Purified, Reconstituted Cardiac Ca\(^{2+}\)-ATPase Is Regulated by Phospholamban but Not by Direct Phosphorylation with Ca\(^{2+}\)/Calmodulin-dependent Protein Kinase*

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Regulation of calcium transport by sarcoplasmic reticulum provides increased cardiac contractility in response to β-adrenergic stimulation. This is due to phosphorylation of phospholamban by cAMP-dependent protein kinase or by calcium/calmodulin-dependent protein kinase, which activates the calcium pump (Ca\(^{2+}\)-ATPase). Recently, direct phosphorylation of Ca\(^{2+}\)-ATPase by calcium/calmodulin-dependent protein kinase has been proposed to provide additional regulation. To investigate these effects in detail, we have purified Ca\(^{2+}\)-ATPase from cardiac sarcoplasmic reticulum using affinity chromatography and reconstituted it with purified, recombinant phospholamban. The resulting proteoliposomes had high rates of calcium transport, which was tightly coupled to ATP hydrolysis (−1.7 calcium ions transported per ATP molecule hydrolyzed). Co-reconstitution with phospholamban suppressed both calcium uptake and ATPase activities by −50%, and this suppression was fully relieved by a phospholamban monoclonal antibody or by phosphorylation either with cAMP-dependent protein kinase or with calcium/calmodulin-dependent protein kinase. These effects were consistent with a change in the apparent calcium affinity of Ca\(^{2+}\)-ATPase and not with a change in \(V_{\text{max}}\). Neither the purified, reconstituted cardiac Ca\(^{2+}\)-ATPase nor the Ca\(^{2+}\)-ATPase in longitudinal cardiac sarcoplasmic reticulum vesicles was a substrate for calcium/calmodulin-dependent protein kinase, and accordingly, we found no effect of calcium/calmodulin-dependent protein kinase phosphorylation on \(V_{\text{max}}\) for calcium transport.

Deletion of the PLB gene in transgenic mice provides a dramatic demonstration of PLB’s role in regulating cardiac contractility (5); PLB-deficient mice have resting hearts that are already contracting maximally and are therefore unable to respond to β-adrenergic stimulation, which normally increases contractility in response to physiological demand.

Many biochemical studies have addressed the mechanism of PLB regulation, and there is a growing consensus that PLB acts to reduce the apparent calcium affinity of Ca\(^{2+}\)-ATPase (K\(_{\text{Ca}}\); Refs. 6–10). The effects seem modest, changing K\(_{\text{Ca}}\) from −0.3 to −1 μM, but this is apparently enough to reduce the amount of calcium pumped into the SR during muscle relaxation, thus prolonging the relaxation time and reducing the amount of calcium released for subsequent contractions. β-Adrenergic stimulation of cardiac tissue results in activation of both cAMP-dependent protein kinase (PKA) and calcium/calmodulin-dependent protein kinase (CaM kinase), which phosphorylate PLB at Ser-16 and Thr-17, respectively (11, 12). Phosphorylation at either site causes dissociation of PLB from Ca\(^{2+}\)-ATPase, thus relieving the suppression of K\(_{\text{Ca}}\) (13). PLB is a small (6000 Da), mostly α-helical transmembrane protein that runs as a pentamer by SDS-polyacrylamide gel electrophoresis (11, 14). The transmembrane domain is thought to consist of a single α-helix (15, 16), whereas the cytoplasmic domain contains sites for phosphorylation and for interaction with Ca\(^{2+}\)-ATPase (17, 18). Model building suggests that the pentamer could be stabilized by coiled-coil interactions between transmembrane helices (19, 20). Spectroscopic measurements show that PLB reduces the mobility of Ca\(^{2+}\)-ATPase, an effect attributed to aggregation of Ca\(^{2+}\)-ATPase in the membrane. Based on these results, it was proposed that PLB pentamers interact with multiple Ca\(^{2+}\)-ATPase molecules, inducing their aggregation and thus inhibiting their activity (21). The detailed molecular mechanism by which these interactions regulate calcium transport, however, remains to be determined.

In addition to PLB-mediated regulation, evidence for direct phosphorylation of cardiac Ca\(^{2+}\)-ATPase by CaM kinase has recently been presented. Using both isolated cardiac SR vesicles (22) and Ca\(^{2+}\)-ATPase expressed in HEK cell membranes (23), soluble CaM kinase II was found to phosphorylate Ser-38 of cardiac Ca\(^{2+}\)-ATPase, causing a 2-fold increase in \(V_{\text{max}}\) but no change in K\(_{\text{Ca}}\). No phosphorylation of the skeletal isoform was observed, even after the analogous residue (His-38) was changed to Ser (23). A membrane-bound CaM kinase is present in cardiac SR, and this direct phosphorylation of Ca\(^{2+}\)-ATPase could therefore be effective, in conjunction with PLB-mediated regulation, for fine control of calcium transport. Earlier studies, however, failed to detect significant CaM kinase phosphorylation of Ca\(^{2+}\)-ATPase in cardiac SR (24–28), and...
the physiological relevance of such phosphorylation remains unclear.

Reconstitution of purified components into liposomes provides a good opportunity for investigating these various hypotheses. Indeed, we previously reconstituted purified, recombinant PLB with affinity-purified, skeletal Ca\(^{2+}\)-ATPase in order to characterize the effects of PLB on calcium uptake, ATPase activity, and membrane permeability (6). In addition, we studied the cytoplasmic and transmembrane domains of PLB and, contrary to previous reports (29-31), found that neither domain was sufficient to reproduce the regulatory effects of intact PLB. Although both skeletal and cardiac Ca\(^{2+}\)-ATPase have been previously shown to interact with PLB (32, 33), we wondered whether the effects of PLB on skeletal Ca\(^{2+}\)-ATPase in our reconstituted system would be enhanced by the use of cardiac Ca\(^{2+}\)-ATPase, which is the physiological substrate for PLB. Previous methods reported for purification of cardiac Ca\(^{2+}\)-ATPase (34-37) did not yield preparations suitable for co-reconstitution with PLB, and we have therefore developed an improved method for purification of cardiac Ca\(^{2+}\)-ATPase, which utilizes affinity chromatography. This method is quite different from previous purification schemes, yielding a highly purified protein after a short, one-step procedure. Furthermore, this purified Ca\(^{2+}\)-ATPase retained high catalytic activity after reconstitution; in fact, rates of calcium transport and coupling of transport to ATP hydrolysis were far better than those previously reported. Regulation by recombinant PLB, co-reconstituted with the cardiac enzyme, was similar to that observed with skeletal Ca\(^{2+}\)-ATPase (6). This seemed an ideal system for investigating the proposed synergistic effects of PLB and CaM kinase on regulation of the activity of Ca\(^{2+}\)-ATPase (22, 23); however, we found that the purified, cardiac Ca\(^{2+}\)-ATPase was not a substrate for CaM kinase II, raising questions about this most recent hypothesis for regulation of calcium transport across cardiac SR.

**MATERIALS AND METHODS**

Preparation of Cardiac SR—Cardiac SR was prepared from canine ventricles by combining several methods (38-40). All operations were performed at 4°C. The atria were excised from two dog hearts, and the fat and connective tissue were removed from the ventricular muscle, which was then cut into 1-cm² pieces. The combined ventricular tissue (100–125 g) was homogenized in 500 ml of 30 mM Tris maleate, 0.3 M sucrose, 0.5 mM DTT, and 3 mM NaN\(_3\), pH 7, using an Omni mixer (Omni International Inc.) with four 15-s cycles of homogenization at full speed separated by an interval of 15 s. The homogenate was centrifuged at 5,600 rpm (Sorvall, GSA rotor) for 10 min, and the supernatant was filtered through 4 layers of cheesecloth. The filtrate was centrifuged at 12,000 rpm (Sorvall, GSA rotor) for 30 min, and the supernatant was filtered through glass wool. The filtrate was centrifuged again at 12,000 rpm, and the supernatant was again filtered through glass wool. The filtrate was centrifuged at 40,000 rpm (Beckman, Ti 45 rotor) for 60 min; the pellets were homogenized in 20 ml Tris maleate, 0.3 M sucrose, 0.6 M KCl, 0.5 mM DTT, and 3 mM NaN\(_3\), pH 7, using a Dounce homogenizer, and the volume was adjusted to 125 ml (based on 100–125 g of starting tissue). After stirring for 25 min at 4°C, the suspension was centrifuged at 45,000 rpm (Beckman, Ti 45 rotor) for 60 min. The top, loose beige pellet containing SR vesicles was collected by carefully adding 10 mM Tris-HCl, 0.3 M sucrose, 1 mM DTT, 1 mM histidine, and 0.2 mM KCl, pH 7.0, to the centrifuge tube. The lower, transparent hard pellet was discarded; it represented 10–20% of the total protein and was composed of glycogen and phosphorylase. The pooled fractions were put into a dialysis bag (3,500-Da cut-off) and dialyzed for 6–8 h at 4°C, resulting in a final protein concentration of approximately 3 mg/ml. To store the protein, an equal amount of lipid was added (egg yolk phosphatidylcholine/egg yolk phosphatidic acid, 10:1 by weight), and the detergent was removed by stirring with Bio-Beads SM2 (Bio-Rad, 50 mg of hydrated beads per mg of C\(_{18}\)E\(_{8}\) for 3 h at room temperature. This detergent-free protein was quick-frozen in liquid nitrogen and stored at -70°C; thus stored, the protein retained full activity for 5–6 months and through 3–4 cycles of freezing and thawing.

**Co-reconstitution of PLB and Ca\(^{2+}\)-ATPase**—Purified cardiac Ca\(^{2+}\)-ATPase and recombinant PLB were co-reconstituted using the same method described in detail for skeletal Ca\(^{2+}\)-ATPase (6). Briefly, 33 μg of PLB and 5 mg of lipid (egg yolk phosphatidylcholine/egg yolk phosphatidic acid, 10:1 by weight) were dried in a thin film. This lipid film was resuspended in 20 ml imidazole, pH 7.0, at concentrations of 20 mg/ml lipid and 132 μg/ml PLB. Next, 0.1 ml of these lipid/PLB vesicles (2 mg lipid, ~13 μg PLB) were solubilized in a final volume of 0.25 ml containing 4 mg of Triton X-100, 20 mM imidazole, 50 mM K\(_2\)oxalate, 10% glycerol, pH 7.0, and 25 μg of purified Ca\(^{2+}\)-ATPase. The detergent was removed by adding 160 mg of Bio-Beads SM2 and stirring at room temperature for 3 h. The remaining solution contained 8 mg/ml Ca\(^{2+}\)-ATPase, 100 mM Ca\(^{2+}\)-ATPase, and 52 μg/ml PLB, which corresponds to a molar ratio of PLB to Ca\(^{2+}\)-ATPase of 10:1. After reconstitution, the vesicles were put on ice and were assayed for calcium uptake and ATPase activity the same day.

**Phosphorylation**—Immediately prior to measuring Ca\(^{2+}\)-ATPase activity, the vesicles were phosphorylated either with the catalytic subunit of PKA or with CaM kinase II. In addition, a monoclonal antibody to PLB (2D12) was used to mimic the effects of PLB phosphorylation. For phosphorylation, 0.24 mg of vesicles (3 μg of Ca\(^{2+}\)-ATPase at 50 μg/ml) were incubated at 30°C for 30 min in 50 mM MOPS, pH 7.0, 10 mM Mg\(_2\)Cl\(_2\), 10% glycerol, 50 mM K\(_2\)SO\(_4\), and 0.1 mM ATP either with 25 units (0.8 μg at 0.47 μM) of the catalytic subunit of PKA (Sigma) or with 0.25 μg (active site concentration of 80 nM) of rat brain CaM kinase II (the gift of Dr. Howard Schulman), 3 μg (3 μM) of calmodulin, and 0.1 mM CaCl\(_2\). Controls omitted the catalytic subunit of PKA or the CaM kinase, respectively. For the antibody, vesicles were simply preincubated at an equimolar ratio of PLB to antibody on ice for 15 min.

**Calcium Uptake—**The rate of calcium uptake was determined by a microfiltration method (44) as described previously (6). Briefly, each assay was performed in duplicate in 0.2 or 0.25 ml of solution containing 2 or 2.5 μg of Ca\(^{2+}\)-ATPase and 50 mM imidazole, 45 mM K\(_2\)SO\(_4\), 5 mM Na\(_2\)HPO\(_4\), 5 mM Mg\(_2\)Cl\(_2\), 5 mM K\(_2\)oxalate, 1 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone, 1 μM valinomycin, 0.5 mM EGTA, and HEPES-KOH to give a pH of 7.0 (45). The reaction was initiated by addition of 5 mM ATP, and 40 μl aliquots were filtered with 0.22-μm GS filters (Millipore Corp.) at 10-30 s intervals. Filters were then washed three times with 2 ml of 10 mM MOPS, 50 mM K\(_2\)SO\(_4\), 5 mM Mg\(_2\)Cl\(_2\) and 5 mM LaCl\(_3\), at pH 7.0, and retained on the filters (i.e. inside the vesicles) was measured by scintillation counting. The initial rates of calcium uptake were calculated by least-square regression of time points where the uptake remained linear.

ATPase—ATP hydrolysis was determined by liberation of phosphate,
as measured by a modified procedure (46) of the malachite green reagent method (47) in the same solution used for the calcium uptake assay, except that 1 μg/ml calcium ionophore A23187 was included. Duplicate aliquots (40–50 μl) were taken immediately after initiating ATP hydrolysis with 5 mM ATP (zero time point) and again after 2 min (pCa 5.4) or 4 min (pCa 6.8). The aliquots were immediately added to 0.5 ml of 20 mM H$_2$SO$_4$, followed by the addition of 0.1 ml of 1.75% NH$_3$Mo$_7$O$_4$ in 3.15 M H$_2$SO$_4$. After 5 min, 0.1 ml of 0.035% malachite green reagent (Sigma) in 0.35% polyvinyl alcohol (dissolved in hot water) was added and, after 5–10 more min, 0.1 ml of 4 M H$_2$SO$_4$ was added (such acidic conditions stop the reaction by preventing the formation of the ternary complex between molybdate, malachite green and inorganic phosphate). Approximately 1 h later, the absorbance was read at 610 nm, and the amount of inorganic phosphate was calculated based on a standard curve generated with 0–8 nmol of K$_2$HPO$_4$.

Electrophoresis and Immunoblotting of PLB and Ca$^{2+}$-ATPase—SDS-polyacrylamide gel electrophoresis was performed with 6–20% gradient acrylamide gels, which were stained with Coomassie Brilliant Blue (Sigma). Autoradiography was measured with a phosphorimager (Molecular Dynamics). Immunoblotting was performed as described previously (6) for PLB and skeletal Ca$^{2+}$-ATPase. Briefly, protein bands were transferred to polyvinylidene difluoride membranes (Bio-Rad) after electrophoresis in 25 mM Tris, 193 mM glycine, pH 8.3, 10% methanol, and 0.01% SDS at 4°C for 1 h at a constant current. After transfer and blocking, the membranes were incubated with monoclonal antibodies to PLB (2D12) and to cardiac Ca$^{2+}$-ATPase (VIE8 from Dr. Kevin Campbell) in blocking buffer. Then the membrane was washed and incubated for 1 h in blocking buffer containing goat-anti-mouse antibody conjugated to horseradish peroxidase (Fisher). After washing with binding buffer, the immunogenic protein bands were developed with 3,3'-diaminobenzidine tetrahydrochloride reagent (Sigma).

RESULTS

Purification of Cardiac Ca$^{2+}$-ATPase—The cardiac Ca$^{2+}$-ATPase was purified from SR vesicles in two easy steps (Fig. 1A). Twenty mg of SR protein was solubilized in 1% C$_{12}$E$_{6}$, giving an ATPase activity of 1.5–2 μmol/mg/min at 37°C, and insoluble material was removed by centrifugation. The supernatant was enriched in a prominent band at $M_r$ = 100,000, corresponding to the Ca$^{2+}$-ATPase, and, at this point, ATPase activity was increased slightly to 2.5–3 μmol/mg/min. The solubilized Ca$^{2+}$-ATPase was then adsorbed to Cibacon Blue agarose and, after elution with buffer containing ADP, the purified protein was obtained. Ca$^{2+}$-dependent ATPase activity was 6 μmol/mg/min, and we obtained 0.5 mg of protein for a yield of 2.5%. The protein was almost homogeneous, accounting for 96.5% of the total Coomassie Blue staining material, although several weak bands could be seen at 45 and 35 kDa.

In exploring conditions for this purification, we initially employed Reactive Red 120 (Sigma) affinity chromatography, which we routinely use for purification of the skeletal Ca$^{2+}$-ATPase. Similar to a previous preliminary report using cardiac SR (42), we found that 50–100 mM salt was required in addition to nucleotide for elution of Ca$^{2+}$-ATPase from this matrix, whereas no salt was required for elution of the skeletal isoform. The resulting yields for the cardiac Ca$^{2+}$-ATPase were variable and always low; furthermore, the purity was poor probably due to nonspecific elution of impurities by the salt. Cibacon Blue has a lower affinity and Ca$^{2+}$-ATPase can therefore be specifically eluted with ADP alone. However, to maximize the efficiency of elution, it was necessary to allow solubilized SR to bind to the column resin in suspension, prior to packing the column in a 1-ml syringe. By binding in suspension, we were guaranteed a homogeneous distribution of bound protein in the packed column and a more reproducible elution pattern. This special requirement for loading the cardiac isoform is probably due to the considerably larger proportion of impurities in cardiac SR relative to skeletal SR.

The purity and homogeneity of the Ca$^{2+}$-ATPase preparation was verified by immunoblot analysis (Fig. 1B). Three different preparations were analyzed by immunoblotting with monoclonal antibodies to both Ca$^{2+}$-ATPase and PLB. Two different Ca$^{2+}$-ATPase antibodies recognized a single band, and no proteolytic fragments were detected. Most importantly, we detected no PLB in the purified preparation of cardiac Ca$^{2+}$-ATPase, despite the high sensitivity of this method. This indicates that Ca$^{2+}$-ATPase and PLB had been effectively dissociated by solubilization and completely separated by affinity chromatography.

Reconstitution—The purified cardiac Ca$^{2+}$-ATPase was reconstituted by adding detergent-solubilized lipid at low protein
to lipid ratios and then removing detergent with Bio-Beads. Calcium uptake after reconstitution was measured at 25 and 37 °C (Fig. 2), and skeletal Ca\(^{2+}\)-ATPase was reconstituted and assayed in parallel. This reconstituted cardiac Ca\(^{2+}\)-ATPase was very active; as far as we know, the uptake rates reported are the highest ever demonstrated for cardiac Ca\(^{2+}\)-ATPase and are only slightly lower than those we obtained with the reconstituted, skeletal Ca\(^{2+}\)-ATPase. The calcium concentration required for half-maximal stimulation was virtually the same for the two isoforms, pCa \(-6.5\) at 25 °C and pCa \(-6.65\) at 37 °C. Furthermore, we found tight coupling between calcium transport and ATPase activity; for the cardiac enzyme, 1.65 and 1.86 calcium ions (at 25 and 37 °C, respectively) were transported for each ATP hydrolyzed at 25 and 37 °C, respectively.

Effects of PLB phosphorylation by PKA on calcium uptake, and results were similar to those obtained with the monoclonal antibody (Fig. 4). As expected, autoradiograms showed that PLB was the only species phosphorylated by PKA. The expected mobility shift induced by phosphorylation of PLB was also apparent (Fig. 4A).

Phosphorylation with CaM Kinase—Previous studies detected no significant phosphorylation of Ca\(^{2+}\)-ATPase in cardiac SR vesicles by CaM kinase II (24–28), but recent reports have suggested that the pump may in fact be a substrate (22, 23). Our highly purified preparation of reconstituted cardiac Ca\(^{2+}\)-ATPase, with retention of full catalytic activity, has allowed us to reassess this issue. Fig. 5 shows the results of CaM kinase phosphorylation of the purified Ca\(^{2+}\)-ATPase, reconstituted with and without PLB, and of Ca\(^{2+}\)-ATPase resident in vesicles isolated from free and junctional SR (40). No significant phosphorylation of Ca\(^{2+}\)-ATPase by CaM kinase was detected in any of the preparations analyzed, whereas intense phosphorylation of PLB was observed (Fig. 5B, lanes 3, 5, and 7). A small level of \(^{32}\)P incorporation into a protein band of similar mobility as Ca\(^{2+}\)-ATPase was detected in the junctional SR but not the free SR (Fig. 5B, lanes 5 and 7). \(^{32}\)P incorporation into this band (99 pmol/mg protein) was only 10% of that expected for phosphorylation of the calcium pump (49) and can therefore be attributed to phosphorylation of a protein specific to junctional SR. Phosphorylation of the ryanodine receptor is also seen in the junctional SR, as has previously been reported (50).

The functional effect of CaM kinase phosphorylation on calcium transport (Fig. 5C) is comparable with the effects of phosphorylation by PKA (Fig. 4C). In particular, stimulation of calcium transport by CaM kinase phosphorylation occurred only at low ionized calcium concentration, and no effects were observed in the absence of PLB. These results are consistent with PLB acting as a substrate for CaM kinase but not Ca\(^{2+}\)-ATPase.
fig. 4. phosphorylation with cAMP-dependent protein kinase. After co-reconstitution and phosphorylation by PKA (0.47 μM), proteoliposomes were run on a 6–18% polyacrylamide gel, stained with Coomassie Blue (A), and analyzed by autoradiography (B). Protein concentrations after reconstitution were rather low (0.1 mg/ml), and the corresponding Coomassie stain was somewhat weak (lanes 2 and 3); the 1st lane of the gel was therefore loaded with larger amounts of detergent-solubilized protein to clearly show the locations of the two proteins. As expected, phosphorylation of PLB produces a slight shift in the mobility of the PLB pentamer (PLB5 in lane 3, which also reveals the presence of the catalytic subunit of PKA (PKA cat sub)). The autoradiograph shows that both oligomeric forms of PLB have been phosphorylated by PKA. PLB, corresponds to the monomeric form of PLB. Effects of PKA phosphorylation on calcium uptakes (C) are very similar to those for PLB antibody (Fig. 3). Control (ctrl) represents reconstituted vesicles with Ca2+-ATPase alone, whereas PLB represents vesicles with both Ca2+-ATPase and PLB.

FIG. 5. cardiac Ca2+-ATPase is not a substrate for CaM kinase II. Four different samples were subjected to phosphorylation by rat brain CaM kinase II, detergent-solubilized, purified cardiac Ca2+-ATPase (lane 1), reconstituted vesicles containing purified cardiac Ca2+-ATPase (lane 2) plus purified recombinant PLB (lane 3), free (longitudinal) cardiac SR (FSR, lanes 4 and 5) and junctional cardiac SR (JSR, lanes 6 and 7). A shows the Coomassie Blue-stained gel, whereas B shows the corresponding autoradiograph to detect 32P incorporation. No phosphorylation of Ca2+-ATPase can be detected even though PLB was heavily phosphorylated whenever it was present (as was the ryanodine receptor, RR), indicating that CaM kinase (CaMK) was active under the conditions used for the experiment; autophosphorylation of CaM kinase accounts for the doublet observed at ~55 kDa. Functional effects on calcium transport are comparable with those of PKA phosphorylation and can be explained by phosphorylation of PLB alone. Phosphorylation was conducted for 10 min at 30 °C in 50 μM of medium containing 50 μM MOPS, pH 7.1, 10 mM MgCl2, 0.3 mM CaCl2, 15 μM calyculin A (phosphatase inhibitor), CaM (1 μg at 1.2 μM) and CaM kinase (0.12 μg at 46 μM concentration of active sites) were included as indicated. Reactions were stopped by adding 20 μl of SDS gel sample buffer, and 50-μl aliquots were run on a 7% polyacrylamide gel using the buffer system of Porzio and Pearson (57). C, controls (ctrl) contained Ca2+-ATPase alone, and PLB contained both Ca2+-ATPase and PBL; conditions for phosphorylation are described under “Materials and Methods.”

rates of only 0.7 μmol/mg/min at 37 °C despite ATPase activities of 10–13 μmol/mg/min before reconstitution (29), suggesting that calcium transport was impaired relative to ATPase activity. This is not surprising as the calcium transport sites are thought to exist within the hydrophobic core of the bilayer, which is precisely the region most disrupted by detergent treatment.

We elected to use the more recently available dye-coupled columns for purification of cardiac Ca2+-ATPase. This method requires full solubilization of Ca2+-ATPase, which is required for co-reconstitution with recombinant PLB, but which runs the same risk of perturbing calcium transport sites.
less, affinity chromatography has the potential of separating in a single step the protein of choice from the many other proteins present in cardiac SR. Furthermore, our routine use of Reactive Red affinity chromatography for skeletal Ca\textsuperscript{2+}-ATPase purification not only provided a pure and highly active preparation, but yielded high quality crystals that were well suited for structure determination by electron crystallography (41). Although we tried Reactive Red chromatography for purification of Ca\textsuperscript{2+}-ATPase from cardiac SR, we found that elution of the enzyme was inefficient and that the resulting preparations were impure, although still highly active. Substitution of Cibacron Blue for Reactive Red, however, allowed preparation of reasonably pure cardiac Ca\textsuperscript{2+}-ATPase with yield and ATPase activity well within the range previously described. Furthermore, the true integrity of our purified protein was documented by reconstitution into proteoliposomes, after which calcium uptake rates and coupling ratios were much higher than those reported previously for the cardiac enzyme (29). Furthermore, the coupling ratio that we obtained, 1.6–1.9 calcium ions per ATP molecule hydrolyzed at pH 5.4, is close to the theoretical value of 2.0.

These excellent calcium uptake rates were obtained even though the ATPase activity after reconstitution was 1.5–2-fold less than that immediately before reconstitution. This reduction in ATPase activity was not due to spontaneous inactivation of the protein during reconstitution, because activities similar to the detergent-solubilized starting material were recovered if reconstituted preparations were solubilized by detergent. There are several other factors that may contribute to this effect. First, the molecules do not all face the same direction in the reconstituted vesicles, and those with their hydrophobic domains inside will not have access to ATP and will therefore not contribute to activity measurements; random insertion of molecules into the bilayer would thus account for the factor of 2. However, reconstitution of skeletal Ca\textsuperscript{2+}-ATPase using Ca\textsubscript{2+}E\textsubscript{r} produces proteoliposomes with ~80% of molecules facing outward, as determined by proteolysis (51) and by fluorescence isothiocyanate labeling.\textsuperscript{2} Second, the presence of detergent tends to stimulate ATPase activity, especially at the higher calcium concentrations (pCa 4.6) used for our enzyme-coupled ATPase assay (52); this stimulation could be due either to a more fluid environment for the Ca\textsuperscript{2+}-ATPase molecule in the detergent micelle (53) or to eliminating calcium accumulation inside the vesicles which normally opposes release of calcium from the low-affinity luminal sites. Finally, it is possible that the transport of calcium across the membrane represents a load that slows the turnover rate of the molecule. In the case of F\textsubscript{0}F\textsubscript{1}-ATPase, there is a 100-fold increase in ATPase activity when F\textsubscript{1} is dissociated from F\textsubscript{o}. Unlike F\textsubscript{0}F\textsubscript{1}, however, Ca\textsuperscript{2+}-ATPase appears to be monomeric (54), and parts of the molecule responsible for ion transport cannot be physically dissociated from those responsible for ATP hydrolysis, making it difficult to test this hypothesis.

Regulation of Cardiac Ca\textsuperscript{2+}-ATPase—The effects of PLB on Ca\textsuperscript{2+}-ATPase are well documented, and there is a general consensus that the apparent affinity for calcium (K\textsubscript{Ca}) is slightly lower when PLB interacts with Ca\textsuperscript{2+}-ATPase (7–10). This effect is reversible either by phosphorylation of PLB or by antibody binding to PLB; both are thought to dissociate PLB from Ca\textsuperscript{2+}-ATPase, thus returning K\textsubscript{Ca} to its normal value (13). Our reconstituted system with purified Ca\textsuperscript{2+}-ATPase and purified, recombinant PLB is ideal for investigating the consequences of PLB phosphorylation, in particular, for quantitating the corresponding effects on K\textsubscript{Ca} and V\textsubscript{max} of Ca\textsuperscript{2+}-ATPase and for addressing the role PLB has in modulating calcium leakage across the membrane. Our results with cardiac Ca\textsuperscript{2+}-ATPase are similar to those we previously obtained with skeletal Ca\textsuperscript{2+}-ATPase (6), namely a 2-fold stimulation by the PLB antibody at low calcium concentrations and no effect at saturating calcium concentrations. This observation applied both to calcium uptake and to ATPase activity, and phosphorylation of PLB by PKA or by CaM kinase produced similar results. Taken together, our results are consistent with the established effect of PLB on K\textsubscript{Ca} but are inconsistent both with PLB forming an ion pore (19, 55) and with PLB affecting V\textsubscript{max} (30, 31, 56). If PLB formed a pore, it would be expected to generate a calcium leak in the SR membrane, thus reducing uptake by the proteoliposomes but not affecting the ATPase activity of the pump, which is not what we observed. Furthermore, in our previous work with skeletal Ca\textsuperscript{2+}-ATPase, we directly measured a leakage across reconstituted vesicles and also failed to detect any increased leakage due to PLB. In the case of V\textsubscript{max}, we were unable to detect any change in either calcium uptake or ATPase activity at the corresponding calcium concentration (pCa 5.4).

More recently, a stimulation of V\textsubscript{max} was demonstrated by another mechanism, direct phosphorylation of cardiac Ca\textsuperscript{2+}-ATPase by exogenous CaM kinase II. A roughly 2-fold stimulation was observed for ATPase activities of detergent-solubilized cardiac and slow-twitch skeletal SR (22); effects on calcium uptake were measured after expression of cardiac Ca\textsuperscript{2+}-ATPase in HEK-293 cells (23). Autoradiographs showed \textsuperscript{32}P incorporation into a protein of 100 kDa that reportedly could be immunoprecipitated with anti-Ca\textsuperscript{2+}-ATPase antibodies. Still not adequately explained, however, was that only a minor fraction of Ca\textsuperscript{2+}-ATPase molecules were phosphorylated, even though ATPase activity was stimulated 2-fold. Given these observations, we were eager to study the relationship between PLB regulation and CaM kinase phosphorylation in our reconstituted preparation. However, we were unable to detect any phosphorylation of cardiac Ca\textsuperscript{2+}-ATPase with CaM kinase II either after reconstitution of purified components or in free (longitudinal) SR vesicles. The fact that we could easily detect phosphorylation of PLB in these same preparations demonstrates that our assay was working correctly and that CaM kinase was active. Accordingly, functional measurements of calcium uptake revealed that the effect of CaM kinase phosphorylation was indistinguishable from that of PKA phosphorylation and that this effect could be attributed solely to PLB. Thus, our results are consistent with those from the earliest group of studies on CaM kinase (24–28, 50). We have no ready explanation for the detection of a low level of Ca\textsuperscript{2+}-ATPase phosphorylation by CaM kinase in more recent studies (22–23); the only significant difference appears to be a somewhat higher concentration of CaM kinase (~4-fold higher) used for these studies. Even if this concentration difference were key, the low level of phosphorylation observed (estimated to be at most 12% of Ca\textsuperscript{2+} pumps present (22)) reinforces our conclusion that direct phosphorylation of Ca\textsuperscript{2+}-ATPase is of no physiological significance. Interestingly, we did observe that junctional SR contains a protein around 100 kDa that is phosphorylated by CaM kinase (Fig. 5). Close inspection of the gels revealed that this band migrates slightly faster than Ca\textsuperscript{2+}-ATPase, however. Thus, this protein appears to be a distinct component of junctional SR, the identity of which may be worth investigating.

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\textsuperscript{2} L. G. Reddy, R. C. Pace, and D. L. Stokes, unpublished results.