Molecular Mechanism of Regulation of Ca\(^{2+}\) Pump ATPase by Phospholamban in Cardiac Sarcoplasmic Reticulum

**EFFECTS OF SYNTHETIC PHOSPHOLAMBAN PEPTIDES ON Ca\(^{2+}\) PUMP ATPase**

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The molecular mechanism of the regulation of Ca\(^{2+}\) pump ATPase by phospholamban in cardiac sarcoplasmic reticulum was examined using synthetic peptides of phospholamban and purified Ca\(^{2+}\) pump ATPase from cardiac sarcoplasmic reticulum. The phospholamban monomer of 52 amino acid residues contains two distinct domains, the cytoplasmic (amino acids 1–30) and the transmembrane (amino acids 31–52) domains. The peptide corresponding to the amino acids 1–31 of phospholamban (PLN 1–31) decreased the V\(_{\text{max}}\) of the Ca\(^{2+}\)-dependent ATPase activity in a dose-dependent manner, while it had no effect on the affinity of the ATPase for Ca\(^{2+}\) (K\(_{Ca}\)). On the other hand, the peptide corresponding to the amino acids 28–47 of phospholamban (PLN 28–47) increased the K\(_{Ca}\) from 0.52 to 1.33 \(\mu\)M without significant change in the V\(_{\text{max}}\) value when reconstituted into vesicles with the ATPase. Essentially the same results as PLN 28–47 were obtained with the peptide corresponding to the amino acids 8–47 of phospholamban (PLN 8–47). The inhibitory effects of PLN 1–31 and PLN 8–47 on the ATPase were reversed by cAMP-dependent phosphorylation of the peptides (Ser\(^{16}\)). These results indicate that phospholamban suppresses Ca\(^{2+}\) pump ATPase at two different sites, the cytoplasmic domain for V\(_{\text{max}}\) and the transmembrane domain for K\(_{Ca}\), and that cAMP-dependent phosphorylation de-suppresses these inhibitory effects on the ATPase.

Phospholamban is an integral membrane protein of cardiac SR,\(^1\) which modulates the Ca\(^{2+}\) pumping function of cardiac SR (Tada and Katz, 1982; Tada and Kodama, 1989). When phosphorylated by cAMP-dependent protein kinase (Tada et al., 1975) or by Ca\(^{2+}\)-calmodulin-dependent protein kinase (Le Peuch et al., 1979), phospholamban stimulates Ca\(^{2+}\) pump ATPase, decreasing the K\(_{Ca}\) (Tada et al., 1974, 1983; Kirchberger et al., 1974). Phospholamban has been purified (Inui et al., 1985; Jones et al., 1985), and its primary structure has been determined by cDNA cloning and sequencing (Fujii et al., 1987). It consists of five identical monomers (Wegener and Jones, 1984; Fujii et al., 1986), and the monomer of 52 amino acid residues contains two distinct domains, the hydrophilic N-terminal domain (domain I) and the hydrophobic C-terminal domain (domain II) (Fujii et al., 1986; Simmons et al., 1986; Tada et al., 1988). The first 20 residues from the N-terminal (domain IA) are predicted to form an \(\alpha\)-helical structure containing the phosphorylation sites serine 16 and threonine 17, catalyzed by cAMP- and calmodulin-dependent protein kinases, respectively. The next 10 residues are less structured (domain IB). The last 22 residues form a hydrophobic transmembrane domain (domain II). A line of evidence suggests that phospholamban acts as a suppressor of Ca\(^{2+}\) pump and that phosphorylation of phospholamban de-suppresses the inhibitory effects of phospholamban on the Ca\(^{2+}\) pump (Kirchberger et al., 1986; Inui et al., 1986; Suzuki and Wang, 1986). Recently, we demonstrated a direct protein-protein interaction between phospholamban and Ca\(^{2+}\) pump ATPase, which is diminished by phosphorylation of phospholamban (James et al., 1989).

In the present study, we examined the effects of synthetic phospholamban peptides on purified Ca\(^{2+}\) pump ATPase from cardiac SR. We have obtained evidence that phospholamban exerts inhibitory actions on Ca\(^{2+}\) pump ATPase at two different sites of phospholamban molecule, and that these two sites modulate Ca\(^{2+}\) pump ATPase in different manners.

**EXPERIMENTAL PROCEDURES**

*Materials—[\(\gamma\)-\(^{32}\)P]ATP was obtained from Du Pont-New England Nuclear. cAMP, cAMP-dependent protein kinase, ATP Tris salt, phosphatidylcholine from egg yolk (type XIII-E), phosphoenolpyruvate, and Kemptide were from Sigma; pyruvate kinase from swine myocardium was from Dojin Chemicals (Tokyo, Japan); and A 23187 was from Calbiochem (La Jolla, CA). Affi-Gel 10 was obtained from Bio-Rad. Hybridomas producing monoclonal antibody against phospholamban (mAb A1) were the generous gift of Dr. J. H. Wang of the University of Calgary, Canada. The antibody was purified as described previously (Kimura et al., 1991).*

Preparation of Cardiac SR Vesicles and Ca\(^{2+}\) Pump ATPase—Cardiac SR vesicles were isolated from dog heart ventricles according to the procedure of Chamberlain et al. (1988). Ca\(^{2+}\) pump ATPase was purified from cardiac microsomes by the method of Van Winkle et al. (1978). The purified Ca\(^{2+}\) pump ATPase was 94% pure and free from phospholamban when estimated from a densitometric scan of SDS-polyacrylamide gel by Microscan 1000 (Technology Resources, Nashville, TN). The phospholipid content in the purified Ca\(^{2+}\) pump ATPase was 1.67 ± 0.14 \(\mu\)mol of P/mg of protein (mean ± S.D.; n = 4), when estimated by the method of Bartlett (1959). As reported by Van Winkle et al. (1978), the purified protein is in leaky vesicles of phospholipids.
Synthesis and Purification of Phospholamban Peptides—Three peptides (Fig. 1) were synthesized by the stepwise solid phase method (Erickson et al., 1976) by using an Applied Biosystems 430A automated peptide synthesizer, which was operated following the program provided by the manufacturer. t-Butoxycarbonyl-amino acid derivatives with benzyl-based side chain protecting groups were introduced via 1-hydroxybenzotriazole and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. Valine, isoleucine, and the residues after 20 cycles were incorporated by double couplings. After completion of peptide chain assembly, the N-terminal t-butoxycarbonyl group was cleaved and replaced with an acetyl group by using acetic anhydride. The protected peptide resin was treated with anhydrous hydrogen fluoride (Sakakibara et al., 1987). After removal of the terminal t-butoxycarbonyl group, it was cleaved from the resin and precipitated by adding ether. Crude peptides were purified by ion-exchange and gel filtration chromatography, which were eluted with buffers containing aqueous chloroform/methanol as described by Boyot et al. (1989).

The purified peptides were subjected to analytical high performance liquid chromatography, amino acid analysis, and fast atom bombardment mass spectrometry. 

Incorporation of Ca" Pump ATPase and Hydrophobic Phospholamban Peptide into Liposomes—Hydrophobic peptide, PLN 28-47 (0.3 to -2.4 mg) or PLN 8-47 (1.2 mg), and phosphatidylcholine (0.03 to -0.24 mg) were solved in 2 ml of chloroform/methanol (1:1), and then the mixture was evaporated in vacuum for 1 h at room temperature. After the evaporated sample was suspended in 606 pl of 10 mM Tris-maleate, pH 6.8, and sonicated at 4 °C for 15 s with a probe-type sonicator (Heat Systems-Ultrasonics, Model W-850, Farmingdale, NY) at dial 2. Purified Ca" pump ATPase (44 pl of 5.91 mg of protein/ml) was then added to the peptide-containing vesicles. Fusion of the peptide-containing vesicles with the leaky vesicles of Ca" pump ATPase was carried out by the freeze-thaw-sonication method (Kasahara and Hinkle, 1977). The sample was frozen in liquid N₂, thawed at 25 °C, and then sonicated as described above. Freeze-thaw-sonication was repeated three times. In the control experiments, the same procedure was carried out without peptides.

Measurement of the Ca"-dependent ATPase Activity of Cardiac SR Vesicles and Purified Ca" Pump ATPase—The Ca"-dependent ATPase activity of cardiac SR vesicles, purified Ca" pump ATPase, and recombinant proteoliposomes were assayed using the linked enzyme method as described previously (Tada et al., 1988) with minor modification. Purified Ca" pump ATPase (40 µg of protein/ml) or cardiac SR vesicles (50 µg of protein/ml) were incubated at 25 °C for 3 min in the medium (final volume of 650 µl) containing 10 mM Tris-maleate, pH 6.8, 100 mM KCl, 1 mM MgCl₂, 1 mM ATP, 0.2 mg of cAMP-dependent protein kinase, and 10 mM Tris-maleate, pH 6.8, in a total volume of 650 µl. After incubation at 30 °C for 5 min, the sample was centrifuged at 90,000 rpm for 15 min at 4 °C in a Beckman TL 100 centrifuge using a TLA 100 rotor. The pellet was resuspended in 650 µl of 10 mM Tris-maleate, pH 6.8, and subjected to the ATPase assay.

For phosphorylation of hydrophilic peptide, PLN 1-31 (2.6 mg) was incubated with or without 13 µM cAMP in the medium containing 100 mM KCl, 1 mM MgCl₂, 1 mM ATP, 28 mg of cAMP-dependent protein kinase, and 10 mM Tris-maleate, pH 6.8. In a total volume of 650 µl containing 100 µM CAMP, 100-2 rotor. The pellet was resuspended in 650 µl of 10 mM Tris-maleate, pH 6.8, and subjected to the ATPase assay.

For phosphorylation of PLN 8-47, the peptide (1.17 mg) was first incorporated into liposomes of phosphatidylcholine (0.117 mg). The peptide containing vesicles was incubated at 30 °C for 10 min with or without 5.9 µM CAMP in the medium containing 100 mM KCl, 1 mM MgCl₂, 1 mM ATP, 11.7 mg of CAMP-dependent protein kinase, and 10 mM Tris-maleate, pH 6.8, in a total volume of 650 µl. The sample was centrifuged at 90,000 rpm for 15 min at 4 °C in a Beckman TL 100 centrifuge using a TLA 100-2 rotor. The pellet was resuspended with 10 mM Tris-maleate, pH 6.8. Washing was repeated five times, and the final pellet was resuspended in the same buffer to give the peptide concentration of 1.8 mg/ml. The phosphorylated peptide was extracted with Ca" pump ATPase (0.4 mg of protein/ml) at 25 °C for 10 min, and an aliquot was subjected to the ATPase assay.

To measure the amount of phosphorylation, the same phosphorylation conditions were used as described above with [γ-³²P]ATP instead of cold ATP. The amount of PLN 1-31 phosphorylation was estimated by the radioactivity of the mAb A1 affinity gel which bound PLN 1-31. The amount of PLN 8-47 phosphorylation was estimated by the radioactivity of the PLN 8-47-containing vesicles.

Miscellaneous Methods—Protein concentration was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis was performed in the system of Laemmli (1970) (12.5% gel with 4.5% stacking gel), employing mini-slab gel electrophoresis apparatus (gel size, 70 mm in length and 1 mm in thickness).

RESULTS

Ca"-dependent Profile of the ATPase Activity of Cardiac SR Vesicles and Purified Ca" Pump ATPase—The Ca"-dependent profiles of the ATPase activity of cardiac SR vesicles were shown in Fig. 2. As reported previously (Tada et al., 1974, 1983), the Kₘ was decreased from 1.31 to 0.70 µM by CAMP-dependent protein kinase treatment. In addition, a significant increase in Vₘₐₓ from 362.4 to 495.0 nmol/mg/min was observed upon CAMP-dependent protein kinase treatment of the vesicles. Purified Ca" pump ATPase from cardiac
Phospholamban Peptide

Effects of Synthetic Phospholamban Peptides on Purified Ca²⁺ Pump ATPase

Three peptides used in this study are shown in Fig. 1. We first examined the effects of PLN 1–31 on purified Ca²⁺ pump ATPase. When the purified ATPase was incubated with various amounts of PLN 1–31, the Ca²⁺-dependent ATPase activity at the free Ca²⁺ concentration of 1.25 μM decreased in a dose-dependent manner (Fig. 3, inset). Maximal inhibition (37%) was observed at peptide concentrations above 0.4 mg/ml. The Ca²⁺-dependent profile of the ATPase activity was determined with various amounts of peptide (Fig. 3). PLN 1–31 at the concentration of 0.4 mg/ml decreased the V₅₀ of 635.0 ± 28.3 nmol/mg-min (n = 4) to 420.2 ± 28.3 nmol/mg-min (n = 4) at 25°C. The results represent the mean ± S.D. of four different experiments. *p < 0.05 when compared with the control value using Student’s t test for unpaired variates.

The reconstitution was carried out by fusing the ATPase-containing vesicles with liposomes.

The effects of PLN 28–47 and PLN 8–47 on purified Ca²⁺ pump ATPase were next examined using the vesicles reconstituted with the ATPase and the peptides. The reconstitution was performed by fusing the peptide-containing vesicles and the ATPase-containing vesicles. PLN 28–47 decreased the affinity of the ATPase for Ca²⁺ in a dose-dependent manner (Fig. 4). When the vesicles were reconstituted at the molar ratio of 100:1 for the peptide to the ATPase, the Kᵥ of the ATPase activity increased from 0.52 to 1.33 μM. However, the peptide had no significant effect on the V₅₀ at this molar ratio of peptide to ATPase (Table I). The peptide at higher molar ratios to the ATPase than 100:1 decreased the V₅₀ with additional increase in the Kᵥ (Fig. 4). Essentially the same results were obtained with PLN 8–47, which contains phosphorylation sites (Fig. 5, Table I). When the peptide-

TABLE I
Effects of synthetic phospholamban peptides on purified Ca²⁺ pump ATPase

<table>
<thead>
<tr>
<th>Peptide</th>
<th>V₅₀</th>
<th>Kᵥ</th>
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<tbody>
<tr>
<td>Control</td>
<td>635.0 ± 28.3</td>
<td>0.49 ± 0.02</td>
</tr>
<tr>
<td>+ PLN 1–31</td>
<td>574.8 ± 28.5</td>
<td>0.57 ± 0.16</td>
</tr>
<tr>
<td>+ PLN 1–31</td>
<td>420.2 ± 28.3</td>
<td>0.51 ± 0.05</td>
</tr>
<tr>
<td>Control’</td>
<td>634.5 ± 112.4</td>
<td>0.52 ± 0.02</td>
</tr>
<tr>
<td>+ PLN 28–47</td>
<td>675.7 ± 43.5</td>
<td>1.33 ± 0.30</td>
</tr>
<tr>
<td>Control’</td>
<td>624.7 ± 63.6</td>
<td>0.51 ± 0.04</td>
</tr>
<tr>
<td>+ PLN 8–47</td>
<td>612.1 ± 40.2</td>
<td>1.14 ± 0.21</td>
</tr>
</tbody>
</table>

V₅₀ and Kᵥ were determined from a Ca²⁺-dependent profile of the ATPase activity using the double-reciprocal plot of Lineweaver and Burk.

p < 0.05 when compared with the control value using Student’s t test for unpaired variates.

The reconstitution was carried out by fusing the ATPase-containing vesicles with liposomes.

The reconstitution was performed by fusing the peptide-containing vesicles and the ATPase-containing vesicles. PLN 28–47 decreased the affinity of the ATPase for Ca²⁺ in a dose-dependent manner (Fig. 4). When the vesicles were reconstituted at the molar ratio of 100:1 for the peptide to the ATPase, the Kᵥ of the ATPase activity increased from 0.52 to 1.33 μM. However, the peptide had no significant effect on the V₅₀ at this molar ratio of peptide to ATPase (Table I). The peptide at higher molar ratios to the ATPase than 100:1 decreased the V₅₀ with additional increase in the Kᵥ (Fig. 4). Essentially the same results were obtained with PLN 8–47, which contains phosphorylation sites (Fig. 5, Table I). When the peptide-

SR showed much lower Kᵥ (0.51 μM) than that of cardiac SR vesicles (Fig. 2). The CAMP-dependent kinase treatment of the purified ATPase change neither Kᵥ nor V₅₀. Phospholamban has been thought to increase the V₅₀ of purified ATPase change neither Kᵥ nor V₅₀. Phospholamban has been thought to increase the V₅₀ of cardiac SR vesicles and three for the purified ATPase. The V₅₀ and Kᵥ of cardiac SR vesicles were 362.4 ± 23.3 nmol/mg-min and 1.31 ± 0.28 μM (n = 5) for controls, and 496.0 ± 39.8 nmol/mg-min and 0.70 ± 0.09 μM (n = 5) for phosphorylation, when determined by double-reciprocal plot. The CAMP-dependent protein kinase treatment of the purified ATPase were fased ± 34.3 nmol/mg-min and 0.51 ± 0.03 μM (n = 3) for controls, 671.6 ± 42.7 nmol/mg-min and 0.48 ± 0.05 μM (n = 3) after the protein kinase treatment.

The same results were obtained when phosphatidylcholine containing vesicles with liposomes.

The reconstitution was performed by fusing the peptide-containing vesicles and the ATPase-containing vesicles. PLN 28–47 decreased the affinity of the ATPase for Ca²⁺ in a dose-dependent manner (Fig. 4). When the vesicles were reconstituted at the molar ratio of 100:1 for the peptide to the ATPase, the Kᵥ of the ATPase activity increased from 0.52 to 1.33 μM. However, the peptide had no significant effect on the V₅₀ at this molar ratio of peptide to ATPase (Table I). The peptide at higher molar ratios to the ATPase than 100:1 decreased the V₅₀ with additional increase in the Kᵥ (Fig. 4). Essentially the same results were obtained with PLN 8–47, which contains phosphorylation sites (Fig. 5, Table I). When the peptide-

Fig. 2. Ca²⁺-dependent profiles of the ATPase activity of cardiac SR vesicles and purified Ca²⁺ pump ATPase. Cardiac SR vesicles (500 μg of protein/ml) (●) or the purified ATPase (400 μg of protein/ml) (△) were preincubated with 0.3 mg/ml CAMP-dependent protein kinase in the presence of 1 μM CAMP, and then the samples were subjected to the ATPase assay as described under "Experimental Procedures." In the control experiments, cardiac SR vesicles (●) or the purified ATPase (△) were preincubated without CAMP. Results represent the mean ± S.D. of five different experiments for cardiac SR vesicles and three for the purified ATPase. The V₅₀ and Kᵥ of cardiac SR vesicles were 362.4 ± 23.3 nmol/mg-min and 1.31 ± 0.28 μM (n = 5) for controls, and 496.0 ± 39.8 nmol/mg-min and 0.70 ± 0.09 μM (n = 5) for phosphorylation, when determined by double-reciprocal plot. The CAMP-dependent protein kinase treatment of the purified ATPase were fased ± 34.3 nmol/mg-min and 0.51 ± 0.03 μM (n = 3) for controls, 671.6 ± 42.7 nmol/mg-min and 0.48 ± 0.05 μM (n = 3) after the protein kinase treatment.

Fig. 3. Effects of PLN 1–31 on purified Ca²⁺ pump ATPase. Purified Ca²⁺ pump ATPase was preincubated with (○) or without (●) PLN 1–31 at 25°C for 10 min, and then the samples were subjected to the ATPase assay. The final concentration of the purified ATPase in the assay medium was 40 μg of protein/ml, and those of PLN 1–31 were 0.2 mg/ml (○) and 0.4 mg/ml (△). The results represent the mean ± S.D. of four different experiments. Inset, Ca²⁺ pump ATPase (0.4 mg of protein/ml) was preincubated with various concentrations (0.6 to −5.5 mg/ml) of PLN 1–31 at 25°C for 10 min, and an aliquot was subjected to the ATPase assay. The Ca²⁺-dependent ATPase activity at 1.25 μM free Ca²⁺ was measured as a function of the final peptide concentration in the assay medium.
ATPase-Since PLN 1-31 and PLN 8-47 have a CAMP-phosphorylation were estimated to be 0.72 protein kinase in the presence of CAMP, the amounts of the ATPase. The effects of PLN 28-47 and PLN 8-47 on the inhibitory effect only on the peptide as described under "Experimental Procedures," and then the peptide and the ATPase were 50 (○), 100 (△), 200 (▼), and 400 (□). In the control (●), the reconstitution was carried out by fusing the ATPase-containing vesicles with liposomes. Data were expressed as the percent of maximal ATPase activity. The maximal ATPase activities at each molar ratio were as follows: ●, 642.0; ○, 684.0; △, 695.0; ▼, 477.5; □, 388.5 nmol/mg-min.

The reconstitution studies using purified Ca\textsuperscript{2+} pump ATPase from cardiac SR and synthetic peptides of phospholamban containing vesicles were incubated with the ATPase-containing vesicles without fusion by freeze-thaw-sonication, PLN 28-47 and PLN 8-47 did not show any inhibitory effects on the ATPase. The effects of PLN 28-47 and PLN 8-47 on the ATPase were compared with a hydrophobic peptide which is unrelated to the domain II of phospholamban. The artificial signal sequence, L8PL2-M5 (Yamamoto et al., 1990), had no effect on the $K_{Ca}$ but slightly decreased the $V_{max}$, when reconstituted with the ATPase at the molar ratio of 100:1 between the peptide and the ATPase (data not shown).

**Effects of Phosphorylated Peptides on Purified Ca\textsuperscript{2+} Pump ATPase**—Since PLN 1-31 and PLN 8-47 have a cAMP-dependent phosphorylation site, we examined the effects of phosphorylation of these peptides on Ca\textsuperscript{2+} pump ATPase. When the peptides were incubated with cAMP-dependent protein kinase in the presence of cAMP, the amounts of phosphorylation were estimated to be $0.72 \pm 0.10$ and $0.81 \pm 0.08$ mol of P/mol of peptide (mean ± S.D.; $n = 3$) for PLN 1-31 and PLN 8-47, respectively. Since PLN 1-31 had an inhibitory effect only on the $V_{max}$ (Fig. 3, Table I), the effect of phosphorylation on the ATPase was examined at 1.25 $\mu$M free Ca\textsuperscript{2+}, the level at which maximal ATPase activity is observed in the presence and absence of PLN 1–31. As shown in Table II, cAMP-dependent phosphorylation reversed the inhibitory effect of unphosphorylated PLN 1–31 on the ATPase activity. Upon phosphorylation, the ATPase activity was increased and was restored to the level of the ATPase alone.

The effect of phosphorylation of PLN 8–47 was examined at 0.5 $\mu$M free Ca\textsuperscript{2+}, at which level submaximal ATPase activity is obtained. Phosphorylation of the peptide reversed the inhibition of the ATPase activity caused by unphosphorylated PLN 8–47 (Table II). Furthermore, when the Ca\textsuperscript{2+}-dependent profile of the ATPase activity was compared between phosphorylated and unphosphorylated PLN 8–47, the shift of the curve to the right by unphosphorylated PLN 8–47 was reversed by cAMP-dependent phosphorylation of the peptide (Fig. 6). The $K_{Ca}$ with the phosphorylated peptide was almost the same as that of purified Ca\textsuperscript{2+} pump ATPase alone (Table III).

**DISCUSSION**

The reconstitution studies using purified Ca\textsuperscript{2+} pump ATPase from cardiac SR and synthetic peptides of phospholamban

<table>
<thead>
<tr>
<th>ATPase activity</th>
<th>nmol/mg-min</th>
<th>%</th>
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<tbody>
<tr>
<td>Control</td>
<td>445.0 ± 10.7</td>
<td>100.0 (n = 4)</td>
</tr>
<tr>
<td>+ PLN 1–31</td>
<td>282.0 ± 25.5′</td>
<td>63.3 ± 9.0′ (n = 3)</td>
</tr>
<tr>
<td>+ PLN 1–31 [P]</td>
<td>411.3 ± 37.9</td>
<td>92.4 ± 9.2 (n = 3)</td>
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<tr>
<td>Control</td>
<td>309.5 ± 41.4</td>
<td>100.0 (n = 4)</td>
</tr>
<tr>
<td>+ PLN 8–47</td>
<td>192.3 ± 17.8′</td>
<td>62.1 ± 5.8′ (n = 4)</td>
</tr>
<tr>
<td>+ PLN 8–47 [P]</td>
<td>275.3 ± 29.4</td>
<td>90.3 ± 10.1 (n = 4)</td>
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*p < 0.05 when compared with the control value using Student’s t test for unpaired variates.

**TABLE II**

Effects of phosphorylated peptides on purified Ca\textsuperscript{2+} pump ATPase

![Figure 4](image1)

**FIG. 4. Effects of PLN 28–47 on purified Ca\textsuperscript{2+} pump ATPase.** Ca\textsuperscript{2+} pump ATPase was reconstituted with various amounts of the peptide as described under "Experimental Procedures," and then the samples were subjected to the ATPase assay. The molar ratios between the peptide and the ATPase were 50 (○), 100 (△), 200 (▼), and 400 (□). In the control (●), the reconstitution was carried out by fusing the ATPase-containing vesicles with liposomes. Data were expressed as the percent of maximal ATPase activity. The results represent the mean ± S.D. of three different experiments.

![Figure 5](image2)

**FIG. 5. Effects of PLN 28–47 and PLN 8–47 on purified Ca\textsuperscript{2+} pump ATPase.** Ca\textsuperscript{2+} pump ATPase was reconstituted with PLN 28–47 (△) or PLN 8–47 (○) at the molar ratio of 100 between the peptide and the ATPase. The samples were then subjected to the ATPase assay. In the control experiments (● for PLN 28–47, ○ for PLN 8–47), the reconstitution was carried out by fusing the ATPase-containing vesicles with liposomes which contained the same amounts of phosphatidylcholine as the peptide-containing vesicles. The results represent the mean ± S.D. of three different experiments.

![Figure 6](image3)

**FIG. 6. Effects of phosphorylated PLN 8–47 on purified Ca\textsuperscript{2+} pump ATPase.** Ca\textsuperscript{2+} pump ATPase was reconstituted with unphosphorylated (○) or phosphorylated (△) PLN 8–47 at the molar ratio of 100 between the peptide and the ATPase, before being subjected to the ATPase assay. The ATPase activity was measured at 1.25 $\mu$M free Ca\textsuperscript{2+} for PLN 1–31 or 0.505 $\mu$M free Ca\textsuperscript{2+} for PLN 8–47. Values are the means ± S.D. from the number of separate experiments indicated in parentheses.
revealed that the actions of phospholamban in the regulation of cardiac SR Ca²⁺ pump ATPase are 2-fold: one is to decrease the V$_{\text{max}}$ of the ATPase activity (Fig. 3, Table I) and the other is to lower the affinity of the ATPase for Ca²⁺ (Figs. 4 and 5, Table I). The results from the peptides with different length indicate that the N-terminal region in the hydrophilic domain of phospholamban (domain I) decreases the V$_{\text{max}}$ and that the transmembrane domain (domain II) lowers the affinity for Ca²⁺. Incorporation of phosphate into the phosphorylation site (Ser⁸) in domain I reverses these two inhibitory effects on Ca²⁺ pump ATPase (Fig. 6, Tables II and III), probably inducing the conformational change of domain I and domain II. Thus, Ca²⁺ pumps in the cardiac SR membranes are suppressed by phospholamban, having higher Kₐ than that of purified Ca²⁺ pump ATPase and of skeletal muscle Ca²⁺ pump ATPase, which is devoid of phospholamban. Phosphorylation of phospholamban de-suppresses those two inhibitory effects on Ca²⁺ pump ATPase, increasing the V$_{\text{max}}$ and lowering the Kₐ of the ATPase.

A number of findings including the studies of reconstitution, mild trypsin digestion, and antibody treatment indicate that phospholamban lowers the affinity of Ca²⁺ pump ATPase for Ca²⁺ (Inui et al., 1986; Kirchberger et al., 1986; Suzuki and Wang, 1986). Although the reconstitution of cardiac SR Ca²⁺ pump has been tried by many investigators, previous attempts failed to reconstitute the shift of the Kₐ of the ATPase by phospholamban (Shamoo et al., 1985; Inui et al., 1986; Kim et al., 1990) except the co-expression of Ca²⁺ pump ATPase and phospholamban in COS-1 cells (Fujii et al., 1990). Even in the latter study, the magnitude of phosphorylation effect on Ca²⁺ uptake by the microsomes from the co-expressed cells was less than that observed in native cardiac SR vesicles. In the present study, we succeeded in demonstrating a shift of Kₐ in the reconstituted system using purified Ca²⁺ pump ATPase and the synthetic phospholamban peptides which contain the main part of domain II (Figs. 4 and 5, Table I). The reconstitution was carried out by fusing the leaky vesicles of the purified ATPase with the peptide-containing vesicles. Successful reconstitution was achieved at high weight ratio of the peptide to liposomes such as 10:1 in the peptide-containing vesicles, and at high molar ratio of the peptide to the ATPase such as 100:1 during the fusion. These conditions seem to allow Ca²⁺ pump ATPase and the hydrophobic peptide to efficiently associate with each other within one vesicle. Under those conditions, the reconstituted ATPase showed higher Kₐ almost equivalent to that of the cardiac SR membranes without changing the V$_{\text{max}}$ (Table I). The shift of the Kₐ observed with the peptide is not an artifact of the reconstitution procedure, because the ATPase reconstituted by the fusion of the ATPase-containing vesicles with liposomes did not show a shift of Kₐ (the control experiments in Figs. 4 and 5, Table I). The shift of Kₐ may be due to a direct interaction between the ATPase and phospholamban peptides, since the shift of Kₐ was not observed neither in the ATPase reconstituted with the artificial signal sequence (L8PL2-M8) nor in the mixture of the ATPase without the freeze-thaw-sonication treatment (data not shown). More importantly, phosphorylation of the peptide reversed a shift of Kₐ (Fig. 6, Tables II and III).

The present study clearly demonstrated that the synthetic peptide containing of domain I (PLN 1-31) decreases the V$_{\text{max}}$ of Ca²⁺ pump ATPase without any effects on the Ca²⁺ affinity and that phosphorylation of the peptide relieves the inhibitory effect of the unphosphorylated peptide (Table II). Similar findings were also reported by Kim et al. (1990) using the synthetic peptide corresponding to amino acid residues 1-25 of phospholamban. Thus, phospholamban may suppress Ca²⁺ pump ATPase by lowering the V$_{\text{max}}$ of the ATPase in the native SR membranes, and phosphorylation of phospholamban may reverse the inhibition. In fact, a statistically significant increase in the V$_{\text{max}}$ of the ATPase upon phosphorylation was observed in native cardiac SR vesicles (Fig. 2). When earlier studies were reevaluated in the light of the present result, a significant increase in the V$_{\text{max}}$ of Ca²⁺ uptake and ATPase activity of cardiac SR vesicles upon phospholamban phosphorylation was observed in a number of reports including ours (Tada et al., 1974, 1979, 1983; Hicks et al., 1979; Kirchberger et al., 1986).

Previously, we demonstrated a direct protein-protein interaction between phospholamban and Ca²⁺ pump ATPase using a cross-linking agent (James et al., 1989). The binding site appears to be located in the N-terminal region of domain I, because phospholamban was labeled by the cross-linker at Lys⁸. The binding of this region of phospholamban to Ca²⁺ pump ATPase might cause a decrease of the V$_{\text{max}}$ of the ATPase. The first 7 N-terminal amino acid residues of phospholamban seem to be important for this effect, since no significant decrease of the V$_{\text{max}}$ was observed with PLN 8-47. Interestingly, the phospholamban-binding site in Ca²⁺ pump ATPase molecule has been determined to be a region of the ATPase just C-terminal to the aspartyl residue that forms the acylphosphoprotein intermediate during its reaction cycle (James et al., 1989). On the other hand, the transmembrane domain of phospholamban lowered the affinity of the ATPase for Ca²⁺ (Table I). Ionic interaction between the two proteins has been proposed to exert the inhibitory actions of phospholamban on the ATPase, since ionic strength or polyanions have profound effects on the affinity of Ca²⁺ pump ATPase to Ca²⁺ (Table II). Ionic interaction between the two proteins may reverse the inhibition of the ATPase. Therefore, domain II of phospholamban may change the hydrophobic microenvironment of the transmembrane domains of Ca²⁺ pump ATPase, thus modifying the Ca²⁺ affinity of the ATPase. Since phosphorylation of PLN 8-47 reversed the shift of Kₐ (Fig. 5), a conformational change of domain II may be induced by phosphorylation of Ser¹⁶ via a conformational change of domain I or via a change in charge of domain I. Polyanions such as heparin may induce the same conformational change of domain II through domain I.

The stoichiometry between phospholamban and Ca²⁺ pump ATPase...
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ATPase has not been clear. The ratio between phospholamban monomer and Ca\textsuperscript{2+} pump ATPase may not exceed 5, ratio at which one phospholamban pentamer exerts its action on one Ca\textsuperscript{2+} pump ATPase by a direct protein-protein interaction. In the present study, however, 300-fold excess PLN 1–31 over Ca\textsuperscript{2+} pump ATPase was necessary to obtain the maximal inhibition of the ATPase (Fig. 3, inset), and 100-fold excess PLN 28–47 or PLN 8–47 to obtain the K\textsubscript{Ca} equivalent to that of native cardiac SR vesicles (Fig. 5, Table I). Since PLN 1–31 is devoid of the transmembrane domain and is not anchored to the membrane, high concentration of the peptide around the Ca\textsuperscript{2+} pump ATPase may be necessary for the peptide to exert its action. For PLN 28–47 or PLN 8–47, excess peptides may be required to obtain a vesicle within which both Ca\textsuperscript{2+} pump ATPase and the peptide properly interact with each other. Another possibility of the requirement of excess peptides is that the pentameric form of phospholamban may have a stronger interaction with Ca\textsuperscript{2+} pump ATPase than the monomeric form. PLN 1–31 dose not form an oligomer because domain II is responsible for oligomeric formation (Fujii et al., 1989). PLN 28–47 and PLN 8–47 existed as multiple forms from monomer to pentamer in the reconstituted membranes, and the pentameric form was less than 5% (data not shown). It has been known that 3 cysteine residues (Cys\textsuperscript{28}, Cys\textsuperscript{41}, and Cys\textsuperscript{47}) of domain II are important for pentameric formation of phospholamban (Fujii et al., 1989). Although it cannot be ruled out that the 5 amino acid residues from the C terminus may play some roles in oligomeric formation, the formation of fewer pentamers may be property of the synthetic peptides. Similar to this property of the peptides, phospholamban has a tendency to stay monomeric when treated with organic solvents (Boyot et al., 1989). Additional experiments will be required to elucidate the relationship between the oligomeric formation of phospholamban and its action on Ca\textsuperscript{2+} pump ATPase and to determine the stoichiometry of phospholamban to Ca\textsuperscript{2+} pump ATPase at which the Ca\textsuperscript{2+} pump of cardiac SR is regulated by phospholamban.

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