Aortic smooth muscle and endothelial plasma membrane Ca\(^{2+}\) pump isoforms are inhibited differently by the extracellular inhibitor caloxin 1b1


PMCA are encoded by four genes, PMCA1–4, which have transcripts that may also be alternatively spliced (4, 12, 33, 43, 45). However, it is not known whether arterial smooth muscle and endothelium express the same PMCA isoforms. The role of PMCA has been examined using transgenic animals. The effect of PMCA4 ablation depends on the strain of the mice used. In one strain, the loss of PMCA4 led to impairment of phasic contractions and caused apoptosis in the portal vein smooth muscle in vitro (29). In cultured cells, functional PMCA has been overexpressed only at very low levels, and hence made a limited contribution to the delineation of the physiology of PMCA (18).

To understand the roles of various transporters in arterial function, several specific inhibitors such as digoxin, ouabain, thapsigargin, cyclopiazonic acid, dichlorobenzamil, and SEA0400 have proved useful (13, 18–20, 32, 48, 49). To understand the role of PMCA in arterial smooth muscle, endothelium, and other tissues, two nonspecific inhibitors have been used extensively: vanadate and eisens. Vanadate also inhibits Na\(^+\)-K\(^+\) and SERCA pumps. It has a markedly higher affinity for the Na\(^+\)-K\(^+\) pump (50% inhibition at 0.04–0.2 \(\mu M\)) than for the PMCA pump (50% inhibition at 3–100 \(\mu M\)) (3, 11, 22, 46). Therefore, any vanadate concentrations that even marginally inhibit the PMCA pump would abolish the Na\(^+\)-K\(^+\) pump activity. This makes it difficult to delineate the roles of PMCA and NCXs in cell function. There are also further complications in using vanadate as an ATPase inhibi-
tor. Cells are not readily permeant to vanadate; hence, its accessibility to ATP binding sites (intracellular) may vary with cell type. Finally, the intracellular milieu contains varying levels of thiol groups that can reduce vanadate to its $V^4$ or $V^5$ valence state (2). Consequently, many studies (1, 27, 39, 40) have shown why the effects of vanadate are difficult to explain. Eosins are nonspecific because they inhibit by binding to a protein domain conserved in Na$^+$-K$^+$, PMCA, SERCA pumps, and other ATPases. Hence, specific inhibitors of PMCA are needed to understand its contribution in signal transduction and the maintenance of cellular Ca$^{2+}$ homeostasis.

On the basis of available hydropathy plots and other biochemical data, the PMCA proteins have 10 transmembrane domains, 5 extracellular domains, and 3 major cytosolic domains (5). The cytosolic domains of the protein contain sites for known functions of the pump, such as high-affinity Ca$^{2+}$ binding, binding of ATP, acylphosphate formation and hydrolysis, and calmodulin activation (5, 33, 44). In the SERCA pump, a role for luminal loops in the transport cycle is suggested from X-ray diffraction studies (47). Because extracellular domains in PMCA correspond to luminal loops in SERCA, we initiated a search for peptides that would bind to these domains and possibly inhibit the PMCA pump activity. The sequences of the extracellular domains of PMCA do not have significant homology with any of the other P-type ATPases and hence are selective targets for the development of PMCA-specific inhibitors. Earlier, we screened a random peptide phage display library using an extracellular domain 2-based synthetic peptide as the target and obtained caloxin 2a1, which inhibited the Ca$^{2+}$-Mg$^{2+}$-ATPase in erythrocyte ghosts (6). Herein we report that endothelial cells express PMCA1 and smooth muscle cells express mainly PMCA4 plus low levels of PMCA1. We exploited the fact that extracellular domain 1 shows the largest differences between PMCA1 and PMCA4 (Swiss protein accession nos. P20020, Q01814, Q16720, and P23634), and modified our screening procedures to obtain the PMCA4 selective caloxin 1b1. Caloxin 1b1 is a selective PMCA inhibitor, which has 10 times higher affinity than caloxin 2a1 and is the first PMCA inhibitor with isoform selectivity. We used caloxin 1b1 extracellularly to examine its effects on arterial smooth muscle contractility and cytosolic [Ca$^{2+}$] ([Ca$^{2+}$]).

**EXPERIMENTAL METHODS**

**Membrane isolation.** Leaky human erythrocyte ghosts were prepared as described previously (10, 28). In this procedure, the leaky ghosts are washed thoroughly with EDTA to remove any bound calmodulin and then aliquots are stored at −80°C in a buffer containing (in mM) 130 KCl, 20 HEPES, 0.5 MgCl$_2$, 0.05 CaCl$_2$, and 2 dithiothreitol at pH 7.4. To obtain vascular smooth muscle membranes, pig aortas were obtained from Maple Leaf Meats (Burlington, ON, Canada) and used for preparation of microsomes as described previously (13). However, 1 mM EGTA was included in the homogenization buffer and in the buffer for suspending the microsomes to ensure that calmodulin was removed. The plasma membrane fraction was then isolated from the microsomes on a sucrose density gradient as described previously, and aliquots were stored at −20°C (13, 14). Skeletal muscle sarcomplasmic reticulum was a gift from Dr. N. Narayanan (University of Western Ontario). Human embryonic kidney (HEK)-293 cells were obtained from American Type Culture Collection (Manassas, VA), cultured, and used for preparing microsomes (15).

**RNA isolation and RT-PCR.** Fresh endothelial cells were obtained from aortas of two pigs with the use of lectin-coated magnetic beads (Dynabeads, Dynal Biotechnology, Lake Success, NY), as described elsewhere for coronary artery endothelium (8). Cells isolated by this method were positive for von Willebrand factor and endothelial NO synthase. RNA was isolated from these cells when still attached to the beads with the use of TRIzol (Invitrogen), following the manufacturer’s instructions. RNA was isolated from pig aortic smooth muscle tissue (200 mg), from pig coronary artery cultured endothelial cells (six 75-cm$^2$ flasks) and HEK-293 cells (three 10-cm culture dishes) using a Qiagen total RNA isolation kit. The isolated RNA was DNase I digested and reverse transcribed using the Thermoscript RT-PCR system (Invitrogen) using 5 μM oligo(dT) primers, following instructions of the manufacturer. PCR was carried out with AmpliTaq (Applied Biosystems) at 2.5 mM MgCl$_2$ with the following primers: PMCA1up (5'-TAGGCACTTTTGTGGTACAG-3'), PMCA1dn (5'-GGCTCTGAATCTTCTTACCTCA-3'), PMCA4up (5'-CCAGCGACA- GCATTACACCATT-3'), and PMCA4dn (5'-TGTAGAGAGCTGT- CCGACTGG-3'). The PCR conditions were as follows: denaturation at 94°C for 40 s, annealing at 58°C for 40 s, and extension at 72°C for 50 s for 30 cycles. Water, NO-RT, and RNA were used as templates for negative controls.

**Screening phage for binding to a synthetic sequence.** The first extracellular domain of human PMCA4 consists of residues 116–147 with the residue 132 being a cysteine (GenBank accession no. NM_001684). The peptide PMCA4–115 (CISLVLSTSFYPAGEENEL) which contained residues 115–131 and an additional NH$_2$ terminal cysteine to link the peptide to different proteins was synthesized commercially (Dalton Chemical Laboratories, Toronto, ON, Canada) and conjugated to keyhole limpet hemocyanin or ovalbumin through the cysteine (Biosynthesis). We panned an M13 phage display library expressing random linear 12 amino acid peptides (Ph.D.12; New England Biolabs), as described previously (6). The phage in the eluate was amplified in two cycles of infection.

**Screening phage for binding to purified PMCA protein.** An aliquot of the erythrocyte ghosts of known protein concentration was centrifuged at 500,000 g for 15 min, and the pellet was resuspended to obtain a protein concentration of 8 mg/ml in solubilation buffer composed of (in mM) 260 KCl, 40 HEPES, 1 MgCl$_2$, 2 dithiothreitol, and 0.1 CaCl$_2$ at pH 7.4 plus a cocktail of protease inhibitors (Complete Mini, EDTA-free; Roche). To this, an equal volume of a soluble fraction containing 0.8% Triton X-100 was added slowly and the tube was inverted 5–10 times to mix. The suspension was centrifuged at 500,000 g for 15 min and the supernatant was retained as the soluble fraction. A bed volume of 200 μl of agarose-calmodulin resin (Sigma-Aldrich) was packed in a column (Bio-Rad) and washed in the wash buffer composed of 0.4% Triton X-100, 130 mM KCl, 20 mM HEPES, 1 mM MgCl$_2$, 0.5 mM CaCl$_2$, and 0.05% phosphatidylserine and phosphatidylycholine. The soluble fraction (1 ml) was mixed with 26 μl of the stock phospholipid solution (2% phosphatidylserine and phosphatidylycholine dissolved in 0.1% Triton X-100), by rocking for 10 min at 4°C and added to the agarose-calmodulin mixture. The unbound flow-through material from the column was removed. The phage was eluted in the wash buffer and mixed with the bound PMCA on a rocker for 60 min. The unbound phage was removed as flow through in four additional washes, each with 1.6 ml of wash buffer. PMCA and the bound phage were eluted using a Ca$^{2+}$-free elution buffer (0.4% Triton X-100, and in mM: 130 KCl, 20 HEPES, 1 MgCl$_2$, 2 dithiothreitol, 5 EGTA, and 0.05% phosphatidyl serine and phosphatidylycholine dissolved in 0.1% Triton X-100). The eluted phage was precipitated and amplified.

Phage titers were performed using *Escherichia coli* XL-1 blue cells. At several stages during screening, phages from individual plaques were picked, amplified, and used for isolating plasmid DNA. DNA
was sequenced at the MOBIX facility at McMaster University using a reverse primer 96-bp downstream of the random library site.

Biochemical assays. \( \text{Ca}^{2+}-\text{Mg}^{2+}\)-ATPase assays were performed by following the hydrolysis of \([\gamma-\text{P}]\text{ATP}\) and in a coupled enzyme assay that monitored the disappearance of fluorescence of NADH. Procedures for both the assays have been described previously (6, 30).

The difference between the total ATPase and the basal \( \text{Mg}^{2+}\)-ATPase was the \( \text{Ca}^{2+}-\text{Mg}^{2+}\)-ATPase activity. Thapsigargin (5 \( \mu \text{M} \)) was also included in the assays for PMCA \( \text{Ca}^{2+}-\text{Mg}^{2+}\)-ATPase but not in the assays for SERCA \( \text{Ca}^{2+}-\text{Mg}^{2+}\)-ATPase. \( \text{Na}^{+}-\text{K}^{+}\)-ATPase was assayed in the same solution as used for the basal \( \text{Mg}^{2+}\)-ATPase, except that ouabain was omitted. Caloxin 1b1 was routinely dissolved at 10 mM in 25% ethanol and stored as aliquots at \(-80^\circ\text{C}\). An equal amount of ethanol was also added to all the control assays. The \( \text{Ca}^{2+}\)-dependent formation of the 140-kDa acid-stable acylphosphatase intermediate of PMCA was determined from \([\gamma-\text{P}]\text{ATP}\) as described previously (30).

\( \text{K}^{+}\)-\( \text{Mg}^{2+}\)-phosphatase was measured as an increase in absorbancy at 405 nm due to hydrolysis of \( \text{p}\)-nitrophenylphosphate in microtiter plates. The wells contained 100 mM imidazole-\( \text{HCl} \), pH 7.8, 5 mM \( \text{p}\)-nitrophenylphosphate, 20 \( \mu \text{g} \) of ghost protein, 5 mM \( \text{MgCl}_2 \), and 0 or 5 mM KCl. The difference in change in absorbancy with and without KCl gave the \( \text{K}^{+}\)-dependent increase. To determine the \( \text{Mg}^{2+}\)-dependent phosphatase, the wells contained 100 mM imidazole-\( \text{HCl} \), pH 7.8, 5 mM \( \text{p}\)-nitrophenylphosphate, 20 \( \mu \text{g} \) of ghost protein, and 0 or 5 mM \( \text{MgCl}_2 \). The difference in change in absorbancy with and without \( \text{MgCl}_2 \) produced the \( \text{Mg}^{2+}\)-dependent increase.

**Contractility studies.** Thoracic aortas from male Wistar-Kyoto rats (Charles River Laboratories, Wilmington, MA) was obtained, cut into 3-mm-wide rings and used for contractility studies in an organ bath containing Krebs solution composed of (in mM) 115.5 \( \text{NaCl} \), 4.6 KCl, 1.2 \( \text{MgSO}_4 \), 1.2 \( \text{NaH}_{2}\text{PO}_4 \), 2.5 \( \text{CaCl}_2 \), 22.0 \( \text{NaHCO}_3 \), and 11.1 \( \text{D}\)-glucose, bubbled with 95% \( \text{O}_2 \) and 5% \( \text{CO}_2 \) as described previously (24). The aortic rings under 2-g tension were contracted three times with 60 mM \( \text{KCl} \) added to the Krebs solution before use in any experiments.

**Cytosolic \( \text{Ca}^{2+}\) measurement.** Smooth muscle and endothelial cells were cultured from pig coronary artery and seeded onto coverslips as described earlier (13). Phenotypic characteristics of the cells used here have been reported previously. While still attached to the coverslips, the cells were loaded with fluo-3 AM and probedcinc and then used for \([\text{Ca}^{2+}\]\) measurement at 37°C as previously described (13).

**Data analysis.** Band intensities of ethidium bromide-stained gels were determined using Kodak 1D Image Analysis Software. The acylphosphates were quantified using a PhosphorImager. To compute values of \( K_i \) for the noncompetitive inhibition, the data were analyzed according to the following equation:

\[
\text{inhibition} = 100 \times \frac{(K_i\ +\ [\text{inhibitor}])}{(K_i\ +\ [\text{inhibitor}])}\nonumber
\]

by nonlinear regression. Curve fitting was carried out with the use of FigP software (Ancaster). Statistical significance was determined using Student’s \( t\)-test, and values of \( P < 0.05 \) were considered to be significant.

**RESULTS**

**Screening phage for binding to PED1 of PMCA4.** Two types of PMCA4-specific targets were used to screen a 12-amino acid linear random peptide phage display library. The target was the synthetic PED1 peptide PMCA4–115 for the first three cycles (see EXPERIMENTAL METHODS for details). PMCA purified from erythrocyte ghosts was the target for cycles 4 and 5. This was followed by cycle 6 using the synthetic target and cycle 7 using the purified PMCA. A consensus sequence was obtained at the end of cycle 7. We independently showed that this consensus sequence emerged as a result of selection rather than as an amplification artifact (data not shown). Next, we determined that the selected phage binds selectively to the synthetic peptide target PMCA4–115 in a panning experiment similar to a protocol used in screening (data not shown). The selected phage had the 12-amino acid sequence TAWSVEVLHLLLHR. In the phage, this sequence continues into the conserved GGG, followed by the remainder of the sequence of the gIII protein. The peptide TAWSVEVLHLLLRRGG-amide was synthesized and termed caloxin 1b1. Another peptide, termed RP1b1 (GAETLSHGLRLGSVW-amide), which had the same amino acid composition but a randomized sequence was synthesized as a control. Stock solutions of the peptides in 25% ethanol were added to attain the required concentrations. Typically, this resulted in 0.5% final ethanol in the reaction solutions. All experiments with caloxin 1b1 were conducted using this vehicle control and an RP1b1 control.

**Effects of caloxin 1b1 on erythrocyte PMCA \( \text{Ca}^{2+}-\text{Mg}^{2+}\)-ATPases.** Difference in ATPase activity in erythrocyte ghosts in saturating \( \text{Mg}^{2+}\) with and without \( \text{Ca}^{2+}\) was defined as the PMCA \( \text{Ca}^{2+}-\text{Mg}^{2+}\)-ATPase. The assay solution contained inhibitors of SERCA (thapsigargin), \( \text{Na}^{+}-\text{K}^{+}\)-ATPase (ouabain) and mitochondrial \( \text{Ca}^{2+}\)-ATPase (azide). Figure 1A shows that caloxin 1b1 inhibited the PMCA \( \text{Ca}^{2+}-\text{Mg}^{2+}\)-ATPase with a \( K_i \) of 46 ± 5 \( \mu \text{M} \) in leaky erythrocyte ghosts. For comparison, the effect of caloxin 2a1 is also shown.

**Afinity of caloxin 1b1 is 10 times higher than that of caloxin 2a1.** The results in Fig. 1A were based on a coupled enzyme assay that monitors the hydrolysis of ATP. In an initial experiment, it was determined that caloxin 1b1 did not influence the coupled enzyme system (data not shown). Thus any effects of caloxin 1b1 are not artifacts of the effects of this peptide on the assay system itself. The effect of caloxin 1b1 on PMCA \( \text{Ca}^{2+}-\text{Mg}^{2+}\)-ATPase was also determined using an assay involving the hydrolysis of \([\gamma-\text{P}]\text{ATP}\), and similar results were obtained (Fig. 3A). As anticipated, caloxin 1b1 also inhibited the \( \text{Ca}^{2+}-\text{Mg}^{2+}\)-ATPase in purified PMCA (Fig. 2).

**Isoform selectivity of caloxin 1b1.** Erythrocyte ghosts express mainly PMCA4 and HEK-293 cells express mainly PMCA1 (42). To determine whether caloxin 1b1 exhibits an isoform preference, we examined its effect on PMCA \( \text{Ca}^{2+}-\text{Mg}^{2+}\)-ATPase in HEK-293 plasma membrane enriched fraction compared with those in erythrocyte ghosts. The assays were conducted in the presence of 5 \( \mu \text{M} \) thapsigargin to inhibit any SERCA activity and 1 mM \( \text{Na}^{+}\)-azide to inhibit any mitochondrial \( \text{Ca}^{2+}\)-ATPase. In initial experiments, we determined that the HEK-293 preparation contained a high activity of basal \( \text{Mg}^{2+}\)-ATPase. The basal \( \text{Mg}^{2+}\)-ATPase activity decreased if the membrane was preincubated with 0.1% Triton X-100 for 30 min to permeabilize the vesicles. In a control experiment, the effect of caloxin 1b1 on PMCA \( \text{Ca}^{2+}-\text{Mg}^{2+}\)-ATPase activity in erythrocyte ghosts was not altered by the detergent (data not shown). Caloxin 1b1 inhibited the PMCA \( \text{Ca}^{2+}-\text{Mg}^{2+}\)-ATPase activity in HEK-293 cells with a higher inhibition constant (\( K_i \); 105 ± 11 \( \mu \text{M} \)) value than that in erythrocyte ghosts (46 ± 5 \( \mu \text{M} \)) (Fig. 1B; \( P < 0.05 \)). Thus caloxin 1b1 had a higher affinity for PMCA4 than for PMCA1.

We also tested the effect of caloxin 1b1 on \( \text{Ca}^{2+}-\text{Mg}^{2+}\)-ATPase activity in the plasma membrane-enriched fraction isolated from pig aortic smooth muscle (Fig. 2). The inhibition with 200 \( \mu \text{M} \) caloxin 1b1 in the aortic smooth muscle (82 ± 10%) was similar to that obtained with the ghosts (79 ± 8%).
Control experiments showing specificity of caloxin 1b1.

Figure 2 contains fluorescence tracings of coupled enzyme assays showing the effect of caloxin 1b1 on PMCA Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase in erythrocyte ghosts. Activity in the absence of any added inhibitor was taken as 100%, and the decrease in activity in the presence of the added inhibitors was used to compute %inhibition. \(K_i\), inhibitor constant. Each value is mean ± SE from 3–4 measurements. The effect of caloxin 2a1 is shown for comparison. B: effect of caloxin 1b1 on Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase in human embryonic kidney (HEK)-293 microsomes and erythrocyte ghosts. The assay solutions for the HEK-293 microsomes contained 5 \(\mu\)M thapsigargin to inhibit any sarcoendoplasmic reticulum Ca\(^{2+}\) pump (SERCA) activity and 1 mM Na\(^+\) azide to inhibit any mitochondrial Ca\(^{2+}\)-ATPase.

**Fig. 1.** Concentration dependence of the effects of caloxin 1b1. A: effects of caloxin 1b1 and RP1b1 on Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase using a coupled enzyme ATPase assay in erythrocyte ghosts. Difference between the activity in the presence and absence of Ca\(^{2+}\) was taken as Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase. Activity in the absence of any added inhibitor was taken as 100%, and the decrease in activity in the presence of the added inhibitors was used to compute %inhibition. \(K_i\), inhibitor constant. Each value is mean ± SE from 3–4 measurements. The effect of caloxin 2a1 is shown for comparison. B: effect of caloxin 1b1 on Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase in human embryonic kidney (HEK)-293 microsomes and erythrocyte ghosts. The assay solutions for the HEK-293 microsomes contained 5 \(\mu\)M thapsigargin to inhibit any sarcoendoplasmic reticulum Ca\(^{2+}\) pump (SERCA) activity and 1 mM Na\(^+\) azide to inhibit any mitochondrial Ca\(^{2+}\)-ATPase.

**Fig. 2.** Traces showing the decrease in fluorescence due to disappearance of NADH in a coupled ATPase assay. The scale \(\Delta F\) indicates change in fluorescence corresponding to 1 nM NADH for ghosts and sarcoplasmic reticulum (SR), 0.8 nM for purified PMCA and 2.4 nM aortic plasma membrane (PM). A slope of the change in fluorescence before the arrow corresponds to Mg\(^{2+}\)-ATPase activity. Rapid decrease in fluorescence is due to a dilution upon addition of Ca\(^{2+}\) at the time indicated by the arrow. Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase activity was determined as the difference between the slopes before and after the addition of Ca\(^{2+}\).
conducted at several caloxin 1b1 concentrations using the assay based on hydrolysis of [γ-33P]ATP, and again inhibition was not observed (Fig. 3C). Caloxin 1b1 did not inhibit the Na+-K+-activated ATPase in a coupled enzyme assay (Fig. 3D) or the K+-Mg+-activated p-nitrophenyl phosphatase (measured as a partial reaction of the Na+-K+-ATPase; Fig. 3E). Caloxin 1b1 (0–200 μM) did not inhibit Mg2+-ATPase activity in erythrocyte ghosts (Fig. 3G) or HEK-293 cells (Fig. 3H) or the Mg2+-dependent hydrolysis of p-nitrophenylphosphate (Fig. 3F). These assays established that the inhibition by caloxin 1b1 was specific for the PMCA Ca2+-Mg2+-ATPase and that it did not inhibit other ATPases.

**Acylphosphate intermediate formation.** Acylphosphate intermediate formation from [γ-33P]ATP in erythrocyte ghosts gave only one major band at 140 kDa (data not shown) in the presence of Ca2+. The band was not observed in the presence of excess of the Ca2+ chelator. Preincubation of the ghosts with RP1b1 did not affect the intensity of the acylphosphate band. The effects of RP1b1 and caloxin 1b1 were compared in 14 gels. Incubation of the ghosts with caloxin 1b1 before the acylphosphate formation reaction increased the intensity of the band to 148 ± 7% (P < 0.05).

**PMCA isoform expression in arterial smooth muscle and endothelium.** To determine the PMCA isoform expression in arterial smooth muscle and endothelium, we tested RNA from pig aortic smooth muscle, freshly isolated pig aortic endothelial cells, and cultured coronary artery endothelial cells. Figure 4 shows the results for RT-PCR using PMCA1 and PMCA4 primers. Expected molecular weights of PMCA4a, PMCA4b, and PMCA1b bands are 902, 727, and 429 bp, respectively. RT-PCR with PMCA4-specific primers gave two bands with weights, these corresponded to PMCA4a (902 bp) and PMCA1b bands are 902, 727, and 429 bp, respectively.

**Fig. 3. Specificity of caloxin 1b1.** A: effect of caloxin 1b1 on Ca2+-Mg2+-[γ-33P]ATPase in ghosts. PMCA Ca2+-Mg2+-ATPase activity was measured as hydrolysis of [γ-33P]ATP in erythrocyte ghosts. Ca2+-Mg2+-ATPase activity was determined as the difference in the activity in the presence and absence of Ca2+. On each day, the value without caloxin 1b1 was taken as 100% and values of inhibition of 4 replicates at each concentration of caloxin 1b1 were computed. %Inhibition values from several days were pooled. Values of inhibition in the graph are means ± SE of 8–12 replicates. The data fit best with a Kᵢ value of 48 ± 4 μM. B: effect of RP1b1 on Ca2+-Mg2+-[γ-33P]ATPase in ghosts. The values are means ± SE of 4 replicates. C: effect of caloxin 1b1 on SERCA Ca2+-Mg2+-ATPase in ghosts. The values are means ± SE of 4 replicates. D: effect of caloxin 1b1 on Na+-K+-ATPase in ghosts in a coupled enzyme ATPase assay based on disappearance of NADH fluorescence. The values are means ± SE of 2 replicates at each concentration compared with 5 replicates without caloxin 1b1. E: effect of caloxin 1b1 on K+-Mg2+-phosphatase activity measured using hydrolysis of p-nitrophenolphosphate to determine the Kᵢ-dependent phosphatase in erythrocyte ghosts. The difference in change in absorbancy with and without KCl gave the Kᵢ-dependent increase. The values are means ± SE of 4 replicates. F: effect of caloxin 1b1 on hydrolysis of p-nitrophenolphosphate to determine the Mg2+-dependent phosphatase in erythrocyte ghosts. The difference in the change in absorbancy with and without MgCl2 gave the Mg2+-dependent increase. The values are means ± SE of 4 replicates. G: effect of caloxin 1b1 on Mg2+-ATPase in erythrocyte ghosts in a coupled enzyme assay. ATPase activity was measured as disappearance of NADH fluorescence. The values are means ± SE of 2–4 replicates at each concentration and 4 replicates without caloxin 1b1. H: effect of caloxin 1b1 on Mg2+-ATPase in HEK-293 cell membranes in a coupled enzyme assay. ATPase activity was measured as disappearance of NADH fluorescence. The values are means ± SE of 2 replicates at each concentration and 4 replicates without caloxin 1b1.
PMCA4b (727 bp). With PMCA1 specific primers, only one band corresponding to PMCA1b (429 bp) was observed.

It was also established in initial experiments that the optimum conditions were identical for PCR using PMCA1- and PMCA4-selective primers. Co-PCR with primers for both PMCA1 and PMCA4 was carried out with the same set of primers using transcripts from endothelium and smooth muscle. A single band corresponding to PMCA1 was obtained using RNA from endothelial cells freshly isolated from pig aorta and from endothelial cells cultured from pig coronary artery (Fig. 4). With the use of the same set of primers, co-PCR with aortic smooth muscle cDNA gave a different band pattern. The observed bands corresponded to PMCA4a, PMCA4b, and PMCA1b (Fig. 4). To determine relative levels of expression, PCR was carried out using different dilutions of the reverse transcripts (Fig. 4). With the use of the diluted cDNA, the band for PMCA4b was more intense than those for PMCA4a and PMCA1. Thus the PMCA4 gene products (PMCA4a + PMCA4b) were significantly more than those for PMCA1. HEK-293 cells used as a control also expressed mainly PMCA1b. For PMCA2 and 3, RT-PCR gave appropriate products with the pig brain that was used as a positive control, but neither smooth muscle nor endothelium gave any products. Thus arterial endothelium expressed mainly PMCA1, and in smooth muscle the expression of PMCA4 gene products was more than those of PMCA1.

Effects of caloxin 1b1 on arterial contractility. Increasing [Ca$^{2+}$]i in smooth muscle and endothelium has contrasting effects on blood vessel contractility-contraction in the former and relaxation in the latter. Because endothelium expresses PMCA1 (Fig. 4) and smooth muscle expresses PMCA4 plus some PMCA1, we next tested the effects of caloxin 1b1 on contractility of rat aorta. We first tested whether caloxin 1b1 would increase the force of contraction of the partially precontracted rat thoracic aorta without the endothelium. Figure 5A shows contraction of a deendothelialized aortic ring at different concentrations of phenylephrine from 0.01 to 1 μM. Only a partial contraction was observed at 0.1 μM phenylephrine. The addition of caloxin 1b1 (200 μM) after 0.1 μM phenylephrine increased the force of contraction (Fig. 5B). The increase in the force of contraction with caloxin 1b1 was observed in all the aortic rings tested. The addition of ethanol (vehicle control; Fig. 5C) or RP1b1 did not increase the force of contraction (not shown). Next, we determined whether caloxin 1b1 would potentiate the endothelium-dependent relaxation. In the rat aorta, carbachol produces an endothelium-dependent relaxation, which is blocked by NO synthase inhibitors (24). Figure 5D shows the relaxation of an artery at different concentrations of carbachol (0.01 to 3 μM) after a full contraction with 1 μM phenylephrine. Only a partial relaxation was observed with 0.3 μM carbachol. The addition of caloxin 1b1 (200 μM) at this point did not increase the relaxation, but instead increased the force of contraction (Fig. 5E). Ethanol (vehicle control; Fig. 5F) and RP1b1 (not shown) had no effect. Thus caloxin 1b1 increased the force of contraction in smooth muscle (mainly PMCA4 + some PMCA1) but did not potentiate endothelium-dependent relaxation (predominantly PMCA1).

Effects of caloxin 1b1 on cytosolic Ca$^{2+}$ in arterial smooth muscle and endothelium. Because caloxin 1b1 showed some isoform specificity, we compared its effects on [Ca$^{2+}$], in smooth muscle (mainly PMCA4 plus some PMCA1) and endothelial cells (predominantly PMCA1) (Fig. 6). Because caloxin 1b1 was designed to work extracellularly, we added it to the medium containing the cells. The addition of 50 and 200 μM caloxin 1b1 produced a slight increase in [Ca$^{2+}$], in both cell types. However, the effects were much greater on smooth muscle cells than on endothelial cells (Fig. 6, A–C). Although other interpretations are also possible, these results are consistent with a greater affinity of caloxin 1b1 for PMCA4 than for PMCA1. In the next experiment, we tested whether caloxin 1b1 could potentiate the effects of a submaximum concentration (100 nM) of the nonfluorescent Ca$^{2+}$ ionophore 4-bromo-A23187. Ca$^{2+}$ ionophore directly mediates Ca$^{2+}$ movement and was therefore used to increase [Ca$^{2+}$]. This would simplify interpretation of the results. The ionophore by itself caused a transient increase in [Ca$^{2+}$]. The addition of 200 μM caloxin 1b1 after the ionophore produced a greater increase in [Ca$^{2+}$], in smooth muscle cells than in endothelial cells (Fig. 6, D and F). The increase in [Ca$^{2+}$], was not observed with 200 μM of the randomized peptide RP1b1 (Fig. 6E) or with ethanol (vehicle control, not shown).
Fig. 5. Effects of caloxin 1b1 on contractility of rat thoracic aortic rings. A–C: endothelium removed. A: dose-response curve for phenylephrine. Phenylephrine additions are shown by arrows at increasing concentrations (0.01, 0.03, 0.1, 0.3, and 1 μM). B: caloxin 1b1 (200 μM), when added after a submaximum concentration of 0.1 μM phenylephrine, increased the force of contraction. C: ethanol when added after a submaximum concentration (0.1 μM) phenylephrine had no effect. RP1b1 also had no effect (not shown). D–F: endothelium intact. D: arteries that were contracted with 1 μM phenylephrine, relaxed in response to increasing concentrations of carbachol (0.01, 0.03, 0.1, 0.3, 1 and 3 μM). E: caloxin 1b1 (200 μM) was added to an artery ring contracted with 1 μM phenylephrine and partially relaxed with 0.3 μM carbachol. It increased the force of contraction. F: ethanol was added to an artery ring contracted with 1 μM phenylephrine (arrow) and partially relaxed with 0.3 μM carbachol (●). It had no effect. RP1b1 also had no effect (not shown). Each of the experiment was replicated on 3 separate days.

Fig. 6. Effects of caloxin 1b1 on [Ca^{2+}]_i in cells cultured from pig SMC and EC. Caloxin 1b1 alone was added to smooth muscle cells (A) and endothelial cells (B). C: comparison of the caloxin 1b1-stimulated increase in [Ca^{2+}]_i in smooth muscle and endothelial cells based on means ± SE of 4 replicates. The effect on smooth muscle cells was significantly greater than on endothelium at each concentration of caloxin 1b1 (P < 0.05). The smooth muscle cells were first stimulated with a submaximum concentration (100 nM) of the nonfluorescent Ca^{2+} ionophore 4-bromo-A23187 and then challenged with caloxin 1b1 (D) or the negative control peptide RP1B1 (E). The endothelial cells challenged with caloxin 1b1 (F).
DISCUSSION

Caloxin 1b1 was designed to bind an extracellular epitope, and it produced its effects when added extracellularly. The results show that caloxin 1b1 produced a greater increase of [Ca\(^{2+}\)] in arterial smooth muscle cells than in endothelial cells. It produced a smooth muscle-dependent contraction and did not potentiate endothelium-dependent relaxation. This is consistent with the K_i value of caloxin 1b1 being lower for PMCA4 than for PMCA1. Caloxin 1b1 is specific in that it inhibited PMCA Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase but not other ATPases. The DISCUSSION focuses on the selectivity of caloxin 1b1, its PMCA isoform preference, and its potential in understanding the role of PMCA pumps in arterial smooth muscle, endothelium, and other tissues.

We determined that fresh or cultured arterial endothelial cells expressed predominantly PMCA1b and aortic smooth muscle expressed higher levels of PMCA4 (a+b) than of PMCA1. Because splicing does not exclude the extracellular domain 1 for which caloxin 1b1 was selected, it is expected to work equally well on PMCA1a or 1b and 4a or 4b. The K_i value of caloxin 1b1 was lower for erythrocytes (PMCA4) than for HEK-293 cells (PMCA1). The results obtained with HEK-293 cells would also apply to endothelial cells because both express predominately PMCA1 (Fig. 4). The assays using the aortic smooth muscle plasma membrane-enriched fraction and 200 μM caloxin 1b1 gave an inhibition value of 82 ± 10%, which was consistent with the greater expression of PMCA4 than PMCA1. Caloxin 1b1 was more effective in increasing [Ca\(^{2+}\)] in arterial smooth muscle than in endothelium. It increased the force of contraction in smooth muscle and did not potentiate the relaxation due to endothelium. The three experiments are consistent with caloxin 1b1 being more effective on PMCA4 than on PMCA1. Recently, the contribution of PMCA to contraction and relaxation of bladder smooth muscle was examined in wild-type mice and PMCA4-ablated mice (26). PMCA4 did not lead to major differences in the contraction time of the arteries to 80 mM KCl but differences in the relaxation times were larger. The contribution of PMCA to relaxation was calculated to be 25–30%, and the remainder was attributed to SERCA pump and NCX. It would be of interest to compare these results with the results of those studies using caloxin 1b1, because the transgenic mice would have time to adapt and the effect of caloxin 1b1 would be acute.

Human bone marrow-derived mesenchymal stem cells show spontaneous [Ca\(^{2+}\)] oscillations (21). It was proposed that both Ca\(^{2+}\) influx via PMCA and NCX, and Ca\(^{2+}\) influx through store-operated Ca\(^{2+}\) channels and probably nonselective cation channel operate in concert to maintain [Ca\(^{2+}\)] oscillations. Application of 2 mM caloxin 2a1 gave a single oscillation with a large amplitude, followed by a complete block of the [Ca\(^{2+}\)] oscillation. This response differed from that obtained with eosin, which gave a large sustained increase in [Ca\(^{2+}\)]. Na\(^+\) removal also gave a large sustained increase in [Ca\(^{2+}\)]. Although a model to explain these results was not presented, it is clear that caloxin 2a1 (a selective PMCA inhibitor) gave different results than eosin (inhibitor of PMCA and Na\(^+\)-K\(^+\)-ATPase) (6, 27, 39, 40). It is anticipated that the higher-affinity and isoform-selective caloxins would allow better understanding of the interactions between different Ca\(^{2+}\) transport mechanisms.

Studies (6, 17, 30, 31) using caloxins have shown that the levels of the acylphosphate intermediate obtained depend on the extracellular domain chosen as target. Caloxin 2a1 (extracellular domain 2) inhibited the acylphosphate formation, caloxin 3a1 (extracellular domain 3) had no effect, whereas caloxins 1a1 and 1b1 (extracellular domain 1) increased it. Implications of this increase in acylphosphate to the PMCA reaction cycle remains to be explored.

Caloxin 1b1 is specific in that it inhibits PMCA but not any other P-type ATPases. A BLAST search of protein sequences in the Swiss-Prot Expert Protein Analysis System shows that the extracellular domains of PMCA do not have significant sequence identities with any other P-type ATPases. Therefore, this selectivity was anticipated. Furthermore, the target sequence used does not have significant identities with any sequences other than those of PMCA proteins. Thus we anticipate that the specificity of caloxin 1b1 would be very high, even when non-ATPases are considered. However, such broad specificity remains to be tested. Transient overexpression experiments with PMCAs led to only small increases in Ca\(^{2+}\). Mg\(^{2+}\)-ATPase activity (18). Tissue-specific transgenes show only less than double functional PMCA over that of wild-type animals (9, 37). Perhaps PMCA cannot be overexpressed to very high levels in mammalian cells because they are central to cell function. Caloxin 1b1 with a K_i of 46 ± 5 μM for erythrocyte ghost Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase and no effect on other P-type ATPases is a much better alternative to the currently used reagents for studies on the physiological role of PMCA in arterial smooth muscle, endothelium, and other tissues. We have shown herein that it can be used with ease in contractility experiments, in those monitoring [Ca\(^{2+}\)], and in various signal transduction studies.

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GRANTS

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DISCLOSURES

Observations in this study form the basis of a patent application in progress.

REFERENCES


