Structural Domains in Phospholemman
A Possible Role for the Carboxyl Terminus in Channel Inactivation

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Abstract—Phospholemman (PLM) is a small (72–amino acid) transmembrane protein found in cardiac sarcolemma that is a major substrate for several protein kinases in vivo. Detailed structural data for PLM is lacking, but several studies have described an ion conductance that results from PLM expression in oocytes. Moreover, addition of purified PLM to lipid bilayers generates similar ion currents, suggesting that the PLM molecule itself might be sufficient for channel formation. To provide a framework for understanding the function of PLM, we investigated PLM topology and structure in sarcolemmal membrane vesicles and analyzed purified recombinant PLM. Immunoblot analyses with site-specific antibodies revealed that the extracellular segment (residues 1 to 17) exists in a protected configuration highly resistant to proteases, even in detergent solutions. The intracellular portion of the molecule (residues 38 to 72), in contrast, was highly susceptible to proteases. Trypsin treatment produced a limit peptide (residues 1 to 43), which showed little change in electrophoretic mobility in SDS gels and retained the ion-channel activity in lipid bilayers that is characteristic of the full-length protein. In addition, we found that conductance through PLM channels exhibited rapid inactivation during depolarizing ramps at voltages greater than ±50 mV. Channels formed by trypsinized PLM or recombinant PLM 1–43 exhibited dramatic reductions in voltage–dependent inactivations. Our data point to distinct domains within the PLM molecule that may correlate with functional properties of channel activity observed in oocytes and lipid bilayers. (Circ Res. 1998;82:367-374.)

Key Words: phospholemman • sarcolemma • ion channel • topology

Phospholemman is a prominent phosphoprotein localized to the plasma membrane of cardiac,1–3, skeletal,4,5 and smooth6–8 muscle cells and liver hepatocytes.9 This phosphoprotein was first observed in cardiac sarcolemmal vesicles after in vitro phosphorylation by PKA and PKC,1,3,10,11 and it is a major substrate for several protein kinases in vivo. Detailed structural data for PLM is lacking, but several studies have described an ion conductance that results from PLM expression in oocytes. Moreover, addition of purified PLM to lipid bilayers generates similar ion currents, suggesting that the PLM molecule itself might be sufficient for channel formation. To provide a framework for understanding the function of PLM, we investigated PLM topology and structure in sarcolemmal membrane vesicles and analyzed purified recombinant PLM. Immunoblot analyses with site-specific antibodies revealed that the extracellular segment (residues 1 to 17) exists in a protected configuration highly resistant to proteases, even in detergent solutions. The intracellular portion of the molecule (residues 38 to 72), in contrast, was highly susceptible to proteases. Trypsin treatment produced a limit peptide (residues 1 to 43), which showed little change in electrophoretic mobility in SDS gels and retained the ion-channel activity in lipid bilayers that is characteristic of the full-length protein. In addition, we found that conductance through PLM channels exhibited rapid inactivation during depolarizing ramps at voltages greater than ±50 mV. Channels formed by trypsinized PLM or recombinant PLM 1–43 exhibited dramatic reductions in voltage–dependent inactivations. Our data point to distinct domains within the PLM molecule that may correlate with functional properties of channel activity observed in oocytes and lipid bilayers.

Cloning of protein cDNAs in several laboratories has led to the conclusion that PLM is part of a family of proteins that have similar structural features and sequence overlap. Proteins of this family are small (5 to 10 kD), traverse the membrane a single time, and have predicted α-helical transmembrane segments that are rich in hydrophobic residues, particularly leucine and isoleucine. The N- and C-termini are predicted to project extracellularly and intracellularly, respectively. In addition, two or more cysteine residues reside within, or near, the inner (intracellular) leaf of the plasma membrane.

Three PLM homologues are known, and all adhere to this structural motif: MAT-8, a protein present in mammary tumors15; a small (γ) subunit of Na+, K+-ATPase present in many tissues16; and CHIF, an epithelial cell–enriched protein that is steroid (mineralocorticoid)–induced.17 PLM, MAT-8, and CHIF have each been shown to induce ionic conductances when expressed in Xenopus oocytes.17–19 PLM expression induces a Cl–selective conductance that is activated by hyperpolarizing voltages, exhibits very slow kinetics, and is noninactivating.18 MAT-8 and CHIF also induce voltage–activated ionic currents with very slow kinetics.17,19 Of the

Received May 8, 1997; accepted November 17, 1997.

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proteins of this family cloned thus far, CHIF is the most similar in structure to PLM; however, unlike PLM and MAT-8, CHIF-induced oocyte channels are K⁺ selective. PLM homologues may form ion channels through the membrane or may be regulators of ion channels that are already present. We have previously found that the addition of PLM to planar bilayers produces ion channel activity that has the basic features of ion channels produced by oocyte expression. On the other hand, convincing data suggesting that the PLM polypeptide may activate an endogenous ion channel also exist. A more detailed structural characterization of PLM would be helpful in determining molecular mechanisms regardless of how the ion pore is assembled.

In order to investigate the structure and membrane topology of PLM and to correlate structural characteristics with ion channel activity measured in lipid bilayers, we analyze in the present study the purified recombinant protein and the native protein in cardiac sarcolemmal vesicles. Our results provide important new structural information concerning three major structural domains of PLM: an extracellular domain (PLM 1–17) that is completely resistant to proteases, a transmembrane region (PLM 18–37) that appears to produce the in vitro conductance of PLM, and a relatively large intracellular domain (PLM 38–72) that is highly susceptible to proteolysis. Furthermore, we show that PLM conductance undergoes inactivation during a depolarizing voltage ramp at voltages greater than +50 mV and that this voltage-dependent inactivation requires an intact C-terminus.

Materials and Methods

Antibodies

Affinity-purified peptide antibodies to the N-terminus (residues 1 to 15) and C-terminus (residues 58 to 72) of PLM were made and used as previously described. Monoclonal antibody B8 to PLM was raised to the recombinant protein expressed in Sf21 insect cells.

Membrane Preparation

Canine cardiac sarcolemmal vesicles were isolated from dog left ventricles by sucrose flotation as described previously and stored at −40°C in 30 mmol/L histidine and 0.25 mol/L sucrose. Procedures for removal of dog hearts were in accordance with institutional guidelines. Protein concentrations were determined by the method of Lowry et al.

Construction of PLM Recombinant Baculovirus

The baculovirus transfer vector pVL-NcoI was constructed from pVL1392 (Invitrogen) by the addition of a synthetic linker containing a unique NcoI restriction site within a sequence context that corresponds exactly to the start codon of the baculovirus polyhedrin open reading frame. Dog heart PLM cDNA corresponding to base pairs 1 to 279 was amplified from the full-length clone in pBluescript and ligated into pVL-NcoI. Plasmid DNA sequences were verified by dideoxy sequencing.

Expression of Recombinant PLM

For standard purification of PLM, Sf21 cells (500 mL) at a density of 1.5×10⁷ cells/mL were grown in suspension with PLM recombinant virus using a multiplicity of infection of 5 to 10. The cell suspension was incubated at 27°C in a 4-L Erlenmeyer flask in an orbital shaker (90 rpm) in Grace’s medium containing 10% fetal bovine serum, 0.1% pluronic F68, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. At 2.5 to 3.0 days after infection, the cells were sedentized and resuspended in 250 mL of 100 mmol/L Na₂CO₃, pH 11.4, followed by centrifugation at 21 000 rpm for 30 minutes in a Beckman 21 rotor. The carbonate-extracted pellets were resuspended in 0.25 mol/L sucrose and stored frozen at −20°C.

Monoclonal Antibody Affinity Purification of PLM

For a typical purification, carbonate-extracted pellets from two 500 mL PLM infections were pooled, yielding 416 mg of protein, which was incubated at room temperature for 20 minutes in 279 mL of 9 mmol/L MOPS, 0.5 mol/L NaCl, and 1% octyl glucoside (pH 7.2). The suspension was sedentized at 40 000 rpm for 20 minutes in a Beckman 50.2 rotor, and the supernatant, containing the detergent-solubilized PLM, was loaded over a 45-mL protein A–agarose (Sigma) column with covalently attached PLM monoclonal antibody B8. Monoclonal antibody density on the protein A–agarose beads was ~8 mg antibody/mL beads. Covalent coupling of antibody to beads was performed using dimethyl pimelimidate. The detergent-solubilized PLM was passed through the column two times at room temperature over a period of 2 to 3 hours. The column was eluted with seven consecutive 45-mL washes of 20 mmol/L MOPS, 0.5 mol/L NaCl, and 1% octyl glucoside (pH 7.2), followed by three consecutive 45-mL washes with 20 mmol/L MOPS and 1% octyl glucoside (pH 7.2). Purified PLM was then eluted with five consecutive 45-mL washes of ice-cold 20 mmol/L glycine and 1% octyl glucoside (pH 2.0), adding enough 1 mol/L MOPS to bring the final pH to 7.1. Peak fractions containing PLM were pooled and concentrated to a final volume of 6.2 mL using a 50-mL Amicon and PM-10 membrane. Purified PLM was stored in small aliquots at −40°C. The protein concentration was 1.95 mg/mL, giving a final yield of 12.1 mg of purified PLM from 416 mg of carbonate-extracted protein.

Native PLM was purified from canine cardiac sarcolemmal vesicles. Membrane protein (128 mg) was solubilized as described above, and PLM was purified in an identical fashion, this time using 4.2 mL of B8-linked immunoaffinity beads.

Protein concentrations were determined by the method of Schaffner and Weissman. SDS-PAGE of purified PLM samples was according to Porzio and Pearson or Laemmli, as indicated.

Proteolysis

Sarcolemmal vesicles (60 μg) or purified PLM (0.2 to 2.0 μg) was incubated with several different proteases in 35 μL of 50 mmol/L MOPS, pH 7.8, and 0.25 mol/L sucrose, without the addition of a reducing agent. Incubations with thermolysin also contained 1 mmol/L CaCl₂ in accordance with the manufacturer’s specifications. Triton X-100 (1%) was included, as indicated in the figure legends. Incubations were conducted at 37°C for 4 hours using 0.25 μg of each protease; reactions were stopped by adding 15 μL of 15% SDS sample buffer containing 40 mmol/L DTT. Twenty-microliter aliquots were loaded onto precast 4% to 20% acrylamide gels (Bio-Rad) and electrophoresed at 150 V. Gels were transferred to nitrocellulose for immunoblotting, and antibody binding bands were detected with [125I]protein A.
Sequence Analysis
To determine the tryptic cleavage sites, 100 μg of recombinant PLM was first incubated at 30°C in 300 μL of 20 mmol/L MOPS, pH 7.4, 0.5 mmol/L EDTA, 10 mmol/L MgCl₂, 15 μmol/L [γ-32P]ATP, and 10 U of the catalytic subunit of PKA (Sigma). After 7 minutes, 500 μmol/L unlabeled ATP was added, and the incubation continued an additional 10 minutes to achieve stoichiometric phosphorylation. The phosphorylated sample was chilled on ice and then exchanged with buffer containing 50 mmol/L MOPS, pH 7.8, and 0.25 mol/L sucrose. Trypsin-TPCK (5 μg) was added, and the sample was incubated for 4 hours at 37°C. After digestion, cleaved fragments were filtered through a Centricon-10 membrane. Peptides passing through the 10 000-D molecular mass cutoff filter were separated by reverse-phase chromatography using an Ultraphere ODS C₁₈ column operated on a Beckman System Gold high-performance liquid chromatograph. Peptides were chromatographed in 0.1% trifluoroacetic acid/50% acetonitrile and eluted with a gradient of increasing concentrations of acetonitrile.

Planar Bilayer Measurements
Channel activities were recorded in planar bilayers composed of ultrapure phosphatidylserine and phosphatidylethanolamine (Avanti Polar Lipids, Inc). Bilayers were painted across a 250-μL ultrapure phosphatidylserine and phosphatidylethanolamine (Avanti Polar Lipids, Inc) and eluted with a gradient of increasing concentrations of acetonitrile.

Preparation of Trypsinized PLM 1–43
Purified recombinant PLM was incubated with trypsin-TPCK (1:20 [trypsin/PLM]) in 50 mmol/L MOPS, pH 7.8, at 37°C for 2 to 4 hours. After digestion, trypsinized PLM was filtered through a Centricon-10 membrane followed by several washes with 20 mmol/L MOPS, pH 7.1, and 1% octyl glucoside to remove small peptides. The limit peptide PLM 1–43 did not pass through the membrane and was recovered in purified form (see “Sequence Analysis” above). Complete trypsinolysis was verified by immunoblot analysis using anti-PLM (C-terminus) antibodies.

Construction of PLM 1–43 Recombinant Baculovirus and Purification of the Truncated Protein
Dog heart PLM cDNA corresponding to base pairs 1 to 189 was amplified from the full-length clone in pBluescript and ligated into the transfer plasmid pVL1393. The recombinant baculovirus was formed by cotransfection with BaculoGold (Pharmlingen) and isolated by plaque purification.

Materials
Trypsin-TPCK, α-chymotrypsin, and papain were purchased from Sigma, and thermycin, pronase, and Staphylococcus aureus V8 protease were from Boehringer-Mannheim. Media and supplements for insect cell cultures were from Sigma.
various treatments seen with antibodies to the PLM N-terminus reflect losses of varying lengths from the C-terminal end. Because the N-terminal sequence contains many potential protease cleavage sites (see Fig 5), the data demonstrate that this region of the molecule is in a protected configuration. The protease resistance of the N-terminus is underscored by the fact that immunoreactivity was maintained even after most other sarcolemmal proteins were totally degraded (top left panel, lanes 6 and 7). Of the proteases tested, only pronase was able to digest the PLM N-terminus; the digestion was complete only when detergent was added (middle right panel).

Purification and Proteolysis of Recombinant PLM
PLM expressed in Sf21 cells was purified to homogeneity by monoclonal antibody affinity chromatography (Fig 2, lanes 3 and 4). Under nonreducing conditions, the protein migrated with an apparent Mr of 24 000 (lane 3); under reducing conditions, the apparent Mr changed to 16 000. This suggests that the protein was isolated as a disulfide-linked dimer. The high level of PLM expression in insect cells was demonstrated by its appearance as a major staining band in the crude carbonate-extracted pellet fraction (lanes 1 and 2). Recombinant PLM was indistinguishable from native cardiac PLM by several criteria. The monomeric forms of both proteins (Mr, 8409) migrated anomalously on SDS-PAGE, with apparent an Mr of 15 000 to 16 000). N-terminal amino acid sequencing of the recombinant protein verified its identity through 54 consecutive residues (data not shown). This analysis established that the N-terminus was not blocked and that normal cleavage of the 20–amino acid signal peptide of PLM occurred in the Sf21 cell line, yielding the same mature protein found in dog heart. Mass spectrometry of purified recombinant PLM (reduced) gave a single species with a predicted Mr, of 8409 (D. Cafiso, unpublished data, 1997). Thus, the anomalous mobility of the PLM monomer in SDS gels appears to be entirely a consequence of its primary structure.

Purified recombinant PLM was subjected to proteolysis by the same proteases used to analyze PLM in sarcolemmal vesicles (Fig 3A). Remarkably, results of proteolysis of purified PLM were qualitatively similar to those obtained with cardiac sarcolemmal vesicles. The PLM N-terminus maintained its protected configuration, whereas the C-terminal portion alone was readily and completely degraded. Only V8 protease exhibited a significant difference in its ability to digest the N-terminus of PLM when applied to the purified recombinant protein compared with the membrane-bound form. Results of protease treatments were similar whether or not a reducing agent was added (data not shown).

Analysis of Native PLM Purified From Cardiac Sarcolemmal Vesicles
The isolation of recombinant PLM as a disulfide-linked dimer was puzzling, since no evidence exists for dimerization of native PLM in the sarcolemmal membrane. To address this issue, we also purified PLM from sarcolemmal vesicles. PLM
was isolated from sarcolemmal vesicles in monomeric form; DTT had no effect on protein mobility (Fig 4, lanes 1 and 2). Recombinant PLM, in contrast, showed the characteristic mobility shift induced by DTT treatment (lanes 3 and 4). It is possible that overexpression of PLM in SF21 cells gives rise to protein dimerization, whereas biosynthesis in heart cells maintains the reduced form.

The pattern of proteolysis of native PLM was similar to that of recombinant PLM (Fig 3B). Both proteins contained a protease-accessible C-terminus and a protease-resistant N-terminus. Thus, the resistance of the PLM N-terminus to proteolysis was conserved among the three preparations of PLM examined.

Sequence Analysis of Limit Tryptic Peptide

The C-terminal cytoplasmic domain (residues 38 to 72) of PLM contributes 49% of the sequence and exhibits numerous potential tryptic cleavage sites (Fig 5). Yet tryptic proteolysis of this region minimally affects protein mobility on SDS-PAGE (Figs 1 and 3). After trypsin treatment, for example, the apparent mobility of PLM only changes by 3 kD, from \( \sim 15 \) D to 12 kD (Fig 3). In order to determine the proteolytic cleavage sites for trypsin, we digested 100 \( \mu \)g of recombinant PLM, and the cleaved fragments were then filtered through a Centricon-10 membrane, separated by reverse-phase chromatography, and sequenced. To aid in the analysis, we first determined the tryptic cleavage site closest to the N-terminus, between lysine 43 and phenylalanine 44, is indicated (arrow), along with the tryptic peptides sequenced (bars with arrows). The clear bar designates the radioactive phosphopeptide sequenced.
that individual ion-channel closures occurred within the two ranges (−75 to −50 mV and +50 to +75 mV) (Fig 7C).

In contrast to our results with the full-length PLM, transient channel closures were almost never seen when trypsinized PLM was used, as exemplified in Fig 7D. In 200 episodes (13 bilayer experiments) we observed only 10 closings, whereas for intact PLM we observed 130 closings during 300 episodes (15 bilayer experiments). These results were consistently obtained and were independent of protein preparation. To confirm the formation of ion channels and loss of voltage-dependent channel inactivations seen when the tryptic peptide (PLM 1–43) was used, we prepared the same truncated PLM mutant by baculovirus expression and immunoaffinity purification. Analysis of purified recombinant PLM 1–43 in lipid bilayers gave channels that exhibited slow kinetics and conductances similar to those seen with the trypsinized protein (Fig 7E). Both anion and cation conductances were seen as for each of the other PLM preparations. Moreover, in 612 tracings (33 bilayer experiments), no inactivations were seen.

**Discussion**

In the present study, we have combined experimental approaches to analyze the structure, topology, and in vitro channel activity of canine cardiac PLM. Our results are the first to examine the PLM molecule in detail and to attempt to correlate its structure with its unusual channel activity. Furthermore, our studies reveal new aspects of PLM channel activity seen in lipid bilayers that may be correlated with channel regulation in vivo.

Several studies have now shown that expression of PLM in *Xenopus* oocytes produces a Cl− conductance. More recently, Moorman et al. found that addition of PLM to lipid bilayers produced ion channels that have features similar to those seen in excised patches of oocytes injected with PLM mRNA. These channels exhibit no closures during a voltage ramp extending from −50 to +50 mV. Anion and cation conductances were both measured in lipid bilayers containing PLM, an unusual property that may promote taurine transport in vivo. An alternative mechanism has also been suggested for PLM channel activity in oocytes involving its coassembly with an endogenous channel; this mechanism is similar to one recently described for the small transmembrane protein Isk.

Although findings from several laboratories yield a potentially complex picture of PLM actions, these studies have not discerned whether the PLM polypeptide can form a channel pore. In the present study, we show that PLM channel activity in lipid bilayers is similar in producing anion and cation conductances whether using native cardiac, recombinant (insect cell) PLM, or PLM 1–43. Determination of the site of trypsin cleavage with characterization of a distinct alteration in PLM channel activity strongly implicates PLM as the source of the channel activity. We have also shown that loss of conductance inactivation is seen using the recombinant form of PLM 1–43. This further extends our data by proving that the change observed with trypsin results from the proteolyzed PLM polypeptide and not from some minor contaminant in the PLM preparation. Thus, whether or not PLM functions as an ion channel in vivo, the in vitro channel activity that we have observed does arise from PLM, specifically from residues 1 to 43 of the PLM molecule.

The oligomeric structure of PLM in native membranes or in artificial lipid bilayers was not investigated in the present study, although a multimer of PLM might be necessary to form an ion channel pore. Covalent (disulfide) linkages were observed in SDS gels for recombinant PLM, whereas the protein purified from cardiac sarcolemmal vesicles was isolated as a monomer. Disulfide linking observed for recombinant PLM prepared from SF21 cells might be the result of impaired redox control in the virus-infected host cell. Importantly, however, both preparations yielded the same channel activity; we did not observe any effect of the reducing agent DTT on channel activity for either the native or recombinant preparation (data not shown). Thus, disulfide bond formation does not appear to play a role in the channels we analyzed in the present study.

The intraluminal localization of the C-terminal portion of PLM was confirmed by protease treatment of sealed sarcolemmal vesicles. This result is consistent with previous studies with intact sarcolemmal vesicles showing that membrane permeabilization was required for significant phosphorylation of PLM by PKA and PKC. Trypsin treatment of PLM led to the complete digestion of the C-terminal segment, producing the limit peptide PLM 1–43. The loss of residues 43 to 72 had very little effect on the mobility of PLM on SDS-PAGE, a pattern observed for all proteases examined. For example, PLM 1–43 continued to run with an Mr of 12,000 in SDS gels, even though the calculated Mr is only 4890. Thus, the highly
aberrant mobility of PLM in SDS gels appears to arise in large part from residues 1 to 43. A loss of channel closings at extremes of bilayer voltage occurred with the loss of residues 44 to 72, suggesting that this cytosolic segment is capable of interacting with the pore-forming region (residues 1 to 43) of the molecule. The mechanism of this regulation has yet to be determined. The N-terminal portion of PLM exhibited a remarkable and unexpected resistance to proteolysis. This resistance was maintained even in purified and, presumably, delipidated PLM preparations, indicating that this portion of the molecule was maintained in a highly protected configuration. We are not aware of any structural features for this sequence that would explain such protease resistance. For example, this part of the molecule is only minimally hydrophobic; there is no obvious homology with so-called P regions of other channels that constitute part of the ion pore (reviewed in Reference 45), nor are there any attached lipids that might confer a membrane association as determined by mass-spectroscopic analysis of recombinant PLM (D. Cafiso, unpublished data, 1997). Interestingly, on the basis of fluorescence-energy transfer measurements, Ben-Efraim et al.12 predicted that the N-terminal portion of Isk is inserted into the lipid bilayer. Whether this region of the PLM molecule forms part of the pore cannot be determined at present, but its remarkable protease resistance makes it an interesting target for future mutagenesis studies.

As a major target for hormone-stimulated phosphorylation in the heart, the physiological function of PLM is likely to be an important one. The observation that PLM expression induces ion currents in Xenopus oocytes13 is fortified in the present study by the finding that purified recombinant PLM, purified cardiac PLM, and the truncated PLM 1–43 all form channels in planar lipid bilayers. The effect of the C-terminus on channel inactivation combined with the protease insensitivity of the N-terminal region suggests that the structure of the molecule is more complex than previously appreciated. The availability of milligram quantities of the highly purified protein will allow future studies to refine our knowledge of PLM structure and thus determine how this minimal polypeptide chain produces ionic conductance.

Acknowledgments

This study was supported by a Predoctoral Fellowship Grant from the American Heart Association, Indiana Affiliate, Inc (Dr Chen) and grants from the National Institutes of Health and the American Heart Association, National Center. We wish to thank David Cafiso at the University of Virginia for performing mass-spectrophotometric analysis of recombinant PLM.

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Circ Res. 1998;82:367-374
doi: 10.1161/01.RES.82.3.367

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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