Phosphorylation of Phospholamban by cAMP-Dependent Protein Kinase Enhances Interactions between Ca-ATPase Polypeptide Chains in Cardiac Sarcoplasmic Reticulum Membranes†

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ABSTRACT: We have used spin-label EPR spectroscopy to examine possible alterations in protein−protein interactions that accompany the activation of the cardiac sarcoplasmic reticulum (SR) Ca-ATPase following the phosphorylation of phospholamban (PLB). Using a radioactive derivative of a maleimide spin label (MSL), we have developed conditions for the selective spin-labeling of the Ca-ATPase in both native cardiac and skeletal sarcoplasmic reticulum membranes. The rotational dynamics of the cardiac and skeletal Ca-ATPase isoforms in native SR membranes were measured using saturation transfer EPR. We report that the phosphorylation of PLB in cardiac SR results in a (1.8 ± 0.2)-fold reduction in the overall rotational mobility of the Ca-ATPase. The alteration in the rotational dynamics of the Ca-ATPase is the direct result of the phosphorylation of PLB, and is not related to the phosphorylation of the Ca-ATPase or any other SR proteins since no alteration in the ST-EPR spectrum is observed as a result of conditions that phosphorylate the cardiac Ca-ATPase with ATP. Neither do the use of conditions that activate the Ca-ATPase in cardiac SR result in the alteration of the rotational dynamics or catalytic properties of the Ca-ATPase in skeletal SR where PLB is not expressed. Measurements of the rotational dynamics of stearic acid spin labels (SASL) incorporated into cardiac SR membranes with a nitroxide at the 5- and 12-positions using conventional EPR indicate that there is virtually no difference in the lipid acyl chain dynamics in cardiac SR membranes upon the phosphorylation of PLB. These results indicate that the decrease in the rotational dynamics of the Ca-ATPase in cardiac SR membranes associated with the phosphorylation of PLB is related to enhanced interactions between individual Ca-ATPase polypeptide chains due to (i) an alteration in the spatial arrangement of cardiac Ca-ATPase polypeptide chains within a defined oligomeric state or (ii) increased protein−protein associations. We suggest that altered interactions between Ca-ATPase polypeptide chains and PLB serves to modulate the activation barrier associated with calcium activation of the Ca-ATPase in cardiac SR membranes.

The β-adrenergic cascade in the heart acts to enhance the rate of calcium resequestration into the sarcoplasmic reticulum (SR)[1] lumen through the direct phosphorylation of phospholamban (PLB) at either Ser_{16} or Thr_{17} by cAMP-dependent protein kinase or calmodulin-dependent protein kinase, respectively (Kranias, 1985; Tada et al., 1988; Jones et al., 1988; Simmerman et al., 1986; Wegener et al., 1989). Phosphorylation of PLB results in a shift in the calcium-dependence of the catalytic activity of the Ca-ATPase, resulting in the activation of the Ca-ATPase at submicromolar free calcium concentrations with little or no alteration in the maximal velocity of the Ca-ATPase (Morris et al., 1991; Sasaki et al., 1992; Toyofuku et al., 1994a,b). The similarity between the calcium-dependence of Ca-ATPase transport activity found in activated cardiac SR (subsequent to the phosphorylation of PLB) and that of the Ca-ATPase found in skeletal SR membranes (in which PLB is not expressed) suggests that unphosphorylated PLB acts as an inhibitor of the Ca-ATPase (Kranias, 1985). Detailed kinetic measurements relating to the binding and activation of the Ca-ATPase by calcium suggest that the modulation of transport activity of the Ca-ATPase by PLB is not the result of an alteration in the calcium affinity, but rather of an alteration in the activation barrier associated with the slow isomerization step of calcium activation (Cantilina et al., 1993).

The addition of monoclonal antibodies raised against PLB to cardiac SR vesicles has been observed to stimulate Ca-ATPase activity under nonsaturating calcium concentrations in an identical manner to that associated with the direct activation of PLB through covalent phosphorylation (Kimura et al., 1991; Morris et al., 1991; Cantilina et al., 1993), suggesting that the phosphorylation of PLB decreases the interaction between PLB and the Ca-ATPase. Consistent with this hypothesis, the chemical cross-linking between the Ca-ATPase and PLB is decreased upon the phosphorylation of PLB in mixed micelles (James et al., 1989). The ability

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[1] Abbreviations: ATP, adenosine 5-triphosphate; cAMP, adenosine 3:5-cyclic monophosphate; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EPR, electron paramagnetic resonance; Er-ITC; erythrosin isothiocyanate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MSL, maleimide spin label (4-maleimido-2,2,6,6-tetramethyl-1-piperidinyloxy); NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; PLB, phospholamban; SDS, sodium dodecyl sulfate; SR, sarcoplasmic reticulum; *n*-SASL, stearic acid spin labels in which a nitroxide is covalently linked at the *n*-position relative to the carboxylate group; ST-EPR, saturation transfer EPR.

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of polyanions (e.g., heparin) to activate the Ca-ATPase suggests that electrostatic interactions are involved in stabilizing the interaction between PLB and the Ca-ATPase (Xu & Kirchberger, 1989).

The physical mechanism associated with the regulation of the Ca-ATPase transport activity in native membranes has previously been investigated through the measurement of changes in the rotational dynamics of the Ca-ATPase upon phosphorylation of PLB using time-resolved phosphorescence anisotropy measurements of erythrosin isothiocyanate (Er-ITC)-labeled cardiac SR membranes (Fowler et al., 1989; Voss et al., 1994). In both of the previous reports, observed alterations in the rotational dynamics of the Ca-ATPase upon the phosphorylation of PLB have been interpreted in terms of altered interactions between the Ca-ATPase and PLB. However, there is currently no consensus relating to the influence of PLB on the rotational dynamics of the Ca-ATPase. The phosphorylation of PLB has been reported to both decrease the segmental rotational dynamics associated with specific domains of the Ca-ATPase with little change in the overall rotational dynamics of the Ca-ATPase (Fowler et al., 1989) or alternatively to dramatically increase the overall rotational dynamics of the Ca-ATPase as a result of a large reduction in the degree of protein−protein interactions (Voss et al., 1994). The latter workers suggest that prior to the phosphorylation of PLB the Ca-ATPase forms dodecameric structures and that the phosphorylation of PLB acts to disrupt these nonfunctional oligomeric complexes (Voss et al., 1994). Evaluation of these contradictory results is complicated by the use of Er-ITC as a probe of protein conformation, since it is unclear if Er-ITC specifically labels the Ca-ATPase in these preparations and if the Er-ITC modified Ca-ATPase is representative of the native structure of the Ca-ATPase (Huang et al., 1995).

In order to further investigate the physical mechanisms associated with the modulation of the calcium-dependence of Ca-ATPase activity by PLB, we have developed conditions for the selective spin-labeling of nonessential cysteines on the Ca-ATPase in native cardiac sarcoplasmic reticulum membranes. We are therefore able to use ST-EPR spectroscopy to monitor possible alterations in the rotational dynamics of the Ca-ATPase associated with the phosphorylation of PLB by cAMP-dependent protein kinase in a fully functional preparation. Parallel ST-EPR measurements involving the selective spin-labeling of the Ca-ATPase in skeletal SR membranes allow us to compare the functional and structural properties of the two homologous Ca-ATPases. Complementary measurements of the lipid dynamics are made using stearic acid spin labels, which permit the interpretation of observed changes in the rotational dynamics of the Ca-ATPase in terms of alterations in protein−protein interactions.

EXPERIMENTAL PROCEDURES

Materials. Anti-PLB mouse monoclonal antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-SERCA1 and Anti-SERCA2 (catalog MA3-910) mouse monoclonal antibodies were purchased from Affinity Bioreagents, Inc. (Neshanic Station, NJ). Peroxidase-conjugated goat anti-mouse IgG was purchased from Pierce (Rockford, IL). Alkaline phosphatase was purchased from Worthington Biochemical Corporation (Freehold, NJ). Calyculin A was purchased from Calbiochem (La Jolla, CA). Dextran T-10 was purchased from Pharmacia (Uppsala, Sweden). SASL probes [i.e., 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy and 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxy] and MSL (i.e., 4-maleimido-2,2,6,6-tetramethyl-1-piperidinyloxy) were purchased from Aldrich (Milwaukee, WI). DTT and KCl were purchased from Research Organics Inc. (Cleveland, OH). Acrylamide, bisacrylamide, TEMED, and ammonium persulfate were purchased from Bio-Rad (Richmond, CA). γ-[^{32}P]ATP was purchased from ICN Biomedicals (Costa Mesa, CA). Sucrose, NaCl, and MOPS were purchased from Fisher Scientific (Pittsburgh, PA). $CaCl_2$ standard solutions were purchased from VWR (St. Louis, MO). cAMP, cAMP-dependent protein kinase, ^{14}C-labeled maleic anhydride, TEMPAMINE, NEM, NaN_3, A23187, EGTA, $MgCl_2$, X-OMAT Kodak X-ray film, and all other reagents were of the highest grade commercially available and were purchased from Sigma (St. Louis, MO).

Preparation of Spin-Labeled Hemoglobin. Human oxyhemoglobin was purified and spin-labeled for use in the determination of semiempirical calibration curves relating microsecond rotational motion to ST-EPR spectral parameters, essentially as previously reported (Thomas et al. 1976). Red blood cells were washed in 0.9% NaCl and lysed in ice-cold distilled water. The resulting red cell ghosts were centrifuged at 48 000g_{max}, leaving a supernatant containing purified hemoglobin. For all subsequent experiments, the hemoglobin was in a buffer containing 0.10 M sodium phosphate (adjusted to pH 7.0 at 4 °C). The hemoglobin concentration was determined from its visible absorbance spectrum (Benesch et al., 1965). In all cases, our samples contained less than 1% methemoglobin. All subsequent steps involving the spin-labeling and preparation of known standards in solutions with defined concentrations of glycerol have been discussed in detail previously (Squier & Thomas, 1986a).

Isolation of Sarcoplasmic Reticulum Membranes. Skeletal SR vesicles were prepared from rabbit skeletal fast twitch muscle essentially as previously described (Fernandez et al., 1980). Cardiac SR vesicles were prepared from porcine hearts using a modified procedure to that described previously for canine hearts (Chamberlain et al., 1983). Briefly, fresh hearts were obtained from the local slaughter house and immediately immersed in ice-cold 0.9% NaCl for transport to our laboratory. All subsequent steps were performed at 4 °C. Ventricles were trimmed of fat and connective tissue and sliced into approximately 1 cm^3 pieces. 32-g portions in 160 mL each of 0.3 M sucrose, 10 mM imidazole hydrochloride (pH 6.9 at 25 °C), 3 mM NaN_3, and freshly prepared 0.5 mM dithiothreitol were individually homogenized using a Silverson (East Longmeadow, MA) model L4R homogenizer with a vertical-slotted disintegrating head for 15 s at speed 5, followed by a 15 s rest, and then 45 s at speed 8. The resulting homogenates were centrifuged at 4800g_{max} for 15 min, and the supernatants were filtered through four layers of cheesecloth and then centrifuged at 27 900g_{max} for 15 min to remove cellular debris. The resulting supernatant was again filtered through cheesecloth and then centrifuged at 119 200g_{max} for 2 h to pellet membrane vesicles. The supernatant was removed by aspiration, and the pellets were resuspended in 50−60 mL of 0.65 M KCl, 0.3 M sucrose, 10 mM imidazole hydrochloride (pH 6.7), 3 mM NaN_3, and freshly prepared 0.5 mM dithiothreitol per heart. After 30 min of incubation on

ice, the solution was centrifuged at 4400g_{max}. The resulting supernatants were then centrifuged 100 min at 251 800g_{max}. The softer opaque pellets were removed by aspiration and resuspended in buffer A, and the final volume was adjusted to 0.1 mL times the initial tissue weight in grams. Approximately 6 mL of this microsomal suspension was then layered over a discontinuous sucrose gradient containing dextran T-10, and the gradients were centrifuged for approximately 8 h, essentially as previously described (Chamberlain et al., 1983). Peak calcium-dependent ATPase activity was exhibited by fractions having a refractive index of 1.368, and this fraction was collected and resuspended in 0.65 M KCl, 10 mM imidazole hydrochloride (pH 6.7), 3 mM NaN_3, and freshly prepared 0.5 mM dithiothreitol. The sample was then centrifuged at 194 200g_{max} for 100 min. The resulting pellets were resuspended in 0.3 M sucrose, 0.2 M KCl, 20 mM MOPS (pH 7.0) and were quick-frozen in liquid nitrogen and stored at −70 °C. Frozen SR preparations retained full ATPase and transport activities for at least 3 months.

Enzymatic Assays. The initial rate of release of inorganic phosphate was measured by the method of Lanzetta et al. (1979) in a solution containing 0.05 mg of protein/mL, 2 μM A23187, 100 mM KCl, 0.1 mM EGTA, 5 mM $MgCl_2$, 25 mM MOPS (pH 7.0), and sufficient calcium to provide the free calcium concentrations desired. The SR protein concentration was determined by the biuret method (Gornal et al., 1949). Free calcium concentrations were calculated using a modified version of a computer program previously described (Fabiato & Fabiato, 1979; Fabiato, 1988), which calculates the multiple equilibria between all ligands in solution. For the activation of the cardiac SR Ca^{2+}-ATPase activity by cAMP-dependent protein kinase, 1 μM cAMP and 60 μg of cAMP-dependent protein kinase/mL were also added to the reaction medium and incubated at 25 °C for 10 min. In all cases, the reaction was initiated by the addition of 5 mM ATP. Calcium-independent (basal) ATPase activity was measured in the presence of 0.1 mM EGTA in the absence of any added calcium.

Steady-state levels relating to the extent of phosphorylation to the Ca-ATPase were determined essentially as previously described (Barrabin et al., 1984). Briefly, SR vesicles were incubated at 0 °C for 20 s in the presence of 0.1 mM γ-[^{32}P]-ATP in a reaction medium containing 5 mM $MgCl_2$, 0.10 mM EGTA, 0.12 mM $CaCl_2$ ($[Ca^{2+}]_{free} \approx 20$ μM), 80 mM KCl, 20 mM MOPS (pH 7.0), and 0.5 mg of SR protein per mL of reaction medium. Reactions were quenched with 7% (v/v) trichloroacetic acid in 4 mM NaH_2PO_4. The resulting protein precipitants were washed three times with 3% (v/v) trichloroacetic acid in 2 mM NaH_2PO_4, and the final precipitant was solubilized in 0.1 N NaOH, 2% Na_2CO_3, 2% (w/v) SDS, and 5 mM NaH_2PO_4. Aliquots were taken from this solution for protein determination (Lowry et al., 1951) and liquid scintillation counting.

SDS−Polyacrylamide Gel Electrophoresis. The proteins in both cardiac and skeletal SR membranes were routinely separated according to size by SDS−PAGE as previously described by Laemmli (1970). Total protein was visualized subsequent to the staining of the gel with Coomassie Blue. The localization of both the Ca-ATPase and phospholamban (PLB) on immunoblots transferred from SDS−PAGE gels involved the use of either a monoclonal antibody directed against the Ca-ATPase or, alternatively, a mouse anti-PLB primary antibody obtained from Upstate Biotechnology (Lake Placid, NY). Visualization of the primary antibody involved the use of peroxidase-conjugated goat anti-mouse IgG purchased from Pierce (Rockford, IL) as the secondary antibody.

Synthesis of ^{14}C-Labeled Maleimide Spin Label. The MSL spin label (4-maleimido-2,2,6,6-tetramethyl-1-piperidinyloxy) used to covalently label the Ca-ATPase in both cardiac and skeletal SR preparations was synthesized essentially as previously described (Gaffney, 1976a). Briefly, this involved the one-step reaction in which 50 mg of ^{14}C-labeled maleic anhydride and 88 mg of TEMPAMINE are separately dissolved in diethyl ether, with the subsequent dropwise addition of the TEMPAMINE solution to the reaction vessel containing maleic anhydride. The solution was stirred for 1 h at 25 °C. The resulting product was collected, washed with diethyl ether, and evaporated to dryness. The yield was 94 mg of product, which after being mixed with 14.7 mg of anhydrous acetate and 1.47 mL of acetic anhydride was stirred for 3 days. The acetic anhydride was removed by vacuum distillation, and the resulting residue was dissolved in benzene. Undissolved solids were removed by filtration, and the benzene was removed by distillation resulting in an oil containing ^{14}C-labeled maleimide spin label (MSL). The resulting product was further purified using thin-layer chromatography and was positively identified using mass spectrometry (MW = 251).

Phosphorylation of PLB. Following previously described procedures for the stable phosphorylation of PLB (Voss et al., 1994), cardiac SR samples were phosphorylated for 3 min in the presence of 60 μg of cAMP-dependent protein kinase/mL, 1 μM cAMP, and 5 mM γ-[^{32}P]ATP (4500 cpm per nmol of γ-[^{32}P]ATP), 50 nM calyculin (a phosphatase inhibitor), 100 mM KCl, 100 μM EGTA, 5 mM $MgCl_2$, 103 μM $CaCl_2$, and 25 mM MOPS (pH 7.0). The calculated free calcium concentration was 0.5 μM $[Ca^{2+}]_{free}$. To stop the reaction, denaturing buffer giving a final concentration of 0.625 M Tris/HCl (pH 8.0), 2% SDS, 0.2 M sucrose, 5% β-mercaptoethanol, and 0.001% bromphenol blue was added and samples were applied to a 7.5% SDS−polyacryamide gel. The gel was dried and used to expose an X-OMAT Kodak X-ray film in the presence of an enhancing screen for 12 h. To test the reversibility of the phosphorylation of PLB, phosphorylated samples were incubated in the presence of 1.5 units of alkaline phosphatase per microgram of SR protein for 30 min at 25 °C, and the extent of phosphorylation was then analyzed using autoradiograms of the sample subsequent to the separation of the various SR proteins using SDS−PAGE. For EPR experiments, 3 mg/mL SR samples were incubated with the phosphorylation medium (or when applicable alkaline phosphatase was included) for 3 min as described above, spun for 5 min at 100 000g_{max} in a Beckman TL-100 ultracentrifuge, and the pellet was resuspended in buffer A containing 25 mM MOPS (pH 7.0), 0.3 M sucrose 100 mM KCl, 100 μM EGTA, 5 mM $MgCl_2$, and 103 μM $CaCl_2$. The free calcium concentration was calculated to be about 0.5 μM $[Ca^{2+}]_{free}$.

Spin-Labeling Sarcoplasmic Reticulum Membranes. Hydrocarbon chain rotational mobility was measured using conventional EPR methods to monitor the rotational dynamics of two fatty acid spin labels with the nitroxide moeity (i.e., *N*-oxyl-4′,4′-dimethyloxazolidine) located at the 5- and 12-positions relative to the carboxylate group, which are designated 5- and 12-SASL (stearic acid spin label), respectively. The spin label was diluted from a stock solution in

dimethylformamide into ethanol before adding to SR (40 mg/mL) at a ratio of less than one spin label per 200 phospholipids, with the final ethanol concentration less than 1% (v/v). To measure the rotational dynamics of the Ca-ATPase protein, SR was labeled with a short chain maleimide spin label, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide, as described previously for skeletal SR (Bigelow et al., 1986; Squier & Thomas, 1986b). Briefly, this involved the incubation of SR membranes (10 mg/mL) in sucrose buffer [i.e., 0.3 M sucrose and 20 mM MOPS (pH 7.0)] with 7.2 nmol of NEM per mg of SR protein for 30 min at 25 °C, followed by incubation with 18 nmol of MSL per mg of SR protein for 50 min. The samples were then diluted 30-fold with cold sucrose buffer and centrifuged at 100 000g_{max} for 1 h. The resulting pellet was homogenized in a solution containing 0.3 M sucrose and 100 mM KCl, 100 μM EGTA, 103 μM $CaCl_2$, 5 mM $MgCl_2$, and 25 mM MOPS (pH 7.0) and was again centrifuged at 100 000g_{max} for 1 h. The final pellet was resuspended in the same buffer, and either functional or dynamic properties relating to this preparation were immediately measured.

The labeling conditions for cardiac SR were analogous to those used to label the Ca-ATPase in skeletal SR but were modified for this preparation to block fast reacting sulfhydryl groups on other SR proteins. All operations involving cardiac SR were carried out at 4 °C. Briefly, the selective labeling of the Ca-ATPase in cardiac SR involved the incubation of cardiac SR microsomes (10 mg/mL) with NEM (30 nmol per mg of SR protein) for 30 min prior to the addition of 8.4 nmol of MSL per mg of SR protein for 50 min. Subsequent steps involving the centrifugation and resuspension of the Ca-ATPase were carried out as described for the skeletal SR labeling procedure.

Co-Localization of Maleimide Spin Label to the Ca-ATPase. 200 μg of protein was applied to a 7%−17% gradient SDS polyacrylamide gel. Two identical lanes were run per sample. One lane was stained with Coomassie Blue to identify discrete bands associated with individual proteins present in our SR preparation. The unstained lane was cut into ten uniform pieces, and the pieces were incubated in 1 N NaOH for 8−10 h at 70 °C. The samples were cooled, and 10 N HCL was added to neutralize the pH of the solution. A fluorogenic solution (Research Products International Corp., Mount Prospect, IL) was added, and radioactivity associated with the ^{14}C-labeled MSL was counted using a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co., Downers Grove, IL).

EPR Spectroscopy. EPR spectra were taken on a Bruker ESP300E spectrometer (Acton, MA) modified by the manufacturer to permit the adjustment of the incident microwave power to the sample by 0.1 dB increments and thereby facilitating the measurement of ST-EPR spectra at constant microwave field intensity. In addition, this spectrometer was equipped with a field-frequency lock (ER033M) and digital phase sensitive detector and preamplifier (ER025) permitting both the rapid determination of the proper phase to obtain the out-of-phase signal associated with measurements of microsecond rotational motions (see below) and the simultaneous detection of both in-phase and out-of-phase signals, thereby facilitating the measurement of absolute changes in the intensity of the out-of-phase signal relative to the in-phase signal. Measurements of the rotational motion of either SASL probes or MSL covalently bound to the Ca-ATPase on the sub-microsecond time scale involved the use of conventional EPR spectroscopy (first harmonic absorption in-phase, designated V_1), using 100 kHz field modulation with a peak-to-peak amplitude (H_m) of 2.0 gauss and a microwave field amplitude (H_1) of about 0.1 gauss. Measurements relating to the sub-millisecond rotational dynamics of the Ca-ATPase involved the use of saturation transfer EPR spectroscopy (second harmonic absorption out-of-phase, designated V_2'), using 50 kHz field modulation (with a modulation amplitude of 5.0 gauss) and a microwave field amplitude (H_1) of 0.25 gauss (Fajer & Marsh, 1982; Squier & Thomas, 1986a). Oxygen was removed from reference and experimental samples using gas-permeable sample cells purged with nitrogen gas for 30 min at 25 °C (Popp & Hyde, 1981). Temperature was controlled to within 0.1 °C with a model B-VT 2000 Eurotherm temperature controller. The accurate and reproducible setting of H_1 involved the use of a reference compound with known saturation properties (i.e., 0.9 mM peroxylaminedisulfonate in 50 mM K_2CO_3) in conjunction with the measurement of the relative cavity Q of the aqueous sample, as previously described in detail (Squier & Thomas, 1986a).

EPR Spectral Analysis. The order parameter (S) associated with lipid rotational dynamics of SR membranes for 5- and 12-SASL was obtained from the following relationship:

$$S = \frac{(T'_{||} - T_o)}{(T_{||} - T_o)} \quad (1)$$

where $T_{||} = 14.3$ G and $T_o = 33.5$ G (Gaffney, 1976b; Squier et al., 1988a). Analysis of the apparent rotational correlation time associated with the overall protein rotational motion of the Ca-ATPase involved a comparison of the observed spectral ST-EPR spectral line shape and integrated intensity for spectra of the spin-labeled Ca-ATPase with reference ST-EPR spectra obtained using spin-labeled hemoglobin in glycerol solvents of defined viscosity. The line shape parameters obtained for spin-labeled hemoglobin using our Bruker ESP300E spectrometer are virtually identical to those previously obtained on a Varian E109 spectrometer (Squier & Thomas, 1986a). However, the spectral intensity measurements are dependent on the method of digitizing the data, and the associated spectral intensity values obtained on our Bruker ESP 300E spectrometer were not directly comparable to those previously reported.

RESULTS

Calcium-Dependent Activation of Ca-ATPase. A comparison of the calcium-dependence of the rate of ATP hydrolysis for both cardiac and skeletal sarcoplasmic reticulum (SR) membranes reveals that the half-maximal activation of native cardiac SR membranes occurs at higher free calcium concentrations (i.e., $K_{1/2} \approx 640 \pm 30$ nM) relative to those concentrations observed for native skeletal SR membranes (i.e., $K_{1/2} \approx 220 \pm 10$ nM; Figure 1). In agreement with previous reports (Inui et al., 1986; Cantilina et al., 1993; Voss et al., 1994), we find that the phosphorylation of phospholamban (PLB) in cardiac SR membranes results in a shift in the calcium-dependence of the calcium-dependent ATPase activity such that the free-calcium concentration associated with the half-maximal activation of the Ca-ATPase occurs at a lower free calcium concentration (i.e., $K_{1/2} \approx 290 \pm 30$ nM), which is similar to that observed in skeletal SR membrane vesicles.

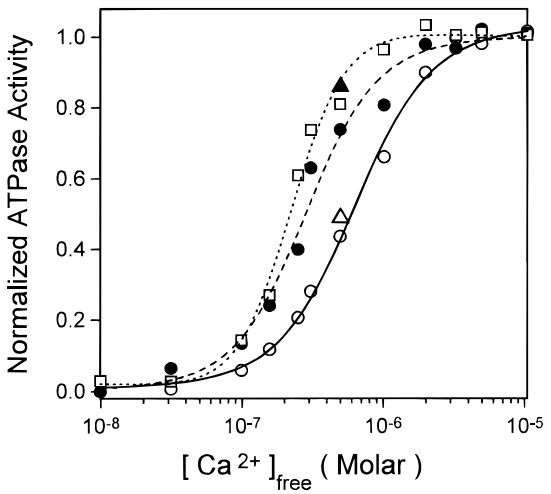

FIGURE 1: Calcium-dependence of the activation of cardiac and skeletal SR membranes. The calcium-dependent ATPase activity associated with the Ca-ATPase in membrane vesicles isolated from fast-twitch skeletal SR (□) and in native (○, ●) or spin-labeled (△, ▲) cardiac SR in the absence (○, △) and presence (●, ▲) of 1.0 μM cAMP and 60 μg of cAMP-dependent protein kinase per mL. The latter conditions are associated with the selective phosphorylation of PLB in cardiac SR membranes, as described in the text. Initial rates of ATP hydrolysis were measured at 25 °C in a medium containing 0.05 mg of SR protein per mL, 0.1 M KCl, 5 mM ATP, 5 mM $MgCl_2$, 25 mM MOPS (pH 7.0), 0.1 mM EGTA, and sufficient $CaCl_2$ to obtain the indicated free calcium concentrations, in which the multiple equilibria between all ligands in solution were taken into account using published binding constants (Fabiato & Fabiato, 1979; Fabiato, 1988). In all cases 6 μM A23187 (a calcium ionophore) was added to uncouple the back-inhibition of ATPase activity that would normally be associated with calcium uptake into the lumen of the SR vesicles. All specific activities were normalized relative to the maximal activity observed at saturating calcium concentrations. Maximal calcium-dependent ATPase activities were 2.2 ± 0.1 and 1.1 ± 0.1 μmol mg^{-1} min^{-1} for skeletal and cardiac SR preparations, respectively.

Calculation of Calcium Binding Affinities and Cooperative Interactions between Calcium Binding Sites on the Ca-ATPase. In order to quantitate the calcium-dependent activation of the Ca-ATPase in cardiac and skeletal SR, we have fit our data (Figure 1) to a standard model involving the cooperative binding of two ligands, allowing us to explicitly analyze for the relative affinities and cooperative interactions between the individual ligand binding sites:

$$Y = \frac{K_1[Ca^{2+}]_{free} + 2K_2[Ca^{2+}]^2_{free}}{2(1 + K_1[Ca^{2+}]_{free} + K_2[Ca^{2+}]^2_{free})} \qquad (2)$$

where Y represents the fraction of high-affinity calcium binding sites occupied with respect to the observed binding isotherm. K_1 is the macroscopic equilibrium constant and corresponds to the sum of the intrinsic equilibrium constants (k_1 and k_2) associated with calcium binding to the two binding sites on the Ca-ATPase, and K_2 represents the intrinsic equilibrium constant for binding calcium to both classes of calcium binding sites ($k_1k_2k_{12}$). This provides a quantitative estimate of the lower limit with respect to the cooperative interactions between the high-affinity calcium sites (i.e., k_{12}; Pedigo & Shea, 1995). An assessment of the relative affinities and cooperative interactions associated with calcium binding and activation of the Ca-ATPase can provide information relating to the possible modulation of the calcium activation of the Ca-ATPase by PLB.

Table 1: Free Energy Changes Associated with Calcium Activation of the ATPase Activity of the Ca-ATPase in Skeletal and Cardiac SR Membranes[a]

sample	ΔG_1[b]	ΔG_2	ΔG_{12}
cardiac SR	-8.1 ± 0.1	-16.9 ± 0.1	-1.5 ± 0.1
cardiac SR + phosphorylation	-8.3 ± 0.3	-17.8 ± 0.1[c]	-2.0 ± 0.3[c]
skeletal SR	-8.4 ± 0.2	-18.1 ± 0.1[c]	-2.1 ± 0.2[c]

[a] Data in Figure 1 were fit using eq 2. [b] ΔG values in kcal mol^{-1} K^{-1}. [c] Observed differences in binding energies relative to cardiac SR (top line) were judged statistically significant using the Student's *t*-test ($P < 0.10$). Errors were propagated (Bevington, 1969).

The fit to the data relating to the calcium-dependence of the ATPase activity (Figure 1) indicates that there is an enhancement in the extent of cooperativity between calcium binding sites on the Ca-ATPase in cardiac SR membranes associated with the phosphorylation of PLB (Table 1). Upon phosphorylation of PLB, the cooperative interactions between the two high-affinity calcium binding sites on the Ca-ATPase in cardiac SR are virtually identical to those of skeletal SR (Figure 1; Table 1). This result indicates that PLB interferes with the normal mechanisms associated with calcium activation of the Ca-ATPase and that the phosphorylation of PLB reestablishes the cooperative interactions between the high-affinity calcium binding sites, which is in agreement with previous kinetic measurements that have demonstrated the mechanism associated with the activation of the Ca-ATPase in cardiac SR membranes at submicromolar calcium concentrations upon the phosphorylation of PLB to involve a reduction in the activation barrier associated with the calcium-dependent activation of the Ca-ATPase (Cantilina et al., 1993).

Therefore, while the kinetic basis relating to the inhibitory action of PLB with respect to the increased energetic barrier associated with the activation of the Ca-ATPase upon the cooperative binding of two calcium ions to the high-affinity sites on the Ca-ATPase is well understood (Cantilina et al., 1993), the physical mechanisms associated with the modulation in the activation barrier to the calcium activation of the Ca-ATPase by PLB remain unclear. Previous measurements suggest that (i) the dissociation of PLB from the Ca-ATPase is associated with the alteration in the calcium-dependence of ATPase activity (James et al., 1989), and (ii) that activation of the Ca-ATPase by PLB may involve either enhanced associations between Ca-ATPase polypeptide chains (Fowler et al., 1989) or the dissociation of large inactive aggregates of the Ca-ATPase (Birmachu et al., 1993; Voss et al., 1994; Arkin et al., 1995). However, there have been no direct measurements in which the sensitivity of both the catalytic activity and the protein rotational motion of a single sample to activating conditions has been assessed.

Selective Labeling of Ca-ATPase with Maleimide Spin Labels. The measurement of the rotational dynamics of membrane proteins is commonly used to assess alterations in the oligomeric state and conformation of membrane proteins (Thomas et al., 1985; Thomas, 1986; Thomas & Mahaney, 1993). In general, these measurements involve the selective and covalent attachment of spectroscopic probes to the protein of interest. Therefore, a quantitative assessment of the rotational dynamics of the Ca-ATPase in native membrane vesicles requires the development of specific conditions in order to selectively attach spectroscopic probes to the Ca-ATPase to the exclusion of other SR proteins.

Furthermore, it is now clear that the specificity of a chemical reagent to a specific functional group on the Ca-ATPase in SR membranes is a function of both the chemical linker and the spectroscopic probe and that an assessment of chemical specificity requires that the actual probe of interest be localized (Bigelow & Inesi, 1992; Wawrzynow & Collins, 1993).

Numerous reports in the literature utilize EPR measurements of the rotational dynamics of the Ca-ATPase spin-labeled with either iodoacetamide- or maleimide-directed spin labels in both native skeletal and cardiac membranes (i.e., IASL and MSL; Coan & Inesi, 1977; Coan et al., 1979; Bigelow et al., 1986; Napier et al., 1987; Squier et al., 1988; Birmachu et al., 1993). In the case of IASL the specificity of labeling has been directly measured subsequent to the incorporation of a radiolabeled IASL spin label on the Ca-ATPase (Wawrzynow et al., 1993). However, this label is sensitive to the segmental rotational dynamics of the Ca-ATPase (Coan & Keating, 1982; Coan, 1983; Mahaney et al., 1995) and is not appropriate for the measurement of the overall rotational dynamics of the Ca-ATPase. In the case of MSL, there are currently no measurements relating to the specificity of labeling the Ca-ATPase in either skeletal or cardiac SR membranes with the actual spin label used to measure rotational motion. However, previous measurements involving the localization of iodoacetamido- and maleimide-directed fluorescent probes on the Ca-ATPase indicate that the labeling sites for the same chromophoric group associated with these reactive moieties are distinct (Squier et al., 1987; Bishop et al., 1988; Bigelow & Inesi, 1991). Furthermore, very different patterns of cysteine reactivity are observed for the Ca-ATPase when different chromophoric groups are attached with maleimide linker groups (Wawrzynow & Collins, 1993), emphasizing the need to determine the specificity of labeling using the actual spectroscopic probe of interest.

We have therefore synthesized a radioactive analog of MSL in which ^{14}C is contained within the maleimide functional group (see Experimental Procedures), permitting us to assess the specificity of labeling of the Ca-ATPase in native cardiac and skeletal SR membranes with MSL. As described in detail in Experimental Procedures, both cardiac and skeletal SR membranes require preincubation with NEM in order to block fast-reacting sulfhydryl groups that would interfere with measurements relating to the overall rotational dynamics of the Ca-ATPase (Squier & Thomas, 1986a,b).

Subsequent to NEM treatment, SR membranes from either cardiac or skeletal membranes are reacted with [^{14}C]MSL for 50 min prior to the separation of the SR membranes from unreacted [^{14}C]MSL by ultracentrifugation. The proteins in both cardiac and skeletal SR microsomes are then separated using SDS−PAGE, and the extent of labeling the Ca-ATPase with [^{14}C]MSL is assessed on the basis of the associated radioactivity with the resolved Ca-ATPase protein (Figure 2). Immunoblots using monoclonal antibodies directed against either the SERCA1 or SERCA2 isoform of the Ca-ATPase found in skeletal and cardiac SR membranes indicate that the predominant protein that migrates with an apparent molecular mass of about 96 kDa on the 7.5% polyacrylamide gels corresponds to the 110 kDa skeletal (data not shown) and cardiac isoform (Figure 3A) of the Ca-ATPase, respectively.

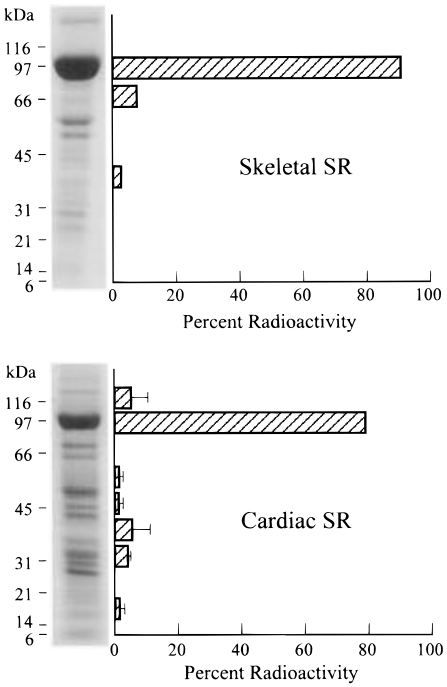

FIGURE 2: Specificity of MSL labeling of the Ca-ATPase in cardiac and skeletal SR membranes. Subsequent to NEM treatment, SR membranes from either skeletal (top) or cardiac (bottom) microsomes were reacted with [^{14}C]MSL for 50 min prior to the separation of the SR membranes from unreacted [^{14}C]MSL, as described in Experimental Procedures. In all cases 200 μg of SR proteins was applied to a 7.5% SDS−polyacrylamide gel, and SR proteins were separated as a function of molecular weight in order to assess the specificity of labeling (Laemmli, 1970). Two identical lanes were run for each sample, and Bio-Rad's Kaleidoscope prestained molecular mass standards were used to facilitate the comparison of the Coomasie-stained gels with the immunoblots. One lane is subsequently stained with Coomassie Blue to identify the relative positions of SR proteins of interest. The unstained lane was cut into pieces corresponding to the width of the bars associated with the relative extent of [^{14}C]MSL associated with each section of the gel. The SR proteins were extracted from the gel upon incubating the samples in 1.0 N NaOH for 8 h at 70 °C. Subsequent to neutralization, the associated radioactivity of each gel slice was counted. This procedure avoids possible quenching of the stimulated emission associated with the activation of the fluorophore by the [^{14}C]MSL by the acrylamide matrix and ensures that the observed radioactivity found in each gel fragment is directly proportional to the amount of MSL present on each protein.

Under our labeling conditions we find that 4.9 ± 0.3 nmol of [^{14}C]MSL is incorporated per mg of skeletal SR membrane protein (i.e., 1.2 ± 0.1 mol of [^{14}C]MSL per mol of Ca-ATPase), and that at least 89% of the protein-bound [^{14}C]-MSL is associated with the Ca-ATPase. In agreement with our previous spin-labeling results involving the skeletal Ca-ATPase, we observe that 95% ± 9% of the associated calcium-dependent ATP hydrolytic activity of the Ca-ATPase is preserved subsequent to the NEM preblock and MSL spin-label incorporation. The labeling conditions associated with skeletal SR membranes used in these experiments are equivalent to those previously described for the same preparation and provide a solid basis for the interpretation of our previous results (Bigelow et al., 1986; Squier et al., 1986b, 1988a,b; Squier & Thomas, 1988).

In the case of cardiac SR membrane vesicles we find that 0.42 ± 0.05 nmol of [^{14}C]MSL is incorporated per mg of cardiac SR membrane protein (i.e., 0.21 ± 0.04 mol of [^{14}C]-MSL per mol of Ca-ATPase) and that more than 80% ± 1% of the protein-bound [^{14}C]MSL is associated with the Ca-ATPase (Figure 2). Under these labeling conditions, we

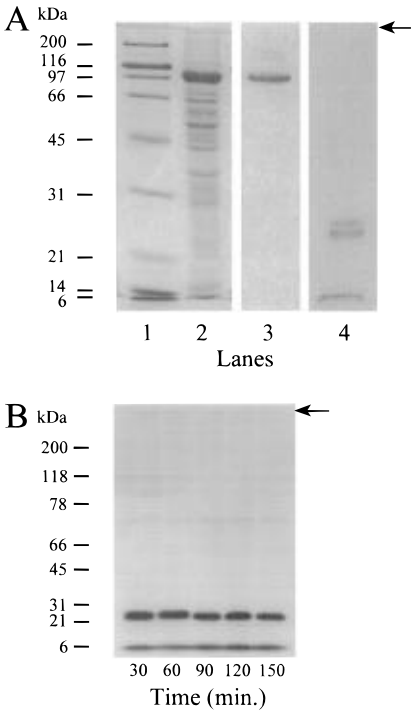

FIGURE 3: Stable phosphorylation of phospholamban in cardiac SR membranes. (A) Identification of the Ca-ATPase in cardiac SR membranes involved the comparison of the Coomasie Blue-stained 7.5% polyacrylamide gel obtained subsequent to SDS−PAGE (lane 2) to immunoblots obtained using antibodies directed against either the Ca-ATPase (lane 3) or PLB (lane 4). The SERCA2 isoform of the Ca-ATPase has an apparent molecular mass of 96 kDa. In agreement with previous observations one observes multiple bands corresponding to different levels of association between the 6 kDa PLB monomers on SDS−PAGE gels (Wegener & Jones, 1984; Tada et al., 1988; Li et al., 1990). We observe that PLB migrates as three major species under our experimental conditions, with molecular masses of 25, 23, and 7.3 kDa. The associated molecular weight markers and their relative molecular masses in kilodaltons are illustrated (lane 1) and correspond to bovine pancrease aprotinin (6.5 kDa), hen egg white lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), bovine carbonic anhydrase (31.0 kDa), hen egg white ovalbumin (45.0 kDa), bovine serum albumin (66.2 kDa), rabbit muscle phosphorylase B (97.4 kDa), *Escherichia coli* β-galactosidase (116.2 kDa), and rabbit skeletal muscle myosin (200 kDa). (B) Representative autoradiograms of cardiac SR samples that were quenched in denaturing buffer [0.625 M Tris/HCl (pH 8.0), 2% (w/v) SDS, 0.2 M sucrose, 5% β-mercaptoethanol, and 0.001% (w/v) bromphenol blue] at the indicated times (in minutes), following the initial separation of cAMP-dependent protein kinase from the cardiac SR membranes by ultracentrifugation (5 min at 100 000*g* in a Beckman TL-100 table top ultracentrifuge). The samples were frozen in liquid nitrogen prior to separation on a 7.5% acrylamide gel using SDS−PAGE. The resulting gels were dried and used to expose an X-OMAT Kodak X-ray film for 12 h. Experimental conditions involved the incubation of cardiac SR microsomes (3 mg/mL) for 3 min in the presence of 60 μg of cAMP-dependent protein kinase/mL, 1 μM cAMP, and 5 mM γ-[^{32}P]ATP in a buffer (buffer A) containing 0.3 M sucrose, 100 mM KCl, 5 mM $MgCl_2$, 25 mM MOPS (pH 7.0), 100 μM EGTA, and 103 μM added $CaCl_2$, resulting in 0.5 μM Ca^{2+}_{free}. In all cases 50 nM calycuylin (a phosphatase inhibitor) was added.

find that 89% ± 12% of the respective calcium-dependent ATP hydrolytic activity of the Ca-ATPase is retained subsequent to MSL incorporation and that the spin-labeled preparation demonstrates an analogous calcium-dependence with respect to the calcium-dependent ATPase activity both before and after the phosphorylation of PLB using cAMP-dependent protein kinase (Figure 1). Both the retention of ATPase activity and the ability of the spin-labeled Ca-ATPase in cardiac SR to undergo activation upon the phosphorylation of PLB permit us to quantitatively assess proposed alterations in the extent of oligomeric interactions between Ca-ATPase polypeptide chains that may correlate with the enhanced catalytic activity of the Ca-ATPase.

Stable Phosphorylation of PLB in Cardiac SR Vesicles. An assessment of possible alterations in the rotational dynamics of the Ca-ATPase polypeptide chains in cardiac SR that result from the phosphorylation of PLB by cAMP-dependent protein kinase requires that (i) PLB be selectively phosphorylated and (ii) the phosphorylation of PLB be sufficiently stable during the time course of our spin-label EPR experiment. We have incubated cardiac SR membranes under conditions that have previously been suggested to result in the selective and stable phosphorylation of PLB [i.e., in the presence of cAMP-dependent protein kinase, cAMP, γ-[^{32}P]ATP, and the phosphatase inhibitor calyculin A (Voss et al., 1994)]. These experiments have emphasized a free calcium concentrations in the sub-micromolar range associated with the half-maximal activation of the cardiac Ca-ATPase prior to the phosphorylation of PLB (Figure 1).

The stability of phosphoprotein formation was assessed subsequent to the removal of cAMP-dependent protein kinase, cAMP, and ATP by centrifugation (as described in Experimental Procedures) and involved the addition of denaturation buffer (0.0625 M Tris, 2% SDS, 0.2 M sucrose, 5% β-mercaptoethanol, 0.001% bromphenol blue at pH 8.0) to the cardiac SR membranes at various times following centrifugation to stop the phosphatase reaction (Figure 3B). These samples were immediately frozen in liquid nitrogen and subsequently analyzed for the associated radioactivity using both autoradiography and liquid scintillation counting of gel slices, as described in Experimental Procedures. Immunoblots using monoclonal antibodies directed against PLB indicate that PLB migrates as a series of oligomeric species on SDS−PAGE gels corresponding to different levels of association between the 6 kDa PLB monomers (Figure 3A), in agreement with previous observations (Wegener & Jones, 1984; Tada et al., 1988; Li et al., 1990; Reddy et al., 1995).

Under our experimental conditions PLB is essentially the only target for cAMP-dependent protein kinase in native cardiac SR membranes (Figure 3B). Phosphoproteins that are labile under these basic conditions (such as the aspartyl phosphate formed by the phosphorylation of the Ca-ATPase by ATP) are not observed. However, a separate analysis of the phosphorylated cardiac microsomes by SDS−PAGE using acidic conditions (pH 6.8) that stabilize all acyl phosphates indicate that the Ca-ATPase is also phosphorylated by γ-[^{32}P]ATP under these experimental conditions (data not shown).

An assessment of the phosphorylation of PLB and its relative stability was determined through the measurement of the time course of the decomposition of phosphorylated PLB (Figure 3B). For comparison purposes, the extent of PLB phosphorylation and its stability in the absence of the phosphatase inhibitor calyculin A were also determined (Figure 4). At all times following phosphorylation we find that inclusion of a phosphatase inhibitor (calyculin A) results in levels of phosphorylated PLB that are substantially higher, indicating the presence of endogenous phosphatases. The level of phosphorylated PLB observed in our preparation is consistent with earlier preparations (about 4−5 nmol per mg; Jakab & Kranias, 1988; Coyler & Wang, 1991) if we do not

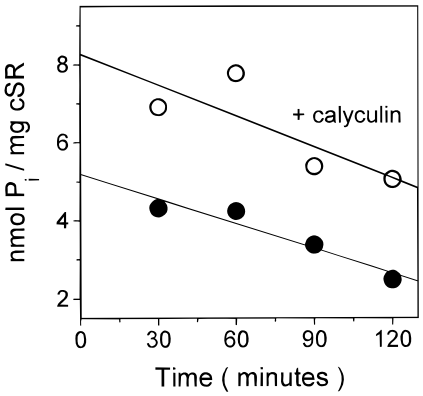

FIGURE 4: Stability of cAMP-dependent phosphorylation of phospholamban. Subsequent to the phosphorylation of PLB by cAMP-dependent protein kinase, cardiac SR microsomes were centrifuged and at various times denaturing buffer [0.625 M Tris/HCl (pH 8.0), 2% (w/v) SDS, 0.2 M sucrose, 5% β-mercaptoethanol, and 0.001% (w/v) bromphenol blue] was added to the SR membranes. Multiple lanes were run for each sample, permitting the localization of PLB on these gels through the use of immunoblots in which antibodies directed against PLB were used to determine the relative mobility of PLB on these gels. The corresponding position on the unstained gel was then cut out and the radioactivity associated with PLB was determined, as described in Experimental Procedures. The initial amount of phosphorylated PLB was estimated by back-extrapolation of a least-squared fit relating to the time-dependent hydrolysis of phosphoserine$_{16}$ on PLB. The associated intercept corresponds to 8.3 $\pm$ 1.2 and 5.2 $\pm$ 0.4 nmol of phosphorylated PLB per mg of cardiac SR in the presence (○) and absence (●) of the phosphatase inhibitor calyculin A. The slopes associated with the hydrolysis of the phosphorylated PLB in the presence and absence of calyculin A were -1.6 ± 0.8 and -1.3 ± 0.3 h^{-1}, respectively.

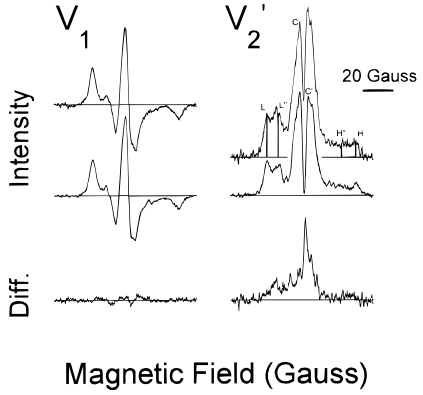

FIGURE 5: Decreased rotational dynamics of the Ca-ATPase in cardiac SR membranes subsequent to the phosphorylation of phospholamban. Conventional (V_1) and ST-EPR (V_2') spectra obtained for MSL-labeled cardiac SR microsomes in buffer A obtained prior to the phosphorylation of PLB (middle spectra) and subsequent to the phosphorylation of PLB (top spectra). In all cases spectra are normalized to the same concentration of spin labels. The difference spectra (bottom) were obtained subsequent to the subtraction of the spectrum taken prior to the phosphorylation of PLB (middle spectra) from the spectra taken subsequent to the phosphorylation of PLB (top spectra). The experimental conditions associated with the phosphorylation of PLB are analogous to those indicated in the legend to Figure 3. All samples were resuspended in buffer A, and EPR spectra were obtained using ~30 mg of cardiac SR/mL at 4 °C. Protein rotational correlation times (τ_r) were obtained using line shape (i.e., L″/L, C′/C, and H″/H) and integrated spectral intensities obtained from empirical reference spectra assuming isotropic rotational motion (Squier & Thomas, 1986) and are compiled in Table 2.

attempt to compensate for the endogenous phosphatase activity present in our preparation. However, a back-extrapolation of the observed extent of phosphorylated PLB (in the presence of calyculin A) indicates that cAMP-dependent protein kinase is able to phosphorylate up to 8.3 $\pm$ 1.1 nmol of PLB per mg of SR protein.

Stoichiometry of Phospholamban Relative to Ca-ATPase in Cardiac SR Membranes. It has been suggested that PLB functions as a pentameric complex in association with Ca-ATPase polypeptide chains (Voss et al., 1994; Arkin et al., 1995). Therefore, we have used the extent of phosphoprotein formation to measure the stoichiometry of PLB polypeptide chains relative to the Ca-ATPase. The latter measurement has previously been used to accurately measure the number of functional Ca-ATPase polypeptide chains in skeletal SR membranes [Barrabin et al., 1984; reviewed by Inesi (1985)]. Using autoradiography to assess the specificity of phosphoprotein formation, we find that the Ca-ATPase is essentially the only acid-stable phosphoprotein formed in cardiac SR membranes when cAMP-dependent protein kinase and cAMP are absent. We find that 2.0 $\pm$ 0.3 nmol of phosphoenzyme intermediate is formed per mg of our cardiac SR preparation. As discussed in the preceding section, cAMP-dependent protein kinase is able to phosphorylate approximately 8.3 $\pm$ 1.1 nmol of PLB per mg of SR protein in our preparation under optimal conditions. This suggests that there are about 4.2 $\pm$ 0.8 mol of PLB per mol of Ca-ATPase, consistent with earlier estimates that suggest that the functional unit of PLB may be a pentamer (Wegener & Jones, 1984; Li et al., 1990).

Phosphorylation of Phospholamban Results in Decreased Rotational Motion of Cardiac SR Ca-ATPase. Spin-label EPR measurements relating to the hydrodynamic properties of the Ca-ATPase provide a sensitive and direct means of detecting alterations in the interactions between Ca-ATPase polypeptide chains (Squier & Thomas, 1986a,b; Squier et al., 1988). Conventional EPR spectroscopy is sensitive to the rotational dynamics of spin labels on the sub-microsecond time scale and to the internal dynamics of the Ca-ATPase near the MSL labeling sites. We observe that the spectral line shape associated with the conventional EPR (V_1) spectrum of spin-labeled Ca-ATPase in cardiac SR membranes is motionally restricted, indicating that the MSL spin label is rigidly bound to the Ca-ATPase (Figure 5). Subsequent to the phosphorylation of PLB by cAMP-dependent protein kinase, we observe no change in the conventional EPR spectral lineshape, as emphasized by the minimal difference spectrum obtained subsequent to spectral subtraction (Figure 5). This indicates that the phosphorylation of either PLB or Ca-ATPase does not enhance the segmental motion associated with the MSL spin label on the sub-microsecond time scale and that the MSL spin label is rigidly bound to the Ca-ATPase permitting the measurement of the overall rotational motion of the Ca-ATPase using ST-EPR.

ST-EPR spectroscopy is sensitive to the slower sub-microsecond rotational dynamics associated with the rotational motion of membrane proteins [reviewed by Thomas et al. (1985)]. Subsequent to the phosphorylation of PLB by cAMP-dependent protein kinase, the extent of phosphorylation of PLB is sufficiently stable for the 2 h necessary to complete our spin-label EPR measurements (Figure 4). After 2 h there is approximately 5.1 nmol of phosphorylated PLB per mg of SR protein remaining.

The ST-EPR spectrum of the MSL spin-labeled Ca-ATPase in cardiac SR membranes is analogous to that previously reported and is characteristic of an integral membrane protein undergoing uniaxial rotational motion with

Table 2: Reversible Decrease in the Rotational Dynamics of the Ca-ATPase in Cardiac SR Membranes upon the Selective Phosphorylation of Phospholamban[a]

sample	τ_c (L″/L)[b]	τ_c(C′/C)	τ_c(H″/H)	τ_c(integral)
cardiac SR	96 ± 18	14 ± 5	117 ± 15	8 ± 1
cardiac SR + phosphorylation	147 ± 27	31 ± 6	211 ± 22[c]	67 ± 10[c]
phosphorylated cardiac SR + phosphatase	112 ± 24	33 ± 8	116 ± 11	15 ± 3

[a] Apparent rotational correlation times (τ_c) and the associated standard error of the mean ($N = 3$) for the spin-labeled Ca-ATPase were obtained from a comparison of the associated ST-EPR spectrum with empirical reference spectra obtained for spin-labeled hemoglobin. [b] τ values in μs. [c] Observed differences in the rotational correlation times relative to cardiac SR (top line) were judged statistically significant using the Student's *t*-test ($P < 0.01$; Anderson, 1987).

respect to the membrane normal with a correlation time of about 100 μs (Birmachu et al., 1993). Subsequent to the phosphorylation of PLB by cAMP-dependent protein kinase (in the presence of calyculin A) we observe substantial increases in both characteristic line shape parameters (defined in Figure 5) and in the integrated spectral intensity that are indicative of decreased rotational mobility of the Ca-ATPase (Table 2). Subsequent to the subtraction of the initial ST-EPR spectrum from that obtained after PLB phosphorylation, we observe a non-zero difference spectrum (Figure 5). The observed decrease in the rotational dynamics of the Ca-ATPase upon phosphorylation of PLB is not related to the phosphorylation of the Ca-ATPase since the observed changes in the ST-EPR spectrum are dependent upon the inclusion of both cAMP and cAMP-dependent protein kinase in the phosphorylation medium (data not shown).

The apparent rotational correlation times (τ_c) associated with the rotational motion of the Ca-ATPase are determined using semiempirical calibration curves obtained from spin-labeled hemoglobin in various glycerol solutions of known viscosity, as described in Experimental Procedures. The apparent correlation times obtained using either the line shape or intensity parameters for the Ca-ATPase do not quantitatively agree (Table 2), consistent with previous ST-EPR measurements relating to the Ca-ATPase which indicate that the observed discrepancy is related to the differential sensitivity of the different ST-EPR spectral parameters to the highly anisotropic rotational motion characteristic of membrane proteins (Kusumi et al., 1978; Robinson & Dalton, 1980; Marsh, 1980; Koole et al., 1981; Fajer & Marsh, 1983a; Horváth & Marsh, 1983; Squier & Thomas, 1986b). However, irrespective of the ST-EPR spectral parameter used to quantify the rotational dynamics of the Ca-ATPase, we observe a significant increase in the rotational correlation time associated with the rotational mobility of the Ca-ATPase when PLB is phosphorylated (Table 2).

While we find that the phosphorylation of PLB results in an increase in the rotational correlation time associated with the Ca-ATPase, irrespective of the ST-EPR parameter used to assess rotational mobility, the associated errors obtained using the integrated spectral intensity for the ST-EPR spectra are larger using the Bruker ESP300E spectrometer than previously reported using the Varian E109 spectrometer (Squier & Thomas, 1986a). Therefore, for purposes of discussion we have emphasized the use of the high-field line shape parameter (i.e., H″/H) which has previously been determined to accurately reflect the overall rotational motion of integral membrane proteins (Thomas et al., 1976; Fajer & Marsh, 1983b; Squier & Thomas, 1986a,b; Vistnes, 1983). We find that, upon phosphorylation of PLB, the rotational correlation time increases from 117 ± 15 μs to 211 ± 22 μs, corresponding to a (1.8 ± 0.2)-fold increase.

Reversible Alteration of Rotational Dynamics of Ca-ATPase upon Phosphatase Treatment of Cardiac SR Membranes. Since the activation of the Ca-ATPase is reversible upon hydrolysis of phosphoserine$_{16}$ on PLB, we have examined the reversibility of the rotational dynamics of the Ca-ATPase upon treatment of the phosphorylated cardiac SR membranes with alkaline phosphatase. This treatment results in the quantitative disappearance of any detectable level of phosphoprotein formation (data not shown). Using ST-EPR spectroscopy to measure the rotational dynamics of the Ca-ATPase, we observe that upon the addition of alkaline phosphatase that the rotational dynamics associated with the Ca-ATPase are analogous to that observed in native cardiac SR membranes prior to phosphorylation (Table 2). These results demonstrate that changes in the overall protein rotational motion of the Ca-ATPase in cardiac SR associated with the phosphorylation of PLB are reversible upon the hydrolysis of phosphoserine$_{16}$ on PLB. The analogous rotational dynamics observed subsequent to the addition of alkaline phosphatase to that observed in our native cardiac SR preparation prior to the addition of cAMP-dependent protein kinase also indicates that there is essentially no endogenous phosphorylated PLB in our preparation prior to the addition of cAMP-dependent protein kinase.

There are alterations in the central region of the ST-EPR spectrum that are not fully reversible upon the hydrolysis of phosphoserine$_{16}$ on PLB. However, the correlation time obtained using the center-field region of the spectrum (i.e., C′/C) is very sensitive to small alterations in the submicrosecond rotational dynamics associated with MSL and cannot be reliably used to assess the overall rotational mobility of the Ca-ATPase in the presence of weakly immobilized spin labels (Squier & Thomas, 1986b).

Phosphorylation of Ca-ATPase in Skeletal SR Membranes Has No Effect on Rotational Dynamics of Ca-ATPase. Fast-twitch skeletal SR membranes contain virtually no PLB (Jorgensen & Jones, 1986), and the homologous Ca-ATPase in skeletal SR membranes permits us to better understand the origin of observed changes in the rotational dynamics of the Ca-ATPase in cardiac SR membranes that arise specifically as a result of the phosphorylation of PLB, since the phosphorylation of the Ca-ATPase at Asp$_{351}$ by ATP or any nonspecific sites on the Ca-ATPase that might be subject to phosphorylation by cAMP-dependent protein kinase would potentially also be phosphorylated in skeletal SR membranes.

The vast majority of the associated spin labels are rigidly associated with the Ca-ATPase in skeletal SR membranes (Figure 6), permitting an assessment of the overall rotational dynamics using ST-EPR (Squier & Thomas, 1986b). Subsequent to the incubation of skeletal SR membranes with cAMP-dependent protein kinase, cAMP, ATP, and calyculin A we observe no significant change in either the conventional (V_1) EPR spectral line shape or the ST-EPR (V_2') spectral line shape or integrated spectral intensity, as emphasized by the minimal intensity associated with the difference spectrum (Figure 6). These results further indicate that the observed alteration in the rotational dynamics of the Ca-ATPase in cardiac SR subsequent to the incubation of the Ca-ATPase under phosphorylating conditions is a result of the phosphorylation of PLB.

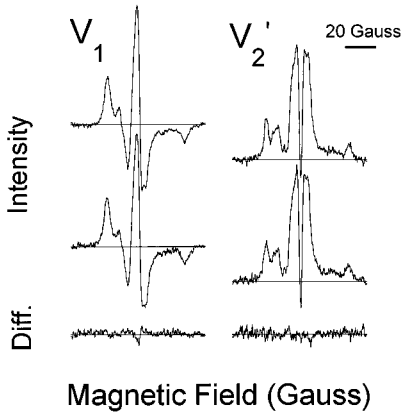

FIGURE 6: Phosphorylating conditions do not change the rotational dynamics of Ca-ATPase in skeletal SR membranes. Conventional (V_1) and ST-EPR (V_2') spectra associated with the rotational dynamics of the MSL-labeled Ca-ATPase in skeletal SR microsomes obtained subsequent (top spectra) and prior (middle spectra) to the addition of 60 μg of cAMP-dependent protein kinase/mL, 1 μM cAMP, and 5 mM γ-[^{32}P]ATP to SR microsomes in buffer A. In all cases spectra are normalized to the same concentration of spin labels. The difference spectra (bottom) were obtained by subtracting the middle spectrum from the top spectrum, which was taken subsequent to the addition of cAMP-dependent protein kinase, cAMP, and γ-[^{32}P]ATP to SR microsomes in buffer A. Experimental conditions used to obtain spin-label EPR spectra are analogous to those indicated in the legend to Figure 5.

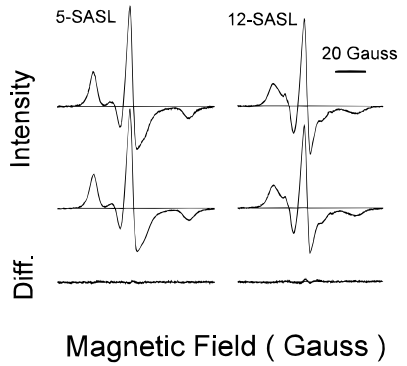

FIGURE 7: Rotational dynamics of phospholipid acyl chains are not altered upon phosphorylation of phospholamban. Conventional spin-label EPR spectra of 5- and 12-SASL probes in cardiac SR microsomes obtained subsequent (top spectra) and prior (middle spectra) to the addition of 60 μg of cAMP-dependent protein kinase/mL, 1 μM cAMP, and 5 mM γ-[^{32}P]ATP to SR microsomes in buffer A. In the case of both 5- and 12-SASL, spectra in the top and middle row are normalized to the same concentration of spins. The difference spectra (bottom) were obtained subsequent to the subtraction of middle spectra from the top spectra.

Phosphorylation of Phospholamban Does Not Alter Lipid Dynamics in Cardiac SR Membranes. Alterations in the overall rotational dynamics of membrane proteins can arise as a result of alterations in the size and shape of the protein of interest or as a result of alterations in the rotational dynamics of the surrounding membrane lipids (Saffman & Delbrück, 1975; Jähnig, 1986; Bigelow et al., 1986; Bigelow & Thomas, 1987). We have therefore also assessed the influence of phosphorylating conditions (defined in the legend to Figure 5) on the rotational dynamics of the membrane lipids in cardiac SR membranes using stearic acid spin labels with nitroxides located at the 5- and 12-positions. We observe no alteration in lipid rotational dynamics subsequent to the phosphorylation of either the Ca-ATPase or PLB (Figure 7), indicating that the observed decrease in the rate of rotational motion associated with the Ca-ATPase upon phosphorylation of PLB results from an alteration in the overall dimensions of the Ca-ATPase relative to the membrane normal.

Two possible models that are consistent with both our data and earlier indications that the Ca-ATPase functions as a dimeric functional unit are illustrated in Figure 8. We suggest that the interactions between PLB and the Ca-ATPase are altered as a result of the phosphorylation of PLB in a manner that increases productive interactions between individual Ca-ATPase polypeptide chains through either the modulation of the oligomeric state (Figure 8A) or through a rearrangement of individual polypeptide chains with respect to one another within a defined oligomeric state (Figure 8B).

DISCUSSION

Summary of Results. We have used spin-label EPR spectroscopy to investigate possible alterations in the conformation and dynamics of the Ca-ATPase in native cardiac SR membranes that are associated with the selective phosphorylation of PLB by cAMP-dependent protein kinase. These measurements have involved (i) the synthesis of a ^{14}C-radiolabeled maleimide spin label (MSL) permitting the development of conditions for the selective labeling of the Ca-ATPase with retention of enzymatic activity (Figure 1), (ii) the quantitation of the extent and the stability of phosphorylation of PLB by cAMP-dependent protein kinase (Figures 3B and 4), and (iii) the use of spin-label EPR spectroscopy to examine possible alterations in both lipid and protein interactions that accompany the activation of the Ca-ATPase in cardiac SR membranes following the phosphorylation of PLB (Figures 5 and 7). We are able to selectively spin label the Ca-ATPase in native cardiac SR membranes with essentially no loss of function (Figures 1 and 2). Consistent with previous observations, we find that PLB is virtually the only target protein that is phosphorylated by cAMP-dependent protein kinase in cardiac SR (Voss et al., 1994) and that the resulting phosphoprotein is sufficiently stable to allow ST-EPR measurements of the rotational dynamics of the Ca-ATPase (Figures 3 and 4). Using ST-EPR, we find that there is a (1.8 $\pm$ 0.2)-fold decrease in the rotational dynamics of the Ca-ATPase associated with the phosphorylation of PLB and the activation of the catalytic activity of Ca-ATPase (Figures 1 and 5). These changes in rotational dynamics and enzyme activity are reversible upon the addition of alkaline phosphatase, which hydrolyzes the phosphoester linkage on PLB (Table 2). No alteration in the lipid acyl chain dynamics is associated with changes in the phosphorylation state of PLB, implying that the observed changes in overall rotational motion of the Ca-ATPase in cardiac SR are the result of an increase in the cross-sectional area of the transmembrane portion of the Ca-ATPase relative to the membrane surface (Saffman & Delbrück, 1975; Jähnig, 1986; Fajer et al., 1989). The alteration in the rotational dynamics of the Ca-ATPase is the direct result of the phosphorylation of PLB and is not related to the phosphorylation of the Ca-ATPase or any other SR proteins since no ST-EPR spectral changes are observed as a result of conditions that phosphorylate the cardiac Ca-ATPase with ATP. Neither does the use of conditions that activate the Ca-ATPase in cardiac SR result in the alteration of the rotational dynamics or catalytic properties of the Ca-ATPase in skeletal SR where PLB is not expressed (Figure 6).

Model Illustrating Possible Physical Mechanisms Associated with Regulation of Ca-ATPase by PLB. The observed

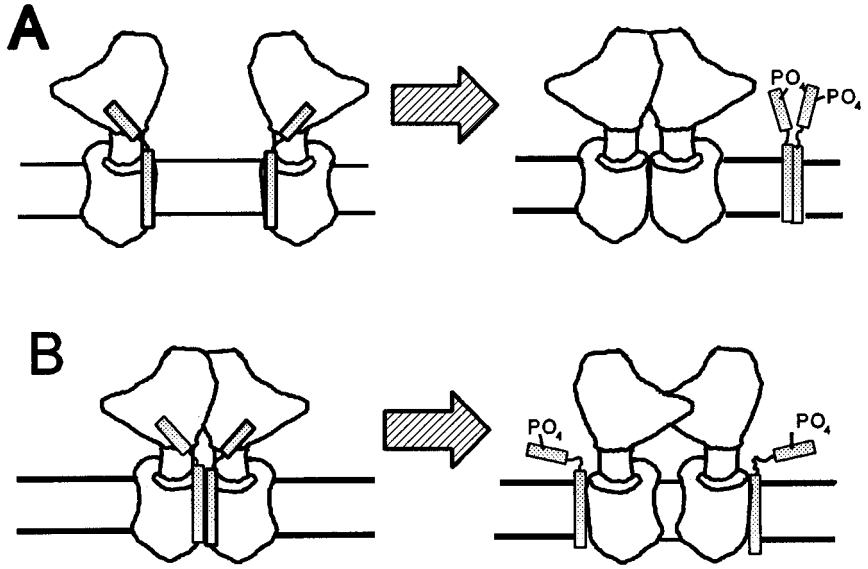

FIGURE 8: Model depicting possible structural rearrangements between Ca-ATPase polypeptide chains associated with phosphorylation of phospholamban. Conformational rearrangements of Ca-ATPase polypeptide chains associated with the phosphorylation of PLB consistent with the decreased rotational dynamics of the Ca-ATPase subsequent to the phosphorylation of PLB (Figure 5) are depicted as involving either changes in the oligomeric state of the Ca-ATPase (A) or alterations in the relative orientations of Ca-ATPase polypeptides within a defined oligomeric complex (B). PLB is indicated as a single structural unit for simplicity and is not meant to address issues relating to the functional oligomeric state of PLB in intact cardiac membranes. The overall shape of the Ca-ATPase was derived from the 14 Å resolution image of the Ca-ATPase obtained using image-enhanced cryoelectron microscopy (Toyoshima et al., 1993). A cross-section of the transmembrane portion of the Ca-ATPase with respect to the membrane surface has an elliptical shape, and the diameters of the major and minor axes are about 58 and 40 Å, respectively. The relative orientation and the extent of the conformational rearrangement between Ca-ATPase polypeptide chains associated with the phosphorylation of PLB are arbitrary and are not meant to indicate a precise knowledge of the spatial arrangement of the Ca-ATPase polypeptide chain within the putative dimeric functional unit.

1.8-fold decrease in the rotational correlation time of the Ca-ATPase associated with the phosphorylation of PLB provides some limitations with respect to possible changes in the dimensions of the oligomeric complex associated with the Ca-ATPase, since there is a direct relationship between changes in the rotational correlation time (τ_c) and changes in the cross-sectional area of the transmembrane portion of the Ca-ATPase relative to the plane of the membrane (Saffman & Delbrück, 1975; Thomas, 1986; Jähnig, 1986; Fajer et al., 1989; Cherry, 1992),

$$\frac{\tau_{c,\text{phosphorylated}}}{\tau_{c,\text{native}}} = \frac{r_{1,\text{phosphorylated}}^2 + r_{2,\text{phosphorylated}}^2}{r_{1,\text{native}}^2 + r_{2,\text{native}}^2}$$

where τ_c is the observed rotational correlation time and r_1 and r_2 represent the dimensions along the half-axes of the ellipsoid associated with the major and minor axes of the functional complex associated with the Ca-ATPase in cardiac SR membranes. The subscripts refer to the Ca-ATPase prior to the addition of cAMP-dependent protein kinase (i.e., $\tau_{c,\text{native}}$, $r_{1,\text{native}}$, and $r_{2,\text{native}}$) and after the phosphorylation of PLB (i.e., $\tau_{c,\text{phosphorylated}}$, $r_{1,\text{phosphorylated}}$, and $r_{2,\text{phosphorylated}}$).

While our results do not address the oligomeric state of the Ca-ATPase, previous measurements indicate that the Ca-ATPase in cardiac SR functions as a dimer (Chamberlain et al., 1983) and that conformational interactions between Ca-ATPase polypeptide chains within defined oligomeric structures of both the skeletal and cardiac isoforms of the Ca-ATPase are involved in the catalytic cycle [Hill & Inesi, 1982; Inesi & Hill, 1983; Bigelow et al., 1992; reviewed by Inesi (1992); Chen et al., 1995]. These previous results suggest that interactions between PLB and the Ca-ATPase may act to regulate structural coupling normally present between Ca-ATPase polypeptide chains within defined oligomeric states. If the phosphorylation of PLB were to result in the modulation of the relative conformations of the Ca-ATPase polypeptide chains within a defined oligomeric state (Figure 8B), it is necessary that the reorientation of Ca-ATPase polypeptide chains with respect to one another act to enhance the overall dimensions of the cross-sectional area of the complex relative to the plane of the membrane, as illustrated in Figure 8B. For example, if phosphorylation of PLB resulted in a reorientation from an initial interaction of the Ca-ATPase along their major axes (i.e., $r_{1,\text{native}} = 29$ Å and $r_{2,\text{native}} = 40$ Å) to an orientation in which the subunits are aligned end-to-end (i.e., $r_{1,\text{phosphorylated}} \approx 65$ Å and $r_{2,\text{phosphorylated}} = 20$ Å), then the calculated rotational correlation time would increase approximately 1.8-fold.

Alternatively, if it is assumed that the Ca-ATPase is a monomeric functional unit when PLB is dephosphorylated (Figure 8A), then $r_{1,\text{native}}$ and $r_{2,\text{native}}$ correspond to about 29 and 20 Å, respectively (Toyoshima et al., 1993). If the phosphorylation of PLB were to result in a dimeric functional unit of the Ca-ATPase (Figure 8A), then a 2-fold reduction in the rotational correlation time would be consistent with the productive association of two Ca-ATPase polypeptide chains along their major axes (i.e., $r_{1,\text{phosphorylated}} = 29$ Å and $r_{2,\text{phosphorylated}} = 40$ Å). In contrast, the association of Ca-ATPase polypeptide chains along their minor axes (i.e., $r_{1,\text{phosphorylated}} = 58$ Å and $r_{2,\text{phosphorylated}} = 20$ Å) would result in more than a 3-fold increase in the rotational correlation time, which is not consistent with our ST-EPR data. Furthermore, any alterations in the degree of association that involve larger changes in the oligomeric state are likewise inconsistent with our data. Regardless of the model, our results indicate that the phosphorylation of PLB results in substantial alterations in the interactions between Ca-ATPase polypeptide chains with respect to one another.

Alternative Interpretations. Our interpretation that the observed increase in the line shape and intensity ST-EPR parameters indicates that the hydrodynamic radius associated with the spin-labeled Ca-ATPase increases assumes that the average angle of the nitroxide spin-label on the Ca-ATPase relative to the membrane normal does not appreciably change upon the phosphorylation of PLB (Fajer et al., 1989; Howard et al., 1993; Hustedt & Beth, 1995). It is therefore important to consider how much the relative angle of the nitroxide relative to the membrane normal would have to change to result in a 1.8-fold reduction in the rotational correlation time. Previous phosphorescence anisotropy measurements (in which the Er-ITC label has been shown to selectively label the Ca-ATPase) suggest that the Ca-ATPase has a rotational correlation time relative to the membrane normal ($\tau_{R_{\|},\text{membrane}}$) of about 60 μs (Birmachu et al., 1993). If one neglects any nonaxial wobble associated with overall protein rotational motion, the actual rotational correlation time of the Ca-ATPase relative to the membrane normal ($\tau_{R_{\|},\text{membrane}}$) is related to the effective ST-EPR rotational correlation time ($\tau_{R_{\|},\text{effective}}$) by the following:

$$\tau_{R_{\|},\text{membrane}} = \frac{\tau^{\text{eff}}_{R_{\|,\text{membrane}}}}{2} \times \sin^2 \Theta$$

where Θ is the angle of the principal spin-label *z*-axis relative to the membrane normal (Robinson & Dalton, 1980; Fajer et al., 1989; Esmann et al., 1994). Since the effective rotational correlation time obtained from our ST-EPR measurements is 117 ± 15 μs, this suggests that the average angle of the principal axis of the nitroxide spin labels is approximately orthogonal to the membrane normal. Upon the phosphorylation of PLB, the effective rotational correlation time increases to 211 ± 22 μs. If this were to involve only a change in Θ (and no change in the rotational correlation time), then the principal axis of the nitroxide spin label would need to change by $46° \pm 3°$. However, since the MSL spin label is rigidly bound to the Ca-ATPase at multiple cysteines and undergoes essentially no rotational motion of the nanosecond time scale, large changes (i.e., $46° \pm 3°$) in the relative orientation of all of the spin label's principle axes relative to the membrane normal are unlikely (Fajer et al., 1989; Bigelow & Inesi, 1992). Furthermore, we have previously observed a quantitative correlation between the extent of cross-linking (which alters the cross-sectional area of the Ca-ATPase), changes in membrane viscosity, and the rotational correlation time of the Ca-ATPase in skeletal SR membranes (Bigelow & Thomas, 1987; Squier et al., 1988a, 1988b), indicating that the observed change in rotational dynamics is more likely to be related to an alteration in the hydrodynamic radius. Nevertheless, in either case it is clear that the phosphorylation of PLB results in a substantial alterations in the tertiary or quaternary structure of the Ca-ATPase in cardiac SR membranes.

Conclusions and Future Directions. The selective spin-labeling of the Ca-ATPase in cardiac SR membranes has permitted us to use ST-EPR to measure changes in the cross-sectional area of the Ca-ATPase. We report that the phosphorylation of PLB in cardiac SR membranes enhances specific protein−protein interactions involving Ca-ATPase polypeptide chains that correlate with enhancements in the calcium-dependent ATPase activity at physiological calcium concentrations. One can rule out models that suggest that the activation of the Ca-ATPase resulting from the phosphorylation of PLB involves either large decreases in the association between Ca-ATPase polypeptide chains (Voss et al., 1994) or any alteration in the hydrodynamic properties of the Ca-ATPase that would involve more than a 2-fold increase in the cross-sectional area of the complex between Ca-ATPase polypeptide chains. In order to further define the physical mechanisms associated with the regulation of the Ca-ATPase by PLB, future studies will emphasize the incorporation of site-specific spin labels and fluorophores on PLB in order to directly measure alterations in both PLB's structure and in the interactions between PLB and the Ca-ATPase subsequent to the phosphorylation of PLB. Additional measurements involving a quantitative analysis of the ST-EPR spectrum of the Ca-ATPase will permit a quantitative interpretation of the observed ST-EPR spectra for the spin-labeled Ca-ATPase in terms of a defined oligomeric state (Hustedt & Beth, 1995).

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