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Effect of a Phospholamban Peptide on the Skeletal Sarcoplasmic Reticulum Ca^{2+} Transport*

Hae Won Kim** and Hee Ran Lee[1]

Department of Pharmacology, University of Ulsan, College of Medicine
Division of Pharmacology and Toxicology, Asan Institute for Life Sciences[1] Seoul 138-040, Korea

ABSTRACT

Phospholamban is the regulator of Ca^{2+}-ATPase in cardiac sarcoplasmic reticulum(SR). The mechanism of regulation appears to involve inhibition by dephosphorylated phospholamban. Phosphorylation of phospholamban relieves this inhibition. Recently, there has been a report that the cytoplasmic domain (amino acids 1-25) of phospholamban is insufficient to inhibit the Ca^{2+} pump. To explore the domains of phospholamban responsible for Ca^{2+}-ATPase inhibitory activity, we examined the effect of a synthetic phospholamban peptide consisting of amino acid residues 1-25 on Ca^{2+} uptake by reconstituted skeletal SR Ca^{2+}-ATPase. The Ca^{2+}-ATPase of skeletal SR was purified and reconstituted in proteoliposomes containing phosphatidylcholine (PC) or phosphatidylcholine: phosphatidylserine (PC : PS). Inclusion of a phospholamban peptide in PC proteoliposomes was associated with significant inhibition of the initial rates of Ca^{2+} uptake at pCa 6.0, and phosphorylation of this peptide by the catalytic subunit of cAMP-dependent protein kinase reversed the inhibitory effect on the Ca^{2+} pump. Similar effects of phospholamban peptide were also observed using PC : PS proteoliposomes. Based on these results, we could conclude that the cytoplasmic domain of phospholamban, containing the phosphorylation sites, by itself is sufficient to inhibit the Ca^{2+} pump of SR.

Key words: Phospholamban, Ca^{2+}-ATPase, Sarcoplasmic reticulum, Reconstitution, Muscle
Abbreviations: SR, sarcoplasmic reticulum; TFA, trifluoroacetic acid

INTRODUCTION

The Ca^{2+}-ATPase of skeletal and cardiac sarcoplasmic reticulum (SR) transports Ca^{2+} from the cytosol into the lumen of the SR, and this reduces the free Ca^{2+} concentration in the sarcoplasm to the submicromolar range and further results in muscle relaxation. In cardiac muscle, calcium uptake into SR is regulated by phosphorylation/dephosphorylation of a 52-amino acid protein, named phospholamban. Phospholamban contains two major domains. The cytoplasmic domain, extending from residues 1-25, contains the phosphorylation sites, is highly basic. The transmembrane domain, extending approximately from residues 26-52, is mostly hydrophobic. It has been reported that dephosphorylated phospholamban is an inhibitor of the Ca^{2+} pump and phosphorylation of phospholamban at serine 16 by the catalytic subunit of the cAMP-dependent protein kinase reverses the phospholamban inhibiton (Kim

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**To whom correspondence should be addressed.

et al., 1990), which acts by increasing the apparent affinity of the Ca^{2+}-ATPase for Ca^{2+}.

Previous studies by us have shown that phospholamban can directly bind Ca^{2+}-ATPases from both cardiac and skeletal SR (kim, 1992), and this direct binding of phospholamban can regulate both Ca^{2+}-ATPases in reconstituted vesicles in a similar manner (Kim *et al.*, 1990; Kim, 1992). It has been also postulated by us and others that the cytoplasmic domain of phospholamban by itself interact directly with the Ca^{2+} pump, which was sufficient to inhibit the enzyme (James *et al.*, 1989; Kim, 1992; Sasaki *et al.*, 1992). Lysine 3 of phospholamban may bind directly to the Ca^{2+}-ATPase (James *et al.*, 1989; Kim, 1992). Mapping studies with monoclonal antibodies implicated phospholamban residues 7-16 in the regulation of the Ca^{2+}-ATPase (Morris *et al.*, 1991).

However, recently, there is a report that the cytoplasmic domain of phospholamban is insufficient to inhibit the cardiac Ca^{2+} pump (Jones and Field, 1993). To further explore the domain of phospholamban responsible for the Ca^{2+}-ATPase inhibition, we examined the effect of a synthetic phospholamban peptide consisting of amino acid residues 1-25 on Ca^{2+} uptake by reconstituted skeletal SR vesicles. Since phospholamban is not expressed in fast twitch skeletal muscle SR, the skeletal SR Ca^{2+}-ATPase is the useful *in vitro* tool to determine the effect of phospholamban peptide on the Ca^{2+} uptake using the reconstituted vesicles.

In the present study, we have obtained evidence that the cytoplasmic domain of phospholamban, containing phosphorylation sites, by itself is sufficient to inhibit the Ca^{2+} pump of skeletal SR.

MATERIALS AND METHODS

Materials

All biochemical reagents were of chemical pure grade. Ammonium salt of [γ-^{32}P] ATP (10~40 Ci/mmol) was purchased from Amersham Corp., $^{45}CaCl_2$ (2 Ci/mmol) was purchased from Du Pont-New England Nuclear, and Na_2ATP was purchased from Boehringer Mannheim. Protein molecular weight standards were obtained from Bio-Rad. Solvents used for synthesis and purification of peptide were of high pressure liquid chromatography grade.

Preparation of sarcoplasmic reticulum vesicles

Skeletal sarcoplasmic reticulum (SR) vesicles were prepared from rabbit back muscle by the method of Nakamura *et al.* (1983). Proteins were measured following the method of Lowry *et al.* (1951), and Ca^{2+}-ATPase activity in SR preparations was assayed by coupling ADP production to NADH oxidation with pyruvate kinase and lactate dehydrogenase (Albers *et al.*, 1968), or by the measurement of inorganic phosphate production, according to Fiske and SubbaRow (1925). The purity of SR preparations, evaluated by various enzyme marker assays, was reported by Kranias *et al.* (1982).

Purification of sarcoplasmic reticulum Ca^{2+}-ATPase

The skeletal SR Ca^{2+}-ATPase was purified from rabbit skeletal SR by the method of Nakamura and Tonomura (1982). To remove Triton X-100 before reconstitution, Bio-Beads SM-2 (0.5~0.6 g/ml) was added to the Ca^{2+}-ATPase preparation (1 mg/ml). The mixture was gently agitated at room temperature for 1 h using a wrist action tube shaker, with a change of Bio-Beads at 30 min. At 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.

Synthesis of a phospholamban peptide

A peptide corresponding to the amino acids 1-25 of phosholamban was synthesized as previously described (Kim *et al.*, 1990). Cleavage of the synthetic peptide from the resin was achieved by acid hydrolysis using hydrogen fluoride (Tam *et al.*, 1983). The crude peptide was purified by high pressure liquid chromatography, and the major peaks were subjected to amino acid composition analysis.

Phosphorylation of a phospholamban peptide

Phosphorylation of a phospholamban peptide occurred by incubating the peptide in 0.1 ml of 50

mM potassium phosphate, pH 7.4, 10 mM $MgCl_2$, 10 mM NaF, 0.5 mM EGTA, 40 units of catalytic subunit of the cAMP-dependent protein kinase and 50 μM[γ-^{32}P] ATP at 30℃ for 5 min.

For investigation of the effect of phospholamban phosphorylation of Ca^{2+} uptake, a phospholamban peptide was phosphorylated under the same conditions as the above using unlabeled ATP. The phosphorylation reaction was initiated by the addition of ATP. Non-phosphorylated peptide was also incubated under identical conditions. The non-phosphorylated and phosphorylated peptide were used for Ca^{2+} uptake in the reconstituted vesicles.

Polyacrylamide gel electrophoresis of proteins

Gel electrophoresis was performed on 15% sodium dodecyl sulfate (SDS)-polyacrylamide gels according to the method of Laemmli (1970). Autoradiograms were obtained from stained, dried gels using Cronex 4 (Dupont) film and intensifying screens (Garrison, 1983).

Reconstitution of the skeletal sarcoplasmic reticulum Ca^{2+}-ATPase

Reconstitution of the SR Ca^{2+}-ATPase was carried out using the freeze-thaw sonication method as previously described (Kim, *et al.*, 1990; Kim, 1992), which was a modification of the method by Wakabayashi and Shigekawa (1985). Phospholipids in 20 mM imidazole-HCl, pH 7.2, and 0.2 M potassium oxalate (buffer A) were vortexed for 5 min and then sonicated to clarity under nitrogen, using a bath-type sonicator (Branson Ultrasonics Co., Model B2200R-4). The Ca^{2+}-ATPase was solubilized at room temperature at a cholate to protein ratio of 4.5:1 (w:w) in buffer A. An aliquot of ice-cold phospholipid suspension (35~40 mg/ml) was added immediately to this cholate/protein mixture to give a final lipid/protein ratio of 40:1 (w:w). The final reconstitution mixture was 0.6 ~ 1.5 ml of buffer A, containing about 0.5 mg/ml Ca^{2+}-ATPase. The mixture was vortexed for 10 s, frozen in liquid N_2 and then either stored overnight in liquid N_2 or allowed to thaw at room temperature. The turbid suspension was then sonicated for 30 s using a probe-type sonicator (Lab-Line Instruments, Inc., Model Disruptor-Ultrasonic) in an ice bath. The free cholate was removed and the external medium was exchanged using the following procedure (Penefsky, 1979). Disposable syringes (1 ml) were filled with a deaerated suspension of Sephadex G-50, which was then washed with 4~5 column volumes of buffer A. The syringes were placed in centrifuge tubes, which were centrifuged at 900 rpm for 2 min, thus forming columns of Sephadex G-50 within the syringes. The vesicle suspension (about 0.4 ml) was applied to the columns, and the syringes were centrifuged again under the same conditions described above. The recovery of the proteins was approximately 90%.

Determination of calcium uptake

Calcium uptake was determined at 37℃, using $^{45}CaCl_2$ and a modification of the Millipore filtration technique described by Martonosi and Feretos (1964). In the Millipore filtration assay, the rate of Ca^{2+} uptake was determined in a medium containing 18 μg of reconstituted vesicles per ml, 0.1 M KCl, 5 mM $MgCl_2$, various amounts of $^{45}CaCl_2$, 0.5 mM EGTA, 5 mM ATP, 6.8 mM oxalate, 5 mM NaN_3 and 40 mM imidazole-HCl, pH 7.0, using Millipore filters (0.22 μm pore size). Calcium uptake was initiated by the addition of 5 mM ATP. The rates of Ca^{2+} uptake were calculated using a least squares linear regression analysis of the 20-, 40-, and 60-s values of Ca^{2+} uptake.

RESULTS

Effect of a synthetic phospholamban peptide on the reconstituted vesicles

A peptide corresponding to amino acids 1-25 of

1 10
Met-Asp-Lys-Val-Gln-Tyr-Leu-Thr-Arg-Ser-

11 20
Ala - Ile- Arg-Arg-Ala-Ser-Thr - Ile-Glu-Met-

21
Pro- Gln-Gln-Ala-Arg

Fig. 1. Amino acid sequence of the synthetic peptide corresponding to amino acids 1-25 of phospholamban.

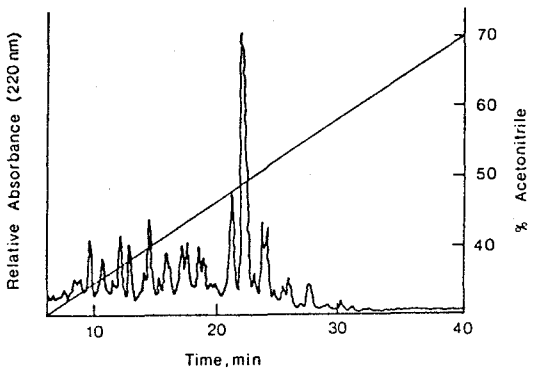

Fig. 2. Analytical high-performance liquid chromatogram of a synthetic phospholamban peptide. Peptides were applied to a Vydac preparative reversed phase (C18) column (1×27 cm, 30 nm pore size) and eluted with a gradient of 30~70 CH_3CN/TFA-H_2O in 30 min at a flow rate of 4.7 ml/min.

Table 1. Effect of a synthetic phospholamban peptide/Ca^{2+}-ATPase ratio on Ca^{2+} uptake in reconstituted vesicles. A synthetic phospholamban peptide was added to the reaction mixture containing the reconstituted vesicles with Ca^{2+}-ATPase. The molar ratios of peptide/Ca^{2+}-ATPase varied between 0-200. At 1 μM free Ca^{2+}, the rate of Ca^{2+} uptake by reconstituted vesicles in the absence of phospholamban was 926.4 nmol/mg/min and it was designated as 100%. Each value represents the mean±SE of five different preparations.

PLB/Ca^{2+}-ATPase	% Ca^{2+} uptake rates
0	100±4.8
10	80.3±5.3
20	84.5±5.0
50	80.5±2.9
100	83.2±3.7
200	78.3±4.5

phospholamban (Fig. 1) was synthesized and purified by high-pressure liquid chromatography (Fig. 2). Major peaks were subjected to amino acid composition analysis. Those peaks that eluted at the same retention time, as a phospholamban peptide, were pooled, concentrated and lyophilized. This peptide had a molecular weight of

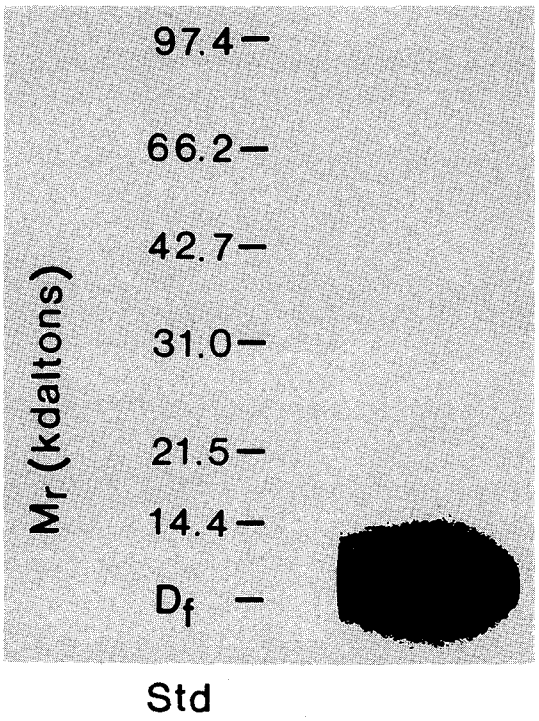

Fig. 3. Autoradiogram of the synthetic peptide by the catalytic subunit of the cAMP-dependent protein kinase. The synthetic peptide (0.1 μg) was phosphorylated at 30℃ for 10 min with [γ-^{32}P] ATP, as described in Materials and Methods. The sample was then subjected to SDS-PAGE followed by autoradiography.

2,900 dalton and could be phosphorylated to a level of 1 mol Pi/1 mol peptide, by the catalytic subunit of the cAMP-dependent protein kinase (Fig. 3) and by Ca^{2+} • calmodulin-dependent protein kinase (data not shown).

To determine the effect of the synthetic phospholamban peptide, the purified skeletal Ca^{2+}-ATPase was reconstituted in the phosphatidylcholine vesicles. Addition of this peptide to the reconstituted Ca^{2+}-ATPase (10~200 peptide/Ca^{2+}-ATPase, molar ratio) resulted in 20% inhibition of the initial rates of Ca^{2+} uptake at pCa 6.0 (Table 1). Thus, the 20% inhibition appeared to be maximal regardless of the ratio of peptide to the Ca^{2+}-ATPase. However, when the phosphorylated peptide was added, the inhibitory effect was relieved (Table 2). In the phosphatidylcholine: phosphatidylserine vesicles, similar effects of

Table 2. Effect of phosphorylation of the synthetic phospholamban peptide on the Ca^{2+}-ATPase in reconstituted vesicles. The synthetic peptide in the unphosphorylated form or after phosphorylation by the catalytic subunit of the cAMP-dependent protein kinase was added to the reaction mixture containing the reconstituted Ca^{2+}-ATPase (peptide: Ca^{2+}-ATPase, 20:1). Phosphorylation conditions were described in Materials and Methods. The initial rates of Ca^{2+} uptake were measured at 1 μM free Ca^{2+} by the reconstituted vesicles, as described in Materials and Methods. The rates of Ca^{2+} uptake by reconstituted vesicles in PC was 931.1±43.5 nmol/mg/min, and in PC:PS was 1518.0±55.1 and those were designated as 100%, respectively. Each value represents the mean±SE of five experiments.

conditions	% Ca^{2+} uptake rates	
	PC	PC:PS
Ca^{2+}-ATPase	100±5	100±4
Ca^{2+}-ATPase+peptide	81±3	80±4
Ca^{2+}-ATPase+P-peptide	98±7	102±3

phospholamban peptide were also observed (Table 2). In control experiments, the rates of Ca^{2+} uptake by the reconstituted vesicles in the presence of the catalytic subunit of the cAMP-dependent protein kinase and ATP were measured. There was no effect compared to the values in the absence of catalytic subunit of cAMP-dependent protein kinase and ATP. Thus, the data with the synthetic peptide further confirmed that phospholamban is an inhibitor of Ca^{2+} pump in the reconstituted vesicles and the cytoplasmic domain of phospholamban is sufficient to inhibit the Ca^{2+} pump of skeletal SR membrane.

DISCUSSION

Effect of a synthetic phospholamban peptide on the Ca^{2+}-ATPase in the reconstituted vesicles

Since chemical cross-linking studies with phospholamban and the Ca^{2+}-ATPase in SR membranes showed a direct interaction between the two proteins (Kim, 1992), a peptide corresponding to amino acids 1-25 of the phospholamban (Fujii *et al.*, 1987) was synthesized. This peptide, which contains the phosphorylation sites, was used to determine whether the hydrophilic portion of phospholamban (amino acids 1-25) is sufficient to affect the reconstituted skeletal Ca^{2+}-ATPase. Addition of this peptide to proteoliposomes containing the Ca^{2+}-ATPase (peptide:Ca^{2+}-ATPase= 10~200:1) was associated with inhibition (20%) of the Ca^{2+} uptake initial rates (Table 1). Phosphorylation of the peptide (1 mol Pi/mol peptide) relieved the inhibitory effect on the Ca^{2+} pump (Table 2). In control experiments, inclusion of the catalytic subunit of the cAMP-dependent protein kinase and ATP with proteoliposomes containing the Ca^{2+}-ATPase had no significant effect on Ca^{2+} uptake. The synthetic peptide of phospholamban is hydrophilic (Fujii *et al.*, 1987) and would not bind to hydrophobic environments such as the liposome membranes. Thus, this peptide inhibit Ca^{2+} uptake in reconstituted vesicles by its direct interaction with the skeletal Ca^{2+}-ATPase as described with the cardiac Ca^{2+}-ATPase (Kim *et al.*, 1990).However, in native cardiac SR membranes it is possible that in addition to the hydrophilic portion of phospholamban, the hydrophobic portion of the protein as well as other polypeptides and factors may interact with the Ca^{2+}-ATPase to regulate Ca^{2+} transport.

Examination of the amino acid sequence of phospholamban reveals that amino acids 1-14 appear to be capable of forming an amphipathic α helix with a hydrophobic surface on one side. This surface could interact with a reciprocal hydrophobic surface on another protein, possibly the Ca^{2+}-ATPase, and such a hydrophobic 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 environment. 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 hydrophobic interaction with another protein such as the Ca^{2+}-ATPase, thereby decreasing the inhibition of the Ca^{2+}-ATPase by phospholamban (Young *et al.*, 1989).

Although our findings do not exclude the possibility that regulation of Ca^{2+} uptake by phospholamban in cardiac SR may be mediated by the membrane-spanning portion of the molecule (residues 26-52 of phospholamban), which has been proposed to form Ca^{2+} channels (Kovacs *et al.*, 1988), they strongly suggest that regulation may be mediated by involvement of the direct physical interaction of the hydrophilic portion of phosholamban with Ca^{2+} pump.

In summary, our findings with the reconstituted vesicles indicate that dephosphorylated phospholamban is associated with the Ca^{2+}-ATPase to exert an inhibitory effect on the Ca^{2+} pump activity, and upon phospholamban phosphorylation the protein interaction is altered, resulting in relief of the inhibition. Therefore, the cytoplasmic domain of phospholamban by itself is sufficient to inhibit the Ca^{2+}-ATPase of skeletal SR.

Proposed model for regulation of the Ca^{2+}-ATPase by phospholamban in cardiac sarcoplasmic reticulum

The proposed model, the phospholamban regulation of the cardiac SR Ca^{2+}-ATPase, is based on the present studies as well as previous ones. The Ca^{2+}-ATPase and phospholamban could be in close proximity for possible direct interaction. As mentioned in the previous section, amino acids 1-14 of phospholamban could form an amphipathic α-helix with a hydrophobic surface on one side and a hydrophilic surface on the other side (Young *et al.*, 1989). Thus, when phospholamban is in the unphosphorylated state, this hydrophobic surface could interact with the hydrophobic surface of the Ca^{2+}-ATPase. The active site of Ca^{2+}-ATPase could then be masked and this would result in inhibition of the Ca^{2+}-ATPase. When phospholamban is phosphoryla-ted, the positively charged groups on the hydrophilic surface of the α-helix would interact with the negatively charged phosphate group. Upon neutralizing the charge group, the conformation of phospholamban would change, thus, weakening the possible hydrophobic interaction and relieving the inhibition.

Previous findings, with the activation of the Ca^{2+}-ATPase upon purification or upon addition of Triton X-100 to the SR (Kim *et al.*, 1990) support the above model. It is possible that the relief of inhibition is due to the removal of phospholamban by purification or due to the breaking of the hydrophobic interaction between the Ca^{2+}-ATPase and phospholamban by addition of detergent. Furthermore, the studies with the phospholamban antibodies also support this model. The binding of the phospholamban antibody may induce a change in the phospholamban conformation, leading to an alteration in its interaction with the Ca^{2+}-ATPase similar to that by phosphorylation, and thus, relieving the inhibition. Since the phospholamban antibody also blocks the phosphorylation of phospoholamban in SR (Suzuki and Wang, 1986), it may be postulated that as a mode of antibody action, the antibody binding may result in a similar change in the conformation of phospholamban, thus, making the phosphorylation sites inacessible to the protein kinases. Another possibility is that the multiple phosphorylation sites of phospholamban are restricted to a small region of phospholamban and the antigenic sites of the antibody are in close proximity to this region, therefore, binding of the antibody results in the physical blocking of the protein kinases from reacting with phospholamban.

In this model, the functional unit of phospholamban would be the monomer and the possible stoichiometry of phospholamban to the Ca^{2+}-ATPase would be 1 : 1. However, this model remains to be tested in future studies.

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=국문초록=

골격근 근장그물 칼슘이동에 대한 Phospholamban 펩타이드의 조절

울산대학교 의과대학 약리학교실, 아산생명과학연구소 약리 • 독물학연구실[1]

김 혜 원 · 이 희 란[1]

Phospholamban은 심근 근장그물 Ca^{2+}-ATPase 조절단백이다. 조절작용기전은 탈인산화된 phospholamban에 의해 Ca^{2+}-ATPase가 억제됨으로 나타나며, 이 phospholamban이 인산화됨으로 Ca^{2+}-ATPase에 대한 억제가 반전됨을 보인다. 최근에 phospholamban의 cytoplasmic domain만으로 Ca^{2+}-ATPase를 억제하기에는 불충분하다는 보고가 있어 본 실험을 계획하였다. Ca^{2+}-ATPase의 활성을 억제하는 phospholamban domain을 밝히기 위하여 합성한 phospholamban 펩타이드(아미노산 1-25)의 Ca^{2+} uptake에 대한 효과를 살펴보았다. 골격근 근장그물에서 Ca^{2+}-ATPase를 분리한 후 phosphatidylcholine이나 phosphatidylcholine과 phosphatidylserine을 포함한 liposome에 재조합시켰다. Phospholamban 펩타이드는 phosphatidylcholine을 이용하여 재조합된 vesicles의 초기 Ca^{2+} uptake rate를 억제하고, cAMP 의존성 protein kinase의 catalytic subunit로 인산화시킨 phospholamban 펩타이드는 이 억제를 반전시킴을 보여 주었다. Phosphatidylcholine과 phosphatidylserine을 포함한 재조합 vesicles에서도 같은 양상을 보였다. 이상의 결과로 미루어 볼 때 인산화 sites를 포함하고 있는 phospholamban의 cytoplasmic domain은 그 자체만으로도 근장그물 칼슘펌프를 억제하기에 충분하다고 결론지을 수 있다.