation produced by caloxin 2A1 reported earlier (Chaudhary et al., 2001; Holmes et al., 2003). Since caloxin 2A1 was obtained as a peptide that binds putative extracellular domain 2 and caloxin 1A1 against putative extracellular domain 1, we conclude that binding of caloxins to different putative extracellular domains may inhibit the plasma membrane Ca^{2+}-pump activity by stabilizing different conformational states of the pump. During switching from E1 to E2 state, the movement of transmembrane helices has been shown to occur in the sarcoplasmic reticulum Ca^{2+}-pump using crystallography studies. In this pump, several extracellular domains are in close proximity and form the extracellular lip of a Ca^{2+} channel needed for the pump action (Toyoshima et al., 2000). Dissociation (or association) of Ca^{2+} accompanies rearrangements in six (1–6) of the ten transmembrane helices. Transmembrane helices 1 and 2 move upwards (+z direction, towards the cytoplasm). The large movements of membrane helices would require conformational changes in luminal domains connecting the transmembrane helices. Luminal loops of the sarcoplasmic reticulum Ca^{2+}-pump correspond to plasma membrane Ca^{2+}-pump putative extrac-

ellular domains. Therefore, the latter may also move during the plasma membrane Ca^{2+}-pump reaction cycle. Caloxin 1A1 specifically inhibits the Ca^{2+}-pump activity by perturbing putative extracellular domain 1 of plasma membrane Ca^{2+}-pump. Thus, this study shows that the putative extracellular domain 1 plays a role in the reaction cycle of plasma membrane Ca^{2+}-pump. It would be interesting if this approach can be extended to understand the roles of the remaining three extracellular domains in the plasma membrane Ca^{2+}-pump reaction cycle.

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