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Reconstitution of the Cytoplasmic Interaction between Phospholamban and Ca^{2+}-ATPase of Cardiac Sarcoplasmic Reticulum

YOSHIHIRO KIMURA and MAKOTO INUI

Department of Pharmacology, Yamaguchi University School of Medicine, Yamaguchi, Japan

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ABSTRACT

Phospholamban (PLN) reversibly inhibits the Ca^{2+}-ATPase of cardiac sarcoplasmic reticulum (SERCA2a) through a direct protein-protein interaction, playing a pivotal role in the regulation of intracellular Ca^{2+} in heart muscle cells. The interaction between PLN and SERCA2a occurs at multiple sites within the cytoplasmic and membrane domains. Here, we have reconstituted the cytoplasmic protein-protein interaction using bacterially expressed fusion proteins of the cytoplasmic domain of PLN and the long cytoplasmic loop of SERCA2a. We have developed two methods to evaluate the binding of the fusion proteins, one with glutathione-Sepharose beads and the other with a 96-well plate. Essentially the same results were obtained by the two methods. The affinity of the binding (K_D) was 0.70 μM. The association was inhibited by cAMP-dependent phosphorylation of the PLN fusion protein and by usage of anti-PLN monoclonal antibody. It was also diminished by substitution at the phosphorylation site of PLN of Ser^{16} to Asp. These results suggest that PLN can bind SERCA2a in the absence of the membrane domains and that the modifications of the cytoplasmic domain of PLN that activate SERCA2a parallel the disruption of the association between the two fusion proteins. It has been shown that the removal of PLN inhibition of SERCA2a rescues cardiac function and morphology in the mouse dilated cardiomyopathy model. Our assay system can be applied to the screening of novel inotropic agents that remove the inhibition of SERCA2a by PLN, improving the relaxation as well as the contractility of the failing heart.

The Ca^{2+}-ATPase of cardiac sarcoplasmic reticulum (SERCA2a) plays a pivotal role in the contraction and relaxation of heart muscle. It pumps Ca^{2+} from the cytoplasm into the lumen of the sarcoplasmic reticulum (SR), resulting in muscle relaxation. In turn, the release of Ca^{2+} from SR induces muscle contraction (Fleischer and Inui, 1989). The activity of SERCA2a is regulated by another SR membrane protein, phospholamban (PLN) (Tada and Katz, 1982). PLN in the dephosphorylated state inhibits SERCA2a by lowering its apparent affinity for Ca^{2+} (Inui et al., 1986). Phosphorylation of PLN by cAMP-dependent protein kinase reduces the inhibition, resulting in facilitation of the Ca^{2+}-pumping activity of SERCA2a, which leads to the acceleration of relaxation and the following increase in contractility of heart muscle. This mechanism constitutes a principal intracellular signaling pathway involved in the positive inotropic effects of β-adrenergic stimulation. Thus, the SERCA2a-PLN system plays a key role in the regulation of cardiac contractility and relaxation (Kadambi and Kranias, 1997; Simmerman and Jones, 1998; Tada et al., 1988).

PLN is a 52-amino acid integral membrane protein consisting of three domains: the N-terminal cytoplasmic helical domain (domain Ia), the less structured cytoplasmic region (domain Ib), and the C-terminal membrane helix (domain II) (Fujii et al., 1987). Chemical cross-linking between purified PLN and SERCA2a molecules has provided evidence that PLN directly binds to SERCA2a (James et al., 1989). Studies employing site-specific mutagenesis followed by transient expression in human embryonic kidney 293 cells have revealed that two, or possibly three, interaction sites in the cytoplasmic domains and the membrane helices of both proteins are involved in the inhibitory action of PLN on SERCA2a (Toyofuku et al., 1994a,b; Kimura et al., 1997, 1998; Asahi et al., 1999, 2001). Based on these observations, we have proposed that PLN and SERCA2a interact via a four (or possibly six)-base circuit through which long range inhibitory interactions are propagated among a series of cytoplasmic and intramembrane interaction sites (MacLennan et al.,

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ABBREVIATIONS: SERCA, Ca^{2+}-ATPase of sarco(endo)plasmic reticulum; SR, sarcoplasmic reticulum; PLN, phospholamban; PCR, polymerase chain reaction; PDZ, PSD-95/Dlg/ZO-1; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; PSD-95, postsynaptic density protein 95; GST, glutathione *S*-transferase.

1998). Disruption of one of these interactions probably removes the inhibitory effect of PLN on SERCA2a.

Studies using gene targeting techniques have shown that excessive inhibition of SERCA2a by mutated PLN impairs cardiac contractility and relaxation (Zhai et al., 2000; Zvaritch et al., 2000), providing a model of heart failure and cardiomyopathy. On the other hand, PLN-knockout mice exhibit increased cardiac performance without catecholamine stimulation, although the enhancement of cardiac function by catecholamines is lost (Luo et al., 1994). Moreover, it has been reported that PLN ablation rescues the impaired cardiac function and morphological changes in mouse dilated cardiomyopathy generated by knockout of a cytoskeletal protein (Minamisawa et al., 1999). Thus, removal of PLN inhibition of SERCA2a could be a good therapeutic option for treatment of heart failure and cardiomyopathy.

In this study, we have developed methods to reconstitute the cytoplasmic interaction between PLN and SERCA2a using fusion proteins of the cytoplasmic domains of the two proteins. We have found that the cytoplasmic domain of PLN physically associates with the cytoplasmic loop of SERCA2 in the absence of the membrane domains and that the modifications that remove the inhibition of the Ca^{2+}-pumping activity of SERCA2a by PLN disrupt the physical association of the two fusion proteins. Our assay method can be applied to the screening of novel inotropic agents that improve the relaxation as well as the contractility of heart muscle by removing the inhibition of SERCA2a by PLN.

Materials and Methods

Construction of $PLN^{1–26}$-PDZ-Myc-His_6 and GST-SERCA2LCL Fusion Proteins. The DNA fragment coding from Met^1 to Gln^{26} of human phospholamban was obtained from a human heart cDNA library by polymerase chain reaction (PCR). An *Nco*I site was introduced using the forward PCR primer 5′-AGCCATGGAGAAAGTCCAATAC-3′, and an *Xba*I site was introduced using the reverse PCR primer 5′-CCTCTAGACGTTGACGTGCTTGTTGAGGCA-3′. The amplified DNA fragments were subcloned into the *Sma*I site of pBluescript (pBS/$PLN^{1–26}$). The construction was confirmed by dideoxynucleotide sequencing. The insert DNA fragment was excised by restriction digestion, and ligated into the *Nco*I and *Xba*I sites of pTrcHis2B (Invitrogen, Groningen, The Netherlands). This construct was named pTrc/$PLN^{1–26}$. The cDNA fragment coding for the three PDZ domains of rat PSD-95 (13–1176) was amplified using PCR. An *Spe*I site was introduced using the forward primer 5′-GCACTAGTCTGTATAGTGACAACCAAG-3′, and an *Xba*I site was introduced using the reverse primer 5′-CTTCTAGATTATACTGAGCGATGATCG-3′. The amplified DNA fragment was digested with *Spe*I and *Xba*I, and subcloned into the *Xba*I site of pTrc/$PLN^{1–26}$. This construct was named pTrc/$PLN^{1–26}$-PDZ. The cDNA fragment coding for the PDZ domains was also digested with *Xba*I, and subcloned into the *Xba*I site and blunted *Sac*I site of pTrcHis2B; this construct was named pTrc/PDZ and was used as control. The fusion proteins ($PLN^{1–26}$-PDZ-Myc-His_6 and PDZ-Myc-His_6) were expressed in *Escherichia coli* DH5α and purified by Ni-NTA column chromatography (QIAGEN, Hilden, Germany).

The large cytoplasmic loop of SERCA2a (SERCA2LCL) was prepared from rabbit heart mRNA by reverse transcription PCR. The DNA fragment coding for the long cytoplasmic loop of SERCA2a (994–2182) was amplified using the forward primer 5′-GCCATTGTTCGAAGCCTCC-3′ and the reverse primer 5′-TTAAGCCACCGCAGTGCCAGA-3′ by touchdown PCR (Don et al., 1991) in which the annealing temperature was shifted from 60° to 50°C by 1°C every 3 cycles. The amplified DNA fragment was subcloned into the *Sma*I site of pGEX-3X (pGEX/SERCA2LCL). The DNA fragments corresponding to the plus and minus strands encoding Ile^{392}-His^{405} of SERCA2a were synthesized on a DNA synthesizer, annealed, and subcloned into the *Sma*I site of pGEX-3X (pGEX/$SERCA2LCL^{392–405}$). The constructions were confirmed by dideoxynucleotide sequencing. The fusion proteins (GST-SERCA2LCL and GST-$SERCA2LCL^{392–405}$) were expressed in *Escherichia coli* DH5α and purified by glutathione-Sepharose column chromatography. Protein concentrations were determined by the method of Bradford (1976).

Oligonucleotide-Directed Mutagenesis. Mutations of Ser^{16} to Asp (S16D) and Thr^{17} to Asp (T17D) were introduced into the PLN sequence inserted into pBluescript (pBS/$PLN^{1–26}$) by the method of Kunkel (1985). After confirming the sequences, the mutant PLN DNA fragments were excised by *Nco*I and *Xba*I digestion, and then subcloned into *Nco*I and *Spe*I sites of pTrc/PDZ.

Binding Assay Using Glutathione-Sepharose Beads. PLN fusion protein was incubated with GST-SERCA2LCL or GST in 300 μl of binding/wash buffer (20 mM HEPES-NaOH, pH 7.5, 100 mM KCl, 2 mM β-mercaptoethanol, and 0.1% Triton X-100) containing 0.5% BSA at room temperature for 2 h with agitation. Twenty microliters of glutathione-Sepharose beads prewashed three times with binding/wash buffer was added and incubated for 1 h. PLN fusion protein bound to glutathione-Sepharose beads was sedimented, washed five times with binding/wash buffer, and then eluted with the use of Laemmli's SDS sample buffer (Laemmli, 1970). The proteins were separated by SDS-PAGE and transferred onto a nitrocellulose sheet. Immunoblotting was performed with anti-PSD-95 polyclonal antibodies (Yamada et al., 1999) as a primary antibody and peroxidase-conjugated anti-rabbit IgG goat antibodies as a secondary antibody. Labeled bands were visualized by enhanced chemiluminescence. For the quantitation of bound $PLN^{1–26}$-PDZ-Myc-His_6 to GST-SERCA2LCL, various amounts of $PLN^{1–26}$-PDZ-Myc-His_6 were incubated with 1 μg of GST-SERCA2LCL or 0.5 μg of GST in 50 μl of binding/wash buffer at room temperature for 2 h, and then glutathione-Sepharose beads were added. Elution, SDS-PAGE, and immunoblotting were done as described above. The amounts of PLN fusion proteins bound to GST or GST-SERCA2LCL were densitometrically determined using an ATTO Lane and Spot Analyzer (ATTO, Tokyo, Japan).

Binding Assay Using a 96-Well Plate. Two micrograms of GST-SERCA2LCL was immobilized in each well of a 96-well plate in 100 μl of plate buffer containing 20 mM HEPES-NaOH, pH 7.5, 100 mM KCl, and 2 mM β-mercaptoethanol at 37°C for 1 h followed by blocking with 0.1% BSA in plate buffer at 37°C for 1 h. One microgram of GST with 1 μg of BSA was immobilized in a well as a control. The plate was washed twice with plate buffer and then twice with plate buffer containing 0.05% Tween 20. One microgram of PLN fusion protein in 100 μl of plate buffer containing 0.05% Tween 20 and 0.5% BSA was added to each well and incubated for 3 h at 25°C. In some experiments, anti-PLN monoclonal antibody A1 (1–25 μg/ml) (Suzuki and Wang, 1986) was included. The same concentrations of mouse IgG were used as a control. After washing five times with plate buffer containing 0.05% Tween 20, bound PLN fusion protein was cross-linked by 4% paraformaldehyde in phosphate-buffered saline for 20 min at room temperature, washed twice with Tris-buffered saline/Tween 20 (20 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.05% Tween 20), and then incubated with 0.5% skim milk in Tris-buffered saline/Tween 20 for 30 min at room temperature. Anti-PSD-95 polyclonal antibodies were used as a primary antibody and peroxidase-conjugated anti-rabbit IgG goat antibodies as a secondary antibody to detect the fusion proteins. Thirty minutes after addition of TMB One solution (Promega, Madison, WI), the absorbance at 655 nm was measured using a model 550 microplate reader (Bio-Rad, Hercules, CA). The absorbance of PLN fusion protein bound to GST was subtracted from the absorbance of that bound to GST-SERCA2LCL.

Phosphorylation of $PLN^{1–26}$-PDZ-Myc-His_6. For the binding assay with glutathione-Sepharose beads, 3 μg of $PLN^{1–26}$PDZ-Myc-

His_6 was phosphorylated by 60 U of the catalytic subunit of cAMP-dependent protein kinase (Sigma, St. Louis, MO) in 95 μl of 13.2 mM HEPES-NaOH, pH 7.5, 66 mM KCl, 1.32 mM β-mercaptoethanol, 1.98 mg/ml dithiothreitol, 0.06% Triton X-100, 1 mM $MgCl_2$, and 1 mM ATP for 10 min at room temperature. The sample was subjected to the binding assay with glutathione-Sepharose beads. For the 96-well plate assay, 1 μg of $PLN^{1–26}$-PDZ-Myc-His_6 was phosphorylated by 8.3 U of the catalytic subunit of cAMP-dependent protein kinase in 10 μl of 20 mM HEPES-NaOH, pH 7.5, 100 mM KCl, 3 mg/ml dithiothreitol, 1 mM $MgCl_2$, and 1 mM ATP for 10 min at 30°C. The sample was subjected to the binding assay using a 96-well plate.

Results

Physical Association between the Cytoplasmic Domains of SERCA2a and PLN. To examine the cytoplasmic interaction between SERCA2a and PLN, we constructed two fusion proteins. One was a protein in which the long cytoplasmic loop of SERCA2a (Ala^{331}-Ala^{726}) was fused to GST (GST-SERCA2LCL). In the other, the cytoplasmic domain of PLN (Met^1-Gln^{26}) was fused to the PDZ domains of PSD-95 followed by Myc and His_6 tags ($PLN^{1–26}$-PDZ-Myc-His_6). Because the cytoplasmic domain of PLN is a small polypeptide, it was easily handled by the addition of the PDZ domains. The latter domains could be detected by polyclonal antibodies (Yamada et al., 1999). The fusion proteins were expressed in *E. coli* and purified by glutathione-Sepharose or Ni-NTA column chromatography. On SDS-PAGE, GST-SERCA2LCL and $PLN^{1–26}$-PDZ-Myc-His_6 migrated at M_r 65,000 and M_r 61,000, respectively (data not shown). We used two methods for the binding assay. In one method, the complex of GST-SERCA2LCL and $PLN^{1–26}$-PDZ-Myc-His_6 was sedimented with glutathione-Sepharose beads. The bound $PLN^{1–26}$-PDZ-Myc-His_6 was evaluated by immunoblotting with anti-PSD-95 antibodies after the elution of proteins by SDS. As shown in Fig. 1, $PLN^{1–26}$-PDZ-Myc-His_6 but not PDZ-Myc-His_6 bound GST-SERCA2LCL. No significant binding was observed with GST instead of GST-SERCA2LCL. Thus, the physical interaction occurs between the cytoplasmic domains of PLN and SERCA2a even in the absence of the intramembrane interaction.

The same results were obtained with the other method in which GST-SERCA2LCL was immobilized to a 96-well plate and the bound $PLN^{1–26}$-PDZ-Myc-His_6 was quantified by anti-PSD-95 antibodies (data not shown). In the 96-well system, the amounts of $PLN^{1–26}$-PDZ-Myc-His_6 bound to GST were less than 15% of those bound to GST-SERCA2LCL. The background binding of PDZ-Myc-His_6 to GST-SERCA2LCL was less than 10% of the specific binding of $PLN^{1–26}$-PDZ-Myc-His_6. Thus, the 96-well system was specific enough to detect the cytoplasmic interaction between SERCA2a and PLN.

When various amounts of $PLN^{1–26}$-PDZ-Myc-His_6 were incubated with GST-SERCA2LCL, the amounts of $PLN^{1–26}$-PDZ-Myc-His_6 bound to GST-SERCA2LCL increased dose dependently and saturated at about 1 μM (Fig. 2). The affinity of the binding (K_D) was 0.70 μM.

Effects of Phosphorylation of PLN on the Interaction between the Cytoplasmic Domains of PLN and SERCA2a. We previously reported that purified PLN in the unphosphorylated state, but not in the phosphorylated state, is chemically cross-linked to purified SERCA2a (James et al., 1989). We examined whether phosphorylation of PLN affects the cytoplasmic interaction of the two proteins without the membrane domains. $PLN^{1–26}$-PDZ-Myc-His_6 was phosphorylated by cAMP-dependent protein kinase and was incubated with GST-SERCA2LCL. The bound fusion proteins were analyzed by immunoblotting of the eluate from glutathione-Sepharose beads. Phosphorylation of $PLN^{1–26}$-PDZ-Myc-His_6 significantly diminished its binding to GST-SERCA2LCL (Fig. 3A). The addition of Mg-ATP or the catalytic subunit of cAMP-dependent protein kinase itself did not affect the binding. Densitometric scanning revealed that the binding was decreased to 50% by cAMP-dependent phosphorylation.

The effects of phosphorylation were also evaluated by assay using a 96-well plate (Fig. 3B). The addition of 0.1 mM Mg-ATP did not affect the binding. The addition of the cata-

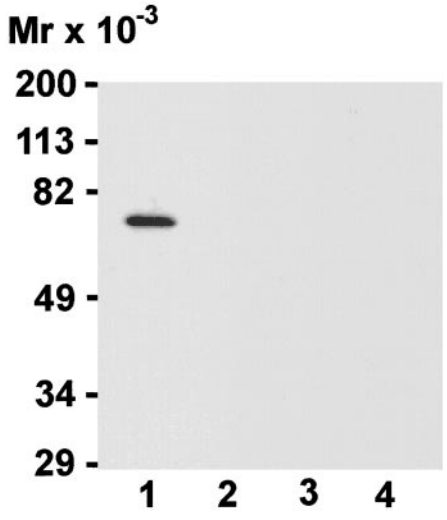

Fig. 1. Physical association between the cytoplasmic domain of SERCA2a and the cytoplasmic domain of PLN. $PLN^{1–26}$-PDZ-Myc-His_6 (lanes 1 and 3) or PDZ-Myc-His_6 (lanes 2 and 4) fusion protein (1 μg) was incubated with 1 μg of GST-SERCA2LCL (lanes 1 and 2) or 0.5 μg of GST (lanes 3 and 4). $PLN^{1–26}$-PDZ-Myc-His_6 or PDZ-Myc-His_6 bound to glutathione-Sepharose beads was analyzed by immunoblotting.

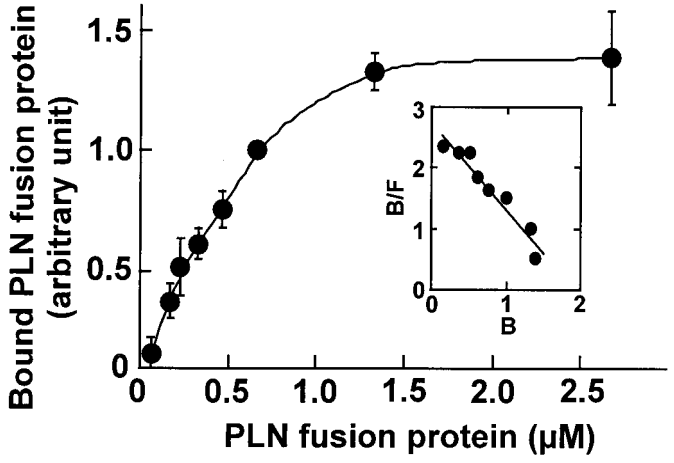

Fig. 2. Dose-dependence curve of the $PLN^{1–26}$-PDZ-Myc-His_6 binding to GST-SERCA2LCL. Various amounts of $PLN^{1–26}$-PDZ-Myc-His_6 were incubated with 1 μg of GST-SERCA2LCL in 50 μl of binding/wash buffer. $PLN^{1–26}$-PDZ-Myc-His_6 bound to glutathione-Sepharose beads was analyzed by immunoblotting. The amount of the binding was evaluated by densitometric scanning of the blot. The data shown are the mean ± S.D. (n = 10). Inset, Scatchard plot of data. The dissociation constant (K_D) was 0.70 μM.

lytic subunit of cAMP-dependent protein kinase slightly increased the binding, although the increase was not statistically significant. Phosphorylation of $PLN^{1–26}$-PDZ-Myc-His_6 reduced the binding to 41% of the control (Fig. 3B). Thus, essentially the same results were obtained with the two methods. The method with the 96-well plate was employed in the following experiments, because the amounts of the $PLN^{1–26}$-PDZ-Myc-His_6 binding were accurately and easily determined by this method.

Effects of Point Mutation of PLN on the Interaction. In addition to the phosphorylation of Ser^{16} by cAMP-dependent protein kinase, PLN is also phosphorylated by calmodulin-dependent protein kinase at Thr^{17}. It has been shown that phosphorylation at Thr^{17} also activates the Ca^{2+}-dependent ATPase and Ca^{2+}-pumping activity of SERCA2a in vitro (Tada et al., 1983). The effects of phosphorylation of Ser and Thr residues can sometimes be mimicked by substitution of these residues to Asp. Therefore, we examined the effects of substitution of Ser^{16} or Thr^{17} of $PLN^{1–26}$-PDZ-Myc-His_6 to Asp on its binding to GST-SERCA2LCL. The amounts of mutants bound to GST-SERCA2LCL decreased to a level almost equivalent to that of phosphorylated $PLN^{1–26}$-PDZ-Myc-His_6 (Figs. 3B and 4).

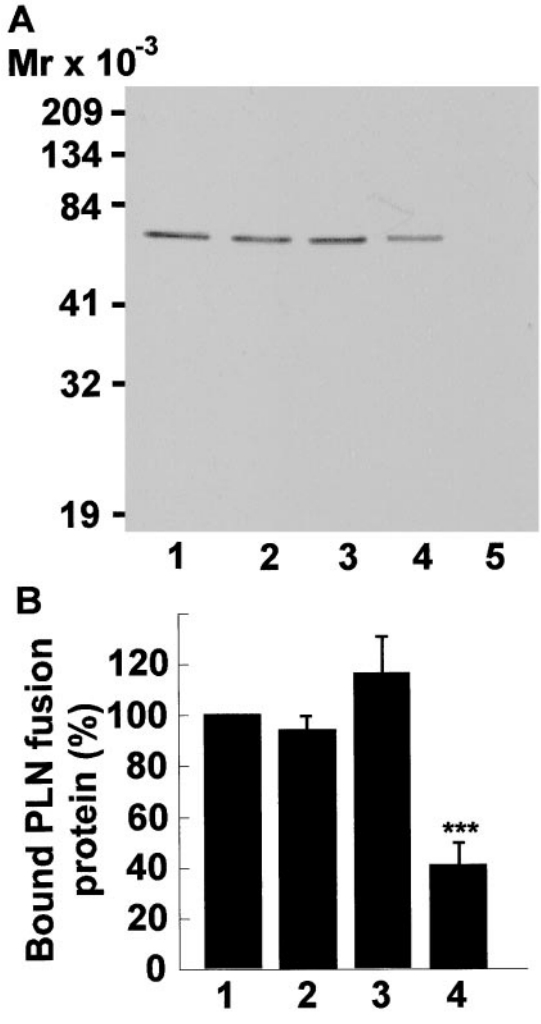

Fig. 3. Effects of phosphorylation of the cytoplasmic domain of PLN on the binding of $PLN^{1–26}$-PDZ-Myc-His_6 to GST-SERCA2LCL. A, $PLN^{1–26}$-PDZ-Myc-His_6 (lanes 1–4) was incubated without 1 mM Mg-ATP (lane 1), with 1 mM Mg-ATP (lane 2), with cAMP-dependent protein kinase (lane 3), or with both (lane 4) for 10 min at room temperature, and then subjected to the binding assay with GST-SERCA2LCL and glutathione-Sepharose beads. In lane 5, PDZ-Myc-His_6 was used instead of $PLN^{1–26}$-PDZ-Myc-His_6. Bound $PLN^{1–26}$-PDZ-Myc-His_6 and PDZ-Myc-His_6 were analyzed by immunoblotting. B, $PLN^{1–26}$-PDZ-Myc-His_6 was incubated alone (control, column 1), with 1 mM Mg-ATP (column 2), with cAMP-dependent protein kinase (column 3), or with both (column 4) for 10 min at 30°C, and then subjected to the binding assay in a 96-well plate upon which GST-SERCA2LCL and GST were immobilized. The amounts of $PLN^{1–26}$-PDZ-Myc-His_6 bound to GST were subtracted from those bound to GST-SERCA2LCL. Data are the mean ± S.D. (n = 7). Statistical significance was determined by analysis of variance followed by the Dunnett t test. ***, $p < 0.001$ compared with control.

Effects of Ca^{2+}, Mg^{2+} and ATP on the Interaction. Because SERCA is a Ca^{2+}-, Mg^{2+}- and ATP-binding protein, we examined the effects of Ca^{2+}, Mg^{2+} and ATP on the binding of $PLN^{1–26}$-PDZ-Myc-His_6 to GST-SERCA2LCL. Free Ca^{2+} up to 10 mM did not affect the binding of $PLN^{1–26}$-PDZ-Myc-His_6 to GST-SERCA2LCL (data not shown), consistent with the fact that GST-SERCA2LCL lacks the Ca^{2+}-binding sites which reside within the membrane helices of SERCA (MacLennan et al., 1997). The addition of 1 mM $MgCl_2$, 1 mM ATP, or 1 mM Mg-ATP did not affect the binding either (data not shown).

Effects of anti-PLN Monoclonal Antibody on the Interaction. Anti-PLN monoclonal antibody A1 has been shown to augment the Ca^{2+}-pumping activity of SERCA2a of cardiac SR vesicles (Suzuki and Wang, 1986; Kimura et al., 1991), probably because of its structural hindrance of the interaction between PLN and SERCA2a. The A1 antibody inhibited the binding of $PLN^{1–26}$-PDZ-Myc-His_6 to GST-SERCA2LCL dose dependently, whereas nonspecific mouse IgG did not (Fig. 5). The binding was reduced to approximately 20% by the addition of 25 μg/ml of the A1 antibody.

Effects of Truncated PLN Binding Region of SERCA2a on the Interaction. Mutation studies have revealed that the amino acids of SERCA2a from Lys^{397} to Val^{402} are essential for functional modulation by PLN (Toyofuku et al., 1994b). We shortened the long cytoplasmic loop of SERCA2a to 14 amino acids, including Lys^{397}-Val^{402} (GST-SERCA2LCL$^{392–405}$). $PLN^{1–26}$-PDZ-Myc-His_6 did not bind to GST-SERCA2LCL$^{392–405}$ (data not shown). In addition, a 10-fold excess of GST-SERCA2LCL$^{392–405}$ over GST-SERCA2LCL did not affect the binding of $PLN^{1–26}$-PDZ-Myc-His_6 to GST-SERCA2LCL (data not shown).

Discussion

We developed methods to evaluate the cytoplasmic interaction between SERCA2a and PLN and demonstrated that the interaction can be reconstituted with the fusion proteins of the cytoplasmic domain of PLN and the long cytoplasmic loop of SERCA2a. We previously demonstrated that Lys^3 in the cytoplasmic domain of PLN is chemically cross-linked to Lys^{397} and Lys^{400} in the long cytoplasmic loop of SERCA2a using purified whole molecules of SERCA2a and PLN (James et al., 1989). Our present results indicate that the cytoplasmic association between SERCA2a and PLN occurs without the intramembrane interaction. A number of studies have shown that modifications to the cytoplasmic domain of PLN, including phosphorylation (Tada and Katz, 1982; Tada et al., 1988), antibody usage (Suzuki and Wang, 1986; Kimura et al., 1991), and mutations (Toyofuku et al., 1994a), profoundly affect the Ca^{2+}-pumping and ATPase activity of SERCA2a,

although the membrane helix of PLN is responsible for the direct inhibitory action of PLN on SERCA2a (Sasaki et al., 1992; Kimura et al., 1996). In the present study, the cytoplasmic modifications previously shown to activate the SERCA2a function were accurately detected as also causing a decrease in the interaction between the two fusion proteins (Figs. 3–5). If our method, especially with the 96-well plate, were applied to drug screening, therefore, a new drug that decreases the cytoplasmic interaction between PLN and SERCA2a, resulting in the activation of SERCA2a, could be discovered.

Although only the fusion proteins were used here, this study made it possible to estimate the affinity of the cytoplasmic interaction between PLN and SERCA2a. The affinity was moderate (K_D = 0.70 μM). Moderate affinity is often observed in the interactions of cytoskeletal proteins, including actin-associated (Tobacman, 1996) and spectrin-based membrane skeleton proteins (Bennett, 1990). A direct protein-protein interaction plays an important role in the functional modulation of these proteins.

The fusion protein of the long cytoplasmic loop of SERCA2a, GST-SERCA2LCL, was used in this study to examine the cytoplasmic interaction with PLN. It has been reported that the recombinant large cytoplasmic loop of SERCA1a, the fast-twitch skeletal muscle isoform of SERCA, which is located between the 4th and 5th membrane helices, retains the ability to bind ATP (Moutin et al., 1998). The amino acid sequence of this region of SERCA is more than 80% conserved between SERCA1a and SERCA2a. Therefore, our construct might also retain the proper conformation in this region. Indeed, our construct retained the ability to bind PLN.

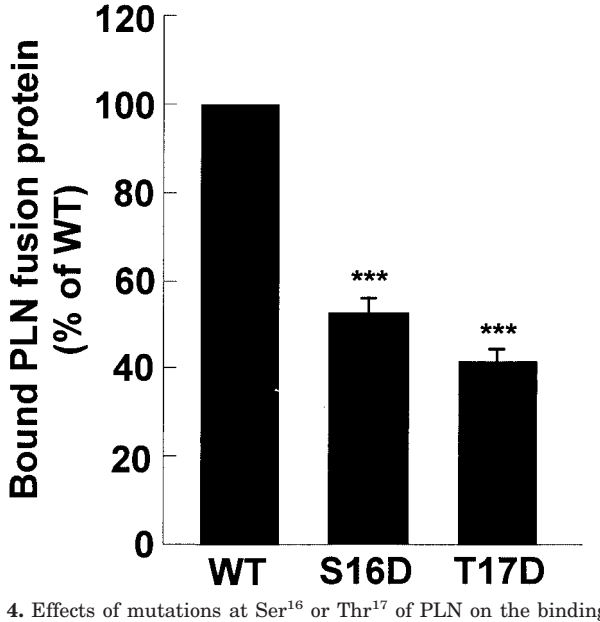

Fig. 4. Effects of mutations at Ser^{16} or Thr^{17} of PLN on the binding of $PLN^{1–26}$-PDZ-Myc-His_6 to GST-SERCA2LCL. $PLN^{1–26}$-PDZ-Myc-His_6 (WT) and two mutants (S16D and T17D, in which Ser^{16} and Thr^{17}, respectively, of PLN were substituted to Asp) were subjected to the binding assay in a 96-well plate upon which GST-SERCA2LCL and GST were immobilized. The amounts of PLN fusion proteins bound to GST were subtracted from those bound to GST-SERCA2LCL. Data are the mean ± S.D. (n = 8). Statistical significance was determined by analysis of variance followed by the Dunnett t test. ***, $p < 0.001$ compared with control.

Crystal structure analysis of SERCA1 at 2.6 Å resolution revealed its PLN binding sequence ($KNDKPI^{402}$) extrudes from the nucleotide binding domain (Toyoshima et al., 2000). Although the PLN binding sequence is essential for the modulation of SERCA2a by PLN (Toyofuku et al., 1994b), the short sequence of 14 amino acids including the PLN binding sequence (GST-$SERCA2LCL^{392–405}$) did not bind to $PLN^{1–26}$-PDZ-Myc-His_6 nor affect the binding of $PLN^{1–26}$-PDZ-Myc-His_6 to GST-SERCA2LCL. Therefore, the PLN binding sequence of SERCA2a may not be sufficient for the physical interaction between the cytoplasmic domains of PLN and SERCA2a. Other region(s) of SERCA2a might be involved in the interaction.

Recently, the physical association between PLN and SERCA2a was examined by immunoprecipitation assay (Asahi et al., 2000). In this study, 5 mM ATP enhanced the interaction between SERCA2a and PLN. In our study, however, 1 mM Mg-ATP did not affect the binding of $PLN^{1–26}$-PDZ-Myc-His_6 to GST-SERCA2LCL. We could not examine higher concentrations of Mg-ATP because of the high background binding of $PLN^{1–26}$ -PDZ-Myc-Hist to GST caused by Mg^{2+} (data not shown). Immunoprecipitation study also showed that an antibody against Ser^{16}-phosphorylated PLN coimmunoprecipitated SERCA2a (Asahi et al., 2000), indicating that physical interactions are retained between PLN and SERCA2a molecules in the absence of functional interactions. Our antibody (A1 antibody) disrupted the association between $PLN^{1–26}$-PDZ-Myc-His_6 and GST-SERCA2LCL (Fig. 5). These discrepancies might be explained by the involvement of the interaction between the membrane domains and between domain Ib of PLN and the loop between transmembrane helices M6 and M7 of SERCA (Asahi et al., 1999, 2001; Kimura et al., 1997, 1998) in the immunoprecipitation study.

It is still controversial whether phosphorylation at Ser^{16} completely removes the inhibitory effects of PLN on the Ca^{2+}-pumping activity of SERCA2a. Some studies have re-

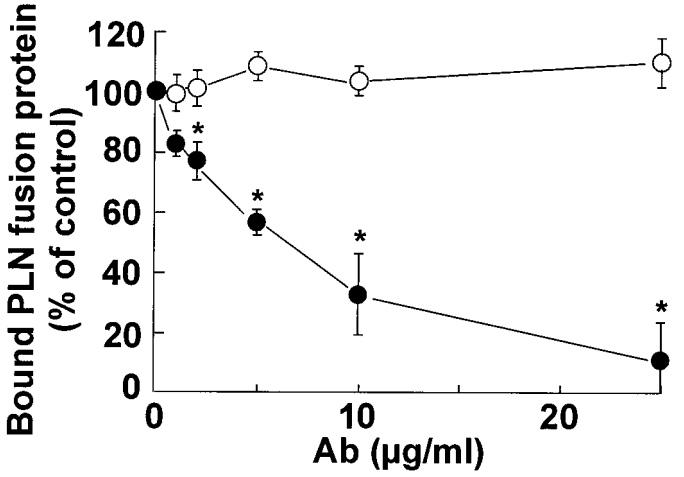

Fig. 5. Inhibition of the binding of $PLN^{1–26}$-PDZ-Myc-His_6 to GST-SERCA2LCL by anti-PLN monoclonal antibody. $PLN^{1–26}$-PDZ-Myc-His_6 was subjected to the binding assay in a 96-well plate upon which GST-SERCA2LCL and GST were immobilized. Various amounts of monoclonal antibody A1 (●) or control mouse IgG (○) were included in the reaction mixture. The amounts of $PLN^{1–26}$-PDZ-Myc-His_6 bound to GST were subtracted from those bound to GST-SERCA2LCL. Data are the mean ± S.D. (n = 3). Statistical significance was determined by the paired t test. *, $p < 0.05$ compared with control mouse IgG.

ported the additive effects of phosphorylation by cAMP- and calmodulin-dependent protein kinases (Tada et al., 1983), whereas others have reported that the complete activation of SERCA2a is achieved solely by cAMP-dependent phosphorylation of PLN (Chu et al., 2000). We have shown that phosphorylation of PLN^{1-26}-PDZ-Myc-His_6 by cAMP-dependent protein kinase decreased the binding to approximately 50% (Fig. 3). The partial dissociation was not caused by incomplete phosphorylation of PLN^{1-26}-PDZ-Myc-His_6 because the reduction of the binding by cAMP-dependent phosphorylation was comparable with that by the mutation of Ser^{16} to Asp (Fig. 4). Furthermore, unphosphorylated PLN^{1-26}-PDZ-Myc-His_6 migrated faster in SDS-PAGE than the phosphorylated fusion protein, and no significant faster migrating band was observed with the phosphorylated sample used in this study (Fig. 3A, lane 4) in high resolution SDS-PAGE (data not shown). Even if cAMP-dependent phosphorylation of PLN completely removed the inhibitory action on SERCA2a, it might not be necessarily accompanied by the complete dissociation of PLN from SERCA2a. Cytoplasmic and intramembrane interaction sites between PLN and SERCA2a influence each other through long range coupling, so the phosphorylation of PLN would affect not only the affinity of the cytoplasmic interaction but also the intramembrane interaction. Therefore, the cytoplasmic interaction may not be linearly correlated with the inhibitory action on SERCA2a. If this were the case, some false negatives would be anticipated when the 96-well plate system was applied to screen a drug that disrupts the functional interaction between PLN and SERCA2a. On the other hand, this screening system has a great advantage over other methods using whole molecules such as immunoprecipitation, in which the cytoplasmic interaction may be masked by the interaction at other sites.

In the failing heart, relaxation as well as contractility is impaired. Delay in the decrement of Ca^{2+} concentration is often observed during diastole in the failing heart, suggesting that extrusion of Ca^{2+} from the cytosol to SR is not sufficient during the diastolic phase (Morgan, 1991; Beuckelmann et al., 1992). In fact, reduced expression and lower activity of SERCA2a have been reported in SR of the failing heart (Mercadier et al., 1990; Arai et al., 1993), as well as a decrease in the phosphorylation level of PLN (Schmidt et al., 1999). Thus, the SERCA2a-PLN system is closely related to the pathophysiology of heart failure. Furthermore, overexpression of superinhibitory PLN mutants has been shown to induce cardiac hypertrophy in mouse, impairing contractility and relaxation of heart muscle (Zhai et al., 2000; Zvaritch et al., 2000), whereas PLN ablation rescues the impaired functional and morphological changes in the heart in the mouse dilated cardiomyopathy model (Minamisawa et al., 1999). Therefore, the removal of PLN inhibition of SERCA2a may be a new strategy to treat heart failure and cardiomyopathy. In this study, we have demonstrated that modifications to the PLN cytoplasmic domain that activate SERCA2a always disrupt the interaction between PLN^{1-26}-PDZ-Myc-His_6 and GST-SERCA2LCL. If a drug could disrupt this interaction, it would activate SERCA2a and be a new therapeutic tool for heart failure and cardiomyopathy. Our assay system will make it possible to discover such a new inotropic agent that would improve the relaxation as well as the contractility of the failing heart.

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Address correspondence to: Makoto Inui, M.D., Ph.D., Department of Pharmacology, Yamaguchi University School of Medicine, 1–1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan. E-mail: minui@yamaguchi-u.ac.jp