Caloxin: a novel plasma membrane Ca\textsuperscript{2+} pump inhibitor

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Plasma membrane Ca\textsuperscript{2+} pumps are encoded by four plasma pump protein models show ten transmembrane and five extracellular domains (4). Except for the first putative extracellular domain, their sequences are conserved in different isoforms. All the known functions of the pump are assigned to the cytosolic domains, although mutagenesis of key residues in the PM Ca\textsuperscript{2+} pump protein shows that transmembrane domains are involved in its activity (5). X-ray diffraction studies of the sarcoplasmic reticulum Ca\textsuperscript{2+} pump also support a role for transmembrane domains in the pump activity (16). In contrast, it is unknown whether extracellular domains in the PM Ca\textsuperscript{2+} pump play any role in its function. Earlier, we raised antibodies against solubilized and purified whole PM Ca\textsuperscript{2+} pump protein (1). However, this approach gives antibodies predominantly against cytosolic domains because these form the bulk of the protein and contain many antigenic epitopes. We have now screened a random peptide phage display library to select for peptides binding to the second extracellular domain sequence of the PM Ca\textsuperscript{2+} pump. This domain links transmembrane domains 3 and 4, and mutagenesis of key residues in domain 4 has been shown to be inhibitory (5). The selected peptide inhibits the pump activity. To our knowledge, this is the first extracellular inhibitor of the PM Ca\textsuperscript{2+} pump.

"Plasma membrane Ca\textsuperscript{2+} pumps are Ca\textsuperscript{2+}-Mg\textsuperscript{2+}-ATPases that use the energy of hydrolysis of ATP to expel cellular Ca\textsuperscript{2+}. Plasma membrane (PM) Ca\textsuperscript{2+} pumps are present in all mammalian cells. However, either PM Ca\textsuperscript{2+} pumps or Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (8, 9, 17) may remove Ca\textsuperscript{2+} from the cell after activation or during homeostasis, and the precise role of PM Ca\textsuperscript{2+} pumps remains to be determined. There are no extracellular PM Ca\textsuperscript{2+} pump inhibitors available to resolve this issue. In contrast, the Na\textsuperscript{+} pump inhibitors ouabain and digoxin have been pivotal in our understanding of how this pump affects cell function (7, 12). The sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) inhibitor thapsigargin, which can diffuse into the cell to act, has also proved very useful in elucidating the role of the SERCA pump (15). PM Ca\textsuperscript{2+} pumps are encoded by four plasma membrane Ca\textsuperscript{2+}-ATPase (PMCA) genes, and their transcripts can be alternatively spliced (2, 4, 10, 14). The isoform PMCA1b is most widely expressed. The PM Ca\textsuperscript{2+} pump protein models show ten transmembrane and five extracellular domains (4). Except for the first putative extracellular domain, their sequences are conserved in different isoforms. All the known functions of the pump are assigned to the cytosolic domains, although mutagenesis of key residues in the PM Ca\textsuperscript{2+} pump protein shows that transmembrane domains are involved in its activity (5). X-ray diffraction studies of the sarcoplasmic reticulum Ca\textsuperscript{2+} pump also support a role for transmembrane domains in the pump activity (16). In contrast, it is unknown whether extracellular domains in the PM Ca\textsuperscript{2+} pump play any role in its function. Earlier, we raised antibodies against solubilized and purified whole PM Ca\textsuperscript{2+} pump protein (1). However, this approach gives antibodies predominantly against cytosolic domains because these form the bulk of the protein and contain many antigenic epitopes. We have now screened a random peptide phage display library to select for peptides binding to the second extracellular domain sequence of the PM Ca\textsuperscript{2+} pump. This domain links transmembrane domains 3 and 4, and mutagenesis of key residues in domain 4 has been shown to be inhibitory (5). The selected peptide inhibits the pump activity. To our knowledge, this is the first extracellular inhibitor of the PM Ca\textsuperscript{2+} pump."

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METHODS

Screening of phage display library. The second putative extracellular domain of PMCA1b in rabbit contains the residues 401–413 (KRFPWLAECTPIYI; GenBank accession no. X59069). Including additional amino acids flanking this sequence, replacing serine for cysteine, and adding a cysteine at the COOH terminus, we synthesized the peptide PMCA398 (WVYKRPWLAESTPIYIQVFVKC). PMCA398 was conjugated to keyhole limpet hemocyanin (KLHL) or ovalbumin. A random 12-amino acid mitogen-activated protein or extracellularly regulated kinase 13 phage display library (PhD12; New England Biolabs) was panned for a phage that bound PMCA398-KLHL conjugate dissolved in phosphate-buffered saline (PBS) and eluted with the PMCA398-ovalbumin conjugate. PBS contained 137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, and 1.5 mM KH2PO4 (pH 7.4). After eight rounds of panning, elution was performed with PBS for 1, 2, 4, 8, 16, and then 120 min, each time replacing the old PBS. DNA from 20 clones of 16- and 120-min eluants was sequenced. Many clones had no inserts, but six clones encoded for the peptide VSNSNWSFPSS. Because in the phage the sequence GGG follows the random peptide, the peptide VSNSNWSFPSSGGG-amide was synthesized and eventually termed caloxin 2A1. Randomization of residues in caloxin 2A1 peptide gave the sequence SWSSFPSSGVSNSNP-amide, and this peptide was synthesized for use as a control.

Biochemical assays. Human erythrocyte leaky ghosts were isolated as previously described (6). The ghosts were suspended in 40 mM imidazole-HCl (pH 7.0) and stored at −80°C until use. The skeletal muscle sarcoplasmic reticulum membrane vesicle preparation was a gift from Dr. N. Narayan of the University of Western Ontario. The Ca2+/Mg2+-ATPase assays were performed at 37°C by following the disappearance of NADH with a fluorometer (excitation at 340 nm and emission at 460 nm) at 37°C in a coupled enzyme assay that was previously described (1, 9). Basal Mg2+-ATPase was first determined in a 135-MM solution that contained 0.2–0.4 mg ghost protein, 0.1 mM ouabain, 100 mM NaCl, 20 mM KCl, 6 mM MgCl2, 30 mM imidazole-HCl (pH 7.0), 0.5 mM EDTA, 0.6 mM NADH, 1 mM phosphoenolpyruvate, excess pyruvate kinase-acetate dehydrogenase, 0.5 mM ATP, 0.5 mM EGTA, and 4 μg/ml calmodulin. Ten microliters of 8 mM CaCl2 was then added, and the total ATPase activity was determined. The difference between the total ATPase and the basal Mg2+-ATPase was the Ca2+-Mg2+-ATPase activity. Na+-K+-ATPase was assayed in the same solution that was used for the basal Mg2+-ATPase except that ouabain was omitted. The Ca2+-dependent formation of 140-kDa acid-stable acylphosphates was determined with SDS-polyacrylamide gels at pH 4.0 as previously described (3, 18). The acylphosphates were quantified with a PhosphorImager by following the radioactivity in each band.

Contractility experiments. Rats were euthanized with 0.5 ml methoxyflurane, and the thoracic aorta was removed. Aortic rings (3 mm long) with endothelium intact were hung in organ baths under 1.5 g of tension in Krebs solution that contained (in mM) 115 NaCl, 5 KCl, 22 NaHCO3, 1.7 CaCl2, 1.1 MgCl2, 1.1 KH2PO4, 0.3 EDTA, and 7.7 glucose. Contractions with phenylephrine (0.5 μM) were monitored as previously described (13). Relaxation of the arteries was first monitored using 1 μM carbachol. The arteries were washed for 60 min and then treated again with phenylephrine. After the arteries had reached a steady contraction, caloxin 2A1 dissolved in Krebs solution was added. After another 20–30 min, 100 μM Nω-nitro-L-arginine methyl ester (L-NAME) was added (11).

Data analysis. Each experiment was replicated at least four times. Values given are means ± SE. Where applicable, Student’s t-test was used, and values of P < 0.05 were considered to be statistically significant. Nonlinear regression was used to determine inhibition constant with the software FigP.

RESULTS

Effect of caloxin 2A1 on Ca2+-Mg2+-ATPase. Figure 1A shows the effect of different concentrations of caloxin 2A1 on Ca2+-Mg2+-ATPase in erythrocyte leaky ghosts. Caloxin 2A1 inhibits the Ca2+-Mg2+-ATPase. It produces 50% inhibition at 0.4 ± 0.1 mM. Figure 1B shows the selectivity of the inhibition. At a concentration of 0.9 mM, caloxin 2A1 inhibits the Ca2+-Mg2+-ATPase in the erythrocyte ghosts by 78 ± 4%, but it has no effect on Mg2+-ATPase or Na+-K+-ATPase in the ghosts or Ca2+-Mg2+-ATPase in the skeletal muscle sarcoplasmic reticulum (Fig. 1B). Thus caloxin 2A1 inhibits Ca2+-Mg2+-ATPase selectively. Figure 1B also shows that the sequence of caloxin 2A1 is needed to cause the inhibition and not just its amino acid composition, because a randomized peptide with the same amino acid composition does not produce an inhibition.

Effect of caloxin 2A1 on Ca2+-dependent acylphosphate formation. The second assay of PM Ca2+-Mg2+-ATPase is based on the Ca2+-dependent formation of the acid-
stable, alkali labile 140-kDa acylphosphate intermediate from [γ-32P]ATP in the erythrocyte leaky ghosts. Because 0.4 ± 0.1 mM caloxin 2A1 produces a 50% inhibition of PM Ca2+-Mg2+-ATPase, it is expected to cause ~90% inhibition at 3.4 mM. At this concentration, caloxin 2A1 also produces nearly complete inhibition of the acylphosphate formation, but the same concentration of the randomized peptide has no effect (Fig. 2).

Effect of caloxin 2A1 on endothelium-dependent relaxation. Inhibition of the PM Ca2+-pump in vascular endothelium would increase the concentration of cytosolic Ca2+, thereby activating the endothelial nitric oxide synthase, which is Ca2+- and calmodulin dependent. The increased nitric oxide relaxes arteries; L-NAME inhibits the nitric oxide synthase (11). In rat aortic rings precontracted with a submaximum concentration (0.5 μM) of phenylephrine, caloxin 2A1 produces a relaxation that is reversed by L-NAME (Fig. 3). Thus caloxin 2A1 produced the biological effect in the endothelium, as expected, from its inhibition of the PM Ca2+-pump, although alternative interpretations cannot be completely ruled out.

DISCUSSION

Caloxin 2A1, a peptide selected for binding to the sequence of the putative second extracellular domain of PM Ca2+-Mg2+-ATPase, inhibits this enzyme in human erythrocyte leaky ghosts and produces an endothelium-dependent relaxation in rat aorta. Here, we focus on sequence identities between PMCA isoforms, the relationship of this work to literature on the mechanism of action of the PM Ca2+-pump, and the potential applications of caloxin 2A1 to the study of cell function.

The sequence of extracellular domain 401–413 in human PMCA1b (KRPWLAECTPIYI) is similar to the corresponding sequences in PMCAs 2, 3, and 4 (Swiss Protein Bank accession nos. P20020, Q01814, Q16720, and P23634), except that in PMCAs 2 and 4, the residue A is replaced by P, and in PMCA3, the sequence KRP is replaced by GRT. Caloxin 2A1 was selective for PM Ca2+-Mg2+-ATPase in that it had no effect on basal Mg2+-ATPase, Na+-K+-ATPase, or SERCA1 Ca2+-Mg2+-ATPase. Additional selectivity of caloxin 2A1 is suggested from the analysis that the target sequence used is absent from all the Na+/Ca2+ exchanger protein sequences reported in the Swiss Protein Bank.

Caloxin 2A1 was selected to bind the second putative extracellular domain. Perturbing other domains may also produce inhibition, because mutagenesis of key PMCA residues in putative transmembrane domains 4, 6, and 8 results in a loss of ATPase activity, and the various extracellular domains connect them (5). The sequences of the extracellular domain 1 show the highest diversity between different PMCA isoforms. It is thus possible for this approach to yield peptides that will at least bind more selectively against the extracellular domain 1. This could then lead to isoform-selective caloxins.

This study shows that caloxin 2A1 produces endothelium-dependent relaxation by activating the endothelial nitric oxide synthase, which is a Ca2+/calmodulin-activated enzyme (11). The PM Ca2+-pump affects virtually every cell, although its role in cell function varies, depending on the expression and level of activity of other transporters. Obviously, this discovery paves the way to examine the role of PM Ca2+-pump in tissues with high levels of PMCA expression such as brain, those with high levels of SERCA expression such as skeletal and cardiac muscle, and those with intermediate levels of expression such as platelets, lymphocytes, endothelium, smooth muscle, pancreas, kidney, and liver.

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Observations in this manuscript form the basis of a patent (patent pending).
REFERENCES


