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Total synthesis and functional properties of the membrane-intrinsic protein phospholamban

THOMAS VORHERR,[1,3] ANTONI WRZOSEK,[2] MICHELE CHIESI,[2] AND ERNESTO CARAFOLI[1]

[1] Institute of Biochemistry, Swiss Federal Institute of Technology (ETH), CH-8092 Zürich, Switzerland
[2] Pharmaceuticals Division, Ciba-Geigy AG, 4002 Basle, Switzerland

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Abstract

The membrane-intrinsic protein phospholamban (PLN), the regulatory protein of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase, was chemically synthesized. The synthesis was accomplished by double couplings and efficient capping procedures, thus eliminating hydrophobic failure sequences. The crude peptide was purified by high-performance liquid chromatographic ion exchange and gel permeation chromatography in chloroform-methanol mixtures. Ion spray mass spectroscopy showed that the product had the correct molecular mass. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis runs produced the typical monomer-pentamer structural pattern. A predominantly helical CD spectrum was obtained in 0.075% $C_{12}E_8$ (67.9% helix, 1.8% beta, 12.2% turn, 18.1% random coil). Synthetic PLN was phosphorylated in detergent solutions by protein kinase A with a stoichiometry close to 1:1 (Pi to PLN monomer). Reconstitution of the isolated skeletal muscle SR Ca^{2+} ATPase in phosphatidylcholine membranes in the presence of PLN using the freezing and thawing technique yielded a preparation with lower Ca^{2+}-dependent ATPase activity. The inhibition was mainly due to a decrease in the affinity (K_m(Ca)) of the ATPase for Ca^{2+} and was partially reversed by PLN phosphorylation with protein kinase A. By contrast, addition of PLN to diluted intact SR vesicles uncoupled the Ca^{2+}-transport reaction, suggesting an ionophoric effect of PLN. Because this effect was observed at very high PLN-to-SR vesicle ratios and was not influenced by PLN phosphorylation, its biological function is doubtful.

Keywords: ATPase; calcium transport; pumps

The rapid and cyclic changes of free Ca^{2+} concentration in the cytosol of myocytes determine the contractile state of muscle. In mammalian myocardium, the majority of the excitatory Ca^{2+} derives from the SR network. Loading of the SR with Ca^{2+} occurs via a Ca^{2+} pumping ATPase, whose activity is regulated by the SR-intrinsic protein phospholamban (PLN) (Tada et al., 1974). Phosphorylation of PLN by various kinases leads to the activation of the Ca^{2+} pump (Tada et al., 1974; Le Peuch et al., 1979; Limas, 1980; Movsesian et al., 1984; Gasser et al., 1986). The increase in the contractile force and in the speed of relaxation of the heart induced by β-agonists can be at least partially explained by the stimulation of the Ca^{2+} pumping ATPase activity caused by the phosphorylation of PLN by protein kinase A. The primary structure of PLN has been deduced from cDNA clones (Fujii et al., 1987): it is generally assumed that the quaternary structure of PLN corresponds to a pentamer of five identical subunits (Gasser et al., 1984; Wegener & Jones, 1984). Each subunit contains 52 amino acids with a highly hydrophobic C-terminal portion probably inserted into the SR membrane. The N-terminal sequence of the monomers is amphiphilic and contains the target sequences for the kinases (Wegener et al., 1986; Fujii et al., 1987). It also contains the domain(s) that interact(s) with the Ca^{2+}-

Reprint requests to: Ernesto Carafoli, Laboratory of Biochemistry III, Swiss Federal Institute of Technology (ETH), 8092 Zürich, Switzerland.

[3] Present address: F. Hoffmann La-Roche AG, 4002 Basle, Switzerland.

Abbreviations: Boc, tertiary butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DIPEA, diisopropyl ethylamine; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DOC, deoxycholate; EDT, ethanedithiol; EGTA, (ethylenebis(oxyethylenenitrilo))tetraacetic acid; Fmoc, 9-fluorenylmethyloxycarbonyl; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); HFIP, hexafluoroisopropanol; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MeOH, methanol; MOPS, 3-morpholino propane sulfonic acid; NMP, *N*-methylpyrrolidone; PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylcholine; PLN, phospholamban; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; SR, sarcoplasmic reticulum; SDS, sodium dodecyl sulfate; TBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; tBu, tertiary butyl; TFA, trifluoroacetic acid; Trt, trityl, PTC, phenylthiocarbamoyl.

ATPase: the direct interaction of the two molecules with each other has been recently demonstrated by cross-linking experiments (James et al., 1989). The isolation of PLN from SR membranes has been traditionally very difficult, only minute quantities being routinely obtained. Several laboratories have used synthetic peptides derived from PLN for functional studies (Kim et al., 1990; Sasaki et al., 1992; Vorherr et al., 1992). However, the total chemical synthesis of the protein has not yet been reported. This is probably due to the presence of patches of hydrophobic amino acids (Val, Leu, Ile), which strongly reduce the coupling efficiency in solid-phase peptide chemistry, and to the strong tendency of PLN to aggregate. As a result, the purification of the protein by the usual reverse-phase HPLC methods is fraught with problems. In this study, appropriate synthetic and separation strategies were used to achieve the total synthesis and the purification of PLN. The physicochemical and functional properties of the synthetic protein were found to correspond to those of native PLN.

Results

Synthesis of PLN

Because the synthesis process started from the hydrophobic C-terminal, double couplings and an efficient capping were used to eliminate hydrophobic failure sequences. The synthesis strategy is summarized in Figure 1. The synthesis was initiated from Fmoc Leu attached to SASRIN-resin to allow cleavage from the support without interfering with the side-chain protection. The activation for the first coupling reaction was performed by TBTU/HOBt using 2 equivalents of diisopropylethylamine. As expected, when single couplings with TBTU/HOBt were performed on small scale pilot experiments, the quantitative Kaiser test showed reduced coupling yields, especially in the hydrophobic portion of the protein (Fig. 2). Thus, in all subsequent experiments it was decided to employ double couplings throughout the synthesis and to use the symmetrical anhydride for the second coupling reaction in the presence of 15% DMSO in the coupling cocktail. This protocol produced reasonable coupling yields (Fig. 2). Capping was performed with acetanhydride to avoid the production of hydrophobic failure sequences containing basic residues. Thus, truncated hydrophobic peptides were easily separated by HPLC ion exchange chromatography. After final acetylation in acetanhydride/methylene chloride/pyridine (1:1:1), cleavage from the support was performed in methylene chloride containing 5% TFA, 0.2% mercaptoethanol, 0.2% thioanisole, and 0.2% dimethylsulfide. No reattachment of the target peptide to the resin, e.g., through Cys side chains, could thus occur. Cleavage of the side-chain protection was performed in a mixture of 4.5% H_2O, 3% thioanisole, 2% EDT, 4.5% phenol (w/v), 2.5% DMSO, and 83.5% TFA for 6.5 h at room temperature. The first purification step by cation exchange chromatography was performed on a CM Serva HPLC column (250 × 10 mm) in chloroform/MeOH/water (4:4:1) with

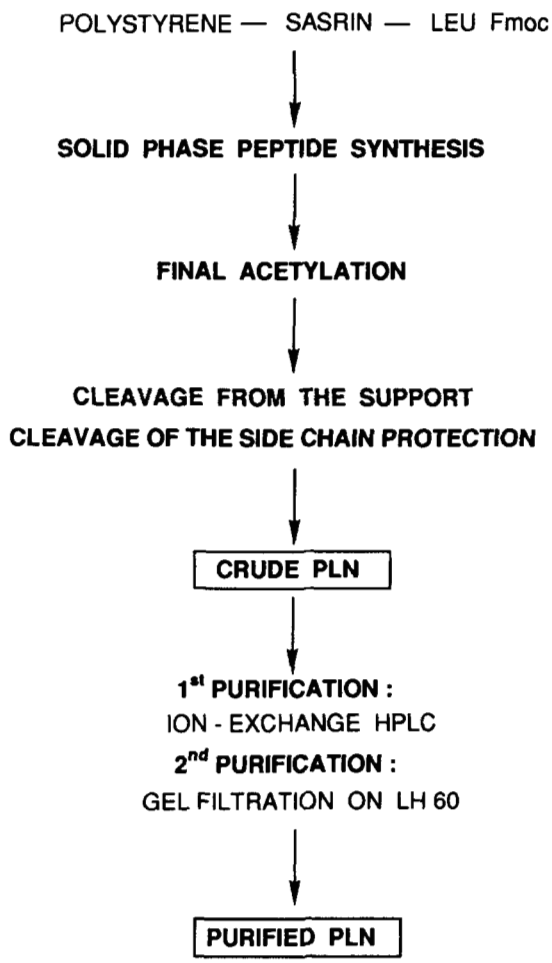

Fig. 1. Scheme for the synthesis and purification of PLN.

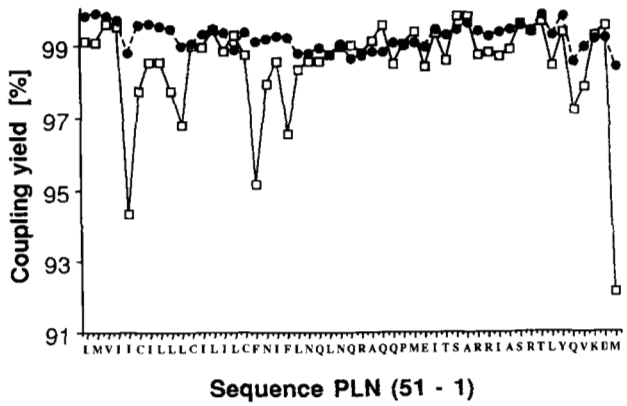

Fig. 2. Coupling yields determined according to the quantitative Kaiser test as described in the manual of the 431 peptide synthesizer of ABI. □, First coupling reaction; ●, Second coupling reaction.

a gradient up to 0.3 M ammonium acetate in the same solvent system (Simmerman et al., 1989). The final purification was achieved by gel filtration on a Sephadex LH60 (1000 × 20 mm) column in chloroform/MeOH (1:1) containing 2.5% H_2O, 0.25% TFA. Ion spray mass spectrometry tests showed a series of ions that corresponded to the expected molecular mass (M_r 6,122.4) (Fig. 3). SDS-PAGE produced the typical monomer–pentamer pattern observed for native PLN (Fig. 4). Complete dissociation of pentameric PLN on SDS gels requires boiling in the presence of high concentrations of SDS (Lamers & Stinis, 1980) or of a mixture of SDS/Triton X-100 (Le Peuch et al., 1980).

CD and ultracentrifugation studies

CD spectroscopy was carried out in a phosphate buffer (pH 7.2) containing 0.075% $C_{12}E_8$, or in organic solvents such as MeOH, TFE, and HFIP. The CD spectrum obtained in $C_{12}E_8$ showed two minima (at 209 and 222 nm) and a maximum at 194 nm (Fig. 5). A similar, typically helical spectrum was obtained in MeOH (data not shown), although the total molar ellipticities were considerably smaller. The secondary structure percentages calculated for the spectra in the two solvents showed slight differences. From the spectrum in the $C_{12}E_8$ solution the following percentages were calculated: α-helix, 67.9%; β, 1.8%; turn, 12.2%; random coil, 18.1%. The spectrum in MeOH had a higher percentage of α-helix and β structure (helix, 73.7%; beta, 10.7%; turn, 15.3%; random coil, 0.3%). These spectra are very similar to those recorded for native PLN by Simmerman et al. (1989). In TFE, and especially in HFIP, the short wavelength minimum was more pronounced, suggesting a less ordered structure (data not shown). However, the typical helical features were still present.

The ultracentrifugation studies in MeOH at 24,000 rpm yielded an average molecular mass of 12,351, consistent with a dimeric state of PLN. The application of ultracentrifugation techniques to molecular mass determination of proteins in nonionic detergents has been described (Ludwig et al., 1982; Ott et al., 1982; Holzenburg et al.,

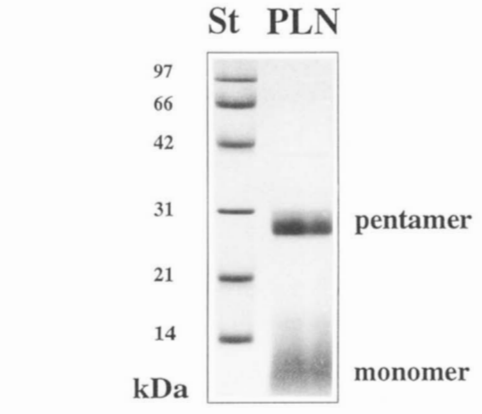

Fig. 4. SDS-PAGE of the synthetic PLN. Synthetic PLN (5 μg) was loaded on a 15% polyacrylamide gel and separated as described by Laemmli (1970). The gels were stained with Coomassie brilliant blue. Other details are found in the Materials and methods. St, molecular mass markers.

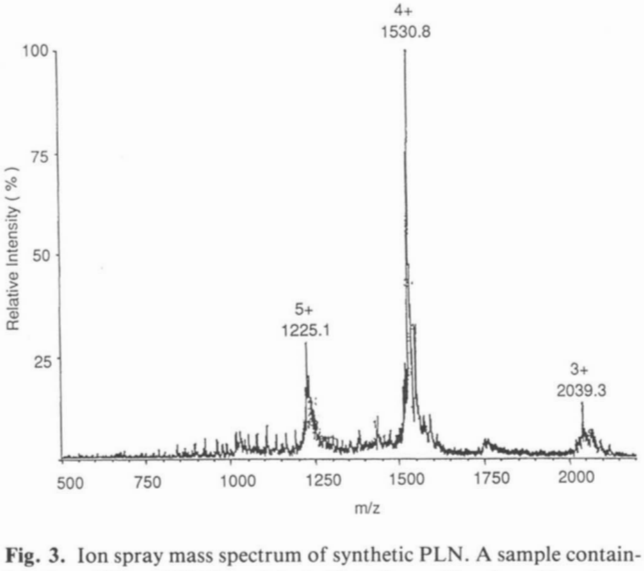

Fig. 3. Ion spray mass spectrum of synthetic PLN. A sample containing about 20 μg of PLN was injected into the capillary in a chloroform: MeOH mixture (1:1). The spectrum shows the $(M + 3H)^{3+}$, $(M + 4H)^{4+}$, and $(M + 5H)^{5+}$ ions of PLN (M_r 6,121.2 ± 3.2).

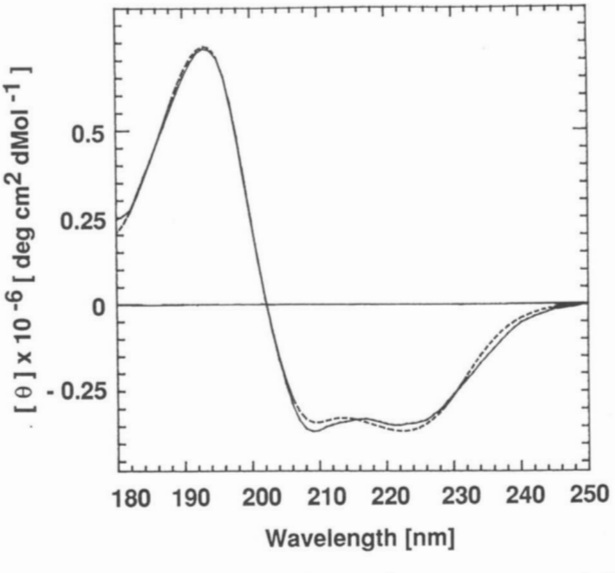

Fig. 5. CD spectra of the synthetic PLN. The spectra were recorded in a buffer containing 10 mM Pi, pH 7.0, and 0.075% $C_{12}E_8$. The PLN concentration was 1 mg/mL. The secondary structure percentages were calculated according to the instructions provided by the manufacturer (helix, 67.9%; β, 1.8%; turn, 12.2%; random coil, 18.1%). The dashed line (- - - -) corresponds to the CD spectrum calculated for the secondary structure percentages indicated. Other details are found in the text.

1989). The studies in detergent solution were preferentially performed in 1% C_8E_5 because the density of the latter is close to 1.0 g cm^{-3}. However, the experiments in detergent solutions resulted in aggregates at pH 7.0 (100–180 kDa), whereas at pH 4.2 in 1% C_8E_5, two PLN components with sedimentation coefficients of 0.75 (ca. 40% of the total) and 2.5 (ca. 60% of the total) were observed. According to sedimentation equilibrium runs at 44,000 rpm, the molecular mass of the first component (0.75 sedimentation coefficient) was estimated to be 5,950 Da, consistent with a monomeric state. Two slopes were observed in the run at 22,000 rpm, and multicomponent analysis according to the method of Chernyak et al. (1982) was carried out. One component consisted most likely of pentamers since the molecular mass was determined to be 30–36 kDa, corresponding, for a globular molecule, to a sedimentation coefficient of 2.5. The second component showed a molecular mass of 59–65 kDa, thus reflecting the presence of higher mass aggregates. These findings are consistent with the properties of PLN in SDS-PAGE runs.

Figure 4 shows that synthetic PLN migrated essentially as a pentamer in SDS gel electrophoresis, with an apparent molecular mass of about 26–28 kDa. As is the case for native PLN, complete monomerization could only be achieved by boiling the protein in the presence of high concentrations of SDS (not shown).

Functional properties of synthetic PLN

The N-terminal amphiphilic domain of PLN contains the serine and threonine residues that become phosphorylated by the appropriate kinases (Wegener et al., 1986; Fujii et al., 1987). Synthetic PLN, maintained in suspension in the presence of a nondenaturing detergent such as $C_{12}E_8$, was phosphorylated by the catalytic subunit of the cAMP-dependent protein kinase. The maximal phosphorylation stoichiometry obtained after 1 h of incubation in the presence of 300 U of the catalytic subunit of the kinase per milliliter was 0.75 ± 0.05 mol of phosphate incorporated per PLN monomer. In solutions containing nondenaturing detergents, some of the PLN was present in the form of high molecular mass aggregates, thus explaining the molar phosphorylation stoichiometry of less than one. Stronger detergents could not be employed because they would have inactivated the kinase.

Various reconstitution protocols using synthetic PLN and either whole SR membranes or the Ca^{2+}-ATPase isolated from the fast-twitch muscles were compared to test the functional effects of the synthetic product. Reconstitution was first attempted by the widely used slow dialysis procedure using synthetic PLN mixed with skeletal muscle SR membranes solubilized by DOC (see the Materials and methods). Functional reconstitution of the pump was achieved; however, no significant effects on the uptake of Ca^{2+} and on the Ca^{2+}-dependent hydrolytic activity were induced by adding PLN to the reconstitution medium at a molar ratio of 5:1 (PLN to ATPase) (not shown). On the other hand, direct addition of PLN to intact skeletal muscle SR vesicles produced a marked inhibition of Ca^{2+} uptake (Fig. 6). The inhibition was dependent on the amount of SR membranes utilized in the assay medium and was evident only when low amounts of SR protein (below 10 μg/mL) were used. The inhibition was also dependent on the free Ca^{2+} concentration in the uptake medium, i.e., it was clearly evident in the presence of submicromolar Ca^{2+}, and barely significant at saturating concentrations (Fig. 7). The effect of synthetic PLN on the Ca^{2+}-dependent ATPase activity was studied under identical conditions. Surprisingly, a stimulation of the hydrolytic activity was observed (Fig. 8), suggesting that the addition of PLN to the SR vesicles rendered them leaky to Ca^{2+} ions. This "uncoupling" effect of PLN was not reversed by the addition of the catalytic subunit of protein kinase A (up to 200 U/mL; not shown) to the assay medium, i.e., by conditions that would promote PLN phosphorylation. A third reconstitution approach was then attempted. PLN was first integrated into the PC membranes, which were then mixed with isolated fast skeletal muscle SR Ca^{2+}-ATPase. Fusion of the liposomal preparations was obtained by the freeze and thaw

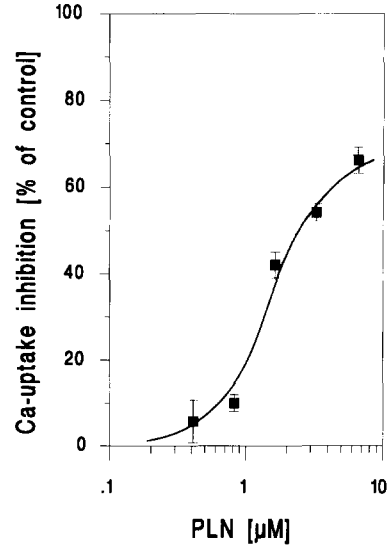

Fig. 6. Inhibition of Ca^{2+} uptake into skeletal muscle SR vesicles by PLN. The Ca^{2+} uptake by rabbit fast skeletal muscle SR vesicles was measured by the Millipore filtration technique at 37 °C in a medium composed of 100 mM KCl, 100 mM sucrose, 50 mM MOPS, pH 6.8, 5 mM $MgCl_2$, 5 mM NaN_3, 3 mM K-oxalate, 0.5 mM EGTA, and 10 μg SR protein/mL. The free Ca^{2+} was adjusted to 0.47 μM by appropriate additions of $CaCl_2$. Synthetic PLN was added as a DMSO solution directly to the uptake medium and incubated with the SR membranes for 5 min (the total DMSO content was adjusted to 1% in all experiments including the controls without PLN). The uptake reaction was then started by the addition of 2 mM Na_2-ATP. The data represents the mean ± SD of three different measurements.

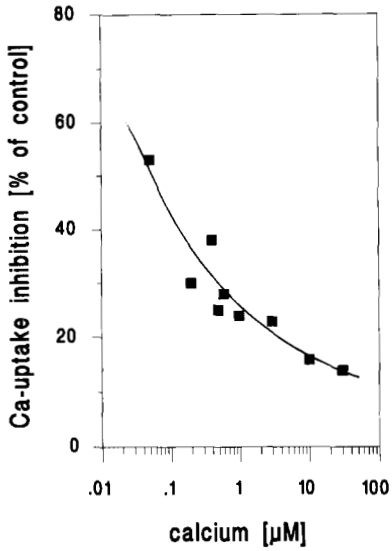

Fig. 7. Effect of Ca^{2+} on the inhibition of Ca^{2+} uptake in SR vesicles by the synthetic PLN. Ca^{2+} uptake by fast skeletal muscle SR membranes was carried out as described in the legend to Figure 6. When required, the uptake medium was supplemented with 2 μM synthetic PLN and with the concentrations of free Ca^{2+} indicated. The results are expressed as % inhibition of the Ca^{2+} uptake rate by PLN.

procedure. The Ca^{2+}-dependent ATPase activity of this reconstituted system was then compared to that of the preparations in which PLN was omitted from the PC reconstitution mixture. Figure 9 shows that in the presence

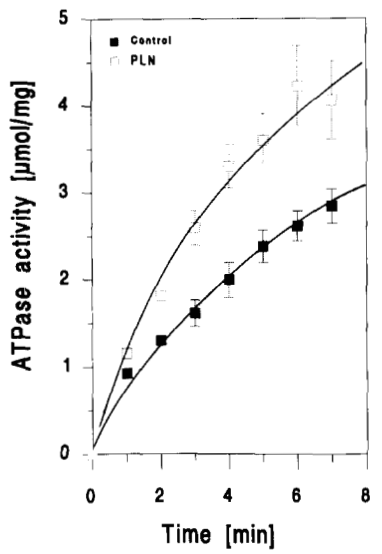

Fig. 8. Effect of synthetic PLN on the Ca^{2+}-dependent ATPase activity of skeletal muscle SR. Fast skeletal muscle SR membranes were incubated in the presence (□) or absence (■) of 2 μM synthetic PLN as described in the legend to Figure 6. The Ca^{2+}-dependent ATPase (see Materials and methods) was followed by measuring the liberation of phosphate from ATP at a free Ca^{2+} concentration of 10 μM as described by Lanzetta et al. (1979). The results represent the mean ± SD of three different measurements.

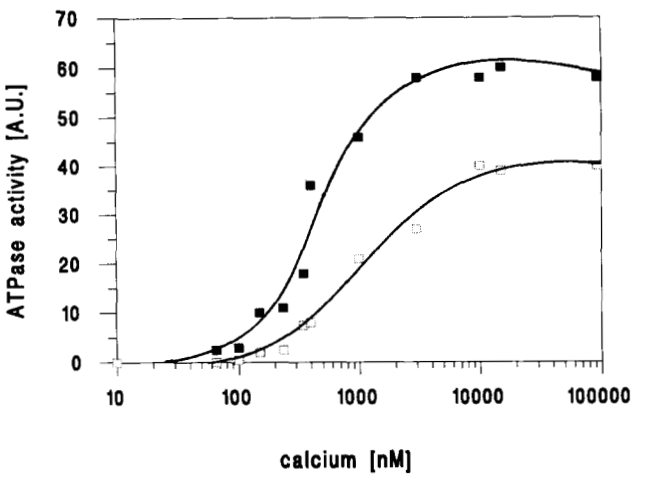

Fig. 9. Effect of synthetic PLN on the Ca^{2+}-dependent activity of isolated skeletal muscle SR after reconstitution. The Ca^{2+}-ATPase from rabbit skeletal muscle SR (see Materials and methods) was incorporated by the freeze-thaw procedure into PC liposomes without (■) or with synthetic PLN (□). The molar ratio of PLN monomer to the ATPase was 80:1. The Ca^{2+}-dependent ATPase activity of the reconstituted systems was then assayed by the coupled enzyme assay at 37 °C at the free Ca^{2+} concentrations indicated. The reaction medium contained 20 mM HEPES-Tris, pH 7.4, 100 mM KCl, 5 mM NaN_3, 5 mM $MgCl_2$, 1 mM EGTA, various amounts of $CaCl_2$, 0.2 mM NADH, 2 mM phosphoenolpyruvate 3 U/mL lactate dehydrogenase, 3 U/mL pyruvate kinase, and 2 μg ATPase/mL.

of co-reconstituting PLN the Ca^{2+}-dependent ATPase activity was inhibited. The effect was dual, i.e., the V_{max} decreased by 20–30% and, most importantly, the affinity for Ca^{2+} decreased from a K_m of about 0.4 to one of 1.0 μM (Fig. 8). The inhibition was partially reversed by phosphorylating PLN with exogenously added protein kinase A: preincubation of PLN in fused vesicles for 10 min at 37 °C in the presence of 20 U/mL of the catalytic subunit of the kinase stimulated the ATPase activity of the pump by 45–60% at a free Ca^{2+}-concentration of 0.4 μM. The Ca^{2+}-stimulated ATPase activity of membranes reconstituted in the absence of PLN was not affected (or even slightly inhibited) by the pretreatment with the catalytic subunit of the kinase.

Discussion

The modulation of the Ca^{2+} pumping function of heart and slow-twitch muscle SR by PLN was discovered nearly 18 years ago (Tada et al., 1974). It has gradually become evident that the modulation process is due to the reversible association of PLN, probably in its pentameric form, with the Ca^{2+} pump. Unphosphorylated PLN binds to the ATPase repressing its Ca^{2+} pumping function (James et al., 1989). Phosphorylation by at least two protein kinases removes PLN from its binding site on the pump, relieving the inhibited state of the latter. The binding site

has recently been identified C-terminally to the aspartic acid that forms the phosphorylated intermediate during the reaction cycle of the ATPase (James et al., 1989).

Biochemical studies of PLN, and thus of the mechanism of its modulating function of the pump, have been hampered by the unusual characteristics of the molecule. This small membrane-intrinsic protein is extremely insoluble and has a strong tendency to aggregate, forming association states eventually favoring the pentameric form (Wegener & Jones, 1984; Gasser et al., 1986). In spite of its abundance in the SR membrane, where PLN is generally thought to be stoichiometric with the Ca^{2+}-ATPase, only microgram amounts have traditionally been produced at the end of laborious purification procedures. The decision to chemically synthesize PLN in amounts adequate for detailed biochemical, and especially structural, studies was thus taken. The synthesis strategy was based on the properties of the 52-residue peptide: a very hydrophobic C-terminal and a very hydrophilic N-terminal. Thus, solid-phase peptide synthesis eliminating hydrophobic failure sequences by double couplings and efficient capping was used, yielding a crude product that could be purified to homogeneity by cation exchange chromatography and gel filtration. Because native PLN was found to completely dissociate into monomers (Boyot et al., 1989) in apolar solvents such as an acidified chloroform/MeOH mixture, these solvent systems were successfully employed for the purification.

The CD investigations showed that the secondary structure of the synthetic product, while varying somewhat from $C_{12}E_8$ to MeOH, had a strong predominance of α-helix. Very similar CD spectra have been obtained with native PLN (Simmerman et al., 1989). The ultracentrifugation studies revealed a dimeric state of PLN in MeOH: thus, the higher percentage of secondary structure calculated from the CD spectra corresponded to a more ordered state in MeOH. The synthetic protein had the expected high tendency to aggregate, showing the traditional pentamer–monomer transition in SDS–polyacrylamide gels. Unexpectedly, the ultracentrifugation experiments showed that PLN, which showed a clear tendency to favor the dimeric form in MeOH, formed high molecular mass aggregates in the detergent solution at neutral pH. However, the results at pH 4.2 were consistent with the monomer–pentamer pattern observed in SDS gels.

A recent study has shown that synthetic PLN peptides tend to remain monomeric after separation by SDS-PAGE (Sasaki et al., 1992). Even the hydrophobic peptides PLN 28–47 and PLN 8–47, which contain the three cysteine residues (Cys^{36}, Cys^{41}, Cys^{46}) that are apparently important for the formation of pentamers (Holzenburg et al., 1989) occur as multiple forms from monomer to pentamer, the proportion of pentamers being only 5%. The work presented here has shown that the complete synthetic PLN molecule occurred either as a monomer or as a pentamer in SDS gels in agreement with the behavior of native PLN. Because all the PLN peptides used in the study mentioned above (Sasaki et al., 1992) lacked the last five C-terminal amino acids, it could be tentatively suggested that the latter play a role in the formation and/or stabilization of the pentameric complex. In fact, another study has shown (Kovacs et al., 1988) that the putative membrane-spanning portion of PLN (tryptic peptide PLN 26–52) also migrated predominantly as a monomer or a pentamer on SDS gels. The functional properties of synthetic PLN were undistinguishable from those of the native protein, i.e., the protein in the membrane environment or after organic solvent extraction. No specific side-by-side comparison between the isolated and the synthetic PLN was carried out in the present study, owing to the difficulty in preparing adequate amounts of the native protein. The synthetic product was a substrate for the phosphorylating activity of protein kinase A. Although no specific tests were performed, it can be reasonably assumed that the calmodulin-dependent kinase would also phosphorylate the synthetic product. Synthetic PLN inhibited the SR Ca^{2+}-ATPase activity when coreconstituted in phospholipid membranes. Interestingly, the experiment was carried out using SERCA1a, i.e., the ATPase isoform, which is expressed in fast-twitch muscles: this confirms the suggestion that both the fast- and slow-twitch muscle Ca^{2+}-ATPase isoforms (i.e., SERCA1a and SERCA2a, respectively; Brandt et al., 1986) could in principle be modulated by PLN. In fact, the domain of the SR Ca^{2+}-ATPase that binds PLN is present in both SERCA1a and SERCA2a, and both isoforms have been shown to bind PLN in cross-linking experiments (James et al., 1989). The reconstitution experiments presented in this work corroborate the conclusions of recent observations on COS cells showing that the Ca^{2+} affinity of the fast-twitch muscle SERCA1a isoform was decreased when the latter was co-expressed with PLN (D.H. MacLennan, Toronto, pers. comm.). Naturally, PLN plays no physiological role in fast-twitch muscles because it is not expressed in them.

The effect of synthetic PLN on the reconstituted Ca^{2+}-ATPase was in line with that recently observed (Sasaki et al., 1992) using synthetic peptides derived from PLN. In that study, different segments of the PLN molecule were found to be responsible for the two major inhibitory effects of native PLN on the SR Ca^{2+}-ATPase, i.e., the membrane-extrinsic hydrophilic domain (residues 1–31) decreased the V_{max} of the enzyme, while the hydrophobic C-terminal portion (residues 28–47 or 8–47) decreased its affinity for Ca^{2+}. By using the same freezing and thawing reconstitution protocol, the present work has shown that the complete synthetic PLN (residues 1–52) possessed both functions, i.e., it decreased the V_{max} and increased the K_m (Ca^{2+}) of the ATPase.

It is intriguing that PLN should exert different effects on the function of SR membranes depending on the reconstitution system. Reconstitution with either the slow

DOC dialysis method, or by the Bio-Beads detergent removal procedure after solubilization with Triton X-100 yielded functionally competent membranes on whose Ca^{2+}-ATPase PLN had no effect whatsoever (Inui et al., 1986; this study). On the other hand, addition of synthetic PLN to diluted, intact SR membrane preparations from skeletal SR induced a marked inhibition of Ca^{2+} uptake that became very evident in the submicromolar Ca^{2+} concentration range. Parallel experiments under the same conditions showed that the Ca^{2+}-dependent ATPase activity became stimulated, i.e., synthetic PLN uncoupled the Ca^{2+}-transport reaction. This action of PLN is reminiscent of previous findings (Kovacs et al., 1988) of the possible ionophoric activity of PLN, i.e., PLN could form ion channels in lipid bilayers. It is interesting to note that also in the previous study, PLN was added directly to the buffer containing intact SR or preformed membranes. Possibly, under these conditions PLN became incorporated in the membrane in a way different from that produced by the freezing and thawing co-reconstitution experiments, i.e., a way that formed transmembrane channels. Because the uncoupling/ionophoric effect of PLN was not influenced by its phosphorylation state, its physiological significance is dubious.

Materials and methods

An Applied Biosystems (Foster City, California) Derivatizer 420A and on-line PTC detection with the model 130A Applied Biosystems Analyzer was used for the derivatization, separation, and identification of the amino acids. Ion spray mass spectrometry spectra were recorded on an API III mass spectrometer from Sciex (Toronto, Canada).

PLN (Ac-M-D-K-V-Q-Y-L-T-R-S-A-I-R-R-A-S-T-I-E-M-P-Q-Q-A-R-Q-N-L-Q-N-L-F-I-N-F-C-L-I-L-I-C-L-L-L-I-C-I-I-V-M-L-L) was synthesized on an Applied Biosystems Peptide Synthesizer model 431 using double couplings with a fivefold excess of amino acid derivative in each coupling step and a capping step after the second coupling. The following side-chain protection for the N-alpha Fmoc protected amino acids was employed: C, N, Q: Trt; D, E, S, T, Y: tBu; R: Pmc; K: Boc. The activation cocktail for the first coupling reaction consisted of amino acid derivative/TBTU/HOBt/DIPEA 1:1:1:2 in DMF according to the protocol developed by Applied Biosystems for HBTU activation. The second coupling was performed using preformed symmetric anhydrides in a mixture of 15% DMSO and 85% NMP. The derivatives were dissolved in the cartridge in a 1:1 mixture of DMSO/NMP, and DCC delivery and transfer were programmed as for the Module H used for loading the first amino acid onto the resin. The capping step was carried out using a solution of 0.5 M acetanhydride, 0.125 M DIPEA, and 0.2% HOBt in NMP according to the capping protocol proposed by the manufacturer. The Fmoc cleavage module was applied two times after the acetylation step. Final acetylation was carried out manually in a 1:1:2 mixture of acetanhydride/methylene chloride/pyridine. The coupling efficiency was monitored by the quantitative Kaiser test as described in the manufacturer's manual. Cleavage from the support in 5% TFA in methylene chloride containing 0.2% thioanisole and 0.2% dimethylsulfide for 10 min at room temperature was followed by cleavage of the side-chain protection in a mixture of 4.5% H_2O, 3% thioanisole, 2% EDT, 4.5% phenol (w/v), 2.5% DMSO, and 83.5% TFA for 6.5 h at room temperature, after filtering from the resin and removal of methylene chloride. A 2.11-g sample of fully protected peptide resin yielded 0.532 g of crude PLN.

Purification of PLN

Cation exchange chromatography was carried out on a CM Serva (250 × 10 mm) HPLC column (250 × 10 mm) in chloroform/MeOH/water (4:4:1) using a gradient up to 0.3 M ammonium acetate in the same solvent system. From 200 mg of crude product 21 mg of PLN were obtained. For the final purification by gel filtration, 20 mg of PLN purified by cation exchange were applied on a LH60 column (1,000 × 20 mm) in chloroform/MeOH (1:1) containing 2.5% H_2O and 0.25% TFA: 14 mg of purified PLN were obtained. The chromatography was carried out on LKB equipment (LKB, Uppsala, Sweden). UV detection was at 276 nm.

CD experiments

The CD spectra were recorded with a Jasco J-720 spectropolarimeter (Eastern, Maryland). The measurements were carried out in a far UV, jacketed, 0.2-mm quartz cuvette over the wavelength range 190–250 nm. Each spectrum was the average of four scans and was corrected for spurious signals generated by the solvent. The solutions were clarified by centrifugation, and the peptide concentrations were determined by amino acid analysis. The calculation of secondary structure percentages was performed using the Jasco software package according to the instructions in the manufacturer's manual.

Ultracentrifugation studies

Sedimentation velocities and sedimentation equilibrium experiments were performed on a Beckman Model E analytical ultracentrifuge (Beckman Instruments, Irvine, California) with absorption optics and a photoelectric scanning system. Sedimentation velocity runs were carried out in an An-D rotor using a 12-mm double sector cell at 22,000–56,000 rpm in phosphate buffer containing 1% C_8E_5 or $C_{12}E_8$, 100 mM NaCl (pH 7.0 and pH 4.2) and in MeOH. Scanner tracings were taken at 280 nm during both sedimentation velocity and sedimentation

equilibrium runs. For the calculation of molecular masses, linear regression (i.e., of the ln A versus r^2 plots; A, absorbance at 280 nm; r, distance of the protein in the cell from the rotor axis) and baseline determination programs were employed (written by H. Berger & A. Lustig, Biocenter, Basle, Switzerland). A partial specific volume of 0.73 $cm^3\ g^{-1}$ was used for the calculations. The buffer density, including the detergent, was taken as 1.0 $g\ cm^{-3}$ (Ott et al., 1989; Holzenburg et al., 1989).

Phosphorylation of PLN by the cAMP-dependent protein kinase 100 μg of PLN in 100 μL of 10 mM MOPS, pH 7.2, 500 mM NaCl, 0.1% $C_{12}E_8$ were mixed with 200 μL of 40 mM MOPS, pH 6.8, 10 mM $MgCl_2$, 8 mM EGTA containing 100 units of the catalytic subunit of the cAMP-dependent protein kinase. The phosphorylation reaction was started by the addition of 50 μL of 2 mM of (γ^{32}P)-ATP (1.5 10^6 cpm/nmol) and carried out for 16 h at 30 °C. The reaction was terminated by adding Laemmli sample buffer. Samples were then subjected to SDS-PAGE according to Laemmli (1970). The protein bands corresponding to PLN were cut from the gel, and the ^{32}P incorporation was quantified by liquid scintillation counting.

Isolation of skeletal muscle SR Ca^{2+}-ATPase

Rabbit fast skeletal muscle SR was isolated according to Eletr and Inesi (1972) and the ATPase was purified to homogeneity by the method of Van Winkle et al. (1978). The ATPase preparation obtained according to this procedure consisted of protein embedded in leaky phospholipid membranes and contained some residual detergent (deoxycholate).

Incorporation of Ca^{2+}-ATPase and PLN into PC liposomes

Basically, the procedure described by Sasaki et al. (1982) was applied. Synthetic PLN (200 μg) and PC (60 μg) were dissolved in 1.2 mL of chloroform/MeOH (1:1) and evaporated under a stream of N_2 at room temperature. The evaporated samples were then suspended in 500 μL of 10 mM Tris-maleate, pH 6.8, and sonicated at 4 °C for 15 s with a probe-type sonicator (Sonifier B-12, microtip output 7; Benson Sonic Power Company, Denburg, Connecticut) to form liposomes. After sonication, 8 μL of the purified Ca^{2+}-ATPase (4.75 mg protein/mL) were added to the PLN-containing vesicles. Incorporation of the Ca^{2+}-ATPase in the PLN–phospholipid vesicles was obtained by the freeze–thaw–sonication method. The samples were frozen in liquid N_2, thawed at room temperature, and sonicated for 15 s in a bath-type sonicator (Heat Systems Ultrasonic model W-375; Farmingdale, New York) with the output control set at 5, 50% duty cycle. This procedure was repeated three times. In control samples, the procedure was carried out in the absence of synthetic PLN.

Co-reconstitution of PLN and skeletal muscle SR membranes solubilized in DOC

Reconstitution of skeletal muscle SR was carried out according to the method described by Hymel and Fleischer (1988). Briefly, skeletal muscle SR (12 mg protein/mL of 0.3 M sucrose, 0.1 M KCl, and 5 mM HEPES, pH 7.1) was mixed with an equal volume of 0.5 M sucrose, 0.8 M KCl, 3 mM $MgCl_2$, 2 mM EDTA, 0.2 mM $CaCl_2$, and 20 mM Tris, pH 7.9, at 0 °C. The membranes were then solubilized by adding DOC (2.5 mg/mL). After incubation for 10 min on ice, the insoluble material was removed by centrifugation (40,000 rpm for 75 min at 0 °C, Beckman rotor 75 TI). Synthetic PLN was added to the supernatant at a ratio of 1:1 (Ca^{2+}-ATPase to PLN pentamer). The solubilized SR was dialyzed at room temperature against 500 volumes of a buffer composed of 0.25 M sucrose, 0.4 M KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM $CaCl_2$, and 5 mM HEPES, pH 7.25, for 24 h. After dialysis, the samples were diluted with an equal volume of dialysis buffer and sedimented (45,000 rpm for 75 min, 20 °C, Beckman 75 TI rotor). The pellet was washed in 25 times the original dialysis volume using dialysis buffer and centrifuged (45,000 rpm for 90 min, 20 °C, Beckman 60 TI rotor). The pellet was resuspended in buffer (0.3 M sucrose, 0.1 M KCl, 5 mM HEPES, pH 7.1), frozen in liquid nitrogen, and stored at −70 °C.

Ca^{2+} uptake measurements

The initial rate of Ca^{2+} uptake by the SR vesicles was determined at 37 °C by the Millipore filtration technique (Chiesi & Inesi, 1979) in a medium containing 100 mM KCl, 100 mM sucrose, 50 mM MOPS, pH 6.8, 5 mM $MgCl_2$, 5 mM NaN_3, 3 mM K-oxalate, 2 mM ATP, 0.4 mM EGTA, and 0.150 mM $CaCl_2$ labeled with $^{45}Ca^{2+}$. The free Ca^{2+} concentration, calculated according to Fabiato and Fabiato (1979), was 0.47 μM. The reaction was initiated by the addition of 2 mM ATP.

ATPase activity assays

The Ca^{2+}-ATPase activity of the SR membranes was measured at 37 °C with a coupled enzyme assay. The basic reaction mixture consisted of 20 mM HEPES-Tris, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.2 mM NADH, 3 mM phosphoenolpyruvate, 3 units of pyruvate kinase/mL of assay medium, 3 units of lactate dehydrogenase/mL of assay medium, and 1 μM of the Ca^{2+} ionophore A23187. Ca^{2+} was added to a final free concentration of 10 μM. The samples were incubated for 5 min at 37 °C in 1 mL of the medium, and the reaction

was started by the addition of ATP to a final concentration of 0.5 mM and monitored in a Shimadzu (Kyoto, Japan) dual-wavelength spectrophotometer (type UV 3000) at 366 nm vs. 550 nm. Alternatively, ATPase activities were measured at the desired free Ca^{2+} concentrations by the Pi liberation method according to Lanzetta et al. (1979) in a buffer composed of 100 mM KCl, 100 mM sucrose, 50 mM MOPS, pH 6.8, 5 mM $MgCl_2$, 5 mM NaN_3, 3 mM K-oxalate, 2 mM ATP, and 0.4 mM EGTA.

Miscellaneous methods

Protein concentrations were measured by a modification of the method of Lowry et al. (1951).

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