Phospholamban-Dependent Effects of $C_{12}E_8$ on Calcium Transport and Molecular Dynamics in Cardiac Sarcoplasmic Reticulum†

Yongli Shi,‡ Brad S. Karon,‡ Howard Kutchai,§ and David D. Thomas*,‡

Department of Biochemistry, University of Minnesota Medical School, Minneapolis, Minnesota 55455, and Department of Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, Virginia 22908

Received June 13, 1996⊗

ABSTRACT: We have studied the effects of the nonionic detergent $C_{12}E_8$ on Ca-ATPase enzymatic activity and oligomeric state (detected by time-resolved phosphorescence anisotropy, TPA) in skeletal and cardiac sarcoplasmic reticulum (SR). In skeletal SR, $C_{12}E_8$ inhibits the Ca-ATPase, both at high (micromolar and above) and low (submicromolar) Ca. In cardiac SR, $C_{12}E_8$ inhibits at high Ca but activates at low Ca. Thus $C_{12}E_8$ activates enzymatic activity only in cardiac SR and only under conditions (submicromolar Ca) where phospholamban (PLB) regulates (inhibits) the enzyme [Lu, Y.-Z., & Kirchberger, M. A. (1994) *Biochemistry 33*, 5056−5062]. TPA of skeletal SR at low and high Ca demonstrates that $C_{12}E_8$ induces aggregation of ATPase monomers and small oligomers. $C_{12}E_8$ also aggregates the Ca-ATPase in cardiac SR at high Ca. In cardiac SR at low Ca, the Ca-ATPase is already highly aggregated, and $C_{12}E_8$ partially dissociates these aggregates. Thus the TPA results provide a simple physical explanation for the functional effects: $C_{12}E_8$ inhibits the ATPase when it aggregates the enzyme (skeletal SR at high and low Ca; cardiac SR at high Ca), and the detergent activates when it dissociates ATPase oligomers (cardiac SR at low Ca). $C_{12}E_8$ stabilizes the E2P conformation of the Ca-ATPase with respect to the E2 conformation, and this stabilization is PLB-dependent. Both the physical and the functional effects of $C_{12}E_8$ on the Ca-ATPase are PLB-dependent, with $C_{12}E_8$ reversing the effects of PLB. The results provide insight into the mechanism by which PLB regulates the Ca-ATPase in cardiac SR.

The Ca-ATPase of sarcoplasmic reticulum (SR)[1] pumps Ca from the sarcoplasm into the SR lumen to allow relaxation of both skeletal and cardiac muscle. Previous studies of the SR Ca-ATPase in skeletal muscle have established the importance of Ca-ATPase oligomeric state to enzymatic function (Squier et al., 1988a; Voss et al., 1991; Karon & Thomas, 1993). Agents that aggregate the Ca-ATPase in skeletal SR, such as chemical cross-linkers, the amphipathic peptide melittin, or the local anesthetic lidocaine, inhibit enzymatic activity (Squier et al., 1988a; Mahaney & Thomas, 1991; Voss et al., 1991; Kutchai et al., 1994). Agents that dissociate the Ca-ATPase, such as the volatile anesthetics ether and halothane, activate the enzyme (Bigelow & Thomas, 1987; Birmachu & Thomas, 1990; Karon & Thomas, 1993).

In cardiac SR, the integral membrane protein phospholamban (PLB) regulates the enzymatic activity of the Ca-ATPase (Lindemann et al., 1983). At submicromolar levels of Ca, PLB−ATPase interaction results in an inhibition of enzymatic activity (James et al., 1989; Sham et al., 1991). PLB−ATPase interaction (at submicromolar Ca) also results in decreased Ca-ATPase rotational mobility (compared to high Ca or PLB phosphorylation) (Voss et al., 1994, 1995; Karon et al., 1995). Upon PLB phosphorylation or addition of micromolar Ca, the resting inhibition of the Ca-ATPase is relieved (the enzyme is activated). This activation correlates with an increase in the rotational mobility of the Ca-ATPase, most likely because the physical interaction between PLB and the Ca-ATPase is disrupted, allowing large Ca-ATPase oligomers to dissociate (Voss et al., 1994, 1995; Karon et al., 1995).

The Ca-ATPase in both skeletal and cardiac SR cycles between two fundamental conformations, E1 and E2, which couple ATP hydrolysis to calcium transport via differences in their affinities and vectorial specificities for ATP and Ca (Scheme 1) (Inesi, 1985; Froehlich & Heller, 1985; Martonosi et al., 1990; Cantilina et al., 1993). In the presence of micromolar calcium, the conformational equilibrium shifts strongly toward E1 (Froud & Lee, 1986; Wakabayashi et al., 1990). ATP-dependent formation of the phosphoenzyme (E1P) occurs and is then followed by calcium translocation, which involves a conformational change of the enzyme from a state with high Ca affinity (E1P) to a state with greatly reduced Ca affinity (E2P) (Froehlich & Heller, 1985). Following Ca translocation, Ca is released from E2P (Beeler & Keffer, 1984), and E2P is hydrolyzed to E2 and P_i, completing the enzyme cycle (de Meis, 1988).

Previous investigations have established a relationship between Ca-ATPase oligomeric state and conformation in both skeletal and cardiac SR. Agents that inhibit the ATPase

† This work was supported by grants to D.D.T. from the National Institutes of Health (GM27906). B.S.K. was supported in part by an NIH Predoctoral Training Grant. H.K. was supported by NIH Grant GM50764.

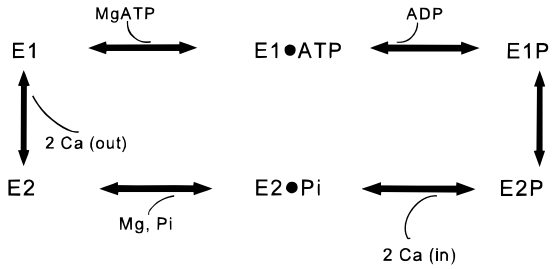
* To whom correspondence should be addressed.
‡ University of Minnesota Medical School.
§ University of Virginia.

⊗ Abstract published in *Advance ACS Abstracts*, October 1, 1996.

[1] Abbreviations: SR, sarcoplasmic reticulum; MOPS, 3-(*N*-morpholino)propanesulfonic acid; ATP, adenosine triphosphate; EGTA, ethyleneglycolbis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ERITC, erythrosin 5-isothiocyanate; FITC, fluorescein 5-isothiocyanate; P_i, inorganic phosphate; TPA, time-resolved phosphorescence anisotropy; EPR, electron paramagnetic resonance; $C_{12}E_8$, octaethyleneglycol mono-*n*-dodecyl ether; LSR, light skeletal sarcoplasmic reticulum; CSR, cardiac sarcoplasmic reticulum.

S0006-2960(96)01408-0 CCC: $12.00

Scheme 1: Widely Accepted Model for the Enzymatic Cycle of the Ca-ATPase[a]



[a] In the presence of micromolar extraluminal (cytoplasmic) Ca, the conformational equilibrium is shifted strongly toward E1, which binds ATP to form a phosphorylated intermediate (E1P). Ca translocation across the SR membrane involves a conformational change of the enzyme from a state with high Ca affinity (E1P) to a state with low Ca affinity (E2P). Ca is released into the SR lumen followed by hydrolysis of the bound phosphate (E2·P) and release of phosphate (forming the E2 conformation).

in skeletal SR by stabilizing the E2 state, such as thapsigargin and cyclopiazonic acid, promote the formation of dimers or small oligomers from monomers (Karon et al., 1994; Mersol et al., 1995). PLB, which oligomerizes the ATPase in cardiac SR, has its regulatory effect by interfering with the E2 to E1 transition (Cantilina et al., 1993). Thus oligomerization of the ATPase correlates with inhibition and with stabilization of the E2 conformation in both skeletal and cardiac SR. To more rigorously test this correlation, and to better understand mechanistic and regulatory differences between skeletal and cardiac SR, we have applied perturbants which have different functional effects in skeletal and cardiac SR.

The nonionic detergent $C_{12}E_8$, at non-solubilizing (0−50 μM) concentrations, has been shown to inhibit the Ca-ATPase in skeletal SR at low (submicromolar) and high (micromolar) calcium and to inhibit the Ca-ATPase in cardiac SR at high Ca. At low (submicromolar) Ca, conditions that allow PLB to interact with and inhibit the ATPase in cardiac SR, non-solubilizing levels of $C_{12}E_8$ activate the cardiac ATPase (Lu & Kirchberger, 1994). Because $C_{12}E_8$ activates only in cardiac SR and only under conditions that allow PLB interaction with the ATPase, it is possible that $C_{12}E_8$ may activate the Ca-ATPase in cardiac SR by dissociating PLB-induced ATPase oligomers. In the present study, to test this hypothesis, we have used time-resolved phosphorescence anisotropy (TPA), as well as functional and biochemical measurements, to determine the effects of $C_{12}E_8$ on ATPase oligomeric state, function, and biochemical conformation in skeletal and cardiac SR. The results provide further insight into the mechanism of PLB regulation in cardiac SR and regulation of the ATPase in skeletal and cardiac SR by oligomeric state.

MATERIALS AND METHODS

Reagents and Solutions. Erythrosin 5-isothiocyanate (ERITC) and fluorescein 5-isothiocyanate (FITC) were obtained from Molecular Probes, Inc. (Eugene, OR) and stored in DMF under liquid nitrogen. ATP, catalase, glucose, and glucose oxidase type IX were obtained from Sigma. Octaethyleneglycol mono-*n*-dodecyl ether ($C_{12}E_8$) was obtained from CalBiochem (La Jolla, CA). Enzyme-linked ATPase assays, phosphorescence anisotropy, and EPR experiments were carried out in a standard buffer containing 60 mM KCl, 50 mM MOPS, 2 mM $MgCl_2$, and either $CaCl_2$ alone or 5 mM EGTA + $CaCl_2$ to reach the desired concentration of free ionized Ca, pH 7.0. Unless otherwise indicated, [Ca] will be used to indicate the concentration of free ionized Ca, calculated as described previously (Voss et al., 1994).

Preparations and Assays. Light SR (LSR) vesicles were prepared from the fast-twitch skeletal muscle of New Zealand white rabbits as described previously (Karon et al., 1994). Cardiac SR vesicles (CSR) prepared from canine ventricular tissue were kindly provided by Dr. Joseph J. Feher (Feher & Briggs, 1983). LSR consisted of approximately 80% Ca-ATPase, or 7.3 nmol of Ca-ATPase/mg of SR protein. CSR consisted of approximately 30% Ca-ATPase, or 2.7 nmol of Ca-ATPase/mg of SR protein. SR lipids were extracted by a modification (Hidalgo et al., 1976) of the method of Folch et al. (1957), using nitrogen-saturated solvents to prevent oxidation. The lipids were stored in chloroform−methanol (2:1) under nitrogen at −20 °C.

Monoclonal anti-PLB Ab 2D12 was kindly provided by Dr. Larry Jones (Cantilina et al., 1993). Experiments in the presence of PLB Ab were performed by incubating Ab (1:2 Ab:Ca-ATPase by weight) at 25 °C for 20 min prior to the start of the ATPase assay, FITC fluorescence, or enzyme phosphorylation experiments.

SR Ca-ATPase activity was measured at 25 °C using an enzyme-linked, continuous ATPase assay, in the standard buffer with the addition of 5−50 μg of SR/mL, 0.42 mM phosphoenolpyruvate, 0.15 mM NADH, 7.5 IU of pyruvate kinase, and 18 IU of lactate dehydrogenase. Mg-ATP (1 mM) was added to start the assay, and the absorbance of NADH was monitored at 340 nm to determine the rate of ATP hydrolysis (Karon et al., 1994). All activity measurements were performed in the presence of 1 μg/mL of the ionophore, A23187, which was added to SR prior to the start of the assays. The ionophore allows the Ca-ATPase activity to be measured in the absence of a Ca gradient, so that any vesicle leakiness produced by the addition of $C_{12}E_8$ will not affect the activity measured (Bigelow & Thomas, 1987). The activity measured in the presence of ionophore gives a maximal Ca-ATPase activity, since the enzyme does not have to work against a concentration gradient. An aliquot of $C_{12}E_8$ stock solution, 5 mM $C_{12}E_8$ in water with 200 μM EDTA, was added to the cuvette 5 min before the start of each assay or experiment involving detergent.

Time-Resolved Phosphorescence Anisotropy. Phosphorescence anisotropy decays were obtained as described previously (Ludescher & Thomas, 1988). The time-dependent phosphorescence anisotropy decay $r(t)$ is given by

$$r(t) = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}} \quad (1)$$

where I_{vv} and I_{vh} are obtained by signal-averaging the time-dependent phosphorescence decays following 2000 laser pulses, with a single detector and a polarizer that alternates between the vertical (I_{vv}) and horizontal (I_{vh}) positions every 2000 pulses. The laser repetition rate was 200 Hz, so a typical $r(t)$ acquisition required 4 min to complete 10 loops, or cycles, of 4000 laser pulses each (2000 in each orientation). G is an instrumental correction factor, determined by measuring the anisotropy of free dye in solution under experimental conditions.

Optical Labeling and Sample Preparation. For phosphorescence experiments, the Ca-ATPase was specifically labeled with ERITC as described previously (Birmachu and Thomas, 1990). Oxygen was enzymatically removed from the samples with 200 μg of glucose oxidase/mL, 30 μg of catalase/mL, and 5 mg of glucose/mL, according to the method of Eads et al. (1984). Deoxygenation was carried out in a sealed cuvette containing 0.2−0.3 mg of SR protein/mL for 15−25 min prior to phosphorescence data collection. $C_{12}E_8$ stock solution was added to the SR in a manner analogous to the technique used for activity measurements. For fluorescence experiments, the Ca-ATPase in cardiac SR was specifically labeled with FITC as described previously (Voss et al., 1995). The fluorescence intensity of 0.5 μM FITC-CSR was measured with a SPEX Fluorolog II fluorimeter ($\lambda_{ex} = 495$ nm, $\lambda_{em} = 520$ nm) by summing the total fluorescence emission over 20 s. This was repeated four times/sample, and the average represents the intensity value for one experiment. The experiments were performed in the standard buffer (high calcium) or a buffer containing 60 mM KCl, 50 mM MOPS, 2 mM $MgCl_2$, and 1 mM EGTA, pH 7.0 (low calcium). Fluorescence changes upon addition of Ca, EGTA, and $C_{12}E_8$, corrected for the effects of sample dilution, were calculated as described previously (Karon et al., 1994).

Data Analysis. Phosphorescence anisotropy decays were analyzed as reported previously (Birmachu & Thomas, 1990), using a nonlinear least-squares fit to a sum of exponentials plus a constant:

$$r(t)/r(0) = \sum_{i=1}^{3} A_i e^{-t/\phi_i} + A_\infty \qquad (2)$$

where ϕ_i are rotational correlation times, A_i are normalized amplitudes (r_i/r_o), A_∞ is the normalized residual anisotropy (r_∞/r_o), and r_o is the initial anisotropy ($r(0) = r_0 = \sum r_i + r_\infty$). The goodness-of-fit for the anisotropy decays was evaluated by comparing χ^2 values for the multiexponential fits and by comparing plots of the residuals (the difference between the measured and the calculated decays). Phosphorescence lifetimes were determined by fitting the total intensity ($I_{vv} + 2I_{vh}$) to a sum of exponentials in a manner analogous to the anisotropy decay fitting (Birmachu & Thomas, 1990). $C_{12}E_8$, at the concentrations used in the experiments, did not have significant effects on phosphorescence lifetimes.

Spin Labeling and Sample Preparation. SR bilayer hydrocarbon chain rotational mobility was measured using a stearic acid derivative containing the spin label at the C-5 position (5-SASL), incorporated into protein-free aqueous dispersions of SR lipids. Incorporation of 5-SASL into SR lipid was accomplished by adding the spin label to extracted lipids in a chloroform−methanol (2:1 v/v) mixture prior to drying with nitrogen. The spin−labeled lipid was lyophilized overnight and then resuspended in the standard buffer to a concentration of 40−50 mM.

EPR Spectroscopy. EPR spectra were acquired using a Bruker ESP-300 spectrometer equipped with a Bruker ER4102 cavity and digitized with the spectrometer's built-in microcomputer using Bruker OS-9-compatible ESP 1620 spectral acquisition software. Spectra were downloaded to an IBM-compatible microcomputer and analyzed using software developed in our laboratory by R. L. H. Bennett. Conventional (V_1) EPR was used to detect submicrosecond motions of the lipid spin-labels. V_1 spectra were obtained using 100 kHz field modulation (with a peak to peak modulation amplitude of 2 gauss), with microwave field intensities (H_1) of 0.14 gauss. Sample temperature was controlled to within 0.5 °C with a Bruker ER 4111 variable temperature controller and monitored with a Sensortek Bat-21 digital thermometer using an IT-21 thermocouple probe inserted into the top of the sample capillary, so that it did not interfere with spectral acquisition.

EPR Spectral Analysis. Fatty acid spin-label spectra were analyzed by measuring the inner ($2T_\perp'$) and outer ($2T_\parallel'$) spectral splittings [see Mahaney and Thomas (1991)]. The effective order parameter (S) was calculated from the spectral splittings according to Gaffney (1976):

$$S = \frac{T_\parallel' - (T_\perp' + C)}{T_\parallel' + 2(T_\perp' + C)} \times 1.66 \qquad (3)$$

where $C = 1.4 - 0.053\ (T_\parallel' - T_\perp')$. SR lipid fluidity ($T/\eta$, inversely related to lipid viscosity) was calculated from the order parameter, such that $S = -0.42[\log(T/\eta)] + 0.56$, as described previously (Squier et al., 1988b).

Enzyme Phosphorylation from Inorganic Phosphate. Prior to phosphorylation, SR vesicles (0.5 mg/mL) were suspended in a buffer containing 10 mM $MgCl_2$, 2 mM EGTA, 100 mM MOPS, pH 6.5. $C_{12}E_8$ was incubated at room temperature with SR in the same buffer in a glass tube for 5 min, and an equal volume of 8 mM [^{32}P]Na_2HPO_4 in the same buffer was added to the samples, which were then vortexed to initiate the phosphorylation reaction. At the indicated time, the reaction was quenched by the addition of 3% perchloric acid + 2 mM H_3PO_4 (final concentrations). The quenched samples were pelleted in a tabletop centrifuge and then washed three times with a solution of 5% trichloroacetic acid, 4 mM H_3PO_4, 6 mM polyphosphate, and 5 mM cold ATP. The final pellets were dissolved in 2 mL of 1 N NaOH overnight and the ^{32}P-phosphoenzyme was assayed by counting the Cerenkov radiation.

PLB Phosphorylation in Cardiac SR. Phospholamban phosphorylation was performed just prior to TPA data collection as described previously (Voss et al., 1994). Samples were split in half to incubate with or without 40 μg/mL of the catalytic subunit of protein kinase A in a buffer containing 50 mM Tris-HCl, 0.1 mM DTT, 2 mM $MgCl_2$, 0.75 mM ATP, 50 nM phosphatase inhibitor calyculin A (LC Laboratories), pH 7.0, at 30 °C. After a 3-min incubation, the sample was immediately centrifuged at 100 000*g* for 5 min. The control and phosphorylated SR vesicles were then suspended in 30 mM MOPS, 250 mM sucrose, 50 nM calyculin A, pH 7.0, and kept on ice.

RESULTS

Effects of $C_{12}E_8$ on Ca-ATPase Activity. Lu and Kirchberger (1994) reported that nonsolubilizing concentrations of $C_{12}E_8$ (0−50 μM) activate Ca-ATPase activity in cardiac SR at low Ca. We also measured the effects of nonsolubilizing levels (0−50 μM) of $C_{12}E_8$ on Ca-ATPase in skeletal and cardiac SR, at low (150 nM) and high (100 μM) Ca. We found that $C_{12}E_8$ activates Ca-ATPase activity in cardiac SR at low Ca, conditions which allow PLB regulation of

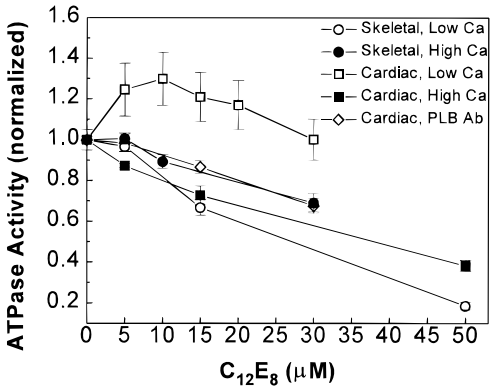


FIGURE 1: Normalized Ca-ATPase activity (normalized to the activity in the absence of $C_{12}E_8$ at each Ca concentration) at 25 °C, as described under Materials and Methods. Ca-ATPase activity (μmol of ATP hydrolyzed/mg of SR protein/min) in skeletal SR at 150 nM Ca (○) and 100 μM Ca (●); in cardiac SR at 150 nM Ca (□), 100 μM Ca (■), and 150 nM Ca + PLB Ab (◇) is shown. Data represent the average of three experiments ± the standard error of the mean.

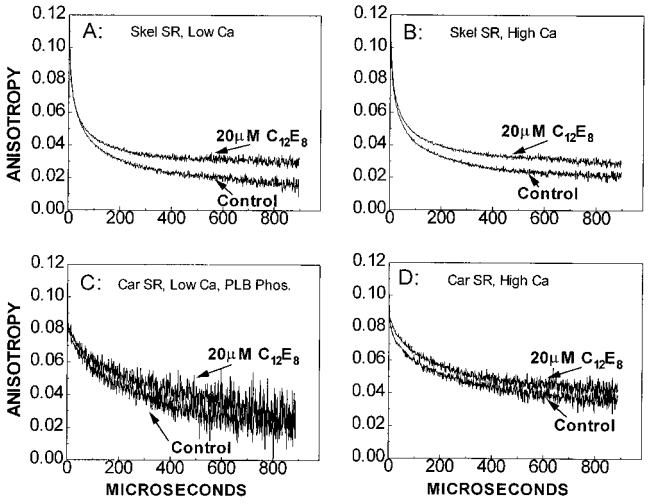


FIGURE 2: Time-resolved phosphorescence anisotropy decays of ERITC-labeled SR at 25 °C in standard buffer in the absence (Control) and the presence of 20 μM $C_{12}E_8$. Panel A: skeletal SR at 150 nM Ca. Panel B: skeletal SR at 100 μM Ca. Panel C: cardiac SR at 150 nM Ca with PLB phosphorylated. Panel D: cardiac SR at 100 μM Ca.

Ca-ATPase (Figure 1). The greatest activation (30%) occurred at 10 μM $C_{12}E_8$. Higher levels of $C_{12}E_8$ ($\geq$30 μM) inhibited the Ca-ATPase. Nonsolubilizing levels of $C_{12}E_8$ inhibited enzymatic activity in skeletal SR at both low and high Ca. In cardiac SR, $C_{12}E_8$ inhibited ATPase activity at low Ca in the presence of PLB Ab (which mimics PLB phosphorylation) and at high Ca (Figure 1). Similar concentrations of $C_{12}E_8$ also inhibited cardiac ATPase at low Ca after PLB was specifically phosphorylated (data not shown).

Effects of $C_{12}E_8$ on the Rotational Dynamics of the Ca-ATPase. Time-resolved phosphorescence anisotropy (TPA) experiments were performed to determine the effects of $C_{12}E_8$ on the oligomeric state of the Ca-ATPase in skeletal and cardiac SR. Addition of $C_{12}E_8$ to ERITC-LSR at 150 nM and 100 μM Ca resulted in a less rapid anisotropy decay, with an increase in the residual anisotropy (A_∞) (Figure 2A,B). Similar effects were observed in cardiac SR at 150 nM Ca after PLB was specifically phosphorylated and at 100 μM Ca (Figure 2C,D). In cardiac SR at 150 nM Ca (in the absence of PLB phosphorylation), addition of $C_{12}E_8$ resulted in a more rapid anisotropy decay, with a decrease in the residual anisotropy (A_∞) (Figure 3).

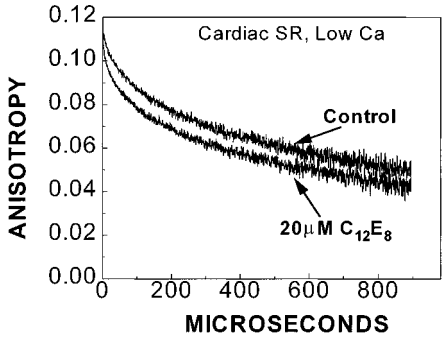


FIGURE 3: Time-resolved phosphorescence anisotropy decays of ERITC-labeled CSR at 25 °C in 150 nM Ca standard buffer in the absence (Control) and the presence of 20 μM $C_{12}E_8$.

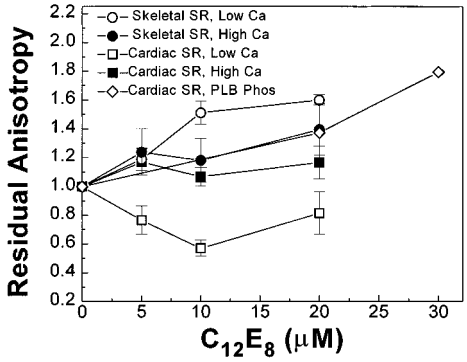


FIGURE 4: Effects of $C_{12}E_8$ at 25 °C on the residual anisotropy (A_∞). A_∞, normalized to the control (no detergent) for each sample (± standard error of the mean, $n = 3$) from fitting TPA data is shown (using eq 2, described under Materials and Methods). Skeletal SR at 150 nM Ca (○) and 100 μM Ca (●); cardiac SR at 150 nM Ca (□) and 100 μM Ca (■); and phosphorylated cardiac SR at 150 nM Ca (◇).

The decays were best fit to a sum of three exponentials plus a constant residual anisotropy (eq 2, $n = 3$), as observed previously (Birmachu & Thomas, 1990; Karon & Thomas, 1993; Mersol et al., 1995). The three components are interpreted to represent the rotational mobility of Ca-ATPase monomers (A_1), small oligomers (A_2), and larger aggregates (A_3). The fraction of immobile Ca-ATPase aggregates, not rotating on the time scale of the TPA experiment (900 μs), can be determined from the value of A_∞ (eq 2) (Birmachu & Thomas, 1990; Karon & Thomas, 1993; Mersol et al., 1995).

$C_{12}E_8$ had little effect on the rotational correlation times (ϕ_i, eq 2) and amplitudes associated with small ATPase oligomers (A_1, A_2, eq 2) observed in skeletal and cardiac SR, which were consistent with those reported previously (Birmachu & Thomas, 1990; Karon et al., 1993, 1995). $C_{12}E_8$ addition to skeletal SR at low and high Ca did increase the value of A_∞. In cardiac SR at low Ca after PLB was phosphorylated, and at high Ca, $C_{12}E_8$ also increased the value of A_∞ (Figure 4). This represents an increase in the fraction of Ca-ATPase molecules present as immobile oligomers (Mersol et al., 1995; and see Discussion). However, in cardiac SR at low Ca, $C_{12}E_8$ decreased the value of A_∞ (Figure 4), indicating a decrease in the fraction of immobilized ATPase (Mersol et al., 1995; and see Discussion). Values of A_3 (large, but not immobile, oligomers) changed slightly in the opposite direction of A_∞ upon the addition of $C_{12}E_8$ in all samples, suggesting that $C_{12}E_8$

Table 1: E2 Stability (in %) by FITC-fluorescence in Skeletal and Cardiac SR[a]

	skeletal SR	cardiac SR	cardiac SR + PLB Ab
EGTA buffer			
+10 μM $C_{12}E_8$	-2 ± 1	0 ± 1	-3 ± 1
+1 mM Ca	-7 ± 1	-7 ± 1	-11 ± 1
Ca buffer			
+10 μM $C_{12}E_8$	0 ± 1	0 ± 1	
+0.5 mM EGTA	$+5 \pm 1$	$+5 \pm 1$	

[a] FITC-SR fluorescence changes in standard buffer containing either 1 mM EGTA or 100 μM Ca at 25 °C, in the absence or presence of PLB Ab (for cardiac SR in EGTA), are shown upon the addition of either 10 μM $C_{12}E_8$, 1 mM Ca (added to EGTA buffer to change [Ca] from 0 to 20 μM), or 0.5 mM EGTA (added to Ca buffer to change [Ca] from 100 μM to 100 nM). Fluorescence changes were calculated as described previously (Karon et al., 1994). Data represent the average of three experiments $\pm$ the standard deviation.

induced the formation of immobile oligomers from larger aggregates (or dissociated immobile oligomers into larger aggregates in the case of cardiac SR at low Ca).

Effects of $C_{12}E_8$ on SR Lipid Fluidity. We used EPR spectroscopy to determine the effects of $C_{12}E_8$ on SR bilayer fluidity (T/η) using protein-free, aqueous dispersions of extracted SR lipids. The lipid dispersions were spin-labeled with a stearic acid derivative labeled at the 5-carbon position, which is sensitive to bilayer fluidity near the headgroup of the lipid (Mahaney & Thomas, 1991). When the ratio of $C_{12}E_8$:lipid was 1:1, $C_{12}E_8$ increased SR bilayer fluidity by 44%, from 0.69 to 0.99. This ratio of $C_{12}E_8$:lipid is similar to the ratio used in TPA and ATPase activity experiments, indicating that $C_{12}E_8$ fluidized the SR membrane under the conditions of TPA and ATPase activity experiments.

Effects of $C_{12}E_8$ on the Fluorescence of FITC-LSR and FITC-CSR. The fluorescence intensity of the bound form of FITC, a fluorescent probe similar in structure to ERITC, is sensitive to conformational changes of the Ca-ATPase between E1 and E2. Addition of EGTA to FITC-SR in the presence of Ca increases the fluorescence intensity (indicating a change in the conformation from E1 to E2), while addition of Ca to FITC-SR in the presence of EGTA decreases the fluorescence intensity (indicating a change in the conformation from E2 to E1) (Froud & Lee, 1986; Sagara et al., 1992).

Addition of 0.5 mM EGTA to FITC-LSR in the presence of 100 μM Ca (change in free [Ca] from 100 μM to 100 nM) increased FITC fluorescence by 5%. Addition of 1 mM Ca to FITC-SR in the presence of 1 mM EGTA (change in free [Ca] from 0 to 20 μM) decreased FITC fluorescence by 7% (Table 1). $C_{12}E_8$ (10 μM) did not affect the fluorescence of FITC-LSR or FITC-CSR in the presence of 100 μM Ca (>99% E1 conformation; Alonso & Hecht, 1990), indicating that $C_{12}E_8$ did not affect the E1 to E2 transition (Table 1). Addition of 10 μM $C_{12}E_8$ in the presence of EGTA decreased FITC-LSR fluorescence intensity by 2%, indicating destabilization of the E2 conformation in skeletal SR (shift in conformational equilibrium toward E1), as observed previously (Andersen et al., 1983). However, $C_{12}E_8$ had no effect on the fluorescence of FITC-CSR (cardiac SR) in the presence of EGTA. Upon addition of PLB Ab to FITC-CSR (in EGTA), $C_{12}E_8$ did induce a 3% fluorescence decrease (indicating destabilization of the E2 conformation). Addition of Ca to FITC-CSR in the presence of PLB Ab (in EGTA) resulted in an 11% decrease in fluorescence intensity, larger than the fluorescence changes observed for cardiac SR in the absence of PLB Ab (Table 1).

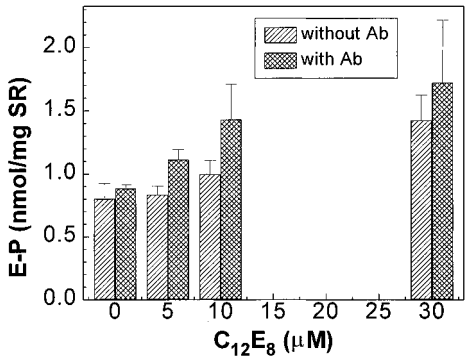


FIGURE 5: Steady state Ca-ATPase phosphorylation by inorganic phosphate at very low Ca (2 mM EGTA, no added Ca) and 25 °C in cardiac SR, as described under Materials and Methods. 0, 5, 10, and 30 μM $C_{12}E_8$ was added to cardiac SR in the presence and absence of PLB Ab. Data represent the average of two to three experiments $\pm$ the standard deviation.

Effects of $C_{12}E_8$ on ATPase Phosphorylation from Inorganic Phosphate. We measured the effects of $C_{12}E_8$ on the formation of E2P from inorganic phosphate in both skeletal and cardiac SR. In skeletal SR, it has been determined that levels of $C_{12}E_8$ that inhibit enzymatic activity stabilize E2P with respect to E2 (Champeil et al., 1986). Similarly, we found that in skeletal SR, $C_{12}E_8$ stabilized E2P with respect to E2 (10 μM $C_{12}E_8$ increased E-P levels at low Ca from 0.71 to 1.29 nmol/mg of SR). In cardiac SR, $C_{12}E_8$ also stabilized E2P with respect to E2, demonstrating that E2P stabilization correlates well with the inhibitory effects of $C_{12}E_8$ in both skeletal and cardiac SR. The presence of PLB Ab increased $C_{12}E_8$-induced stabilization of E2P (Figure 5).

DISCUSSION

Effects of $C_{12}E_8$ on Ca-ATPase Activity. We have found that $C_{12}E_8$ *inhibits* Ca-ATPase activity under most conditions in both skeletal and cardiac SR. The only exception is in cardiac SR at low Ca in the absence of PLB phosphorylation (Figure 1). This is the only condition under which PLB inhibits the Ca-ATPase; thus apparently $C_{12}E_8$ activates the Ca-ATPase by relieving the inhibition caused by unphosphorylated PLB. These results are consistent with a previous study (Lu & Kirchberger, 1994). One possible explanation for this effect is that $C_{12}E_8$ activates cardiac Ca-ATPase by dissociating PLB-induced Ca-ATPase oligomers, as proposed previously (Lu & Kirchberger, 1994). In the present study, we have tested this hypothesis by investigating the effects of $C_{12}E_8$ on the oligomeric state of the Ca-ATPase.

Effects of $C_{12}E_8$ on the Oligomeric State of the Ca-ATPase. Perturbants that increase the rotational mobility of the Ca-ATPase, by promoting dissociation of larger oligomers into smaller oligomers, consistently enhance its activity (Bigelow et al., 1986; Bigelow & Thomas, 1987; Squier & Thomas, 1988; Squier et al., 1988a; Birmachu & Thomas, 1990; Karon & Thomas, 1993; Voss et al., 1991, 1994). In contrast, perturbants that promote the formation of larger oligomers of the Ca-ATPase consistently decrease its activity (Voss et al., 1991, 1994; Mahaney et al., 1992). PLB regulation of Ca-ATPase activity appears to involve modulation of the oligomeric state of the enzyme (Voss et al., 1994, 1995).

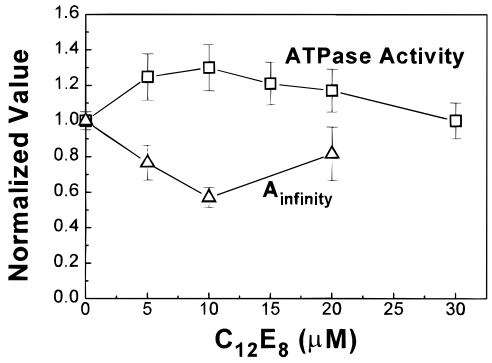


FIGURE 6: Normalized Ca-ATPase activity (□, from Figure 4) of cardiac SR at 150 nM Ca, showing that enzymatic activation by low levels of $C_{12}E_8$ correlates with a decrease in A_∞ while enzymatic inhibition by higher levels of $C_{12}E_8$ correlates with an increase in A_∞.

We have employed TPA measurements of ERITC-labeled SR to investigate the effects of $C_{12}E_8$ on the oligomeric state of Ca-ATPase in the SR membrane. We found that addition of $C_{12}E_8$ to skeletal SR at low and high Ca; to cardiac SR at low Ca when PLB is phosphorylated, and to cardiac SR at high Ca, results in a slower anisotropy decay and an increase in A_∞ (Figure 2). By contrast, addition of $C_{12}E_8$ to cardiac SR at low Ca results in a faster anisotropy decay and a decrease in A_∞ (Figure 3).

A change in A_∞ may be due to (a) a change in the fraction of immobile ATPase oligomers or to (b) a change in the angle between the membrane normal and the probe's absorption and emission dipoles (Mersol et al., 1995). By fitting TPA data to a more precise model of uniaxial rotational that considers both the fraction of immobile oligomers and probe angles; it has been shown that other perturbants (such as halothane, thapsigargin, and lidocaine) affect the rapid submicrosecond motion of the ERITC probe bound to skeletal and cardiac ATPase by changing the mole fraction of immobile Ca-ATPase oligomers, but do not affect the probe angles (Karon et al., 1995; Mersol et al., 1995). In the present study, when we fit TPA decays of ERITC-CSR in the absence and presence of $C_{12}E_8$ to the more precise uniaxial model (Mersol et al., 1995), we found that the detergent increased (or decreased at low Ca in the absence of PLB phosphorylation) the fraction of immobile ATPase oligomers, but did not affect the probe dipole angles.

$C_{12}E_8$-induced increases in A_∞ therefore represent ATPase oligomerization, which correlates with enzymatic inhibition. In cardiac SR at low Ca, the decrease in A_∞ induced by detergent represents dissociation of ATPase oligomers, which correlates with enzymatic activation. The effects of detergent at low Ca in cardiac SR are similar to the effects of PLB phosphorylation (i.e., enzyme activation with dissociation), so it is likely that $C_{12}E_8$ dissociates PLB-induced ATPase oligomers resulting in enzymatic activation. At higher levels, the ATPase in cardiac SR (at low Ca) is oligomerized by $C_{12}E_8$ (A_∞ increases, Figure 4). This correlates with inhibition by higher levels of detergent in cardiac SR at low Ca (see Figure 6). This may represent two separate effects of $C_{12}E_8$. At low levels in cardiac SR the detergent appears to dissociate ATPase from PLB (and activates), and at higher levels it may act directly on the ATPase to aggregate/inhibit.

$C_{12}E_8$ does fluidize the SR membrane, and this may contribute to activation of the Ca-ATPase in cardiac SR at low Ca. However, increased membrane fluidity is associated with *increased* ATPase activity and rotational mobility (dissociation of ATPase oligomers) (Bigelow & Thomas, 1987; Birmachu & Thomas, 1990). Thus membrane fluidity effects cannot explain detergent-induced inhibition and oligomerization of the ATPase, or activation of ATPase activity *only* in cardiac SR at low Ca. Therefore, dissociation of PLB-induced ATPase oligomers by $C_{12}E_8$ is likely the essential step in activation of the Ca-ATPase in cardiac SR at low Ca; while the detergent may act directly on the ATPase to aggregate/inhibit at high Ca and in skeletal SR.

Biochemical Effects of $C_{12}E_8$. It has previously been reported that $C_{12}E_8$ increased the rate of the E2 to E1 transition and stabilized E2P (Andersen et al., 1983; Champeil et al., 1986; Gould et al., 1986; de Foresta et al., 1989). We found that in skeletal SR, $C_{12}E_8$ increased FITC-LSR fluorescence at low but not high Ca (Table 1). This is consistent with $C_{12}E_8$ acting on the E2 conformation (destabilizing E2) to increase the rate of the E2 to E1 transition, while not affecting the E1 to E2 transition (i.e., not acting on E1). We also found that $C_{12}E_8$ stabilized the E2P conformation of the ATPase with respect to the E2 conformation, which has been shown previously to be the mechanism of inhibition (Champeil et al., 1986).

In cardiac SR, $C_{12}E_8$ does not affect the FITC fluorescence in the absence of PLB Ab. In the presence of Ab, there is an increase in FITC-CSR fluorescence (representing a destabilization of E2). The total change in FITC-CSR fluorescence when adding Ca to EGTA buffer (E2 to E1) is also greater in the presence of PLB Ab. $C_{12}E_8$ increased the amount of E2P formed from inorganic phosphate at low Ca (stabilized E2P with respect to E2), and this effect was also greater in the presence of PLB Ab. It has been shown that the main biochemical effect of PLB in cardiac SR is to interfere with the E2 to E1 transition (Cantilina et al., 1993). This may explain why the total fluorescence change of FITC-CSR is greater in the presence of PLB Ab, and why PLB partially prevents $C_{12}E_8$ stabilization of E1 and E2P with respect to E2. By stabilizing E2, PLB may prevent $C_{12}E_8$-induced stabilization of E2P in cardiac SR at low Ca. At higher levels, $C_{12}E_8$ does inhibit the ATPase in cardiac SR at low Ca. This correlates with oligomerization of the ATPase and E2P stabilization by higher levels of detergent. Thus the ATPase can be oligomerized and inhibited in both E2 (PLB) and E2P (detergent) conformations, and dissociating oligomers with low levels of detergent (as occurs in cardiac SR at low Ca) is associated with both activation of enzymatic activity and reduced formation of E2P (as compared to the PLB Ab sample).

Relationship between Oligomeric State, Biochemical Conformation, and Function of the Ca-ATPase. In both skeletal and cardiac SR, agents that stabilize E2-like conformations of the Ca-ATPase oligomerize the enzyme. PLB induces ATPase oligomerization in cardiac SR, and this is also associated with inhibition, presumably due to stabilization of the E2 conformation. $C_{12}E_8$ inhibits, aggregates, and stabilizes the E2P conformation in skeletal SR at low and high Ca, in cardiac SR at low Ca when PLB is phosphorylated, and in cardiac SR at high Ca. This is further evidence that oligomerization is involved in stabilization of E2-like states and thus regulation of the Ca-ATPase in SR. In cardiac SR at low Ca, $C_{12}E_8$ dissociates PLB−ATPase oligomers, activating the ATPase. PLB−ATPase interaction protects against $C_{12}E_8$-induced stabilization of the E1 and

E2P conformations, as evidenced by the increased FITC fluorescence changes and E2P stabilization after the addition of PLB Ab to cardiac SR at low Ca. *This provides the first direct evidence that PLB-induced oligomerization of the ATPase in cardiac SR is related to regulation of enzymatic function by means of E2 stabilization.*

Conclusion. $C_{12}E_8$ inhibits skeletal SR at low and high Ca, cardiac SR at low Ca when PLB is phosphorylated, and cardiac SR at high Ca. $C_{12}E_8$ activates cardiac SR at low Ca in the absence of PLB phosphorylation. $C_{12}E_8$ promotes the formation of larger Ca-ATPase oligomers in skeletal SR and cardiac SR under all conditions (described above) that inhibit enzymatic activity. However, the detergent dissociates ATPase oligomers in cardiac SR at low Ca; which correlates with activation of enzymatic activity. PLB regulation of ATPase activity also occurs only in cardiac SR at low Ca. Thus $C_{12}E_8$ likely dissociates PLB-induced ATPase oligomers resulting in enzymatic activation, providing further evidence that PLB regulation of cardiac SR involves modulation of ATPase oligomeric state. $C_{12}E_8$ stabilizes the E1 and E2P conformations with respect to E2, and stabilization of E2P correlates well with $C_{12}E_8$-induced inhibition and oligomerization of the Ca-ATPase. This demonstrates that oligomerization may play a role in the regulation of the ATPase in skeletal and cardiac SR by stabilizing E2-like conformations.

ACKNOWLEDGMENT

We thank Dr. Joseph J. Feher for the generous gift of cardiac SR and Dr. Larry Jones for the gift of PLB Ab. We also thank Razvan Cornea, Nicoleta Cornea, Robert Bennett, and John Matta for their help in preparation of the manuscript.

REFERENCES

Alonso, G. L., & Hecht, J. P. (1990) *J. Theor. Biol. 147*, 161−176.
Andersen, J. P., Maire, M. L., Ulrich, K.-H., Champeil, P., & Moller, J. V. (1983) *Eur. J. Biochem. 134*, 205−214.
Beeler, T., & Keffer, J. (1984) *Biochim. Biophys. Acta 773*, 99−105.
Bigelow, D. J., & Thomas, D. D. (1987) *J. Biol. Chem. 262*, 13449−13456.
Bigelow, D. J., Squier, T. C., & Thomas, D. D. (1986) *Biochemistry 25*, 194−202.
Birmachu, W., & Thomas, D. D. (1990) *Biochemistry 29*, 3904−3914.
Birmachu, W., Nisswandt, F. L., & Thomas, D. D. (1989) *Biochemistry 28*, 3940−3947.
Cantilina, T., Sagara, Y., Inesi, G., & Jones, L. R. (1993) *J. Biol. Chem. 268*, 17018−17025.
Champeil, P., Maire, M. L., Andersen, J. P., Guillain, F., Gingold, M., Lund, S., & Moller, J. V. (1986) *J. Biol. Chem. 261*, 16372−16384.
de Foresta, B., le Maire, M., Orlowski, S., Champeil, P., Lund, S., Moller, J. V., Michelangeli, F., & Lee, A. G. (1989) *Biochemistry 28*, 2558−2567.
de Meis, L. (1988) *Methods Enzymol. 157*, 190−206.
Eads, T. M., Thomas, D. D., & Austin, R. H. (1984) *J. Mol. Biol. 179*, 55−81.
Feher, J. J., & Briggs, F. N. (1983) *Biochim. Biophys. Acta 727*, 389−402.
Folch, J., Lees, M., & Sloane-Stanley, G. H. (1957) *J. Biol. Chem. 226*, 497−509.
Froehlich, J. P., & Heller, P. F. (1985) *Biochemistry 24*, 126−136.
Froud, R. J., & Lee, A. G. (1986) *Biochem. J. 237*, 197−206.
Gaffney, B. J. (1976) in *Spin Labeling Theory and Practice* (Berliner, L. G., Ed.) pp 567−571, Academic Press, New York.
Gould, G. W., East, J. M., Froud, R. J., Mowhirter, J. M., Stefanova, H. I., & Lee, A. G. (1986) *Biochem. J. 237*, 217−227.
Hidalgo, C., Ikemoto, N., & Gergely, J. (1976) *J. Biol. Chem. 251*, 4224−4232.
Inesi, G. (1985) *Annu. Rev. Physiol. 47*, 573−601.
James, P., Inui, M., Tada, M., Chiesi, M., & Carafoli, E. (1989) *Nature 342*, 90−92.
Karon, B. S., & Thomas, D. D. (1993) *Biochemistry 32*, 7503−7511.
Karon, B. S., Mahaney, J. E., & Thomas, D. D. (1994) *Biochemistry 33*, 13928−13937.
Karon, B. S., Geddis, L. M., Kutchai, H., & Thomas, D. D. (1995) *Biophys. J. 68*, 936−945.
Kutchai, H., Mahaney, J. E., Geddis, L. M., & Thomas, D. D. (1994) *Biochemistry 33*, 13208−13222.
Lindemann, J. P., Jones, L. R., Hathaway, D. R., & Henry, B. G. (1983) *J. Biol. Chem. 258*, 464−471.
Lu, Y.-Z., & Kirchberger, M. A. (1994) *Biochemistry 33*, 5056−5062.
Ludescher, R. D., & Thomas, D. D. (1988) *Biochemistry 27*, 3343−3351.
Mahaney, J. E., & Thomas, D. D. (1991) *Biochemistry 30*, 7171−7180.
Mahaney, J. E., Kleinschmidt, J., Marsh, D., & Thomas, D. D. (1992) *Biophys. J. 63*, 1513−1522.
Martonosi, A. N., Jona, I., Molnar, E., Seidler, N. W., Buchet, R., & Varga, S. (1990) *FEBS Lett. 268*, 365−370.
Mersol, J. V., Kutchai, H., Mahaney, J. E., & Thomas, D. D. (1995) *Biophys. J. 68*, 208−215.
Sagara, Y., Wade, J. B., & Inesi, G. (1992) *J. Biol. Chem. 267*, 1286−1292.
Sham, J. S. K., Jones, L. R., & Morad, M. (1991) *Am. J. Physiol. 261*, H1344−H1349.
Squier, T. C., & Thomas, D. D. (1988) *J. Biol. Chem. 263*, 9171−9177.
Squier, T. C., Hughes, S. E., & Thomas, D. D. (1988a) *J. Biol. Chem. 263*, 9162−9170.
Squier, T. C., Bigelow, D. J., & Thomas, D. D. (1988b) *J. Biol. Chem. 263*, 9178−9186.
Voss, J., Hussey, D., Birmachu, W., & Thomas, D. D. (1991) *Biochemistry 30*, 7498−7506.
Voss, J., Jones, L. R., & Thomas, D. D. (1994) *Biophys. J. 67*, 190−196.
Voss, J., Mahaney, J. E., Jones, L. R., & Thomas, D. D. (1995) *Biophys. J. 68*, 1787−1795.
Wakabayashi, S., Ogurusu, T., & Shigekawa, M. (1990) *Biochemistry 29*, 10613−10620.

BI9614085