Phospholamban Inhibitory Function Is Activated by Depolymerization*

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Phospholamban (PLN), a homopentameric, integral membrane protein, reversibly inhibits cardiac sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA2a) activity through intramembrane interactions. Here, alamine-scanning mutagenesis of the PLN transmembrane sequence was used to identify two functional domains on opposite faces of the transmembrane helix. Mutations in one face diminish inhibitory interactions with transmembrane sequences of SERCA2a, but have relatively little effect on the pentameric state, while mutations in the other face activate inhibitory interactions and enhance monomer formation. Double mutants are monomeric, but loss of inhibitory function is dominant over activation of inhibitory function. These observations support the proposal that the SERCA2a interaction site lies on the helical face which is not involved in pentamer formation. Four highly inhibitory mutants are effectivly devoid of pentamer, suggesting that pentameric PLN represents a less active or inactive reservoir that dissociates to provide inhibitory monomeric PLN subunits. A model is presented in which the degree of PLN inhibition of SERCA2a activity is ultimately determined by the dissociation constant for the PLN pentamer (which is mutation-sensitive), and by the dissociation constant for the PLN/SERCA2a heterodimer (which is likely to be mutation-sensitive).

Phospholamban (PLN)\textsuperscript{1} is a 52-amino acid, integral membrane protein (1) that interacts with and reversibly inhibits the activity of the cardiac sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA2a). In this role, it is a major regulator of the kinetics of cardiac contractility (2). PLN has the mobility of a homopentamer in SDS gels, but the pentamer is dissociated to monomers by boiling in SDS (3). It is an open question whether the functionally inhibitory unit is a pentamer or a monomer, and whether pentamers and monomers are in dynamic equilibrium in the sarcoplasmic reticulum membrane.

Much attention has been directed toward the phosphorylation sites on Ser\textsuperscript{16} and Thr\textsuperscript{17} in cytoplasmic domain Ia of PLN and their role in regulating the inhibitory function of PLN (4, 5). In earlier studies we showed that the PLN cytoplasmic interaction site is formed by charged and hydrophobic amino acids 1–20 (6), while the complementary SERCA2a interaction site consists of amino acids Lys-Asp-Asp-Lys-Pro-Val\textsuperscript{142} (7).

In a later evaluation of potential transmembrane interaction sites (8), we coexpressed SERCA2a with PLN transmembrane sequences 31–52 (PLN domain II) or with PLN domain II constructs to which NH\textsubscript{2}-terminal, cytoplasmic epitopes such as PLN\textsuperscript{1–20} or hemagglutinin were fused. We found that the inhibitory interaction site lies entirely in the transmembrane sequences of PLN and SERCA2a, but can be modulated, through long range interactions, by the noninhibitory cytoplasmic interaction site. We also discovered the phenomenon of “supershifting,” in which the apparent affinity of specific PLN mutants for SERCA2a is enhanced, so that Ca\textsuperscript{2+} concentrations well above the physiological range are required to reverse the inhibitory action of the mutant PLN. In the present study, we associate gain of function with PLN mutants that promote PLN depolymerization. Since the most inhibitory mutants of PLN are devoid of pentamer, we deduce that the pentamer represents a less active or inactive reservoir of subunits and that the PLN monomer is the functional, inhibitory form.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis, Expression, and Assay**—For mutagenesis, a 172-base pair fragment containing the coding sequence for rabbit PLN (bases 6 to 162 (9) was amplified in a recombinant polymerase chain reaction (10) using primers with 5'-add-on sequences containing EcoI (Stratagene) after digestion with XbaI and SalI. Mutagenesis with this construct as a template was carried out as described previously (11, 12). Wild type and mutant PLN cDNAs were ligated into the XbaI and SalI sites of the pMT2 expression vector (13) and amplified. Plasmid DNA was purified by adsorption to and elution from Qiagen tip-500 columns. PLN and SERCA2a cDNAs were cotransfected (8 μg of each cDNA per dish) into HEK-293 cells using the calcium phosphate precipitation method (14). Cells were harvested 48 h after transfection, and microsomes were prepared and assayed for Ca\textsuperscript{2+} transport activity as described previously (6). Data were analyzed as described previously (8).

**Immunoblotting**—Immunoblotting of SERCA2a and wild type and mutant forms of PLN expressed in HEK-293 cell microsomes was carried out with 10 μg of microsomal proteins. Proteins were solubilized in SDS buffer at room temperature, separated on 12.5% SDS-PAGE, transferred electrophoretically to nitrocellulose membranes, and incubated with monoclonal antibody IIDS\textsuperscript{6} against SERCA2a or antibody ID11 against PLN (15), as described previously (8), except that PROTRAN\textsuperscript{™} nitrocellulose membranes with a pore size of 0.05 microns (Schleicher & Schuell) were used. Antibody binding was detected by chemiluminescence using an ECL Western blotting detection system (Amersham Corp). Oligomer/monomer ratios were calculated by scanning densitometry of individual lanes in the exposed films of the immunoblots of the gels.
RESULTS AND DISCUSSION

In earlier studies (8) we demonstrated that inhibitory interactions between PLN and SERCA2a occur through intramembrane interactions. To characterize these inhibitory interactions, we mutated each of PLN transmembrane (domain II) amino acids, Leu31 through Leu52, to Ala and expressed the mutant PLN cDNAs with SERCA2a cDNA in HEK-293 cells. Microsomes were isolated from transfected cells and Ca\(^{2+}\) dependence of Ca\(^{2+}\) transport was measured for each isolate. The reduction in the apparent affinity of SERCA2a for Ca\(^{2+}\) (\(\Delta K_{Ca}\) expressed in pCa units) provided a measure of the inhibitory function of wild type or mutated PLN (Fig. 1A). The \(\Delta K_{Ca}\) for PLN was \(-0.33\) pCa units. PLN mutants, L31A, N34A, F35A, I38A, L42A, I48A, V49A, and L52A, had diminished inhibitory function relative to PLN, as indicated by \(\Delta K_{Ca}\) values between 0 and about \(-0.2\) pCa units. PLN mutants, F32A, I33A, L37A, I40A, L43A, L44A, I45A, L50A, and L51A, gained inhibitory function relative to PLN, as indicated by \(\Delta K_{Ca}\) values between about \(-0.43\) and \(-0.8\) pCa units. PLN mutants, C36A, L39A, C41A, I45A and C46A, had unaltered function, as indicated by \(\Delta K_{Ca}\) values not significantly different from \(-0.33\) pCa units. Loss or gain of PLN function could not be correlated with different levels of expression (Fig. 1B). Moreover, supershifting was not induced by overexpression of PLN (8).

We have assumed that residues Leu\(^{31}\) to Leu\(^{52}\) form an \(\alpha\)-helix (16–19), and in Fig. 1A, we have numbered them in four linear sequences (1–7, 1–7, 1–7, and 1) corresponding to repeating positions in an \(\alpha\)-helix with 3.5 residues per turn. Loss and gain of function were expected if loss of function were associated with mutations on one face of the PLN domain II helix and gain of function were associated with mutations on the opposite face.

Wild type PLN is about 75% pentameric in SDS-PAGE (Fig. 1B, Table I), but is dissociated to a monomer by boiling in SDS under reducing or nonreducing conditions (9). Mutants of Leu\(^{37}\), Ile\(^{40}\), Leu\(^{44}\) and Ile\(^{47}\) are monomeric without boiling, as suggested by their mobility in SDS-PAGE (16, 19). These studies have led to structural models in which these residues lie on one face of the PLN domain II helix and play a role in pentamer formation (18, 19). In Fig. 1B, we show the results of SDS-PAGE analysis of the pentamer stability of unboiled samples of PLN and each of the 22 PLN mutants. Estimates of the percent monomer (Table I) show that the mutants can be divided into four broad classes.

**TABLE I**

| Class | Mutants | % monomer | n | Function |
|-------|---------|-----------|---|---------|
| Wild type | mean ± S.E. | 26 ± 3 | 7 | N |
| 1 | N34A | 18 ± 6 | 5 | L |
| | I38A | 24 ± 10 | 4 | L |
| | I45A | 13 ± 7 | 6 | N |
| | V49A | 11 ± 3 | 5 | L |
| | L52A | 17 ± 6 | 5 | L |
| 2 | L31A | 54 ± 9 | 6 | L |
| | F35A | 33 ± 13 | 4 | L |
| | C36A | 50 ± 13 | 4 | N |
| | L39A | 39 ± 10 | 4 | N |
| | L42A | 40 ± 11 | 7 | L |
| | C46A | 51 ± 13 | 7 | N |
| | I48A | 38 ± 15 | 4 | L |
| 3 | F32A | 69 ± 13 | 6 | G |
| | I33A | 71 ± 15 | 5 | G |
| | C41A | 74 ± 10 | 7 | N |
| | L43A | 78 ± 7 | 8 | G |
| | M50A | 65 ± 5 | 4 | G |
| | L51A | 85 ± 4 | 6 | G |
| 4 | L37A | 94 ± 1 | 4 | G |
| | I40A | 100 ± 5 | 4 | G |
| | L44A | 97 ± 3 | 4 | G |
| | I47A | 95 ± 2 | 4 | G |

**Phospholamban Activation**

In Fig. 1A, effects of mutations and double mutations in domain II of PLN on the affinity of SERCA2a for Ca\(^{2+}\). For each mutant, the normal amino acid residue is defined on the left in a single-letter code, its position in the sequence is identified by a number, and the newly introduced amino acid residue is defined on the right in a single-letter code. K\(_{Ca}\) is the Ca\(^{2+}\) concentration at which half-maximal Ca\(^{2+}\) uptake rates were observed. The vertical dashed line on the left represents the K\(_{Ca}\) value for SERCA2a expressed in the absence of PLN (K\(_{Ca}\) = 6.55), and the vertical dashed line to its right represents K\(_{Ca}\), the K\(_{Ca}\) value for SERCA2a alone (the apparent affinity of SERCA2a for Ca\(^{2+}\) is decreased in the presence of PLN). Data are mean ± S.D.: *, p < 0.05 versus −PLN, and †, p < 0.05 versus +PLN, as judged by analysis of variance followed by the Scheffe F test. The repeated numbers, 1–7 (in brackets), represent the location of each residue in a 3.5 residue/turn helix, as predicted for PLN domain II (18). B, effects of double mutations on the stability of PLN pentamers. SERCA2a and wild type and mutant forms of PLN, expressed in HEK-293 cell microsomes, were separated by SDS-PAGE at room temperature and subjected to immunoblotting. Individual lanes are representative of several experiments that were averaged to obtain the values for percent monomer, presented in Table I. PLN\(_{N}\) and PLN\(_{M}\) refer to pentameric and monomeric forms of phospholamban, respectively. The repeated numbers, 1–7 on the abscissa, represent the location of each residue in a 3.5 residue/turn helix (18). C, effects of double mutations on the stability of PLN pentamers. Lanes: 1, control; 2, PLN−; 3, PLN+; 4, N34A/I40A; 5, F35A/I44A.

Monomer percent was determined by scanning densitometry of pentameric and monomeric bands in individual lanes from SDS-PAGE gels, as described under “Experimental Procedures.” Inhibitory function is indicated: N, normal; L, loss of inhibitory function; G, gain of inhibitory function.
Class 1 has the same or less monomer than wild type PLN; Class 2 has roughly twice as much monomer as PLN; Class 3 has roughly three times as much monomer as PLN; and Class 4 has roughly four times as much monomer as PLN, being virtually 100% monomer. As in Fig. 1A, we have numbered the PLN mutants in Fig. 1B in four linear sequences of seven residues. Loss and conservation of pentamer stability, like loss and gain of function, is cyclical and progressive, repeating every three or four residues, as would be expected if loss of stability were associated with mutations on one face of the PLN domain II helix. The cyclical changes of loss and gain of function and of pentamer stability are clearly in phase.

When the location of each mutated amino acid is plotted on a helical wheel representing the PLN domain II helix (Fig. 2), it is apparent that different classes of mutants are clustered. Loss of function mutants lie on one face of the helix (the proposed exterior face of each helix in a PLN pentamer (18, 19)). Measurement of percent monomer places all of these residues in Classes 1 or 2. Gain of function mutants all lie on the opposite face of the helix (the proposed interior face of each helix in a PLN pentamer (18, 19)). Measurement of percent monomer places all of these mutants in Classes 3 or 4. Measurement of percent monomer in the five mutations that did not alter function placed them in Classes 1, 2, or 3.

We propose that the loss of function that occurs with each of the eight mutants, L31A, N34A, F35A, I38A, L42A, I48A, V49A, and L52A, on the exterior face of the helix, reflects the fact that each of these amino acids is essential for the interaction of the PLN domain II helix with complementary amino acids in the hydrophobic, transmembrane helices in SERCA2a. Ile45 and Cys41 lie on this helical face, but their function was unaltered by mutation. Ile45 (forming a Class 1 mutant) may have no suitable complement in the helical transmembrane sequences of SERCA2a and may play no role in interhelix interactions. The C41A mutant is substantially depolymerized (Class 3), but function is unaltered. This suggests that depolymerization is not the only requirement for activation of inhibitory function. The next steps must be formation of a PLN monomer/SERCA2a complex, followed by the formation of inhibitory interactions between specific amino acids. The ultimate stability of the inhibited PLN monomer/SERCA2a complex will also determine the degree of gain of inhibitory function.

Mutation of Cys36, Leu39, or Cys46 did not alter inhibitory function, but doubled the extent of depolymerization, placing these mutants in Class 2. These residues lie on an ill-defined boundary between the two functional domains where, in these cases, mutation does not seem to be particularly critical for either inhibitory function or pentamer formation. The definition of this boundary is of interest, because the Class 3, gain of function mutant, F32A, is clearly not a nonfunctional resident of the “boundary” area. Perhaps there is a distortion of the transmembrane helix near its cytoplasmic boundary, which aligns Phe32 with the interior face of the rest of the helix or the bulky side chain of Phe32 might, itself, distort the boundary.

All three Cys to Ala mutations destabilized the pentamer (Fig. 1B and Ref. 20), but retained normal function (Fig. 1A). Since Cys is “tiny”, whereas residues at each of the other 19 positions are “small” (Val, Asn) or “bulky” (Leu, Ile, Met, Phe) (21), the mutation of Cys to Ala would not alter the bulk of the side chain significantly. This may account, in part, for their unaltered function. However, we do not know enough about the full effects of these mutant proteins on the formation of an inhibited PLN monomer-SERCA2a complex to understand their full meaning, nor do we understand why mutation of all three of these residues destabilizes the pentamer so effectively, when they are not believed to be key stabilizing residues in the PLN pentamer (18, 19).

The nine Class 3 and 4 mutants, I33A, F32A, L37A, I40A, L43A, L44A, I47A, M50A, and L51A, proposed to lie on the interior face of the helix (18), are gain of function mutants. We propose that gain of function is a direct consequence of enhanced monomer formation. The 2.5-fold increase in inhibitory function that accompanies the 3–4-fold enhancement of monomer formation provides kinetic evidence that PLN monomers are the functional inhibitory form, while PLN pentamers represent a noninhibitory or less inhibitory reservoir. In chemical terms, the concentration of PLN monomers, a function of the dissociation constant of the PLN pentamer, will be a key determinant of the concentration of the inhibited PLN monomer-SERCA2a complex ([MS]) and of the degree of SERCA2a inhibition.

We tested the hypothesis that loss of function PLN mutants, with nonfunctional substitutions at specific sites that are essential for interaction with SERCA2a, would be dominant over gain
of function mutants, activated by an increase in their monomer concentration, by creating double mutants. In agreement with our hypothesis, the double mutants N344A/I404A and F35A/L44A were found to be virtually inactive (Fig. 1A), even though both were monomeric (Fig. 1C). The mutants form monomers, they may form PLN monomer-SERCA2a complexes, but inhibitory interactions clearly do not occur in the complexes.

Although loss of PLN function is likely to result from disruption of interactions between amino acids in transmembrane helices of PLN and SERCA2a, as proposed, it is also possible that it could result from pentamer stabilization. Data in Table I illustrate that the loss of function mutants, N344A, I38A, V49A and L52A, formed slightly more stable pentamers, in line with their loss of function. The mutant I45A also formed more stable pentamers, however, and function was not altered in this case. These discrepancies will, no doubt, be clarified by further study of the subtleties of the formation of the PLN monomer-SERCA2a complex and the formation of inhibitory interactions within the complex.

Gain of inhibitory function might occur through transmembrane or cytoplasmic interaction sites or both. Gain of function was first observed in studies involving coexpression of SERCA2a with PLN domain II constructs to which NH2-terminal, cytoplasmic epitopes such as PLN 1–20 or hemagglutinin were fused (8). An examination of Fig. 2B in Ref. 8 shows that PLN1–20–PLN30–52 was a Class 3 mutant that gained function, while Flag-PLN25–52 was a Class 2 mutant with unaltered function. These observations suggest that at least one form of gain of function results from PLN domain II interactions with SERCA2a. As a further test of this hypothesis, we constructed the Met-PLN25–52 mutant, L37A, to increase the probability that this simple domain II construct would be maximally monomeric, in line with structural predictions for the PLN transmembrane domain. The L37A mutation enhanced the inhibitory properties of Met-PLN25–52 by ΔK(M) of about −0.17 pCa units (8) to ΔK(M) of about −0.3 pCa units (data not shown). We conclude that gain of inhibitory function can be accomplished with only the transmembrane domain of PLN, but we recognize that other means of increasing the concentration of the inhibited PLN monomer-SERCA2a complex would also result in gain of function.

Fig. 3 illustrates our model for PLN interaction with SERCA2a. We deduce that PLN monomers (M) are the functional species and that their dissociation from pentamers (P) is an essential step in SERCA2a (S) inhibition by PLN. The fact that PLN is about 25% depolymerized under normal conditions implies that PLN monomers are normally in relatively abundant supply. The dissociation constants for both the PLN pentamer (Kd1) and the PLN monomer/SERCA2a heterodimer (Kd2) will control both the PLN monomer concentration [M] and the concentration of the monomer-inhibited form of SERCA2a [MS], defined as follows.

\[
P \rightarrow 5M \quad (K_{d1} = [P][M]) \quad \text{(Eq. 1)}
\]

\[
M + S \rightarrow MS \quad (K_{d2} = [MS][M][S]) \quad \text{(Eq. 2)}
\]

The base line of inhibition of wild type SERCA2a by wild type PLN will be proportional to [MS], but [MS] can be altered by mutations or by physiological perturbations. Mutations that enhance PLN monomer formation will alter Kd1 and increase both [M] and [MS]. Mutations that increase the affinity of the interaction between M and S will alter Kd2 and increase [MS], while mutations that decrease affinity will decrease [MS]. Binding of Ca\(^{2+}\) to the transmembrane helices of SERCA2a (22) and phosphorylation of the cytopltoplasmic domain of PLN (1, 5, 23) alter Kd2, enhancing PLN/SERCA2a heterodimer dissociation and activating SERCA2a. Fig. 3 illustrates the complexity of SERCA2a regulation by PLN, which has only been recognized recently (8) and demonstrates that the PLN/SERCA2a interaction provides a novel model of regulation in which an oligomer acts as a reservoir for the generation of active monomer subunits.

Our observations have important physiological and medical relevance. If mutations of Phe32, Ile33, Leu37, Ile40, Leu44, Ile47, Met50, and Leu51 were to occur naturally, we predict that they would increase [MS] to levels that would inhibit Ca\(^{2+}\) removal from the cytoplasm of myocardial cells. Although other Ca\(^{2+}\) removal systems might compensate (24), we predict that the resulting disruption in Ca\(^{2+}\) regulation would lead to cardiomyopathy. We also predict that any mutations in the PLN transmembrane helix, in SERCA2a transmembrane helices, or elsewhere in PLN or SERCA2a, that would increase the affinity between M and S, would also increase [MS], leading to gain of PLN inhibitory function and to cardiomyopathy.

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REFERENCES
1. Fuji, J., Ueno, A., Kitano, K., Tanaka, S., Kadoma, M., and Tada, M. (1987) J. Clin. Invest. 79, 301–304
2. Luo, W., Grupp, I. L., Harrer, J., Ponniah, S., Grupp, G., Duffy, J. J., Doetschman, T., and Kranias, E. G. (1984) Circ. Res. 55, 401–409
3. Jones, L. R., Simmnerman, H. K. B., Wilson, W. W., Gard, P. R. N., and Wegener, A. D. (1985) J. Biol. Chem. 260, 7721–7730
4. Tada, M. (1992) Ann. N. Y. Acad. Sci. 671, 92–102
5. James, P., Inui, M., Tada, M., Chiesi, M., and Carufolo, E. (1989) Nature 342, 90–92
6. Toyofuku, T., Kurzyludowski, K., Tada, M., and MacLennan, D. H. (1994) J. Biol. Chem. 269, 3088–3094
7. Toyofuku, T., Kurzyludowski, K., Tada, M., and MacLennan, D. H. (1994) J. Biol. Chem. 269, 22929–22932
8. Kimura, Y., Kurzyludowski, K., Tada, M., and MacLennan, D. H. (1996) J. Biol. Chem. 271, 21726–21731
9. Fuji, J., Lytton, J., Tada, M., and MacLennan, D. H. (1988) FEBS Lett. 227, 51–55
10. 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
11. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
12. Maruyama, K., and MacLennan, D. H. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3314–3318
13. Kaufman, R. J., Davies, M. V., Pathak, V. K., and Hershey, J. W. (1989) Mol. Cell. Biol. 9, 946–958
14. Kingston, R. E., Chen, C. A., and Okahaya, H. (1990) in Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., and Struhl, K., eds) pp. 9.1.1–9.1.9, John Wiley and Sons, New York
15. Mayer, E. J., McKenna, E., Garsky, V. M., Burke, C. J., Mach, H., Middaugh, C. R., Sardana, M., Smith, J. S., and Johnson, R. E. (1996) J. Biol. Chem. 271, 1669–1677
16. Arkin, I. T., Adams, P. D., MacKenzie, K. R., Lemmon, M. A., Brunger, A. T., and Engelmann, D. M. (1994) EMBO J. 13, 4757–4764
17. Arkin, I. T., Rothman, M., Ludlam, C. P., Aimoto, S., Engelmann, D. M., Rothchild, K. J., and Smith, S. O. (1995) J. Mol. Biol. 248, 824–834
18. Adams, P. D., Arkin, I. T., Engelmann, D. M., and Brunger, A. T. (1995) Nat. Struct. Biol. 2, 154–162
19. Simmnerman, H. K. B., Kohayashi, Y. M., Autry, J. M., and Jones, L. R. (1996) J. Biol. Chem. 271, 5941–5946
20. Fuji, J., Maruyama, K., Tada, M., and MacLennan, D. H. (1989) J. Biol. Chem. 264, 12950–12955
21. Taylor, W. R. (1986) J. Theor. Biol. 119, 205–218
22. Clarke, D. M., Loo, T. W., Inesi, G., and MacLennan, D. H. (1986) Nature 329, 476–478
23. Simmnerman, H. K. B., Collins, J. H., Theibert, J. L., Wegener, A. D., and Jones, L. R. (1986) J. Biol. Chem. 261, 13333–13341
24. Odermatt, A., Taschner, P. E. M., Khanna, V. K., Busch, H. F. M., Karpati, G., Jablecki, C. K., Breuning, M. H., and MacLennan, D. H. (1996) J. Clin. Invest. 94, 191–194