Phospholamban Regulates the Ca\(^{2+}\)-ATPase through Intramembrane Interactions*  

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There is clear evidence for direct regulatory protein-protein interactions between phospholamban (PLN) and the Ca\(^{2+}\)-ATPase of cardiac sarcoplasmic reticulum (SERCA2a) in cytoplasmic domains, but there is less clear evidence for regulatory interactions in the transmembrane domains of the two proteins. We have now coexpressed SERCA isoforms with the transmembrane sequence of PLN and with epitope-tagged transmembrane sequences of PLN to study intramembrane interactions in the absence of cytoplasmic interactions. Coexpression of the transmembrane sequence of phospholamban (Met-PLN\(^{28–52}\)) with SERCA1a, SERCA2a, and SERCA3 inhibited Ca\(^{2+}\) transport by lowering apparent Ca\(^{2+}\) affinity. Addition of the hemagglutinin (HA) epitope to the transmembrane sequence of PLN (HA-PLN\(^{28–52}\)) or deletion of PLN residues 21-29 (PLN\(^{1–20}\), PLN\(^{30–52}\) “supershifted” apparent Ca\(^{2+}\) affinity to values lower than those observed with native PLN without uncoupling Ca\(^{2+}\) transport from ATP hydrolysis. Inhibition by PLN\(^{1–20}\)-PLN\(^{30–52}\) or by Flag-PLN\(^{28–52}\) was reversed by PLN antibody or by Flag antibody, demonstrating that inhibition by these constructs is reversible and that the inhibitory constructs are properly oriented in the membrane. These results suggest that PLN modulates the apparent Ca\(^{2+}\) affinity of SERCA2a through intramembrane interactions, which are disrupted at long range and in concert with disruption of the well characterized cytoplasmic interactions.

The hallmark of phospholamban (PLN)\(^{1}\) inhibition of sarco-(end)plasmic reticulum Ca\(^{2+}\)-ATPases (SERCAs) is a decrease in apparent Ca\(^{2+}\) affinity, which can be reversed by phosphorylation of PLN at Ser\(^{16}\) or Thr\(^{17}\) (1, 2). This function makes PLN a key element in the inotropic response of the heart to β-adrenergic agonists. The 52 amino acids contained in each subunit of PLN homopentamers are predicted to be divided into three domains (3). PLN domain Ia (amino acids 1-20) is highly charged and largely helical (4), domain Ib (amino acids 21-30) is polar and unstructured, and domain II (amino acids 31-52) is neutral, very hydrophobic, and helical. Protein-protein interactions between the cytoplasmic domains of PLN and SERCA2a have been demonstrated before, but not after, phosphorylation of PLN and in the absence of elevated Ca\(^{2+}\) (5). It is clear that there is a functional interaction site between the cytoplasmic domains of PLN and SERCA2a, since mutation of any of 13 of the 20 amino acids making up PLN domain Ia (6) or of any of residues KDDKPV\(^{402}\) in the cytoplasmic domain of SERCA2a (7) diminishes the ability of PLN to inhibit SERCA2a.

In earlier studies, the addition of soluble synthetic PLN\(^{1–31}\) suppressed \(V_{\text{max}}\) without affecting Ca\(^{2+}\) affinity of purified SERCA2a, while the in vitro reconstitution of purified SERCA2a with an unphysiological 100-fold molar excess of synthetic PLN\(^{28–47}\) lowered the apparent Ca\(^{2+}\) affinity of SERCA2a (8). Attempts to reproduce these experiments revealed that the addition of excess PLN\(^{28–52}\) uncoupled Ca\(^{2+}\) transport from ATP hydrolysis (9). Thus these experiments did not provide very strong evidence for a transmembrane interaction site between SERCA2a and PLN. Moreover, studies by Kirchberger et al. (10) showed that mild proteolysis, which removed most of the cytoplasmic sequence of PLN, activated SERCA2a by raising its apparent Ca\(^{2+}\) affinity. The interpretation of the results of this study, however, is clouded by the fact that tryptic digestion of a membrane system could have pleiotropic effects.

In order to evaluate the functional effects of intramembrane interactions between PLN and SERCA2a, we added different epitope tags to the NH\(_2\) terminus of PLN domain II and coexpressed the constructs with SERCA isoforms, thereby achieving in vivo reconstitution between the two proteins. We have found that PLN domain II inhibits SERCA2a by lowering its apparent affinity for Ca\(^{2+}\). We propose that interactions between transmembrane domains of PLN and SERCA2a are inhibitory but are modulated through long range coupling to the cytoplasmic interaction sites in the two molecules.

**MATERIALS AND METHODS**

Synthesis and Expression of PLN Constructs—PLN constructs in which domain I was deleted or replaced with sequences expected to form epitopes for Flag (IBI), hemagglutinin (HA) (11), Myc (12), or PLN were synthesized in a recombinant polymerase chain reaction (13) using primers with 5’-add-on sequences containing restriction endonuclease sites and encoding proposed epitope sequences. Six PLN constructs were evaluated: PLN\(^{1–52}\), Met-PLN\(^{28–52}\) (no epitope), Flag-PLN\(^{28–52}\), HA-PLN\(^{28–52}\), Myc-PLN\(^{30–52}\), and PLN\(^{1–20}\).PLN\(^{30–52}\). The epitope sequences (italicized and in one-letter code) are: Flag, MDYKDDDK-L\(^{26}\); HA, MYPYPDVDA-L\(^{26}\); Myc, MEQKLISEEDL-L\(^{26}\); and PLN\(^{1–20}\).MEKVQLRTRSLAIRASIEM-N\(^{30}\). Although the Flag and PLN\(^{1–20}\) sequences formed functional epitopes, the HA and Myc sequences did not. All constructs were used, however, because they were all functional as PLN analogues.

PLN construct cDNAs and SERCA cDNAs were cotransfected into 21726
HEK-293 cells using the calcium phosphate precipitation method (6). In a typical experiment, 8 μg of PLN cDNA and 8 μg of SERCA2a cDNA (1:1) were added to each dish. In some cases, however, 4 μg of PLN and 12 μg of SERCA2a cDNA were mixed with 8 μg of pMT2 DNA (1:2 ratio) or 12 μg of Met-PLN28–52 were mixed with 4 μg of SERCA3 cDNA (3:1 ratio). In other cases, 0.32 (1:10), 1.6 (1:2), 3.2 (1:1), 6.4 (2:1), 9.6 (3:1), or 12.8 (4:1) μg of PLN cDNA were cotransfected with 3.2 μg of SERCA2a cDNA, pMT2 DNA being added to make a total of 16 μg of DNA per transfection. Reducing SERCA2a cDNA from 8 μg to 3.2 μg reduced V_max of Ca^{2+} transport, but not K_{Ca}. Microsome Preparation and Ca^{2+} Uptake—Microsomes were prepared and assayed for Ca^{2+} transport activity and data were analyzed as described previously (6).

Assay of ATPase Activity—ATPase activity was measured under conditions identical to those used for measurement of Ca^{2+} transport (6). The malachite green procedure for phosphate determination, developed from a phosphate 80 mM NaCl, (14–16), was adapted to this assay to require measurement of the inorganic phosphate liberated from ATP during Ca^{2+} transport by transfected microsomes. The reaction was started by the addition of 80 μl of microsomes (1 mg/ml) to 600 μl of Ca^{2+} transport reaction mixture. After 5, 10, 15, 20, and 25 min, the reaction was stopped by the addition of 100 μl of reaction mixture to 45 μl of malachite green reagent mixture in a 96-well microplate. The malachite green reagent mixture was made by mixing 0.122% malachite green hydrochloride in 6.2 mM HSO_4, 5.76% ammonium molybdate tetrahydrate, and 11% Tween 20 in a volume ratio of 100:66:2. Color development was quenched after 10 s by the addition of 15 μl of 15% sodium citrate disodium. Inorganic phosphate liberated in the ATPase reaction was quantified by comparison of absorbance at 570 nm with standard curves generated with known amounts of Na_2HPO_4 in the presence of 5 mM ATP. At 15 min, 150 μl of the same microsomal sample was withdrawn and filtered for measurement of 45Ca^{2+} uptake. Background Ca^{2+} uptake and ATP hydrolysis, defined as those values obtained from cells transfected only with pMT2 vector DNA, were subtracted from each data point. Under these conditions, ATP hydrolysis was found to be linear for at least 25 min.

Phosphorylation of Microsomes—Microsomes (0.8 mg/ml) were phosphorylated by 33.3 units/ml of cAMP-dependent protein kinase catalytic subunit (Sigma) in 0.15 M KCl, 0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, 0.2 mM MgCl_2, 3 mM β-mercaptoethanol, and 25 μM ATP for 5 min at room temperature. The Ca^{2+}-dependent protein kinase catalytic subunit was omitted in controls. The samples were then assayed for Ca^{2+} transport activity as described previously (6).

Treatment of Microsomes with Anti-PLN or Anti-Flag Antibody—For detection of functional changes induced by anti-PLN antibody, microsomes transfected with SERCA2a and PLN or PLN^N2–20, PLN^N50–52 (1 mg/ml) were incubated with 0.25 mg/ml anti-PLN antibody 1D11, a generous gift from Dr. Robert Johnson, Merck Sharp and Dohme, in 10 mM Tris-HCl, pH 7.5, 0.2 mM MgCl_2, 0.25 mM sodium phosphate, and 4.67 mM Tris-HCl, pH 7.5, 0.2 mM CaCl_2, 3 mM β-mercaptoethanol, and 0.015 M NaCl for at least 30 min on ice. Alternatively, microsomes (0.8 mg/ml) from cells cotransfected with SERCA2a and Flag-PLN^N28–52 were incubated for 1 h on ice with 0.8 mg/ml anti-Flag antibody M2 (IBI) in a buffer containing 70 mM KCl, 117 mM NaCl, 1 mM CaCl_2, 1.4 mM MgCl_2, 5.33 mM sodium phosphate, and 4.67 mM Tris-HCl, pH 7.5. Buffer was added to control samples to achieve the same salt composition in samples plus and minus antibody. Ca^{2+} transport was assayed as described previously (6).

Immunoblotting was carried out as described previously (6), except that the low-molecular-mass protein, dialyzed, SDS–PAGE, and Western blotting detection system (Amersham Corp.) were used.

RESULTS

PLN, when coexpressed with SERCA2a, shifts the curve of Ca^{2+} dependence of Ca^{2+} uptake toward a lower apparent Ca^{2+} affinity (Fig. 1, A–D). Apparent K_{Ca} (the Ca^{2+} concentration that gives half-maximal Ca^{2+} transport activity) was shifted from pCa 6.63 to pCa 6.31 when PLN and SERCA2a cDNAs were cotransfected in a 1:2 or 1:1 ratio (Fig. 1, Table I). Increasing the ratio of PLN cDNA to SERCA2a cDNA in the transfection system to 2:1, 3:1, or 4:1 did not shift apparent K_{Ca} beyond pCa 6.21 under conditions in which an increase in cDNA in the transfection assay induced an increase in PLN synthesis as judged by Western blotting (Fig. 2A).

Met-PLN^N28–52 when coexpressed with SERCA2a, also shifted K_{Ca} significantly to pCa 6.46 (Table I). Unfortunately, we could not assess the level of expression of Met-PLN^N28–52, since we had no antibody against domain II. We added epitope tags to PLN domain II in an attempt to measure synthesis of the PLN derivatives but found that they also resulted in very effective inhibitory constructs. Myc-PLN^N30–52 lowered apparent Ca^{2+} affinity to pCa 6.29, Flag-PLN^N28–52 lowered apparent Ca^{2+} affinity to pCa 6.22, and HA-PLN^N28–52 “supershifted” apparent Ca^{2+} affinity to pCa 6.00.

Our most effective inhibitor of SERCA2a was PLN^N1–20, PLN^N50–52. When the ratio of PLN^N1–20, PLN^N30–52 cDNA to SERCA2a cDNA in the cotransfection system was 1:2, the PLN construct supershifted apparent K_{Ca} to pCa 5.76. When PLN^N1–20, PLN^N30–52 and SERCA2a cDNA were transfected in a weight ratio of 1:1, the K_{Ca} measured in three of five experiments was supershifted to less than 5.61 pCa units, and in two of five experiments no Ca^{2+} uptake was observed, even at 10 μM free Ca^{2+} (Fig. 1B; Table I).

In all of these experiments SERCA2a was expressed at about the same level in the absence and in the presence of coexpressed PLN as judged by Western blot analysis (Fig. 2, A and B). The expression level of PLN^N1–20, PLN^N30–52, however, appeared to be lower than that of PLN as judged by Western blotting with the same antibody (Fig. 2B). Fig. 2B shows that PLN^N1–20, PLN^N30–52 is a mixture of oligomers and monomer much like intact PLN, and in Fig. 2C we show that Flag-PLN^N28–52 is also a mixture of oligomers and monomer. Since supershifts occurred without apparent overexpression of PLN^N1–20, PLN^N30–52 and since we could not achieve supershifting by adding more PLN cDNA to the transfection reaction, we conclude that the supershifts result from a qualitative change in the efficiency of the inhibitory peptides and are not related to overexpression.

The PLN constructs also suppressed Ca^{2+} transport by SERCA1a (16) and SERCA3 (17), lowering the apparent affinity for Ca^{2+} in each case (Fig. 1, C and D; Table I). Although SERCA3 has a lower apparent Ca^{2+} affinity than either SERCA1a or SERCA2a (18–20), NH2-terminal-truncated and epitope-tagged PLN constructs lowered its Ca^{2+} affinity even further. The rank order for potency of suppression of Ca^{2+} uptake by the various PLN constructs was the same for SERCA1a and SERCA2a, but the order of inhibition for Flag-PLN^N28–52 and HA-PLN^N28–52 was inverted for SERCA3.

To evaluate the possibility that the transmembrane domain of PLN might uncouple Ca^{2+} uptake from Ca^{2+}-ATPase (9) we measured Ca^{2+}-dependent ATPase activity and Ca^{2+} uptake simultaneously. As shown in Table II, the inhibition of Ca^{2+} uptake by PLN constructs was mirrored by inhibition of Ca^{2+}-dependent ATPase activity, even though values of Ca^{2+} uptake and ATP hydrolysis varied from experiment to experiment depending on expression levels. Those samples which exhibited no Ca^{2+} transport activity also had no ATPase activity. Thus it is unlikely that PLN constructs uncoupled Ca^{2+} translocation from ATP hydrolysis.

Since PLN^N1–20, PLN^N30–52 could potentially be phosphorylated by cAMP-dependent protein kinase (PKA), and the question of whether phosphorylation of PLN domain Ia could exert a regulatory influence over domain II in this chimeric peptide. Unfortunately, PLN^N1–20, PLN^N30–52 was not phosphorylated by PKA under conditions in which PLN was phosphorylated, leading to an increase in Ca^{2+} uptake at low Ca^{2+} concentration.

Monoclonal antibodies against PLN domain Ia can also reverse PLN inhibition of SERCA2 activity (21–23), and these
Fig. 1. Effects of phospholamban domain II constructs on the Ca\(^{2+}\) affinity of SERCA1a, SERCA2a, and SERCA3. Microsomes from HEK-293 cells cotransfected with PLN construct cDNAs and SERCA cDNAs were assayed for Ca\(^{2+}\) dependence of Ca\(^{2+}\) transport activity. A, effects of epitope-tagged PLN domain II constructs on Ca\(^{2+}\) dependence of Ca\(^{2+}\) uptake by SERCA2a. ○, SERCA2a alone (n = 10); ●, SERCA2a coexpressed with PLN (n = 9); ▲, SERCA2a coexpressed with Flag-PLN (n = 8); ▼, SERCA2a coexpressed with HA-PLN (n = 6); □, SERCA2a coexpressed with Myc-PLN (n = 4). B, effects of truncated or deleted PLN constructs on the Ca\(^{2+}\) dependence of Ca\(^{2+}\) uptake by SERCA2a. ○, SERCA2a alone (n = 11); ●, SERCA2a coexpressed with PLN (n = 9); ▲, SERCA2a coexpressed with Met-PLN (n = 5). When PLN\(^{-20}\)-PLN\(^{35}\) cDNA was transfected in a 1:1 ratio with SERCA2a, no Ca\(^{2+}\) uptake was observed, even at 10 \(\mu\)M Ca\(^{2+}\) in two experiments. The relative Ca\(^{2+}\) uptake values were converted to 0 in these experiments. C, effects of PLN domain II constructs on Ca\(^{2+}\) dependence of Ca\(^{2+}\) uptake by SERCA1. ○, SERCA1a alone, (n = 4); ●, SERCA1a coexpressed with PLN (n = 3); ▲, SERCA1a coexpressed with Flag-PLN (n = 4); ▼, SERCA1a coexpressed with HA-PLN (n = 3); □, SERCA1a coexpressed with Met-PLN (n = 3); ■, SERCA1a coexpressed with Myc-PLN (n = 3). D, effects of PLN domain II constructs on Ca\(^{2+}\) dependence of Ca\(^{2+}\) uptake by SERCA3. ○, SERCA3 alone (n = 11); ●, SERCA3 coexpressed with Flag-PLN (n = 4); ▲, SERCA3 coexpressed with HA-PLN (n = 3); ▼, SERCA3 coexpressed with Met-PLN (n = 4); □, SERCA3 coexpressed with Myc-PLN (n = 3). All data are means ± S.E.

| PLN constructs coexpressed with SERCA constructs | SERCA2a | n | SERCA1a | n | SERCA3 | n |
|-----------------------------------------------|---------|---|----------|---|---------|---|
| None                                         | 6.63 ± 0.06 | 23 | 6.47 ± 0.05 | 4 | 6.02 ± 0.06 | 11 |
| PLN (1:10)                                   | 6.42 ± 0.13 | 3 | ND \(^{a}\) | ND | ND | ND |
| PLN (1:2)                                    | 6.31 ± 0.02 | 2 | ND | ND | ND | ND |
| PLN (1:1)                                    | 6.31 ± 0.08 | 21 | 6.21 ± 0.02 \(^{c}\) | 3 | 5.88 ± 0.08 | 2 |
| PLN (2:1)                                    | 6.21 ± 0.05 \(^{a}\) | 3 | ND | ND | ND | ND |
| PLN (3:1)                                    | 6.20 ± 0.06 \(^{a}\) | 2 | ND | ND | ND | ND |
| PLN (4:1)                                    | 6.25 ± 0.02 | 2 | ND | ND | ND | ND |
| Met-PLN (1:2)                                | 6.46 ± 0.06 | 7 | 6.37 ± 0.05 \(^{c}\) | 4 | 5.85 ± 0.09 \(^{d}\) | 4 |
| Myc-PLN (1:2)                                | 6.29 ± 0.09 | 4 | ND | ND | ND | ND |
| Flag-PLN (1:2)                               | 6.62 ± 0.11 \(^{a}\) | 8 | 6.15 ± 0.01 \(^{c}\) | 4 | 5.71 ± 0.08 \(^{c}\) | 4 |
| HA-PLN (1:2)                                 | 6.00 ± 0.06 | 6 | 5.89 ± 0.10 \(^{c}\) | 3 | 5.77 ± 0.10 \(^{c}\) | 3 |
| PLN (1:2)-PLN (1:2)                          | 5.76 ± 0.25 | 7 | 5.61 ± 0.08 \(^{c}\) | 3 | ND | 3 |
| PLN (1:2)-PLN (2:1)                          | -5.81 | 5 | 5.55 ± 0.07 \(^{c}\) | 3 | 5.60 ± 0.01 \(^{c}\) | 3 |

\(^{a}\) Amount of SERCA2a cDNA was 3.2 \(\mu\)g rather than 8 \(\mu\)g per transfection.

\(^{b}\) ND, not determined.

\(^{c}\) \(p < 0.05\) vs. control by analysis of variance followed by Scheffe F-test.

\(^{d}\) Met-PLN (1:2) DNA was transfected in a weight ratio of 3:1 over SERCA3 cDNA.

\(^{e}\) Statistical analysis was not done because Ca\(^{2+}\) uptake was completely inhibited in two experiments, even at 10 \(\mu\)M free Ca\(^{2+}\).

PLN\(^{28-52}\) were enhanced by either the anti-PLN antibody 1D11 or the anti-Flag monoclonal antibody M2 at low Ca\(^{2+}\) concentrations (Table III). These antibodies had little effect on the Ca\(^{2+}\) uptake activity of microsomes transfected with SERCA2 alone.
Our PLN constructs also suppressed \( \text{Ca}^{2+} \) transport by SERCA1a and SERCA3, lowering their apparent affinity for \( \text{Ca}^{2+} \). The site of cytoplasmic interaction with PLN, KDD-KPV\(^{402} \) in SERCA2, is conserved as KNDKPI\(^{402} \) in SERCA1a but altered to QGEQLV\(^{402} \) in SERCA3 (17), consistent with the fact that SERCA1a activity is inhibited by native PLN, while SERCA3 activity is scarcely affected by its coexpression with PLN (7, 20). By contrast, the sequences of most of the transmembrane helices are highly conserved among the three SERCA isoforms (17). Even though SERCA3 had a lower apparent affinity for \( \text{Ca}^{2+} \) than either SERCA1a or SERCA2a (18, 19), epitope-tagged or NH\(_2\)-terminal-truncated PLN constructs, but not PLN itself, lowered the apparent affinity of SERCA3 for \( \text{Ca}^{2+} \) even further. The rank order for potency of suppression of \( \text{Ca}^{2+} \) uptake by the various PLN constructs was similar for SERCA1a, SERCA2a, and SERCA3. These results are consistent with the view that it is a transmembrane interaction site and not the cytoplasmic interaction site that is responsible for lowering the apparent \( \text{Ca}^{2+} \) affinity of all three SERCA molecules.

It has been suggested that the transmembrane domain of PLN might uncouple \( \text{Ca}^{2+} \) uptake from \( \text{Ca}^{2+} \)-ATPase (9). In our experiments, however, the inhibition of \( \text{Ca}^{2+} \) uptake by PLN constructs was mirrored by their inhibition of \( \text{Ca}^{2+} \)-dependent ATPase activity, and those samples which exhibited no \( \text{Ca}^{2+} \) transport activity had no ATPase activity. Thus those of our PLN constructs which were tested did not uncouple \( \text{Ca}^{2+} \) translocation from ATP hydrolysis in SERCA2.

We could not determine whether phosphorylation of PLN domain Ia could exert a direct regulatory influence over domain II in chimeric peptides, since PLN\(^{1-20} \), PLN\(^{30-52} \) was not phosphorylated by PAK. This lack of recognition of the PKA phosphorylation site in PLN\(^{1-20} \), PLN\(^{30-52} \) is consistent with a change in the exposure of the domain Ia sequence to cytoplasmic molecules in this construct. A monoclonal antibody against PLN domain Ia, however, activated PLN\(^{1-20} \), PLN\(^{30-52} \)-inhibited SERCA2a just as it does for PLN-inhibited SERCA2a (21–23). An antibody against the Flag epitope also reversed the inhibition of SERCA2a by Flag-PLN\(^{28-52} \). Thus it is apparent that the inhibition of SERCA2a, induced by different PLN constructs, can be reversed by modulation of the cytoplasmic domain of the chimeric PLN constructs, just as modulation of the cytoplasmic domain of intact PLN by phosphorylation or antibody interaction can modulate PLN inhibition of SERCA2a.

Conclusions concerning the orientation of two of our chimeras can be drawn from these studies. The activating PLN and Flag antibodies can only be effective from the cytoplasmic side of the sealed \( \text{Ca}^{2+} \)-impermeable microsomes used in this study. Accordingly, the epitopes in the chimeric molecules PLN\(^{1-20} \), PLN\(^{30-52} \) and Flag-PLN\(^{28-52} \) must be cytoplasmic and the transmembrane sequence must be in the same orientation in the chimeras and in native PLN.

From these results we propose a model of PLN-SERCA2a interaction in which PLN interacts with SERCA2a in at least two sites, one in the cytoplasmic sequences of PLN and SERCA2a and one within the transmembrane sequences of PLN and SERCA2a. We propose that the interaction between the transmembrane sequences of PLN and SERCA2a inhibits SERCA2a by altering its apparent \( \text{Ca}^{2+} \) affinity. We also propose that interaction between PLN domain Ia and the cytoplasmic domain of SERCA2a is not by itself inhibitory (8), but it can modulate the inhibitory interactions in the transmembrane domains through long range coupling. If the cytoplasmic interaction is disrupted by PLN phosphorylation or binding of antibody, the inhibitory intramembrane interactions are also disrupted. If the inhibitory transmembrane interaction sites are
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Table II

Effects of phospholamban domain II constructs on the stoichiometry between Ca\(^{2+}\) translocation and ATP hydrolysis.

| Constructs coexpressed with SERCA2a | pCa | n | Ca\(^{2+}\) uptake in microsomes | ATP hydrolysis in microsomes | Ca\(^{2+}\)/ATP ratio |
|-------------------------------------|-----|---|-------------------------------|-----------------------------|---------------------|
| None                                | 6.75| 7 | \(6.9 \pm 4.5\)               | \(4.8 \pm 3.1\)             | 1.5 \(\pm 0.5\)     |
| PLN                                 | 5.5 | 7 | \(20.4 \pm 11.1\)             | \(30.5 \pm 5.5\)            | 2.0 \(\pm 0.7\)     |
| HA-PLN\(^{28-52}\)                  | 5.5 | 4 | \(1.7 \pm 1.1\)              | \(1.9 \pm 1.4\)             | 1.0 \(\pm 0.3\)     |
| HA-PLN\(^{1-20,28-52}\) (1:1)      | 5.5 | 6 | \(15.4 \pm 8.0\)             | \(10.1 \pm 4.9\)            | 1.7 \(\pm 0.8\)     |
| PLN\(^{1-20,28-52}\) \(1:1\)       | 5.5 | 5 | \(0.7 \pm 0.6\)               | \(0.6 \pm 1.0\)             | ND\(^{b}\)          |
| HA-PLN\(^{1-20,28-52}\) (1:1)      | 5.5 | 5 | \(15.9 \pm 4.5\)             | \(9.0 \pm 2.3\)             | 1.8 \(\pm 0.5\)     |
| None                                | 6.75| 5 | \(0.7 \pm 0.1\)               | \(0.1 \pm 0.4\)             | ND\(^{b}\)          |
| HA-PLN\(^{1-20,28-52}\) (1:1)      | 5.5 | 5 | \(6.2 \pm 8.1\)               | \(2.0 \pm 2.9\)             | ND\(^{b}\)          |

\(^{a}\) Ca\(^{2+}\)/ATP ratio was not determined because both Ca\(^{2+}\) uptake and Ca\(^{2+}\)-ATPase activity were at background levels.

\(^{b}\) Ca\(^{2+}\)/ATP ratio was not determined because we detected neither Ca\(^{2+}\) uptake nor ATPase activity in two of five samples in which 1a-PLN\(^{30-52}\) and SERCA2a were cotransfected in a ratio of 1:1, and we detected very little activity in one sample, even at pCa = 5.5.

Table III

Effects of anti-Flag antibody (A) and anti-PLN antibody 1D11 (B) on the inhibition of SERCA2a by phospholamban domain II constructs. Microsomes were incubated with or without anti-Flag antibody M2 (A) or anti-PLN antibody 1D11 (B) and subjected to Ca\(^{2+}\) transport assay as described under "Materials and Methods." Data are mean values \(\pm\) SD.

A

| Constructs coexpressed with SERCA2a | pCa | n | Ca\(^{2+}\) uptake in microsomes | Increase |
|-------------------------------------|-----|---|-------------------------------|---------|
| Control                             |     |   | Control                       | Antibody-treated | % |
| None                                | 6.75| 3 | \(5.4 \pm 2.5\)               | \(5.0 \pm 2.7\)   | \(-9.2 \pm 8.3\) |
| PLN                                 | 6.5 | 4 | \(16.0 \pm 14.5\)             | \(14.9 \pm 13.6\) | \(-7.3 \pm 6.6\) |
| HA-PLN\(^{28-52}\)                 | 5.5 | 3 | \(30.6 \pm 26.0\)             | \(29.1 \pm 26.1\) | \(-6.6 \pm 7.0\) |
| Flag-PLN\(^{28-52}\)               | 5.5 | 3 | \(1.3 \pm 0.6\)               | \(1.3 \pm 0.6\)   | \(-1.1 \pm 15.7\) |
| None                                | 6.75| 4 | \(0.9 \pm 0.3\)               | \(1.9 \pm 0.8\)   | \(106.4 \pm 46.6\) |
| PLN                                 | 6.5 | 4 | \(3.3 \pm 1.4\)               | \(5.7 \pm 1.6\)   | \(81.9 \pm 36.8\) |
| HA-PLN\(^{28-52}\)                 | 5.5 | 4 | \(14.0 \pm 6.5\)              | \(14.9 \pm 5.9\)  | \(13.7 \pm 34.6\) |

B

| Constructs coexpressed with SERCA2a | pCa | n | Ca\(^{2+}\) uptake in microsomes | Increase |
|-------------------------------------|-----|---|-------------------------------|---------|
| Control                             |     |   | Control                       | Antibody-treated | % |
| None                                | 6.75| 3 | \(4.2 \pm 2.3\)               | \(4.3 \pm 2.4\)   | \(4.3 \pm 10.4\) |
| PLN                                 | 6.5 | 3 | \(6.6 \pm 3.4\)               | \(7.0 \pm 3.4\)   | \(7.0 \pm 7.9\) |
| HA-PLN\(^{28-52}\)                 | 5.5 | 3 | \(12.7 \pm 3.4\)             | \(12.7 \pm 7.8\)  | \(5.6 \pm 10.8\) |
| None                                | 6.75| 3 | \(0.8 \pm 0.2\)               | \(3.7 \pm 0.8\)   | \(353 \pm 113\) |
| PLN                                 | 6.5 | 3 | \(1.4 \pm 0.5\)               | \(5.9 \pm 1.6\)   | \(367 \pm 177\) |
| HA-PLN\(^{1-20,28-52}\) (1:2)      | 5.5 | 3 | \(12.3 \pm 1.8\)             | \(14.6 \pm 2.1\)  | \(20 \pm 14\) |
| None                                | 6.75| 3 | \(1.3 \pm 0.9\)               | \(17.0 \pm 7.0\)  | \(1668 \pm 1093\) |
| HA-PLN\(^{1-20,28-52}\) (1:2)      | 5.75| 3 | \(4.5 \pm 2.5\)               | \(20.6 \pm 9.1\)  | \(363 \pm 66\) |
| None                                | 5.0 | 3 | \(12.6 \pm 5.2\)             | \(17.9 \pm 8.2\)  | \(40 \pm 11\) |

\(^{a}\) p < 0.05 vs. control by analysis of variance followed by Scheffe F-test.

 disrupted by elevated Ca\(^{2+}\) concentrations, leading to Ca\(^{2+}\) binding to the high affinity Ca\(^{2+}\) binding and translocation sites in the transmembrane domain of SERCA molecules, then the regulatory cytosolic interaction sites are also disrupted.

In a striking analogy, long range interactions between the catalytic ATP hydrolytic site in the cytoplasmic headpiece domain of SERCA1 and the Ca\(^{2+}\) binding and translocation sites in the transmembrane domain, mediated through a stalk sector, are an integral feature of Ca\(^{2+}\) transport by SERCA molecules (24, 25). It is now clear that PLN also has functional cytosolic (domain Ia) and transmembrane (domain II) domains, which are separated by a stalk sector (domain Ib) and that long range interactions occur between these functional domains. This long range coupling may occur entirely within the PLN molecule or it may be mediated by conduction through the SERCA2a molecule from its cytoplasmic to its transmembrane sites of interaction with PLN. In this case, a four-site regulatory circuit, possibly involving the catalytic site in the cytoplasmic domain of SERCA2a and the Ca\(^{2+}\) binding and translocation sites in the transmembrane domain of SERCA2a, might best describe the interactions between SERCA2a and PLN.

We propose that PLN interacts only weakly with SERCA3 because the lack of compatible cytosolic interaction sites obviates the enmeshing of the transmembrane interaction sites. The transmembrane sequences of PLN constructs in which domain I is replaced or in which domain Ia is located nearer to the membrane surface (PLN\(^{1-20,28-52}\)) may interact directly with the transmembrane sequence of SERCA3, because steric hindrances created by the poor fit of the cytosolic interaction sites in the full length molecule are bypassed in the truncated molecule.

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REFERENCES
1. Tada, M., and Kadoma, M. (1989) Bioessays 10, 157–163
2. Simmerman, H. K. B., Collins, J. H., Theibert, J. L., Wegener, A. D., and Jones, L. R. (1986) J. Biol. Chem. 261, 13333–13341
3. Fujii, J., Ueno, A., Kitano, K., Tanaka, S., Kadoma, M., and Tada, M. (1987) J. Clin. Invest. 79, 301–304
4. Mortishire-Smith, R. J., Pitzenberger, S. M., Burke, C. J., Middaugh, C. R., Garsky, V. M., and Johnson, R. G. (1995) Biochemistry 34, 7603–7613
5. James, P., Inui, M., Tada, M., Chiesi, M., and Carafoli, E. (1989) Nature 342, 90–92
6. Toyofuku, T., Kurzydlowski, K., Tada, M., and MacLennan, D. H. (1994) J. Biol. Chem. 269, 3081–3094
7. Toyofuku, T., Kurzydlowski, K., Tada, M., and MacLennan, D. H. (1994) J. Biol. Chem. 269, 22929–22932
8. Sasaki, T., Inui, M., Kimura, Y., Kuzuya, T., and Tada, M. (1992) J. Biol. Chem. 267, 1674–1679
9. Reddy, L. G., Jones, L. R., Cala, S. E., O’Brien, J. J., Tatulian, S. A., and Stokes, D. L. (1995) J. Biol. Chem. 270, 9390–9397
10. Kirchberger, M. A., Borchman, D. and Kasnathan, C. (1986) Biochemistry 25, 5484–5492
11. Field, J., Nikawa, J., Broek, D., MacDonald, B., Rodgers, L., Wilson, I. A., Lerner, R. A., and Wigler, M. (1988) Mol. Cell. Biol. 8, 2159–2165
12. Evan, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985) Mol. Cell. Biol. 5, 3610–3616
13. Higuchi, R. (1990) in PCR Protocols: A Guide to Methods and Applications (Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., eds), pp. 177–183, Academic Press, San Diego, CA
14. Geladopoulos, T. P., Sotiropoulis, T. G., and Evangelopoulos, A. E. (1991) Anal. Biochem. 192, 112–116
15. Lanzetta, P. A., Alvarez, L. J., Reina, P. S., and Candia, O. A. (1979) Anal. Biochem. 100, 95–97
16. Brandi, C. J., Delon, S., Martin, D. R., and MacLennan, D. H. (1987) J. Biol. Chem. 262, 3768–3774
17. Burk, S. E., Lytton, J., MacLennan, D. H., and Shull, G. E. (1989) J. Biol. Chem. 264, 18561–18568
18. Toyofuku, T., Kurzydlowski, K., Lytton, J., and MacLennan, D. H. (1992) J. Biol. Chem. 267, 14490–14496
19. Lytton, J., Westlin, M., Burk, S. E., Shull, G. E., and MacLennan, D. H. (1992) J. Biol. Chem. 267, 14483–14489
20. Toyofuku, T., Kurzydlowski, K., Tada, M., and MacLennan, D. H. (1994) J. Biol. Chem. 268, 2809–2815
21. Suzuki, T., and Wang, J. H. (1996) J. Biol. Chem. 261, 7018–7023
22. Jones, L. R., and Field, L. J. (1993) J. Biol. Chem. 268, 11486–11488
23. Mayer, E. J., McEnna, E., Garsky, V. M., Burke, C. J., Middaugh, C. R., Sardana, M., Smith, J. S., and Johnson, R. G., Jr. (1996) J. Biol. Chem. 271, 1669–1677
24. MacLennan, D. H. (1990) Biophys. J. 58, 1335–1365
25. MacLennan, D. H., Clarke, D. M., Loo, T. W., and Skerjanc, I. (1992) Acta Physiol. Scand. 146, 141–150
