Functional Reconstitution of the Cardiac Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase with Phospholamban in Phospholipid Vesicles*

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The Ca\(^{2+}\)-ATPase in cardiac sarcoplasmic reticulum (SR) is under regulation by phospholamban, an oligomeric protein. To determine the molecular mechanism by which phospholamban regulates the Ca\(^{2+}\)-ATPase, a reconstitution system was developed, using a freeze-thaw sonication procedure. The best rates of Ca\(^{2+}\) uptake (700 nmol/min/mg reconstituted vesicles) were observed when cholate and phosphatidylcholine were used at a ratio of cholate/phosphatidylcholine/Ca\(^{2+}\)-ATPase of 2:80:1. The EC\(_{50}\) values for Ca\(^{2+}\) were 0.05 μM for both Ca\(^{2+}\) uptake and Ca\(^{2+}\)-ATPase activity in the reconstituted vesicles compared with 0.63 μM Ca\(^{2+}\) in native SR vesicles. Phospholamban in the reconstituted vesicles was associated with a significant inhibition of the initial rates of Ca\(^{2+}\) uptake at pH 6.0. However, phosphorylation of phospholamban by the catalytic subunit of the cAMP-dependent protein kinase reversed the inhibitory effect on the Ca\(^{2+}\) pump. Similar findings were observed when a peptide, corresponding to amino acids 1–25 of phospholamban, was used. These findings indicate that phospholamban is an inhibitor of the Ca\(^{2+}\)-ATPase in cardiac SR and phosphorylation of phospholamban relieves this inhibition. The mechanism by which phospholamban inhibits the Ca\(^{2+}\) pump is unknown, but our findings with the synthetic peptide suggest that a direct interaction between the Ca\(^{2+}\)-ATPase and the hydrophobic portion of phospholamban may be one of the mechanisms for regulation.

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§ The abbreviations used are: SR, sarcoplasmic reticulum; EGTA, ethyleneglycol-bis(oxyethylenenitrito)tetraacetic acid; SDS, sodium dodecyl sulfate; MOPS, 3-(N-morpholino)-2-hydroxypropanesulfonic acid.
Phospholamban was purified from canine cardiac SR by a modification of the method described by Martonosi and Feretos (25). In the Millipore filtration assay, the phosphorylating capacity of the phospholamban was determined. Fifty µg of SR or 10 µg of reconstituted vesicles per ml or, as indicated in the figure legends, 0.1 M KCl, 5 mM MgCl₂, various amounts of CaCl₂, 0.5 mM EGTA, 5 mM ATP, 2.5 mM oxalate, 5 mM Na₂HPO₄, and 40 mM histidine-HCl, pH 7.0, or 40 mM imidazole-HCl, pH 7.0, using Millipore filters with a pore size of 0.45 µm for SR or 0.22 µm for reconstituted vesicles (GS). Calcium uptake was initiated by the addition of 5 mM ATP. The rates of Ca²⁺ uptake were calculated by least squares linear regression analysis of the 20-, 40-, and 60-s values of Ca²⁺ uptake. The initial rates of calcium uptake were linear with the change of Bio-Beads and at the end of incubation, the sample was pelleted and prepared for electron microscopy using a graded series of ethanol concentrations and embedded in Epon. Thin sections were stained in lead citrate and uranyl acetate and examined in a Zeiss EM 10 transmission electron microscope.

Purification of Cardiac Sarcoplasmic Reticulum Ca²⁺-ATPase-The Ca²⁺-ATPase was purified from cardiac SR by the method of Nakamura et al. (19). The yield was 3-4 mg based on 100 mg of starting SR protein. This preparation could be stored at -80 °C for several months without loss of activity. To remove Triton X-100 before reconstitution, Bio-Beads SM-2 (0.5-0.6 g/ml) were added to the Ca²⁺-ATPase preparation (1 mg/ml), as described previously (26). The mixture was gently agitated at room temperature for 1 h using a stirrer attached with a stirring bar, with a change of Bio-Beads just before the change of Bio-Beads and at the end of incubation, the sample was recovered from the Bio-Beads using a 0.1 ml Hamilton syringe with a 0.006-inch inner diameter needle.

Purification of Phospholamban by Affinity Chromatography-Phospholamban was purified from cardiac SR by a modification of the method described by Bidlack et al. (17). Cardiac SR was prepared as described previously (27). Isolated SR vesicles were suspended at a concentration of 5 mg/ml in 0.25 M sucrose. Deoxycholate was added at a ratio of 2 mg of deoxycholate/5 mg of SR (0.2% final deoxycholate concentration). The suspension was incubated at room temperature for 15 min with stirring. After incubation, the suspension was centrifuged at 120,000 X g for 45 min at 20 °C. The supernatant was then applied to a Sephadex G-75 (fine) column (1.5 X 180 cm), equilibrated with Buffer A (10 mM Tris-HCl, pH 7.8, 0.04% cholate). The sample was eluted from the column using Buffer A and 0.6 M NaCl, and the absorbance at 280 nm was monitored. Phospholamban was eluted as a single protein peak, determined by SDS-polyacrylamide gel electrophoresis. The fractions containing phospholamban were pooled and dialyzed against 4 liters of 100 mM Tris-HCl, pH 7.4, 0.25 M sucrose, and 0.04% sodium cholate, overnight. The dialysate was concentrated using polyethylene glycol.

Phospholipid Analysis-The total lipid extracts were prepared as described previously (28), and the lipid classes were separated by the method of Rouxer et al. (29). The separated phospholipid spots were scraped and the phosphorus group of the phospholipids was determined as described by Kahovcova and Oudavic (30). Total phosphorus determination was performed by the method of Hess and Derr (31).

Phosphorylation of Phospholipids in the Purified Ca²⁺-ATPase-Incorporation of radioactivity from [γ-³²P]ATP into phospholipids was determined by thin layer chromatography and subsequent autoradiography, as described previously (20).

Phospholamban Affi-Gel 10 Affinity Column—Affi-Gel 10 (Bio-Rad) was prepared as recommended. The gel was washed with 10 volumes of 2-propanol, followed by 10 volumes of cold water and equilibrated with 10 mM MOPS, pH 7.0, 0.1% Zwittergent 3-14, and 20 mM diithiothreitol. The equilibrated gel was suspended at a concentration of 50 µg of SR or 10 µg of reconstituted vesicles per ml or, as indicated in the figure legends, 0.1 M KCl, 5 mM MgCl₂, various amounts of CaCl₂, 0.5 mM EGTA, 5 mM ATP, 2.5 mM oxalate, 5 mM Na₂HPO₄, and 40 mM histidine-HCl, pH 7.0, or 40 mM imidazole-HCl, pH 7.0, using Millipore filters with a pore size of 0.45 µm for SR or 0.22 µm for reconstituted vesicles (GS). Calcium uptake was initiated by the addition of 5 mM ATP. The rates of Ca²⁺ uptake were calculated by least squares linear regression analysis of the 20-, 40-, and 60-s values of Ca²⁺ uptake. The initial rates of calcium uptake were linear with the change of Bio-Beads and at the end of incubation, the sample was pelleted and prepared for electron microscopy using a graded series of ethanol concentrations and embedded in Epon. Thin sections were stained in lead citrate and uranyl acetate and examined in a Zeiss EM 10 transmission electron microscope.

Preparation of Antibodies—Antibodies to purified phospholamban were raised in two rabbits, approximately 2 kg each. Both rabbits were injected subcutaneously with phospholamban emulsified in Freund's complete adjuvant: 150 µg of phospholamban or 120 µg of phospholamban on days 1, 150 µg of phospholamban on days 2 and 35. Serum was collected on day 45. For affinity purification, the serum were applied to the phospholamban Affi-Gel 10 column in 20 mM sodium phosphate buffer, 150 mM NaCl, pH 7.2 (PBS). The column was washed with 20 column volumes of PBS, and the phospholamban adsorbed to the affinity matrix was reconstituted with 2 cm 300 mM glycine-HCl, containing 0.1 mg of bovine serum albumin/ml, pH 3.2. The eluant was neutralized immediately with a saturated solution of Na₂CO₃, and the affinity-purified antibody was concentrated and equilibrated with PBS, using Centricon 30 membrane filters (Amicon Division).

A synthetic peptide based on a portion of the primary sequence of the cardiac Ca²⁺-ATPase amino acids (192-205) was synthesized by Biosearch. A cysteine was added at the amino terminus to facilitate attachment of the oligopeptide to the carrier and the affinity column. Some of the synthetic oligopeptide was conjugated to keyhole limpet hemocyanin, and polyclonal antibodies were raised as described above.

Electron Microscopy—Liposomes and proteoliposomes reconstituted with the purified Ca²⁺-ATPase or Ca²⁺-ATPase and phospholamban were pelleted and prepared for electron microscopy using routine procedures. Pellets were treated with 2.5% glutaraldehyde, postfixed with 1% osmium, dehydrated in ethanol, and embedded in Epon. Thin sections were stained in lead citrate and uranyl acetate and examined in a Zeiss EM 10 transmission electron microscope.

Immuno-gold Electron Microscopy—Reconstituted vesicles, containing the Ca²⁺-ATPase or the Ca²⁺-ATPase and phospholamban, were bound to nitrocellulose paper (modified from Ref. 33) and were used to immunolocalize phospholamban or the Ca²⁺-ATPase. In control studies liposomes, which did not contain any protein, were used in parallel with the reconstituted vesicles. Briefly, small pieces of nitrocellulose paper were placed into the wells of enzyme-linked immunosorbent assay plates and were covered with vesicle suspensions for 60 min to allow vesicles to bind to the paper. This and all subsequent procedures were carried out at 4 °C. Best results were obtained using 1 mg of protein/ml, since this resulted in a preparation with only one or two layers of vesicles bound to the surface of the nitrocellulose. The vesicle suspension was withdrawn and then the wells were filled with PBS/hemoglobin containing 100 mM sucrose (PBS/Hb/sucrose) to block all the nonspecific binding sites. The membranes were washed with PBS/Hb/sucrose. The experimental samples were then incubated with the phospholamban polyclonal antibody or a polyclonal antibody raised against a synthetic peptide of the cardiac SR Ca²⁺-ATPase for 3 h, washed with PBS/Hb/sucrose (five times for 5 min), and incubated for 3 h with 5 nm gold conjugated to goat anti rabbit IgG (gold/goat anti rabbit IgG), Janssen Auorprobe, obtained from Ted Pella Inc.). Control samples were left in wash solution, during the 3-h period that experimental samples were incubated with the phospholamban antibody or the goat anti rabbit antibody. All samples were then washed with PBS/Hb/sucrose (three times for 5 min), followed by 100 mM sodium cacodylate (two times for 5 min) and prepared for electron microscopy. The nitrocellulose paper was fixed with 2.5% buffered glutaraldehyde and postfixed with 2% osmium tetroxide. After each fixative, the tissues were washed with 100 mM sodium cacodylate, as described above. Samples were dehydrated in a graded series of ethanol concentrations and embedded in Epon. Thin sections were stained with lead citrate and uranyl acetate and examined in a Zeiss EM 10 transmission electron microscope.

[The rest of the text continues as before.]
Hinkle (38). Liposomes were prepared from crude soybean phospholipids (50 mg/100 μl), which were dissolved in chloroform. The chloroform was evaporated under a stream of nitrogen, the dried phospholipids were dissolved in ethanol, dried again under nitrogen to remove residual chloroform, and subsequently lyophilized. Phospholipids in 40 mM imidazole-HCl, pH 7.0, 0.1 M KCl, and 0.25 M sucrose (Buffer B) were sonicated to clarity at 0°C with a probe-type sonicator (Branson sonifier, model W140) or with a bath-type sonicator (Laboratory Supplies Co., model G112SP1G). An aliquot of this phospholipid suspension was added immediately to the purified SR Ca\(^{2+}\)-ATPase, which was solubilized at 0°C, as indicated, to give an appropriate lipid/protein ratio. The final reconstitution mixture was 0.5-1.2 ml, and it contained 0.15-0.6 μg of Ca\(^{2+}\)-ATPase, various concentrations of phospholipids, and Buffer B. The mixture was then frozen in liquid N\(_2\), thawed at room temperature, and sonicated in an ice bath with a Branson sonifier for 30 to 60 s. To exchange the external medium and to remove the free detergent, the resultant proteoliposome suspension (approximately 0.4 ml) was passed through a plastic column containing 1.2 ml of Sephadex G-50, which had been equilibrated with Buffer B according to the Sephadex column centrifugation method of Penefsky (39).

To reconstitute the Ca\(^{2+}\)-ATPase with phospholamban, the purified phospholamban was combined with the purified Ca\(^{2+}\)-ATPase, cholate was added, and this mixture was then combined with the phospholipid suspension. Reconstituted vesicles were subsequently isolated as described above.

Miscellaneous—Calcium content of all solutions was determined by atomic absorption spectroscopy using a Perkin-Elmer 4100 atomic absorption spectrophotometer. Calcium-EGTA buffers contained various concentrations of EGTA and CaCl\(_2\). Free Ca\(^{2+}\) concentrations at pH 7.0 were based upon the EGTA association constants reported by Martell and Smith (40), and they were calculated by the use of a computer program, which takes into consideration the effect of oxalate (41).

Protein concentration of reconstituted vesicles or purified phospholamban was determined by the method of Bensadoun and Weinstein (42), which is a modification of the method described by Schaffner and Weissman (43), using bovine serum albumin as a standard. Protein concentration of the purified Ca\(^{2+}\)-ATPase was determined by the Bradford method (44). Deoxycholic acid (free acid, Sigma) was recrystallized before use.

RESULTS

Characterisation of the Purified Ca\(^{2+}\)-ATPase from Cardiac Sarcoplasmic Reticulum.—The purified cardiac SR Ca\(^{2+}\)-ATPase appeared homogeneous on 7.5% SDS-polyacrylamide gels and it was essentially free of phospholamban (19). The Ca\(^{2+}\) dependence of the purified Ca\(^{2+}\)-ATPase is shown in Fig. 1. The EC\(_{50}\) for Ca\(^{2+}\) was 6.00 ± 0.01 μM (n = 4) for the purified enzyme compared with 0.63 ± 0.04 μM (n = 7) for the native cardiac SR (Fig. 1). This increase in the affinity of the Ca\(^{2+}\)-ATPase for Ca\(^{2+}\) could be due to several factors: the presence of Triton X-100 remaining associated with the purified enzymic preparation, the absence of the trans-calcium inhibition which is present in native SR, the removal of phospholamban, which has been suggested to be an inhibitor of the Ca\(^{2+}\)-ATPase (26, 45), the removal of other regulatory components, or a combination of these factors. To determine the effect of Triton X-100, the purified Ca\(^{2+}\)-ATPase was subjected to treatment with Bio-Beads SM-2. There was no effect on the enzymic activity assayed over a wide range of Ca\(^{2+}\) concentrations (Fig. 1). Furthermore, an amount of Triton X-100, equivalent to that present in the purified Ca\(^{2+}\)-ATPase preparation (19), was added to native SR membranes. At this Triton concentration the initial rates of Ca\(^{2+}\) uptake were less than 10% of the rates obtained with native membranes, presumably due to leakiness of the SR vesicles. The EC\(_{50}\) of the Ca\(^{2+}\)-ATPase for Ca\(^{2+}\) decreased from 0.63 ± 0.04 μM (n = 7) in native SR to 0.40 ± 0.03 μM (n = 4) in Triton-treated SR. Thus, the increase in the affinity of the purified Ca\(^{2+}\)-ATPase for Ca\(^{2+}\) may be at least partially due to relief of the trans-calcium inhibition, present in native SR.

Since phospholipids are important in biological membranes, the phospholipid composition of the purified Ca\(^{2+}\)-ATPase was assessed by thin layer chromatography. The major phospholipid was phosphatidylcholine followed by phosphatidylethanolamine and phosphatidylinositol (Table I). Interestingly, the levels of phosphatidylinositol (12.8%), associated with the purified Ca\(^{2+}\)-ATPase preparation, were higher than those present in SR membranes (9%). To determine whether the phosphatidylinositol associated with the Ca\(^{2+}\)-ATPase could be phosphorylated, in a similar fashion as that previously reported for the purified phospholamban (20), the catalytic subunit of the cAMP-dependent protein kinase was used in the presence of [γ-\(^{32}\)P]ATP. Phospholipids were extracted and analyzed by thin layer chromatography and autoradiography. There was no detectable \(^{32}\)P labeling of the phosphatidylinositol 4-monophosphate or phosphatidylinositol 4,5-bisphosphate forms, suggesting that the endogeneous phosphatidylinositol was not phosphorylated by the catalytic subunit of the cAMP-dependent protein kinase.

Reconstitution of the Cardiac Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase.—To determine the optimal conditions for reconstitution of the cardiac SR Ca\(^{2+}\)-ATPase, various detergents and ratios of detergent/ATPase as well as various phospholipids and ratios of phospholipid/ATPase were tested. Triton X-
100, deoxycholate, and cholate were used to solubilize the purified Ca\(^{2+}\)-ATPase preparation. When deoxycholate was used, the reconstituted vesicles did not exhibit any Ca\(^{2+}\) uptake activity. When Triton X-100 was used, the reconstituted vesicles exhibited Ca\(^{2+}\) uptake activities of 210 nmol/mg/min, while when cholate was used the Ca\(^{2+}\) uptake activities were 466 nmol/mg/min. Thus, among the three detergents used, cholate appeared the most suitable for solubilization of the Ca\(^{2+}\)-ATPase preparation. Subsequently, the optimal ratios of cholate/Ca\(^{2+}\)-ATPase and of phospholipids/Ca\(^{2+}\)-ATPase were determined. The amount of cholate added to the purified Ca\(^{2+}\)-ATPase, in the initial solubilization step, was varied to yield a ratio of 0.5–4 cholate/ATPase, while the amounts of Ca\(^{2+}\)-ATPase and phospholipids were kept constant (lipid/protein ratio of 40). It was found that the optimal ratio (w/w) of cholate/protein was 2 (Fig. 2). Subsequently, the amounts of cholate and protein were fixed at a ratio of 2, and the ratio of lipid/protein (w/w) was varied between 20 and 160. The optimal rate of Ca\(^{2+}\) uptake (550 nmol/mg/min) was obtained by vesicles reconstituted in a lipid/protein ratio of 80 (data not shown). In control experiments, the reconstituted vesicles did not exhibit any detectable Ca\(^{2+}\) uptake activity, when ATP was omitted from the reaction mixture. Furthermore, liposomes, which were processed in parallel with reconstituted vesicles, did not show any Ca\(^{2+}\) uptake activity.

To determine the most appropriate phospholipid for reconstitution of the Ca\(^{2+}\)-ATPase into liposomes, phosphatidylcholine, phosphatidylethanolamine, phosphatidylyserine, or mixtures of phospholipids, similar to those reported to be present in canine cardiac SR (46), such as phosphatidylcholine/phosphatidylethanolamine (7:2) or phosphatidylcholine/phosphatidylethanolamine/phosphatidylinositol/phosphatidylserine (7:2:2:1), were used to make liposomes. The rates of Ca\(^{2+}\) uptake in reconstituted vesicles were similar when any one of the phospholipids (except phosphatidylyserine) or the mixtures of phospholipids were used. When phosphatidylyserine was used for reconstitution of the Ca\(^{2+}\)-ATPase, the rates of Ca\(^{2+}\) uptake were less than 10% of the levels obtained with the reconstituted vesicles containing any one of the phospholipids or mixtures of phospholipids. Since there were no differences between the other phospholipids or mixtures of phospholipids, phosphatidylcholine was chosen as the phospholipid to be used in the reconstitution experiments.

Proteoliposomes were obtained by the freeze-thaw sonication and column-centrifugation methods, using a mixture of cholate/Ca\(^{2+}\)-ATPase/phosphatidylcholine at a ratio of 2:1:80 (w/w). Formation of vesicles was verified by electron microscopy (Fig. 3). The liposomes were heterogeneous and tended to aggregate (Fig. 3A). Some of the vesicles appeared to contain non-membranous lipid material, and the outlines of the membranes were not distinct. This might be partially due to an oblique plane of sectioning. The reconstituted vesicles with the Ca\(^{2+}\)-ATPase (Fig. 3B) or the Ca\(^{2+}\)-ATPase and phospholamban (Fig. 3, C and D) were much less aggregated than the liposomes (Fig. 3A), which did not contain any protein. Furthermore, the vesicles did not appear to be as spherical as the pure liposomes, which may be a result of incorporation of the purified Ca\(^{2+}\)-ATPase into the lipid bi-
Reconstituted vesicles with the Ca\textsuperscript{2+}-ATPase and phospholamban (Fig. 3, C and D) were not distinguishable from the reconstituted vesicles with the Ca\textsuperscript{2+}-ATPase alone (Fig. 3B). These vesicles were small and irregularly shaped, some of which clearly formed bilayers (Fig. 3, C and D).

These reconstituted vesicles were capable of ATP-dependent oxalate-facilitated Ca\textsuperscript{2+} transport at a rate of 700 nmol/mg/min compared with native SR, which exhibited a rate of 800 nmol/mg/min. The time course of Ca\textsuperscript{2+} uptake by reconstituted vesicles, at various amounts of protein, is shown in Fig. 4. Calcium uptake increased fairly rapidly with time and amount of proteoliposomes. The rates of Ca\textsuperscript{2+} uptake appeared to be linear up to 1 min at various protein concentrations (0.8–24 \mu g/ml). Calcium uptake rates by reconstituted vesicles were also measured at various pH values, and the maximal rate of Ca\textsuperscript{2+} uptake was observed at pH 7.4 (data not shown).

The initial rates of Ca\textsuperscript{2+} uptake and the Ca\textsuperscript{2+}-ATPase activity by reconstituted vesicles were assayed at various [Ca\textsuperscript{2+}] (Fig. 5). The concentration of calcium yielding half-maximal activation of the Ca\textsuperscript{2+}-ATPase activity (EC\textsubscript{50}) was 0.053 ± 0.002 (n = 4) \mu M Ca\textsuperscript{2+} for the reconstituted vesicles compared with 0.63 ± 0.04 (n = 7) \mu M for native cardiac SR membranes. Similarly, the EC\textsubscript{50} value for Ca\textsuperscript{2+} uptake by the reconstituted vesicles was 0.047 ± 0.003 (n = 4) \mu M Ca\textsuperscript{2+}.

To determine whether the purified proteins were incorporated into the liposome vesicles, indirect immunogold labeling of reconstituted vesicles was performed. The affinity-purified polyclonal antibody, raised against the purified cardiac phospholamban, or a polyclonal antibody, raised against a synthetic peptide (amino acids 192–205) of the cardiac SR Ca\textsuperscript{2+}-ATPase, was used as the primary probe. Liposomes reconstituted with the cardiac SR Ca\textsuperscript{2+}-ATPase and the purified phospholamban were adsorbed to nitrocellulose, treated with the affinity-purified phospholamban antibody (Fig. 6B) or the

![Fig. 4: Time course of Ca\textsuperscript{2+} uptake by reconstituted vesicles.](image)

![Fig. 5: Ca\textsuperscript{2+} dependence of Ca\textsuperscript{2+} uptake and of Ca\textsuperscript{2+}-ATPase activity by the reconstituted vesicles.](image)

![Fig. 6: Immunogold-labeled proteoliposomes.](image)
Ca\(^{2+}\)-ATPase peptide antibody (Fig. 6C), and then treated with the secondary goat anti-rabbit/gold antibodies. The number of gold particles associated with the vesicles and the labeling pattern with primary antibodies suggested that the Ca\(^{2+}\)-ATPase and the phospholamban were associated with the membranes (Fig. 6, B and C). The outlines of the vesicles were not as distinct in these preparations as they were in the thin sections of the pelleted membranes (Fig. 3). This was possibly the result of subjecting the vesicles, which were reconstituted at a very low protein to lipid ratio, to the fairly lengthy incubation time necessary for immunolabeling. As a control, liposomes reconstituted with the cardiac SR Ca\(^{2+}\)-ATPase and without phospholamban were treated with the affinity-purified phospholamban antibody (Fig. 6A). There was an extremely low level of labeling associated with this preparation, which might suggest that a tiny amount of phospholamban remained associated with the purified Ca\(^{2+}\)-ATPase, but this was below the detection limits of immunoblot techniques.

The Effect of Phospholamban on the Cardiac Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase in Reconstituted Vesicles—Reconstitution of the Ca\(^{2+}\)-ATPase with phospholamban, isolated in the presence of N-octylglucoside, resulted in 20% inhibition of the Ca\(^{2+}\) uptake rates at pCa 6.0 compared with proteoliposomes prepared in the absence of phospholamban (Table I). When the proteoliposome preparation was phosphorylated by the catalytic subunit of the Ca\(^{2+}\)-ATPase, the initial rates of Ca\(^{2+}\) uptake were similar to those of reconstituted vesicles containing the Ca\(^{2+}\)-ATPase alone. In control experiments the reconstituted Ca\(^{2+}\)-ATPase alone was incubated in the presence of the catalytic subunit of the Ca\(^{2+}\)-ATPase, ATP, and conditions identical to those used for phosphorylation. There was no effect compared to the rates of vesicles with the Ca\(^{2+}\)-ATPase alone. These findings suggest that phospholamban in the dephosphorylated state is an inhibitor of the Ca\(^{2+}\) pump, and upon phosphorylation of phospholamban this inhibition may be relieved. However, since the extent of inhibition was relatively small, another phospholamban preparation was used to confirm these findings.

Phospholamban was purified by gel filtration, as described previously by Bidlack et al. (17), except that cholate was used to elute the protein bound to the Sephadex column. This phospholamban preparation also resulted in 20% inhibition of the initial rates of Ca\(^{2+}\) uptake at pCa 6.0 (566 nmol Ca\(^{2+}\)/

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**TABLE I**

Reconstitution of Ca\(^{2+}\)-ATPase with phospholamban isolated in N-octylglucoside

<table>
<thead>
<tr>
<th>Protoliposome preparation</th>
<th>Ca(^{2+}) uptake rates</th>
<th>%</th>
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<tbody>
<tr>
<td>Ca(^{2+})-ATPase</td>
<td>717 ± 23</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>Ca(^{2+})-ATPase + PLB</td>
<td>574 ± 7</td>
<td>80 ± 1</td>
</tr>
<tr>
<td>Ca(^{2+})-ATPase + P + PLB</td>
<td>681 ± 16</td>
<td>95 ± 1</td>
</tr>
<tr>
<td>Ca(^{2+})-ATPase (+ catalytic subunit + ATP)</td>
<td>746 ± 16</td>
<td>104 ± 2</td>
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**DISCUSSION**

This study presents the successful reconstitution of the Ca\(^{2+}\)-ATPase, purified from cardiac sarcoplasmic reticulum (SR), into phosphatidylcholine liposomes. The rate of Ca\(^{2+}\) uptake by the reconstituted vesicles (700 nmol/mg/min) was compatible with the rate of Ca\(^{2+}\) uptake by native cardiac SR vesicles (800 nmol/mg/min) and by reconstituted vesicles containing the skeletal SR Ca\(^{2+}\)-ATPase (57). The reconstitution was achieved after removal of Triton X-100 from the purified Ca\(^{2+}\)-ATPase by Bio-Beads SM-2 and subsequent solubilization by cholate. The freeze-thaw sonication procedure, previously used for the reconstitution of the skeletal SR Ca\(^{2+}\)-ATPase, was employed. When optimal conditions for reconstitution of the two types of ATPase were compared, the cholate/Ca\(^{2+}\)-ATPase/phosphatidylcholine ratio was 2:1:80 for the cardiac enzyme and 4.5:1:40 for the skeletal enzyme. The optimal pH for the proteoliposome formation was 7.0–
7.2 for the cardiac SR Ca\textsuperscript{2+}-ATPase and 7.2–7.5 for the skeletal SR Ca\textsuperscript{2+}-ATPase. However, both reconstituted Ca\textsuperscript{2+}-ATPases exhibited maximal rates of Ca\textsuperscript{2+} uptake at pH 7.4. Successful reconstitution of the cardiac SR Ca\textsuperscript{2+}-ATPase did not require the presence of potassium oxalate in the buffers, used throughout the freeze-thaw sonication procedure, while this was essential for the skeletal SR Ca\textsuperscript{2+}-ATPase.

The Ca\textsuperscript{2+}-ATPase in native cardiac SR membranes is regulated by the status of phospholamban phosphorylation, and evidence from several laboratories indicates that the effects of β-adrenergic agents on cardiac muscle may be mediated at least in part through phospholamban. However, the mechanism by which phospholamban modulates the Ca\textsuperscript{2+}-ATPase in SR remains unclear. Regulation may be mediated via a direct physical interaction between phospholamban and the Ca\textsuperscript{2+}-ATPase or via phospholamban through an intermediary component. To understand more clearly this regulatory mechanism, phospholamban and the Ca\textsuperscript{2+}-ATPase were purified and reconstituted into liposomes. Reconstitution of the Ca\textsuperscript{2+}-ATPase with phospholamban resulted in inhibition of the initial rates of Ca\textsuperscript{2+} uptake assayed at pCa 6.0. The inhibition was relieved by phosphorylation of phospholamban in the reconstituted vesicles by the catalytic subunit of the cAMP-dependent protein kinase. These results indicate that the Ca\textsuperscript{2+}-ATPase in native cardiac SR membranes may be suppressed by phospholamban, in agreement with previous studies (26, 45), and that phosphorylation of phospholamban relieves this suppression. However, the inhibitory effect of phospholamban on the Ca\textsuperscript{2+}-ATPase appeared to be relatively small. This small inhibition may be due to the orientation of phospholamban with respect to the Ca\textsuperscript{2+} pump in the reconstituted vesicles or to an intermediary component required to exert the effect of phospholamban in native SR membranes, which may be missing from the purified preparation. To determine whether the hydrophilic portion of phospholamban, which contains the phosphorylation sites, could affect the reconstituted Ca\textsuperscript{2+}-ATPase, a peptide corresponding to amino acids 1–25 of the phospholamban (47) was synthesized. Addition of this peptide to proteoliposomes containing the Ca\textsuperscript{2+}-ATPase was associated with inhibition of the initial rates of Ca\textsuperscript{2+} uptake at pCa 6.0. The degree of inhibition by the synthetic peptide was similar to that obtained with phospholamban, and phosphorylation of the peptide relieved the inhibitory effect on the Ca\textsuperscript{2+} pump. Our findings with phospholamban and the synthetic peptide were obtained under optimal assay conditions for the Ca\textsuperscript{2+} pump, and it is not presently known whether the regulation exerted affects the maximal velocity or the affinity of the Ca\textsuperscript{2+} pump for calcium or both.

The phospholamban peptide, synthesized in this study, is hydrophilic (47), and this peptide may inhibit Ca\textsuperscript{2+} uptake in reconstituted vesicles by its direct interaction with the cytosolic portion of the Ca\textsuperscript{2+}-ATPase. We have recently suggested that amino acids 1–14 of phospholamban appear capable of forming an amphipathic α-helix with a hydrophobic surface on one side (48). This surface could possibly interact with a reciprocal hydrophilic surface on another protein, possibly the Ca\textsuperscript{2+}-ATPase, and such a hydrophilic interaction could be quite strong in hydrophilic environments. The opposite side of the α-helix of phospholamban is very hydrophilic and could interact with the aqueous environments. This hydrophilic surface also contains four positively charged groups, which may interact with the negatively charged phosphate groups when phospholamban becomes phosphorylated. Such an interaction could weaken the hydrophilic interaction with another protein such as the Ca\textsuperscript{2+}-ATPase, thereby decreasing the inhibition of the Ca\textsuperscript{2+}-ATPase by phospholamban (48). Although our findings do not exclude the possibility that regulation of Ca\textsuperscript{2+} uptake by phospholamban may be mediated by the membrane-spanning portion of the molecule (residues 26–52 of phospholamban), which may form Ca\textsuperscript{2+} channels (49, 50), or by other polypeptides and factors, we strongly suggest that regulation may be at least partially mediated by a direct physical interaction of the hydrophilic portion of phospholamban with the Ca\textsuperscript{2+} pump. This suggestion is supported by previous findings on the stimulation of the Ca\textsuperscript{2+}-ATPase by mild trypsin treatment, which resulted in loss of the phosphorylation sites on phospholamban (45) and by our recent studies, using chemical cross-linkers (48).

In summary, our findings with the reconstituted vesicles indicate that dephosphorylated phospholamban may exert an inhibitory effect on the Ca\textsuperscript{2+}-ATPase and upon phospholamban phosphorylation the inhibition is relieved. However, the functional unit of phospholamban (monomer versus oligomer) as well as the stoichiometry of phospholamban to Ca\textsuperscript{2+}-ATPase, required for regulation of the Ca\textsuperscript{2+} pump, remain to be determined.

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REFERENCES

Regulation of Cardiac SR Ca\textsuperscript{2+}-ATPase

Functional reconstitution of the cardiac sarcoplasmic reticulum Ca2(+) -ATPase with phospholamban in phospholipid vesicles.
H W Kim, N A Steenaart, D G Ferguson and E G Kranias


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