Phospholamban: Protein Structure, Mechanism of Action, and Role in Cardiac Function

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I. INTRODUCTION

The molecular mechanisms regulating myocardial contractility through β-adrenergic stimulation, the familiar “fight-or-flight” response, have been the object of scientific investigations for several decades. β-Adrenergic stimulation of intact hearts increases both the rate and magnitude of contraction and the rate of relaxation of cardiac muscle (228). These effects are believed to result from variations in the cytosolic calcium concentration produced by alterations in calcium fluxes across the two principal membrane systems, the sarcolemma and the sarcoplasmic reticulum (212). Since the discovery in the 1960s of the role of cAMP as a second messenger and its activation of a protein kinase, the mediation of β-adrenergic effects by cAMP-dependent phosphorylation of specific proteins has been well established (58, 123, 171). In heart tissue, targets of this kinase have provided important leads in identifying key proteins of the myocardial regulatory machinery (86). Thus it has been proposed that the accelerated rate of cardiac contraction in response to β-adrenergic stimulation may be caused by increased calcium influx after cAMP-dependent phosphorylation of a sarcolemmal channel protein (86, 212), whereas the accelerated rate of relaxation of β-adrenergic-stimulated cardiac tissue has been suggested to result mainly from an increased rate of calcium uptake by the sarcoplasmic reticulum in response to phosphorylation of phospholamban (PLB), the principal protein phosphorylated in cardiac sarcoplasmic reticulum (208).

After the discovery of PLB, early reviews described the apparent role of PLB in regulation of calcium transport across the sarcoplasmic reticulum and sketched a complex protein structure (3, 39, 111, 191, 208, 210, 216, 217). Interest in PLB grew as structure-function details were revealed experimentally, which suggested that PLB might itself exhibit channel activity (7, 115, 171), and recent compilations have updated descriptions of PLB structure (31, 37a, 197a). Other recent summaries have focused on the role of PLB in the regulation of myocardial function by catecholamines (36, 59, 95, 114, 200, 207) and the role of PLB in skeletal and smooth muscle (137). The present

Simmerman, Heather K. B., and Larry R. Jones. Phospholamban: Protein Structure, Mechanism of Action, and Role in Cardiac Function. Physiol. Rev. 78: 921–947, 1998.—A comprehensive discussion is presented of advances in understanding the structure and function of phospholamban (PLB), the principal regulator of the Ca2+-ATPase of cardiac sarcoplasmic reticulum. Extensive historical studies are reviewed to provide perspective on recent developments. Phospholamban gene structure, expression, and regulation are presented in addition to in vitro and in vivo studies of PLB protein structure and activity. Applications of breakthrough experimental technologies in identifying PLB structure-function relationships and in defining its interaction with the Ca2+-ATPase are also highlighted. The current leading viewpoint of PLB's mechanism of action emerges from a critical examination of alternative hypotheses and the most recent experimental evidence. The potential physiological relevance of PLB function in human heart failure is also covered. The interest in PLB across diverse biochemical disciplines portends its continued intense scrutiny and its potential exploitation as a therapeutic target.
review attempts to provide a complete historical perspective of PLB structure and function studies as a framework for understanding better the current approaches to revealing the properties and physiological role of this unique protein.

II. BRIEF HISTORY OF DISCOVERY OF PHOSPHOLAMBAN

Phospholamban was first identified in cardiac microsomes as a 22-kDa protein serving as the principal substrate for cAMP-dependent protein kinase (125, 213, 247). Lower-molecular-mass forms of phosphorylated PLB (7–12 kDa) were later identified in sarcoplasmic reticulum vesicles (15, 87, 245) and shown to be interconvertible with the 22-kDa form; boiling in SDS (124) or treatment with Triton X-100 (128) caused dissociation of the 22-kDa form into the low-molecular-weight forms, which could be reassocciated to the 22-kDa form by freezing overnight (124). These observations led to an early proposal that PLB was a homodimer (128). Other intermediate forms of phosphorylated PLB were subsequently observed in sarcoplasmic reticulum, prompting descriptions of PLB as a heterotrimer (140) or a homotetramer (105). The development of a method to purify PLB in the dephosphorylated form, however, ultimately permitted determination that PLB is a pentamer of identical subunits (240). More recent studies of purified PLB and mutagenic variants have revealed considerable detail of PLB structure and subunit interactions, summarized in section iii.

In part because of the complexity of PLB structure, its precise function and mechanism of action have been elusive. Phospholamban was originally implicated in the regulation of calcium transport across the cardiac sarcoplasmic reticulum by the correlation of cAMP-induced stimulation of calcium transport by the Ca$^{2+}$-ATPase, with the predominant phosphorylation of PLB by the cAMP-dependent protein kinase (110, 215, 213, 215). Because of its role as the principal substrate of cAMP-dependent protein kinase in cardiac sarcoplasmic reticulum vesicles, Tada et al. (212, 213) named the protein phospholamban, meaning “phosphate receptor.” The level of calcium transport stimulated by cAMP-dependent mechanisms was commensurate with the degree of phosphorylation of PLB (212, 217). Phospholamban was also found to be the principal substrate of an endogenous myocardial calcium/calmodulin-dependent protein kinase (128) and is one of the few proteins avidly phosphorylated in sarcoplasmic reticulum by both cAMP-dependent and calcium/calmodulin-dependent protein kinases (16, 89, 106). The enhanced rate of calcium transport stimulated by calcium/calmodulin-dependent protein kinase parallels the activity of the Ca$^{2+}$-ATPase and phosphorylation of PLB (174). Phosphorylation of PLB by the cAMP-dependent and calcium/calmodulin-dependent protein kinases occurs independently and additively (16, 77, 128, 209, 240), as does the concomitant stimulation of calcium transport and ATPase activity (116, 179, 209), although one study found nonadditivity in stimulation of ATPase activity (32). Cardiac sarcoplasmic reticulum also contains an endogenous phosphatase activity, which is capable of reversing these phosphorylation effects (108, 117, 118, 147, 214, 240). The concentration of calcium required for half-maximal activation of the calcium pump and calcium transport ($K_{Ca}$ value) is decreased by ~50% after cAMP-dependent phosphorylation of PLB (65, 215) and further decreased by additional phosphorylation of PLB by calcium/calmodulin-dependent protein kinase (116, 209). Although the decrease in $K_{Ca}$ produced by phosphorylation may not seem very impressive upon first consideration, in actuality, at low ionized calcium concentration (submicromolar calcium concentration) where the calcium pump is marginally active, phosphorylation of PLB by protein kinases induces a substantial increase in calcium transport to levels increased by fourfold or greater (65, 190, 213). These results suggested that PLB phosphorylation increases the apparent affinity of the Ca$^{2+}$-ATPase for calcium (208). Several other lines of evidence suggested that dephosphorylated PLB acts as an inhibitor of the Ca$^{2+}$-ATPase and that phosphorylation releases the inhibition (78, 107, 202). Sites of interaction between PLB and the Ca$^{2+}$-ATPase and the functional consequences of this interaction have been examined with the recent aid of mutagenesis techniques and the development of practicable expression systems, described in section v.

The physiological role of PLB in cardiac health and disease has been the source of much speculation. Phospholamban clearly is a mediator in the regulation of myocardial function by catecholamines through the cAMP cascade. Consistent with this, PLB is present in slow skeletal muscle as well as cardiac muscle, which both exhibit cAMP-dependent stimulation of calcium transport, whereas PLB has not been identified in fast skeletal muscle, which lacks this response pathway (92, 109). By use of antibodies to the purified protein, PLB has also been detected in microsomal membranes prepared from several types of smooth muscle (180), including membranes prepared from large and medium-sized arteries (35, 42, 44, 178), although the highest content nevertheless remains in the heart. A more widespread distribution of PLB in smooth muscle suggests it may play a broader regulatory role in the cardiovascular system. For example, in PLB knockout mice, tissue contractility is affected in both cardiac (144) and smooth muscle (123a). Recent development of sensitive immunological techniques, viable reconstitution methods, and cell expression and transgenic model systems has enabled testing of current hypotheses regarding the physiological function of PLB, described throughout this review.
III. PURIFICATION AND STRUCTURE OF PHOSPHOLAMBAN

Identification and structural analysis of PLB in heart were initially problematic because of the difficulty in isolating the purified membrane protein. For definitive localization of PLB, reproducible methods for preparation of purified sarcolemmal and sarcoplasmic reticulum vesicles were required (87), and before their implementation, the use of impure and incompletely characterized membrane fractions contributed to confusion regarding the identification, localization, and role of PLB in regulating intracellular calcium flux. Part of the confusion came in distinguishing PLB from a 15-kDa sarcolemmal phosphoprotein also present in crude cardiac membrane preparations (86), which was subsequently purified, cloned, and named phospholemman (173). In highly purified preparations of cardiac sarcolemmal and sarcoplasmic reticulum vesicles, the highest content of PLB was found in free and junctional sarcoplasmic reticulum vesicles, and the lowest amount in the sarcolemmal fraction (90, 150, 176). Furthermore, immunoelectron microscopic studies on the subcellular localization of PLB in intact cardiac muscle, using two different antibodies highly specific for PLB, demonstrated a uniform distribution of PLB throughout the sarcoplasmic reticulum and absence from the surface sarcolemma and t tubules (93, 94). Thus an early proposal that PLB was a component of the sarcolemmal slow calcium channel, i.e., “calciductin” (23, 72, 183), or another unique sarcolemmal protein (47, 72, 80, 124, 138) was subsequently shown to be unlikely (150, 176) but reflective of the general difficulty in obtaining pure membrane fractions and the more specific problem in identification and isolation of purified authentic PLB (99). Contributing to this, earliest purification efforts generally used harsh conditions of organic solvents or strong detergents to solubilize PLB, which usually was phosphorylated to aid in monitoring the purification (14, 23, 25, 30, 67, 106, 129, 130). The “proteolipids” isolated from these efforts exhibited acidic isoelectric points but disparate amino acid compositions, suggesting that homogeneous preparations of authentic PLB were probably not achieved. An additional claim that PLB remained strongly associated with the Ca\(^{2+}\)-ATPase after detergent solubilization (129, 130) was not universally observed (78, 121).

A breakthrough in the characterization and understanding of PLB came with the purification of the dephosphorylated protein by conventional chromatographic methods in sufficient amounts for further detailed biochemical studies (79, 240). Jones and co-workers (90, 91, 240) purified PLB by sulfhydryl group affinity chromatography after solubilization of the protein from canine cardiac microsomes using the zwitterionic detergent Zwittergent 3–14. Tada and co-workers (79, 211) purified the protein from canine cardiac microsomes in the nonionic detergent C\(_{12}\)E\(_{8}\) using a combination of size-exclusion and ion-exchange chromatographies. The isolated PLB protein (90, 91) differed in two major respects from the proteolipids characterized previously: it was basic (pI = 10, dephosphorylated; pI = 6.7, phosphorylated) not acidic (pI = 3.7–4.1) (14, 15, 129, 130), and it was cysteine rich (6 mol%) not cysteine deficient (14, 129, 130). The cysteine residues were not disulfide bonded (193). Electrophoretic analysis of highly purified PLB demonstrated a series of phosphorylation-induced mobility shifts in SDS polyacrylamide gels that was additive upon simultaneous phosphorylation by cAMP-dependent and calcium/calmodulin-dependent protein kinases, providing evidence for a protein structure of multiple identical phosphorylatable subunits (240). Confirming this, electrophoretic studies of sarcoplasmic reticulum membranes phosphorylated in vitro (32, 57, 77, 81, 82) and in vivo (175, 242) were consistent with the notion that PLB monomers could be simultaneously phosphorylated in situ by both kinases. Upon phosphorylation by cAMP-dependent protein kinase, PLB exhibited a dramatic change in pl, from 10 (dephospho) to 6.7 (phospho) (90). This large change in charge is undoubtedly important to the function of PLB and its ability to reversibly regulate the calcium pump. Proteolytic cleavage, peptide mapping, and phosphoamino acid analysis of purified PLB supported the model of PLB as a pentamer (240) of identical low-molecular-weight subunits, each of which can be phosphorylated at distinct sites by cAMP-dependent protein kinase and calcium/calmodulin-dependent protein kinase (241). A largely hydrophobic, protease-resistant domain on each subunit appeared to be responsible for anchoring PLB in the sarcoplasmic reticulum membrane and for holding the multiple subunits together, and a smaller protease-sensitive domain on each subunit contained all of the phosphorylation sites. Only serine was phosphorylated by cAMP-dependent protein kinase, whereas calcium/calmodulin-dependent protein kinase phosphorylated exclusively threonine (193, 241), contradicting early observations from less pure preparations that PLB is phosphorylated only at serine residues (128, 130). An early structural model of PLB (193), based on the partial amino acid sequence and the biochemical characterizations described above, is depicted in Figure 1.

The cGMP-dependent protein kinase phosphorylates cardiac and smooth muscle PLB in vitro on the same serine as the cAMP-dependent protein kinase (24, 71, 179), and experiments with intact cardiac myocytes (185) and smooth muscle cells (35, 98, 163) demonstrate that PLB is phosphorylated in vivo by cGMP-dependent mechanisms, although conflicting results with intact cells were obtained in one study (71). Calcium/phospholipid-dependent protein kinase C was also reported to phosphorylate PLB in vitro on the same protease-sensitive peptide as the other kinases (80, 163). However, PLB is not an efficient in vitro substrate for protein kinase C compared with cAMP- and calcium/calmodulin-dependent protein ki-
nases, and the localization of this protein kinase activity to cardiac sarcolemma and its absence from sarcoplasmic reticulum casts doubt on the physiological relevance of protein kinase C phosphorylation of PLB (176). Wegener et al. (242) found no evidence for in vivo PLB phosphorylation by protein kinase C in perfused guinea pig hearts, an observation also demonstrated in intact guinea pig hearts (40), in rat cardiac myocytes after administration of phorbol ester (63), and in perfused rat hearts after α-adrenergic stimulation (133).

Phosphorylation of PLB pentameric subunits by cAMP-dependent protein kinase occurs by a random mechanism (131), whereas results of gel-mobility shift analysis have been interpreted to suggest that calcium/calmodulin-dependent phosphorylation proceeds by a cooperative mechanism (82). Cross-linking and affinity-labeling experiments have suggested that PLB binds calmodulin (139), that calmodulin binding to PLB is inhibited by prior phosphorylation of PLB by either cAMP-dependent or calcium/calmodulin-dependent protein kinases (156), and that calmodulin binding may be a prerequisite for PLB phosphorylation by the calcium/calmodulin-dependent protein kinase (198). However, the role of this proposed calmodulin binding to PLB in the functional regulation of the Ca^{2+}-ATPase by PLB is unsupported, and the binding of calmodulin to PLB is marginal compared with the major calmodulin binding protein of the sarcoplasmic reticulum, the ryanodine receptor (189). Endogenous phosphatases of the sarcoplasmic reticulum are non-discriminatory in their ability to dephosphorylate the serine phosphorylated by cAMP-dependent protein kinase and the threonine phosphorylated by the calcium/calmodulin-dependent protein kinase (118, 147), although dephosphorylation at the cAMP-dependent site was suggested to occur by a cooperative mechanism (131). Early studies noted that phosphatidylinositol potentiated phospholamban phosphorylation by cAMP-dependent protein kinase (203) and that the phosphorylation of phosphatidylinositols by cAMP-dependent protein kinase occurred concurrently with PLB phosphorylation (83). However, these findings have not been confirmed, and a role for phosphatidylinositol or other specific phospholipids in the PLB regulatory pathway has not been identified.

Amino acid sequence analysis of purified PLB after phosphorylation revealed that the serine and threonine residues phosphorylated uniquely and exclusively by cAMP-dependent and calcium/calmodulin-dependent protein kinases, respectively, are adjacent (193), refuting an early proposal espousing two distinct PLB polypeptides of slightly different molecular weights, each bearing a
FIG. 2. Amino acid sequences of PLB monomer from different species, deduced by cDNA cloning. Amino acid sequences are presented in 1-letter amino acid code, with residue numbers indicated in top margin. Approximate cytoplasmic and transmembrane domains are bracketed. Solid circle and box designate serine-16 and threonine-17, respectively, the 2 phosphorylated residues. Dots indicate identical residues in all isoforms. Sequences were obtained from the following references: dog (53), pig (231), rabbit (50), rat (76), mouse (55), human (54), chicken (227).

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single site phosphorylated by one or the other protein kinase (25). Elucidation of the complete PLB primary structure by Fujii and co-workers by amino acid sequencing (49) and cloning of the cDNA (53) firmly established the molecular mass of each monomer to be 6,080 Da and confirmed the hypothesis that oligomeric PLB is a pentamer of 5 identical subunits (90, 240, 241), each containing only 52 amino acids (Fig. 2). Serine-16 and threonine-17 were identified as the residues phosphorylated by cAMP-dependent protein kinase and by calcium/calmodulin-dependent protein kinase, respectively (53, 193). Consistent with earlier experimental evidence that PLB was composed of two domains (241), analysis of the PLB sequence by hydropathic profiling and secondary structure predictive algorithms (53, 193) suggested a structure comprised of a hydrophilic, cytoplasmically oriented amino-terminal domain containing the phosphorylation sites and a carboxy-terminal hydrophobic domain anchoring the protein in the membrane (Fig. 2). The hydrophobic region was predicted to form a helix sufficiently long to traverse the sarcoplasmic reticulum membrane and, when assembled into the pentameric oligomer, to form a domain resembling a channel structure (193) (Fig. 1). The sequence of PLB from several species is shown in Figure 2, illustrating the highly conserved primary structure of the protein.

Determination of the amino acid sequence of PLB (49, 53, 193) promoted the development of more specific methods for its detection, quantitation, and characterization, including the development of specific cDNA and antibody probes. Alternative methods for preparation of PLB were soon reported thereafter. Immunoaffinity chromatography from deoxycholate-solubilized sarcoplasmic reticulum vesicles yielded PLB that exhibited pentamers in SDS (201), like the protein purified from Zwittergent 3-14 (90) or C₁₂E₈ (49), supporting the belief that oligomeric assembly is an inherent property of PLB and is independent of the method of PLB preparation. Knowledge of the PLB sequence enabled access to recombinant methods for its de novo synthesis. In vitro translation of mRNA transcribed from cDNA of the PLB sequence was used to confirm that PLB spontaneously forms pentamers both in the presence and in the absence of microsomes (33, 194). Interestingly, PLB expressed in Escherichia coli caused cell lysis, restricting the utility of this synthetic route, but strengthening the hypothesis that PLB may exhibit pore-forming activity (33, 115). With the use of mass spectrometry and direct sequence analysis to monitor purification efforts, a successful protocol was eventually developed for isolation of authentic PLB in organic solvents (18); the PLB was phosphorylatable with cAMP-dependent protein kinase and formed pentamers in SDS, indicating retention of these “functional” properties. However, aggregation was a problem after purification of PLB with use of organic solvents (18). Although by the original method for purification of PLB a considerable amount of protein could be isolated sufficient for detailed biochemical studies (3–5 mg of highly purified PLB were isolated routinely from sarcoplasmic reticulum vesicles prepared from 18–20 dog hearts; Refs. 90, 91), it is now possible to purify PLB by monoclonal antibody affinity chromatography after expression of the protein in insect cells using the baculovirus cell expression system (181). By this method, 15–20 mg of recombinant wild-type or mutant PLB are typically isolated at a time (181, 182, 194). The recombinant protein behaves identically as the native protein: it is isolated as a pentamer with oligomeric mobility forms indistinguishable from the natural protein (Fig. 3) and retains the phosphorylation-induced mobility shifts, it exhibits a blocked amino terminus, and it reconstitutes functionally with the cardiac or skeletal muscle calcium pumps like native PLB (181, 182). Recently, PLB has been prepared by complete chemical synthesis using standard peptide synthesis and purification methods in organic solvents and exhibited biochemical characteristics similar to native PLB (152, 235). The ready availability of large quantities of recombinant and synthetic PLB should greatly facilitate future studies directed toward its biochemical and structural characterization.
noting that the complete PLB sequence is predominantly helical in aqueous solutions (196) and is almost entirely helical in 1:1 chloroform/methanol (151). However, the transmembrane hydrophobic helix at the carboxy terminus comprises only one-half or less (≤50%) of the total residues and structure of PLB (53). This suggests that other residues in the sequence must also form a helix to account for the predominantly helical structure of the overall protein. The results further suggest that anchoring the PLB cytoplasmic domain to the carboxy-terminal half of the protein alters short-range interactions to promote this helical structure.

Given the apparent instability of structure in the PLB cytoplasmic domain, it should perhaps not be surprising that phosphorylation effects on secondary structure have so far been inconclusive. Circular dichroism measurements of the cytoplasmic domain in organic solvents suggested that phosphorylation results in a decrease in helicity by 6% (221), 11% (70), and 33% (160), although in aqueous solution little (10% decline) (220) or no change in structure (10, 196, 221) is attributable to phosphorylation. Results from nuclear magnetic resonance in trifluoroethanol suggest an unwinding of helix at residue 12 and the following residues after phosphorylation (160), but in aqueous buffers, the formation of a salt bridge between arginine-14 and serine-16(P) may induce or stabilize a nascent helix in this region (160, 177). The transient and inducible nature of the cytoplasmic PLB structure, which is sensitive to its environment, may partly account for these discrepant experimental observations concerning which specific residues in this peptide adopt a helical configuration and how phosphorylation may affect the secondary structure.

The propensity of PLB monomers to self-associate forming an oligomeric structure was recognized using electrophoretic analysis soon after its discovery. However, deviation from ideal electrophoretic behavior often seen with membrane proteins and low-molecular-weight peptides caused early confusion regarding the number and nature of PLB subunits that associate. Subsequently, gel filtration in SDS with postcolumn detection by laser light scattering (238) supported the model of a pentameric quaternary structure deduced from the electrophoretically resolved oligomeric mobility forms (Fig. 3 showing pentameric through monomeric mobility forms) and from the 10 discrete phosphorylation-induced mobility shifts in the pentamer after graded phosphorylation by cAMP- and calcium/calmodulin-dependent protein kinases (32, 77, 240, 242) (Fig. 4). The preferred assembly into pentamers was also suggested by gel filtration in deoxycholate and by sucrose density centrifugation in the presence or absence of octyl glucoside (61). The agreement in this latter hydrodynamic study may be serendipitous, however, because no attempt was made to correct for detergent binding to the protein. Although chemical cross-linking experi-
ments failed to detect higher order oligomers than dimers of PLB in sarcoplasmic reticulum vesicles (251), the observation that purified PLB or the hydrophobic domain of PLB residues 26–52 displays conductance properties classically attributed to channels suggested that PLB at least transiently forms a pentameric pore structure in membranes (115). Recent experiments using electron paramagnetic resonance spectroscopy on lipid-reconstituted recombinant PLB have verified that it is present primarily as a pentamer in the lipid bilayer (34). However, in the dephosphorylated form, a substantial fraction of monomers is also present, contributing ~20% of the total PLB protein and giving a molar ratio of ~1:1 for pentamers to monomers. Remarkably, the proportion of pentameric PLB species in the membrane approaches 100% after PLB phosphorylation. This suggests that electrostatic repulsion between the cytoplasmic head groups of PLB may inhibit pentamer formation. Phosphorylation of PLB, which changes the isoelectric point from 10 to 6.7 (90), may reverse this charge repulsion and allow complete oligomerization. This change in oligomeric size due to phosphorylation is not detected under SDS-PAGE or high ionic strength conditions, probably because the electrostatic effects are screened by SDS or high concentrations of ions (34).

The specific residues that interact to create the pentameric structure of PLB are confined to the carboxy-terminal hydrophobic domain (8, 193, 194, 241). To probe the involvement of polar residues within this domain in the formation of PLB quaternary structure, site-specific mutants of PLB were studied by Fujii et al. (51), who first expressed the protein in mammalian cells. Replacement of the polar glutamine residues at sequence positions 22, 23, 26, and 29 and the asparagine residues at positions 27 and 30 with either alanine or the respective acid forms of each amide residue had no effect on the thermal stability of the PLB pentamer as monitored by electrophoresis, indicating their lack of involvement in PLB oligomers (51). However, mutation of the cysteines at positions 36, 41, and 46 to serine, alanine, or phenylalanine indicated that PLB quaternary structure is sensitive to changes at these sites and is most intolerant of changes in cysteine-41 (51). Pentamer formation and stability decreased as the size of the substituted side chain increased, implicating a steric or surface area-related component in disruption of the oligomeric interactions.

With the design of other mutagenesis experiments to focus on the role of PLB hydrophobic residues, it was noted that the heptad repeat of leucines at positions 37, 44, and 51 conforms to the structural motif of a leucine zipper, and the further heptad repeat of isoleucines at positions 40 and 47 renders an overall 3–4 residue repeat spacing characteristic of a coiled coil (195). Specific replacement of any of these individual residues with alanine prevented PLB pentamer formation, indicating their essential involvement in the oligomeric assembly (194). Experiments to probe the sensitivity of the PLB pentamer to individual mutations of other nonzipper residues to alanine enabled mapping of the surface orientations of these sequence positions. The quaternary structure of PLB was thus proposed to be a coiled-coil symmetric structure containing a central pore defined by the specific hydrophobic surface interactions of the five stabilizing leucine/isoleucine zippers, which are oriented to the interior and form the backbone of the pentamer (194, 195) (Fig. 5).

An alternate approach by Arkin et al. (8) used saturation mutagenesis of a chimeric protein construct in which Staphylococcal nuclease (a monomeric soluble domain) was fused to the amino terminus of PLB. It was found that the chimeric fusion protein formed pentamers like native PLB and was independently observed that the mutationally sensitive residues of PLB forming the oligomers lined up on faces of a 3.5-residue/helical turn coiled-coil configuration (8). To map the orientation of the cysteine sulfhydryls in the membrane-embedded domain of PLB, exchange of the sulfhydryl S-H to S-D was examined with a synthetic peptide composed of residues 25–52 of PLB, which was purified from organic solvents (9). The results supported the model of a stable, membrane-associated oligomer of PLB and suggested that only one sulfhydryl, that of cysteine-41, is able to exchange and thus is oriented toward the outer surface of the structure where it may exchange with water diffused in the bilayer. However, the pentameric association of the peptide was not verified in this study (9). Although PLB models generated from the two surface mapping studies agree on the general orientation of the PLB
helices (37a), the model interpreted from the sulphydryl exchange results suggests that four of the seven PLB helical faces are occluded from lipid exposure (9), whereas the model derived from the specific mutagenesis results suggests that only two helical faces, those of the leucine/isoleucine zippers, are completely unavailable for lipid interaction (194). A recent detailed study, which uses a computational method in combination with computerized analysis, has compared the merits of the two models by critically assessing the role of the leucine/isoleucine zipper region in pentamer stability (64a). High-resolution methods such as crystallography and nuclear magnetic resonance will ultimately be needed to resolve the fine details of PLB quaternary structure, however (37a). Indeed, recently, the crystal structure has been determined for a five-stranded coiled-coil protein, the cartilage oligomeric matrix protein (149), which bears a remarkable similarity to the model proposed for PLB (194, 195).

IV. PHOSPHOLAMBAN GENE STRUCTURE, EXPRESSION, AND REGULATION

The PLB gene is highly conserved and present in single copy in the genome of mammalian (54, 85, 144) and avian (227) species. One exon encodes the 52-amino acid protein in the rabbit (54), rat (85), mouse (60a, 144), and chicken (227). An unusually long intron is present in the 5′-untranslated region in all these species. A highly conserved region within the first 113 base pairs of the 5′-flank of the PLB gene was found in mammals (85). Potential consensus cis-promoter elements similar to known muscle-specific promoters have been identified (54, 85, 227), and a recent study suggests that a GATA-4 motif may be especially important for cardiac-specific PLB gene expression (60a). In this same study (60a), enhancer regulatory elements were located at least 600 base pairs upstream from the transcription start point, and evidence was presented that the PLB intron may contain repressor elements capable of modulating PLB gene expression. In humans, the PLB gene has been mapped to chromosome 6q22.1, distant from the cardiac Ca2+-ATPase gene, which is located on chromosome 12 (54, 172a). Thus transcriptional regulation of the calcium pump and its regulator, PLB, are not controlled by a simple, spatially linked mechanism. Only a single type of PLB molecule has been found in all species examined; there do not appear to be any alternative splice isoforms of the protein. The remarkable conservation of the PLB sequence is evident in Figure 2.

Phospholamban gene expression has been probed in various tissue sources from several species. Messenger RNA of different sizes has been isolated from the dog (229), rabbit (50), pig (231), and chicken (227), most likely representing use of different poly(A) attachment sites in the 3′-untranslated regions. Among mammalian, avian, amphibian, and fish species, protein expression levels of PLB and calcium transport activity were compared, and the results provided an early suggestion that the proportion of PLB and Ca2+-ATPase expressed may not be constant, but may differ depending on the species source (244). In this study, however, the existence of PLB in hearts of lower vertebrates (frog and carp) was suggested only by phosphorylation and was not verified with use of a specific antibody or by other more direct methods. Isolation and comparison of PLB cDNAs from mammalian smooth muscle and cardiac tissue demonstrate the identical nucleotide sequence (76, 231), as expected for the presence of a single-copy gene (54, 85, 144). However, expression of the gene is tissue specific, with less PLB mRNA seen in smooth muscle than in cardiac muscle (42, 76), consistent with immunological probes of PLB tissue distribution and protein expression levels (42, 180) and by relative PLB phosphorylation levels in aortic versus cardiac tissue (24, 239). Disparate expression of PLB relative to the Ca2+-ATPase has also been observed in pig smooth muscle tissues (42). Stomach, ileum, pulmonary artery, and aorta from pig all showed comparable levels of...
of calcium pump mRNA and protein (SERCA2 isoform), whereas PLB mRNA and protein varied over a 12-fold range, with depleted amounts of PLB detected in the pulmonary artery and aorta (42). However, much more PLB was detected in canine and bovine aorta (41, 180). It is obvious from these results that expression of the cardiac calcium pump (SERCA2) and PLB are not always coordinately regulated, as further exemplified from results of hormonal treatments, as discussed below.

Three Ca\(^{2+}\)-ATPase genes (SERCA1, SERCA2, and SERCA3) exist encoding the 100,000-kDa sarcoplasmic reticulum/endoplasmic reticulum calcium pumps in intracellular membranes (21). In heart and slow-twitch skeletal muscle, only the SERCA2a pump is expressed significantly (137), which originates as an alternative splice variant from the SERCA2 gene and is the main isoform coupled to PLB. In smooth muscle, the SERCA2b isoform is mainly expressed, which is identical to SERCA2a except for the presence of an extended tail of 49 amino acids replacing the carboxy terminal four residues of SERCA2a. In fast-twitch skeletal muscle, the SERCA1 gene is predominately expressed, whereas the SERCA3 gene product is found at low levels in a wide variety of tissues (21, 137). SERCA1a is the predominant alternative splice isoform of the calcium pump expressed in adult fast-twitch skeletal muscle (21).

Phospholamban expression can be induced in fast-twitch skeletal muscle, a tissue devoid of PLB (92, 109), by chronic low-frequency stimulation (19, 126), which changes the phenotype of the muscle from fast twitch to slow twitch. Under these conditions, the skeletal muscle isoform of the Ca\(^{2+}\)-ATPase (SERCA1a) is concurrently switched to the isoform found in slow-twitch and cardiac tissues (SERCA2a) (19, 126). With the use of quantitative immunoblotting with specific antibodies, proportions of PLB and SERCA2a were found to be nearly identical in canine cardiac, slow-twitch, and chronically stimulated fast-twitch tissues (20). However, functional analysis of the regulatory effect of the PLB on the Ca\(^{2+}\)-ATPase in each type of tissue indicated that PLB is not tightly coupled to the calcium pump in the slow-twitch or chronically stimulated fast-twitch muscle, in that calcium transport and Ca\(^{2+}\)-ATPase activity in sarcoplasmic reticulum vesicles from these tissues were poorly stimulated (2-fold or less) by a monoclonal antibody that reverses the PLB inhibitory effect (20). A recent analysis of the time course of mRNA and protein expression during chronic stimulation of fast-twitch skeletal muscle indicated a close correspondence between the appearance of PLB mRNA and PLB protein, whereas induction of the SERCA2a protein lagged behind expression of its mRNA (68). A follow-up study indicated that the transcription rates of the SERCA2 and PLB genes in chronically stimulated fast-twitch skeletal muscle are discordantly regulated (68a). Incompletely understood is why some tissues, viz. slow skeletal muscle, (20) and certain smooth muscles (178, 239), exhibit ratios of expression of PLB to SERCA2 that are equal to those present in heart, but nonetheless have poor functional coupling between the two proteins (230). Other functions for PLB in these tissues have been proposed, but none has yet been demonstrated. There is one report that PLB may regulate a chloride channel in sarcoplasmic reticulum (37). Recent results with PLB knockout mice (136a, 196b) and transgenic mice overexpressing PLB in skeletal muscle (196a) suggest that PLB plays a physiological role in skeletal muscle, but its regulatory effects on the calcium pump and contractility in this tissue nevertheless remain much less impressive than that in cardiac muscle. Fortunately, several cell culture systems useful for expression of recombinant PLB and SERCA2, employing COS-1 cells (224, 231), HEK-293 cells (223), and SF21 insect cells (11, 12), maintain efficient coupling between the two expressed proteins and are now utilized widely for investigational purposes.

Discordant regulation of the expression of PLB and the Ca\(^{2+}\)-ATPase in heart is exemplified from results of thyroid hormone treatment. Treatment of rabbits with thyroid hormone resulted in a 67% elevation in Ca\(^{2+}\)-ATPase mRNA and a 39% decrease in PLB mRNA levels, whereas hypothyroidism evoked a 49% reduction in Ca\(^{2+}\)-ATPase mRNA with no effect on the level of PLB mRNA (165). Similar trends in response to thyroid hormone have been obtained in rats by monitoring PLB and Ca\(^{2+}\)-ATPase mRNA levels (104) or by measuring the PLB and Ca\(^{2+}\)-ATPase proteins directly (13, 113). In these latter three studies, calcium uptake into cardiac sarcoplasmic reticulum was increased with hyperthyroidism and decreased with hypothyroidism, as originally observed by Suko (199). With hyperthyroid rat hearts, the rate of cardiac relaxation was increased, and in sarcoplasmic reticulum vesicles isolated from these hearts, the apparent affinity of the calcium pump for calcium was also increased, as predicted from the decreased levels of PLB expression found relative to expression of the calcium pump (13, 113). Therefore, it seems likely that alteration of the ratio of PLB to SERCA2a with the thyroid hormone state produces significant mechanical effects, but changes in levels of other proteins including ratios of myosin isoforms (113a) should also be considered as accounting for contractile effects. Observations of the effects of thyroid hormone on heart have led to the hypothesis that there may be two ways in which PLB regulates Ca\(^{2+}\)-ATPase activity: 1) a quick-acting, short-term mechanism involving PLB phosphorylation and derepression of calcium pumping activity, and 2) a slower acting but longer term process involving a change in the molecular ratio of PLB to the Ca\(^{2+}\)-ATPase brought about by control of gene expression (104, 113).

Phospholamban gene expression during embryonic development of the heart appears to be regulated...
uniquely. Phospholamban mRNA has been detected in murine embryos very early in gestation at the time when spontaneous contractions are first observed (55). In chick embryos, expression of PLB mRNA is suppressed relative to cardiac α-actin transcripts, suggesting that PLB follows an independent expression program despite the presence of several putative muscle-specific promoter elements in the 5′-flanking region of its gene (222). In the developing rat heart, mRNA for the Ca\(^{2+}\)-ATPase appears 3 days before the expression of PLB mRNA, and the mRNA of the two proteins are expressed in opposite patterns along the embryonic cardiac tube, with the calcium pump mRNA most abundant in the upstream part of the cardiac tube and the richest amount of PLB mRNA in the downstream compartments. The results suggest that there are region-specific programs of PLB and Ca\(^{2+}\)-ATPase gene expression that may account for the functional differences in contractile properties between cardiac compartments (157). Both Mahony and Jones (148) and Szymanska et al. (204) have observed that the protein levels of PLB and SERCA2a are decreased in neonatal mammalian heart compared with adult heart, which may account in part for the decreased contractile reserve of neonatal heart and its blunted response to β-adrenergic agonists.

V. MECHANISM OF CALCIUM PUMP REGULATION IN SARCOPLASMIC RETICULUM

Concurrent with the identification of PLB as the principal membrane substrate for cardiac protein kinases, the effect of PLB phosphorylation on calcium transport by the calcium pump (SERCA2a) of sarcoplasmic reticulum was investigated. The predominant, physiologically relevant effect of PLB phosphorylation is to increase the apparent affinity of the transport ATPase for calcium (usually expressed as a decrease in the \(K_{Ca}\) value, or the ionized calcium concentration required for half-maximal activation of the pump), with little or no effect of phosphorylation on the maximal velocity of the enzyme (\(V_{\text{max}}\)) as measured at saturating ionized calcium (>1 μM) concentration. Both calcium transport and ATP hydrolysis by the pump are affected equally. This effect of PLB phosphorylation to increase the calcium affinity of the calcium pump, with little or no effect on the \(V_{\text{max}}\), has been demonstrated consistently and reproducibly by numerous investigators after phosphorylation of PLB by cAMP-dependent protein kinase (27, 32, 65, 78, 101, 107, 172, 179, 215, 246), calcium/calmodulin-dependent protein kinase (60, 172, 174), or both kinases together (32, 116, 172). Identically, monoclonal antibodies directed to the phosphorylation domain of PLB increase the sensitivity of calcium activation of SERCA2a, with little or no effect on the \(V_{\text{max}}\) of the enzyme (as measured at saturating calcium concentration) (20, 22, 88, 101, 152, 159). Kirchberger and co-workers (5, 5a) have recently challenged the tenet that the main regulatory effect of phospholamban is on the calcium affinity of the calcium pump; however, the vast preponderance of data concur that the \(K_{Ca}\) value is primarily affected. Although some investigators have reported that synthetic peptides corresponding to the cytoplasmic domain of PLB have substantial inhibitory effects on the SERCA enzymes measured at saturating calcium concentration (74, 187), this effect has not been found by others (28, 88, 181, 234) and, moreover, does not appear to be functionally relevant because intact PLB does not significantly affect the \(V_{\text{max}}\) of the enzyme as cited above. Direct phosphorylation of the calcium pump by calcium/calmodulin-dependent protein kinase was recently reported to stimulate the \(V_{\text{max}}\) of the enzyme, independently of any involvement of PLB (223, 249). Phosphorylation of the calcium pump and \(V_{\text{max}}\) effects were not observed previously by other investigators, however, and these results were recently disputed (discussed in detail by Reddy et al., Ref. 182). Mutagenesis work with recombinant SERCA2a suggested that the site of ATPase phosphorylation was serine-38 (223), but in-depth investigation of the effect of serine-38 phosphorylation revealed that any apparent \(V_{\text{max}}\) effects attributed to it resulted from artifacts of incubation (172). Thus the major regulatory effect on the cardiac calcium pump is mediated by phosphorylation of PLB, and this regulatory effect is to increase the calcium sensitivity of the enzyme. As a result of studies of the types above, it was proposed that PLB in the dephosphorylated state is an inhibitor of the Ca\(^{2+}\)-ATPase (78) and that phosphorylation of PLB removes this inhibition by increasing the enzyme’s apparent calcium affinity (reviewed in Ref. 210). Subsequent studies have been directed toward elucidating the mechanism of this calcium pump suppression by PLB.

Early studies assessing the mechanism of PLB action on the cardiac calcium pump used transient state analysis of ATPase activity and calcium transport to distinguish several kinetic steps in the reaction cycle (120, 218). In particular, when PLB in cardiac sarcoplasmic reticulum vesicles was phosphorylated by cAMP-dependent protein kinase, a marked increase occurred in the rate of a step associated with calcium binding to the enzyme and the rate at which the acylphosphoprotein intermediate was formed (218). However, from these types of kinetic studies one could not discern if phosphorylation of PLB directly affected the equilibrium calcium-binding affinity of the pump or, instead, caused an apparent increase in calcium affinity by accelerating a kinetic step. It was important to distinguish these two alternatives, because they have different mechanistic implications relevant to how phospholamban regulates the calcium pump.

This issue was finally resolved by Inesi and co-workers (22) by measuring calcium binding to the calcium
pump in cardiac sarcoplasmic reticulum vesicles directly, under equilibrium binding conditions. The measurements were made in the presence and absence of a monoclonal antibody to PLB, which removes the PLB inhibitory effect in analogy to phosphorylation (190). When calcium transport into cardiac sarcoplasmic reticulum vesicles was measured, the monoclonal antibody shifted the calcium concentration dependence for activation of transport to the left, but in the same sarcoplasmic reticulum vesicles, the antibody had no effect on the calcium affinity of the ATPase, when measured under equilibrium calcium binding conditions (Fig. 6). From these results and others, it was concluded that PLB affects the kinetics of enzyme activation by bound calcium rather than the actual calcium binding affinity (22). In support of this, it was found that under equilibrium binding conditions, the calcium affinity of SERCA2a in cardiac sarcoplasmic reticulum vesicles (containing active dephosphorylated PLB) is identical to that of SERCA1a in fast-twitch skeletal muscle sarcoplasmic reticulum vesicles, in which no PLB is present (22). Consistent with this, SERCA2a has the identical calcium sensitivity in calcium transport assays as SERCA1a, when expressed independently of PLB (146, 222a). It was also found that SERCA2a, like SERCA1a, has two high-affinity calcium binding sites per ATP catalytic site and that PLB does not alter this fixed stoichiometry (22). The positive cooperativity of calcium binding to SERCA is identical in cardiac and skeletal sarcoplasmic reticulum vesicles (22), which discounts the earlier proposal that PLB acts by altering calcium cooperativity (65; see also Refs. 107 and 174 in which no changes in calcium cooperativity of ATPase activation were found after phosphorylation of PLB by cAMP- or calcium/calmodulin-dependent protein kinases, respectively.) On the basis of these results, it was proposed that PLB acts by inhibiting the slow isomeric transition after binding of the first calcium to the pump, without changing the overall equilibrium constant for calcium binding (22). Phosphorylation of PLB greatly accelerates this slow transition. Future studies will continue to address the molecular mechanism by which phosphorylation of PLB accelerates the rate-limiting steps of enzyme turnover, giving an apparent increase in calcium affinity.

Suzuki and Wang (202) first demonstrated that incubation of cardiac sarcoplasmic reticulum vesicles with a monoclonal antibody recognizing only PLB increased the calcium pump activity in identical fashion as the stimulation occurring with PLB phosphorylation, confirming that PLB, and not some other phosphorylatable substrate, specifically modulates the Ca\(^{2+}\)-ATPase. Stimulation of the ATPase by the PLB antibody also provided additional evidence that PLB, in the dephosphorylated state, acts as an inhibitor of the enzyme at low ionized calcium concentration (202). Monoclonal antibodies recognizing the cytoplasmic domain of PLB (22, 88, 152, 159) thus substitute for PLB phosphorylation in reversing the suppressor effect of PLB on the ATPase. Such antibodies have become a valuable experimental tool. For example, their use in intact cells allows selective disruption of the PLB effect free from the potential influence of phosphorylation of other components, as would occur during \(\beta\)-adrenergic stimulation (190). In biochemical experiments requiring prolonged incubations, such as those measuring equilibrium calcium binding to the calcium pump in membranes, interference from phosphorylating phospholamban are obviated by use of the antibodies (22).

To achieve a better understanding of the molecular interactions between PLB and the cardiac calcium pump, attempts have been made to functionally reconstitute the two proteins using purified components. Initial efforts by Inui et al. (78) to solubilize and recompose cardiac sarcoplasmic reticulum successfully reconstituted the calcium pump activity and calcium loading rate but failed to reestablish the coupled modulatory effect of PLB phosphorylation on calcium transport; however, the reconstituted cardiac calcium pump transported calcium with threefold...
Increased apparent calcium affinity, supporting the novel concept that PLB, when properly coupled, acts as an inhibitor of the calcium pump and that phosphorylation releases this suppression. Subsequent attempts at reconstitution of purified native PLB or synthetic PLB with skeletal muscle or cardiac ATPases achieved only limited success in that significant effects of added PLB on the $V_{\text{max}}$ of the ATPases were noted (100, 187, 205, 206, 235), which is not observed with intact cardiac membranes.

In more recent studies, some progress in reconstitution has been achieved. Reddy et al. (181) successfully reconstituted recombinant PLB purified from insect cells with SERCA1a isolated from rabbit skeletal muscle sarcoplasmic reticulum. In initial studies, purified SERCA1a was utilized because this enzyme was readily available and PLB is equally effective in inhibiting SERCA1a and SERCA2a (62, 224). Successful functional coreconstitution of purified proteins into phospholipid vesicles was demonstrated by satisfying the following criteria (181): PLB inhibited the ATPase at low (pCa 6.8) but not high (pCa 5.4) ionized calcium concentration, the inhibitory effect was reversed by a monoclonal antibody to PLB, and both ATPase activity and calcium transport by the pump were inhibited by PLB, as occurs with native sarcoplasmic reticulum vesicles. Intact PLB was required to meet these criteria for a successful reconstitution. Maximal calcium pump inhibition occurred at a molar stoichiometry of approximately three PLB monomers per Ca$^{2+}$-ATPase monomer in the reconstituted system (181). Neither the cytoplasmic domain of PLB (residues 1–31 acetylated at the amino terminus) nor the membrane-spanning domain (residues 26–52) resulted in successful reconstitution. On the basis of the results obtained through reconstitution, it was concluded that both the cytoplasmic and transmembrane regions of PLB are essential for normal calcium pump regulation (181).

Applying the same reconstitution method, Mayer et al. (152) recently reported success using synthetic PLB and SERCA1a.

In a subsequent study, Reddy et al. (182) reported on the rapid purification of the canine cardiac Ca$^{2+}$-ATPase by Cibacron blue affinity chromatography and successful functional coreconstitution of SERCA2a with recombinant PLB. The same reconstitution criteria mentioned above were fulfilled and, in addition, phosphorylation of PLB by either cAMP- or calcium/calmodulin-dependent protein kinase reversed the PLB inhibition. No significant phosphorylation of the purified cardiac calcium pump by cAMP- or calcium/calmodulin-dependent protein kinase was observed, whereas PLB was readily phosphorylated, accounting for the stimulation of calcium transport at low ionized calcium concentration (182). The phospholipid vesicles reconstituted with SERCA2a retained functional and structural integrity, in that two calcium ions were transported per ATP molecule hydrolyzed, which agrees with the two calcium/catalytic site stoichiometry reported by Cantilina et al. (22) for native cardiac sarcoplasmic reticulum vesicles. Thus there is no evidence that PLB alters the coupling efficiency of the calcium pump as part of its reaction mechanism, as hypothesized in an early study (128). Although some success has been achieved in these latter reconstitution studies (152, 181, 182), there is still room for improvement in that the inhibition of SERCA provided by PLB is no greater than 50% with use of these systems. In intact cardiac sarcoplasmic reticulum vesicles, dephosphorylated PLB inhibits the calcium pump by 80% or more at the same low ionized calcium concentrations utilized. With the recent availability of milligram quantities of purified PLB and SERCA2a protein reagents, however, the efficiency of the reconstitution procedures should improve as new approaches are tried.

In an earlier attempt at reconstitution of SERCA2a purified from canine cardiac sarcoplasmic reticulum, it was reported that a synthetic peptide composed of residues 1–31 of PLB (cytoplasmic domain) inhibited the $V_{\text{max}}$ of the enzyme by 36% and that a synthetic peptide containing residues 28–47 of PLB, corresponding to most of the transmembrane domain, lowered the calcium affinity (187). However, the reliability of the reconstitution system was not completely verified by the criteria defined above, and very high concentrations of peptides (100–300 molar ratios of peptides to ATPase) were required for effects, suggesting that nonspecific interactions may have occurred. Recently, another group (74, 75, 197) has reported that the cytoplasmic domain of PLB (residues 1–25) inhibits the $V_{\text{max}}$ of SERCA1a from rabbit skeletal muscle by a substantial amount (53%). In some studies (74, 75), the nonacetylated peptide was used, but in a more recent study, the acetylated peptide was required for the effect (197). The physiological relevance of all of these results remains questionable, however, because as cited above, PLB in native cardiac sarcoplasmic reticulum has no significant effect on the $V_{\text{max}}$ of the enzyme measured at saturating calcium concentration. Furthermore, using the well-characterized reconstitution system of Reddy and coworkers described above (181, 182), no significant effect of a synthetic peptide containing residues 1–31 of PLB (acetylated at the amino terminus) was found on SERCA1a (181) or SERCA2a (L. G. Reddy, D. L. Stokes, and L. R. Jones, unpublished data) activities by monitoring both ATP hydrolysis and calcium transport by the enzymes. Similar lack of effect of high concentrations of a synthetic peptide containing amino acids 1–32 of PLB on SERCA1a activity in fast skeletal muscle sarcoplasmic reticulum vesicles was noted by others (28, 234). In an earlier report, it was shown that sarcoplasmic reticulum vesicles isolated from mouse atrial tumor cardiomyocytes are essentially devoid of PLB but exhibit appreciable SERCA2a protein and calcium transport activity (88). With the use of this "natural" reconstitution system, it
was shown that the cytoplasmic domain peptide (residues 2–25) of PLB had no effect on calcium transport by these cardiac sarcoplasmic reticulum vesicles (88), nor did a peptide composed of PLB residues 1–31, which was acetylated at the amino terminus like native PLB (unpublished data). Both peptides were ineffective when calcium transport was measured at both high and low ionized calcium concentrations, in line with the view that the cytoplasmic domain of PLB by itself is insufficient to inhibit the calcium pump of cardiac sarcoplasmic reticulum in any way that reflects the action of native PLB in the sarcoplasmic reticulum membrane.

Attempts have been made to uncouple PLB from the Ca$^{2+}$-ATPase in cardiac sarcoplasmic reticulum vesicles by different biochemical methods to better understand the nature and sites of interaction. Trypsin treatment, which degrades PLB between lysine residue 3 and arginine residue 25 (103) removing the cytoplasmic domain (241), increased the calcium sensitivity of calcium transport similar to PLB phosphorylation, suggesting that this region of PLB is involved in the inhibition of the calcium pump (73, 107). Mechanistic interpretations of these experiments, however, are complicated by the fact that trypsin also degrades the calcium pump. In an attempt to localize the cytoplasmic residues of PLB that interact with the Ca$^{2+}$-ATPase, an anti-PLB monoclonal antibody that reverses the PLB inhibition was used in a competitive binding assay to map the PLB epitope to amino acids 7–16 (159). However, it is still not tested if site-specific antibodies to other regions of the cytoplasmic domain of PLB are capable of disrupting the inhibitory interaction with the Ca$^{2+}$-ATPase. These results substantiate the importance of the cytoplasmic domain of PLB for reversal of the inhibition of the calcium pump by phosphorylation or antibody. In vitro reconstitution studies suggest, however, that anchoring of the cytoplasmic domain to the transmembrane region of PLB is required for effective functional coupling of PLB to ATPase inhibition (88, 181). In coexpression studies, coupling of PLB to ATPase inhibition could be achieved when severely truncated or altered cytoplasmic domains were attached to the transmembrane domain (102). These results were explained by the proposal of the existence of a circuit of regulatory interactions involving communication between cytoplasmic and transmembrane domains in both PLB and SERCA (102, 103).

The large change in the isoelectric point of PLB induced by phosphorylation (90) suggests that charge-charge protein interactions may be important for the ability of PLB to inhibit the calcium pump. A change in surface membrane potential, in fact, occurs after phosphorylation of PLB and, under high ionic strength conditions, both the surface membrane potential and PLB phosphorylation effects on the apparent calcium affinity of the Ca$^{2+}$-ATPase are attenuated, supporting the notion that electrostatic interactions between the cytoplasmic domain of PLB and the pump are involved in the regulatory mechanism (26). Consistent with this concept, the polyanion heparin sodium stimulates calcium uptake into cardiac sarcoplasmic reticulum vesicles to the same level as PLB phosphorylation (250), as does the application of a small concentration of negatively charged detergent (26). Tannin, a plant phenol, also stimulates the Ca$^{2+}$-ATPase and calcium uptake activities by decreasing the $K_{Ca}$ value with no effect on the $V_{max}$, leading to the hypothesis that the acidic groups of tannin interact with basic groups of PLB to disrupt its inhibitory effect on the calcium pump (27).

Another polyphenol, quercetin, reverses phospholamban inhibition of the Ca$^{2+}$-ATPase in cardiac sarcoplasmic reticulum vesicles by inducing a similar increase in calcium sensitivity, and indirect evidence has been presented suggesting that quercetin interacts with the nucleotide binding site of the ATPase (154). Cross-linking experiments by James et al. (84) with a PLB photoaffinity labeling probe first indicated that lysine residue 3 of PLB in the cytoplasmic domain binds to a region of the Ca$^{2+}$-ATPase just downstream of the acylphosphorylation site at aspartate residue 351. Taken together, the results suggest that PLB may inhibit the Ca$^{2+}$-ATPase through electrostatic interactions involving basic residues of the PLB cytosolic domain and acidic residues of the Ca$^{2+}$-ATPase near the ATP binding site and the phosphorylated aspartic acid (see mutagenesis results discussed below). The dynamic equilibrium between phospholamban pentamers and monomers in the plane of the lipid membrane also appears to be controlled by electrostatic interactions. Phosphorylation of PLB or buffers of high ionic strength promotes pentameric stability of the protein (34).

Important new insights on mechanisms of calcium pump regulation have been provided through the use of cellular coexpression of recombinant PLB with SERCA enzymes. Fujii et al. (52) found that coexpression of PLB with SERCA2a in COS-1 cells lowered the calcium sensitivity of the enzyme in calcium transport assays conducted with microsomal membrane preparations, confirming by recombinant methods that PLB is an inhibitor of the calcium pump. The effect of PLB on the calcium affinity of the smooth muscle isoform of the Ca$^{2+}$-ATPase (SERCA2b) has been compared with that of the cardiac isoform (SERCA2a) in another cell transfection system, and both isoforms were found to exhibit an approximately twofold decrease in apparent calcium affinity when coexpressed with PLB (230). The efficient functional coupling of PLB to SERCA2b in the transfection system (230) suggests that the relatively poor coupling between the two proteins in intact smooth muscle (178, 239) is not because of structural differences between SERCA2a and SERCA2b. With use of the recombinant coexpression system, the issue of the role of PLB in regulating the calcium sensitivity of the cardiac calcium pump vis-à-vis the $V_{max}$ of the enzyme was reassessed by Odermatt et al. (172). It
was observed that phosphorylation of PLB in microsomes from HEK-293 cells coexpressing PLB and SERCA2a decreased the \( K_{ca} \) value in transport assays by approximately twofold but had no effect on the \( V_{max} \) of the enzyme at saturating calcium concentration. Similar results were observed when PLB was phosphorylated by cAMP-dependent protein kinase, by calcium/calmodulin-dependent protein kinase, or by both kinases together, and when native cardiac vesicles were analyzed in parallel experiments, the same effect on the \( K_{ca} \) value, but not \( V_{max} \), was obtained (172). Likewise, coexpression of phospholamban with SERCA2a in Sf21 insect cells increased the \( K_{ca} \) value obtained for activation of ATPase activity or calcium transport with no effect on the \( V_{max} \) of the enzyme (12). Overexpression of PLB in cultured neonatal rat cardiomyocytes by infecting cells with PLB-encoding adenovirus also increases the \( K_{ca} \) value with no effect on the \( V_{max} \) of the enzyme (60b). Thus, with the use of the purified proteins coreconstituted biochemically (181, 182), with the use of molecular coreconstitution in cultured cells (12, 60b, 172), and with the use of native sarcoplasmic reticulum vesicles (22, 172), recent carefully controlled studies confirm the conclusion that the main functional effect of PLB is to regulate the calcium sensitivity of the SERCA2 enzyme.

Mutational analyses have also been conducted with cellular coexpression systems to localize molecular domains important for PLB/calcium pump interactions. To localize residues in the Ca\(^{2+}\)-ATPase required for functional coupling to PLB, PLB was coexpressed individually with SERCA1a, SERCA2a, and SERCA3 and found to decrease the calcium sensitivity of SERCA1a and SERCA2a, but not SERCA3 (224). In the PLB uncoupled states, SERCA1a and SERCA2a had identical high calcium affinities, but the apparent calcium affinity of SERCA3 was already low and unaffected by PLB. By analysis of SERCA2/SERCA3 chimeric proteins, regions of the Ca\(^{2+}\)-ATPase molecule critical for the functional coupling to PLB were localized by Toyofuku et al. (224) to two cytoplasmic domains, one containing residues 336–412 (acylphosphorylation domain) and the other comprised of residues 467–762 (nucleotide binding/hinge domain). Mutagenesis experiments by Toyofuku et al. (226) further specified the essential amino acids in the acylphosphorylation domain of the Ca\(^{2+}\)-ATPase interacting with PLB as residues 397–402 and pointed to the importance of charged side chains in the interaction. Interestingly, the SERCA2a peptide labeled by the PLB photoaffinity probe by James et al. (84) encompasses the critical ATPase interaction site later identified in the mutagenesis experiments of Toyofuku et al. (226), suggesting that lysine residue 3 of PLB in the cytoplasm may bind directly to residues 397–492 of the ATPase.

Cellular coexpression of recombinant proteins has also been used to identify amino acids of PLB important for functional interaction with the cardiac Ca\(^{2+}\)-ATPase. Phospholamban mutants coexpressed with the cardiac Ca\(^{2+}\)-ATPase localized 13 amino acid residues between sequence positions 2–18 of PLB as important for the functional association between the two proteins (225). Point mutation of four positively charged residues, one negatively charged residue, four hydrophobic residues, two alanine residues, and the phosphorylated residues (serine 16 and threonine 17) in this region all resulted in loss of functional coupling between the membrane-spanning domains of PLB and the cardiac Ca\(^{2+}\)-ATPase (225). This result emphasizes the fact that considerable structural specificity is required to maintain functional PLB coupling and that hydrophobic as well as electrostatic interactions are important. Based on these results, the authors (225) concluded that this cytoplasmic region of PLB (residues 2–18) is essential for functional association with the Ca\(^{2+}\)-ATPase. Somewhat surprisingly, however, in a subsequent study it was observed by Kimura et al. (102) that the cytoplasmic domain of PLB could be replaced with a foreign epitope or deleted entirely with functional coupling of the PLB transmembrane domain to SERCA pumps preserved. In fact, some of the mutant PLB constructs examined, which lacked the native cytoplasmic domain, were actually stronger inhibitors of SERCA2a activity than was wild-type PLB. For example, addition of the hemagglutinin epitope to the transmembrane sequence of PLB containing residues 28–52 “supershifted” the apparent calcium affinity to values lower than those observed with native PLB. From these observations, Kimura et al. (102) proposed that the interaction between the membrane-spanning domains of PLB and SERCA2a inhibits SERCA2a by lowering its apparent calcium affinity. It was also suggested that the cytoplasmic domain of PLB is by itself not inhibitory, but instead modulates the inhibitory interactions in the transmembrane domains through a long-range coupling process (102).

A puzzling but at the same time intriguing question is the role of the PLB pentamer in calcium pump regulation (31). Recent experiments (12, 103) have led to the conclusion that the PLB monomer, not the pentamer, may actually bind to and inhibit the calcium pump in the sarcoplasmic reticulum membrane. With the use of either the Sf21 insect cell system (11, 12) or HEK-293 cells (103) for coexpression of PLB with SERCA2a, changing leucine residue 37 in the leucine zipper region of PLB (Fig. 5) to alanine (L37A-PLB) depolymerized the protein in the plane of the phospholipid membrane (34), while at the same time giving a stronger inhibition of the calcium pump at low ionized calcium concentration than did coexpression of wild-type, pentameric phospholamban. Whereas coexpression of wild-type PLB with the Ca\(^{2+}\)-ATPase increased the \( K_{ca} \) value required for pump activation by approximately twofold, coexpression of L37A-PLB with the Ca\(^{2+}\)-ATPase increased the \( K_{ca} \) value by four-
The inhibition of the Ca$^{2+}$-ATPase by L37A-PLB was shown to be reversed by addition of the PLB monoclonal antibody (12), demonstrating that the basic mechanism of inhibition of the Ca$^{2+}$-ATPase by wild-type PLB and L37A-PLB was similar. From such results, it was hypothesized that PLB monomers and pentamers are in dynamic equilibrium in the lipid bilayer (34) and that PLB monomers preferentially bind to the calcium pump and inhibit calcium transport in the sarcoplasmic reticulum membrane (12, 103).

Kimura et al. (103) performed scanning-alanine mutagenesis of the membrane-spanning residues of PLB and confirmed the observation (194) that leucines 37, 44, and 51 and isoleucines 40 and 47 repeating every three or four residues along one face of the transmembrane helix were the pentamer-stabilizing residues (Fig. 5). These investigators then examined the functional consequences of the PLB point mutations by coexpressing all of the mutated proteins with SERCA2a in HEK-293 cells. A remarkable cyclical pattern was noted, in that mutations on one face of the helix diminished or completely prevented inhibitory interactions with the calcium pump, whereas mutations on the opposite face of the helix activated inhibitory interactions with the calcium pump (103). Most of the mutations that enhanced inhibitory interactions with the calcium pump were the monomer-producing mutations located in the leucine/isoleucine zipper region (194) (L37A-PLB, I40A-PLB). These monomeric mutants were termed "supershifters" (102, 103) because they decreased the apparent calcium affinity of SERCA2a about twofold more effectively than did wild-type PLB. Based on these results, Kimura et al. (103) independently concluded that PLB monomers are the active species inhibiting SERCA2a in the sarcoplasmic reticulum membrane. An equally important observation of Kimura et al. (103) was the identification of several "loss-of-function" mutants of PLB (i.e., L31A-PLB, N34A-PLB, L42A-PLB) that arose from changing residues on the face of the transmembrane helix opposite from the pentamer-stabilizing face. These loss-of-function mutants were no longer able to inhibit the calcium pump effectively, suggesting for the first time the presence of essential amino acid residues in the transmembrane region of PLB that might be required for direct functional interactions with the transmembrane segments of SERCA2a. In fact, on the basis of these results (103) and those from their earlier study (102), it was concluded that the transmembrane region of PLB containing the loss-of-function mutational sites is the domain directly responsible for inhibiting the Ca$^{2+}$-ATPase and lowering its apparent calcium affinity. The cytoplasmic domain of PLB, on the other hand, was proposed to modulate the inhibitory interactions in the transmembrane domain through long-range coupling (102). This would explain why the cytoplasmic domain of PLB by itself is insufficient to inhibit the ATPase (88, 181). Identification of the transmembrane residues of SERCA2a that interact directly with the putative inhibitory transmembrane helical face of PLB will be essential for a complete understanding of the mechanism by which PLB regulates the activity of the calcium pump (197a).

Given the evidence that suggests that not only the PLB cytoplasmic domain but also the transmembrane domain serves a functional role beyond tethering the cytoplasmic domain to the sarcoplasmic reticulum, what are the structural details of the PLB/Ca$^{2+}$-ATPase interaction? Related questions, given the model that dephosphorylated monomeric PLB is capable of interacting directly with the Ca$^{2+}$-ATPase to inhibit its calcium pumping activity, involve determining the stoichiometry of PLB to the Ca$^{2+}$-ATPase and, moreover, the role of oligomeric complexes of the Ca$^{2+}$-ATPase itself (4) to its own function. Early indirect estimates using phosphate incorporation into both PLB and the Ca$^{2+}$-ATPase for quantitation suggested a 1:1 stoichiometry of PLB pentamers to Ca$^{2+}$-ATPase monomers (141, 209), but a more recent indirect study using densitometric determination of the Ca$^{2+}$-ATPase concentration and mobility shift estimates of the PLB concentration indicated 0.4 mol of PLB pentamer for every mole of Ca$^{2+}$-ATPase monomer (32). The concept of a fixed stoichiometry between PLB and the calcium pump in the sarcoplasmic reticulum membrane may be a "red herring," however, because of the relatively weak binding affinity between the two proteins (78, 121, 182), the ease of dissociation of the functional complex in the plane of the sarcoplasmic reticulum membrane (142, 192), the apparent calcium affinity of SERCA2a about twofold between the two proteins (78, 121, 182), the ease of dissociation of the functional complex in the plane of the sarcoplasmic reticulum membrane (142, 192), and the uncoordinated expression of the two proteins in vivo under certain conditions (104, 112).

Although the true stoichiometry between PLB and the Ca$^{2+}$-ATPase remains ill defined, it has been speculated that dephosphorylated PLB might restrict Ca$^{2+}$-ATPase conformational freedom and thereby suppress its activity (45). In support of this hypothesis, recent experiments by Thomas and co-workers (236) using time-resolved phosphorescence anisotropy indicate that there is a distribution of oligomeric complexes of the Ca$^{2+}$-ATPase in sarcoplasmic reticulum, with large stationary aggregates and slowly rotating oligomers in addition to the highly dynamic monomers. Phosphorylation of PLB increased the rotational mobility of SERCA2a as a result of a decrease in large aggregates, supporting a model in which PLB phosphorylation releases the Ca$^{2+}$-ATPase from a kinetically unfavorable associated state (236), perhaps the kinetic state after the binding of the first calcium ion (22). Reciprocal aggregation (pentamer formation) of phospholamban by phosphorylation (34) with resultant deaggregation and activation of the Ca$^{2+}$-ATPase (236) (Fig. 7) is consistent with the recent PLB mutagenesis studies cited above in which expression
of PLB with depolymerizing mutations gives stronger inhibition of the Ca\textsuperscript{2+}-ATPase than does expression of wild-type PLB (12, 103). The inhibition by PLB due to induction of Ca\textsuperscript{2+}-ATPase lateral aggregation was further supported by a study of comparative molecular dynamics in mouse atrial tumor versus ventricular sarcoplasmic reticulum, which exploited the absence of PLB in the atrial membranes as a control for the ventricular membranes that contain PLB in levels similar to other mammals (237). The model proposed from these results suggests a structural and functional perturbation of the Ca\textsuperscript{2+}-ATPase through electrostatic interactions with PLB that affect self-association of the Ca\textsuperscript{2+}-ATPase (236, 237), resembling the general mechanism of many signal transduction processes that involve lateral association of membrane proteins (45). Mutagenesis of PLB (12, 103) combined with molecular dynamics results (34, 236, 237) suggest that the PLB monomer may be the most active species promoting Ca\textsuperscript{2+}-ATPase aggregation/inactivation. Phosphorylation of PLB drives its equilibrium toward pentamers (34), which may be relatively ineffective inhibitors of the Ca\textsuperscript{2+}-ATPase compared with monomers (103). Whether the pentamer by itself (in the absence of any monomers) is capable of inhibiting the Ca\textsuperscript{2+}-ATPase remains to be determined. In this model of PLB interaction with the calcium pump, the pentamer in the membrane can be viewed as a reservoir for monomers (103), which dissociate from the pentamer in the dephosphorylated form (34), diffuse in the plane of membrane, and then bind to and inhibit the Ca\textsuperscript{2+}-ATPase at low ionized calcium concentrations (12, 103) by an aggregation-based mechanism (236, 237) (Fig. 7). It should be pointed out that Chu et al. (29) recently over-expressed the monomeric mutant C41F-PLB in transgenic mouse ventricle and noted no stronger over-suppression of contractility than that achieved with over-expression of wild-type PLB. This negative result is predictable, however, because C41F-PLB, although monomeric (51, 194), is no more effective than wild-type PLB in inhibiting the calcium pump (225). This is probably because the interaction domain with the ATPase is partially perturbed with this mutation, even though the protein is monomeric (103).

Other experiments appear to provide results that conflict with the finding that dephosphorylated PLB inhibits SERCA2a by aggregating the enzyme. Studies using either time-resolved phosphorescence polarization (48) or spin-label electron paramagnetic resonance (167) have reported a decrease in the rotational dynamics of the Ca\textsuperscript{2+}-ATPase associated with phosphorylation of PLB, prompting the suggestion that PLB phosphorylation actually enhances interaction between Ca\textsuperscript{2+}-ATPase chains either by increasing protein-protein interactions or by altering the conformation of Ca\textsuperscript{2+}-ATPase chains within a stable oligomeric state (167). These findings, however, are more consistent with PLB being an activator of the Ca\textsuperscript{2+}-ATPase rather than an inhibitor, and also are difficult to reconcile with the observation that SERCA1a and SERCA2a have identical calcium affinities when PLB is not present (146) or is inactivated (22). Moreover, other recent results using time-resolved phosphorescence anisotropy to investigate the effects of the nonionic deter-

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**FIG. 7.** Cartoon model of PLB mechanism of action. Dephosphorylated PLB monomer inhibits SERCA2a (12, 103) by binding to cytoplasmic and membrane domains of pump, stabilizing enzyme in E\textsubscript{2} conformation, and causing enzyme inhibition and pump aggregation (236). Phosphorylation of PLB reverses calcium pump inhibition favoring association of PLB monomers into pentamers (34). Dark shading in membrane region of PLB indicates face of transmembrane helix interacting with calcium pump (103), and open area designates face of helix containing leucine/isoleucine zipper residues (194). $K_1$ and $K_2$ are hypothetical dissociation constants for PLB pentamer to monomer conversion and for PLB monomer binding to SERCA2a, respectively, as explicitly proposed in Ref. 103. [Adapted from Cornea et al. (34) and Kimura et al. (103).]
gent C$_N$E$_S$ on calcium transport and molecular dynamics of the Ca$^{2+}$-ATPase (192) support earlier evidence that the mechanism of PLB regulation of calcium transport involves modification of the Ca$^{2+}$-ATPase oligomeric state (206, 237). To resolve these discrepancies and better define the correlation between PLB and Ca$^{2+}$-ATPase oligomerization, molecular dynamics within the sarcoplasmic reticulum, and the regulation of calcium transport, further experiments will be required utilizing the latest technologies for detecting and quantitating protein-protein interactions.

The intriguing pentameric quaternary arrangement of PLB subunits and its resemblance to a pore-forming structure (7, 8, 193–195) invite the question whether PLB may regulate calcium flux independent of Ca$^{2+}$-ATPase activity by functioning as a channel (64a, 115). Supporting the structural model of PLB as a channel, the crystal structure of a five-stranded coiled-coil domain in the cartilage oligomeric matrix protein was recently determined (149) and bears a striking similarity to the transmembrane hydrophobic pore structure proposed for PLB (194, 195). The observed 2–6 Å internal diameter of the pore in this crystal structure and the presence of 13 water molecules distributed within the hydrophobic pore (149) provide an experimental basis to validate the plausibility of the structural model of PLB as a calcium channel. Furthermore, the regular rings of conserved glutamine residues and constrictions of the internal pore diameter caused by side chains at positions a and d of the coiled-coil residue pattern may suggest the physical basis for ion selectivity and gating (149).

Single-channel recording experiments have, in fact, demonstrated calcium-selective channel behavior for PLB (115), and early attempts to directly express PLB in cell-based expression systems resulted in cell lysis (33), consistent with the ability of PLB to spontaneously form membrane pores. However, there is no evidence yet that PLB functions as a channel or passive pore in the sarcoplasmic reticulum membrane (12, 181). The good correlation of calcium uptake with changes in ATPase activity in vitro vesicle systems supports a simple model in which the Ca$^{2+}$-ATPase is regulated by phosphorylation-reversible interaction with PLB and does not require for explanation a complex model involving putative PLB calcium channel activity (10). The fact that the PLB monomer is a stronger inhibitor of calcium transport than is the pentamer (12, 103) also argues against any kind of channel activity being required for calcium pump inhibition. Nevertheless, the recent appreciation that coiled-coil structures in proteins can fulfill many functions, from molecular stalks and scaffolding (e.g., kinesin and fibrin) to dimerization and selective interaction (e.g., bZip or basic region leucine zipper transcription factors) to a dynamic hinge (e.g., influenza hemagglutinin) (37a, 145), suggests that all of the functional attributes of PLB may not yet be fully revealed.

VI. PHOSPHOLAMBAN PHOSPHORYLATION AND FUNCTION IN INTACT SYSTEMS

Beyond in vitro studies that indicate the important role of PLB and its phosphorylation in regulating Ca$^{2+}$-ATPase activity and calcium transport across the sarcoplasmic reticulum, in vivo experiments demonstrate that PLB is a key regulator of myocardial relaxation and, as more recently demonstrated, force development as well (144). Early studies of microsomal membranes isolated from $^{32}$P-perfused mammalian hearts demonstrated that PLB is the major sarcoplasmic reticulum protein phosphorylated in intact cardiac muscle in response to $\beta$-adrenergic stimulation (122, 134). Phosphorylation of PLB correlated temporally, over a time course of seconds, with the ability of the $\beta$-agonist isoproterenol to accelerate the rate of relaxation of the heart (134) and occurred as a consequence of activation of cardiac $\beta_1$-adrenergic receptors selectively (2, 248). When Ca$^{2+}$-ATPase activity was analyzed in sarcoplasmic reticulum vesicles isolated from hearts subjected to $\beta$-adrenergic stimulation, in which care was taken to prevent dephosphorylation of PLB during the preparation of membranes, it was observed that both Ca$^{2+}$-ATPase (134) and calcium uptake (119) activities were increased as a result of PLB phosphorylation. Furthermore, the stimulation of calcium pump activity occurred most prominently at low ionized calcium concentration (55, 119, 134), showing that for PLB phosphorylated in vivo, like the protein phosphorylated in vitro, the main effect is on the calcium sensitivity of the ATPase, not on the $V_{\text{max}}$. In other experiments, it was demonstrated that dephosphorylation of PLB, rather than of other cardiac protein substrates such as troponin I, C-protein, or phospholamban (56, 155, 219, 252), most closely paralleled termination of the mechanical effects of $\beta$-adrenergic stimulation. Furthermore, cholinergic stimulation, which reverses the functional effects of $\beta$-adrenergic stimulation, attenuated phosphorylation of PLB and Ca$^{2+}$-ATPase activity at the same time that it increased the half time of relaxation (136). Thus, during $\beta$-adrenergic stimulation of the heart, phosphorylation of PLB occurs rapidly, is regulated dynamically, and causes an increased rate of calcium transport into the sarcoplasmic reticulum. The resultant increase in rate of lowering of the cytoplasmic calcium concentration can account for the increased rate of myocardial relaxation produced by $\beta_1$-agonists (215).

The amino acid residues of PLB phosphorylated in beating mammalian myocardium in response to $\beta$-adrenergic stimulation were localized by monoclonal antibody affinity purification of the $^{32}$P-labeled protein from perfused guinea pig hearts followed by phosphoamino acid analysis and direct protein sequencing (242). It was observed that only serine-16 and threonine-17 were phosphorylated, indicating that in intact myocardium, $\beta$-adren-
ergic stimulation results in PLB phosphorylation by both cAMP-dependent protein kinase (at serine-16) and calcium/calmodulin-dependent protein kinase (at threonine-17). During β-adrenergic stimulation with isoproterenol, phosphorylation at serine-16 precedes that at threonine-17, but at steady state, both sites are phosphorylated in approximately equimolar amounts (242). Talosi et al. (219) subsequently showed that during termination of β-adrenergic effects, dephosphorylation at serine-16 also precedes that at threonine-17, and proposed that the phosphorylation state of serine-16 correlates most closely with the mechanical effects. Immunoblotting of microsomes from hearts subjected to β-adrenergic stimulation revealed 10 discrete mobility steps induced in the pentamer form of the protein (Fig. 4), confirming the additive phosphorylation of both sites on each monomer composing the pentamer during the time course of β-agonist stimulation (242) and refuting an earlier proposal that there is no dephospho form of PLB in vivo in the basal unstimulated state (127). Phosphorylation of PLB by cAMP-dependent protein kinase and calcium/calmodulin-dependent protein kinase was also observed by use of the back-phosphorylation technique in hearts from live animals administered isoproterenol (96). Thus dual phosphorylation of PLB occurs in vivo, as well as in vitro, with PLB regulating relaxant properties of the heart in a graded fashion by its incremental phosphorylation.

A vexing problem concerning PLB phosphorylation in intact muscle is that whereas PLB is readily phosphorylated in vitro at threonine-17 by calcium/calmodulin-dependent protein kinase independent of any requirement for prior phosphorylation at serine-16 by cAMP-dependent protein kinase, in intact muscle under physiological conditions, PLB is only phosphorylated significantly by agents that elevate cAMP (135). Positive inotropic agents that increase intracellular calcium concentration sufficiently high to activate calcium/calmodulin-dependent protein kinase (high extracellular calcium concentration, calcium channel agonists, α-adrenergic agonists), but which do not increase cAMP, have no effect on PLB phosphorylation in intact hearts (133, 135, 166, 232, 233, 243). This paradox was recently addressed by Mundina-Weilennmann et al. (164), who took advantage of antibodies that distinguish between serine-16-phosphorylated PLB and threonine-17-phosphorylated PLB (38) to reassess mechanisms regulating PLB phosphorylation at these two sites in perfused hearts. Notably, when hearts were perfused at elevated extracellular calcium concentration in the presence of the phosphatase inhibitor okadaic acid, phosphorylation of threonine-17 of PLB readily occurred, and without any requirement for phosphorylation at serine-16. A significant decrease in the half-relaxation time coincided with this unique phosphorylation of threonine-17 (164). Thus phosphorylation of PLB at threonine-17 alone is sufficient to increase the cardiac relaxation rate, as originally predicted from results of calcium uptake assays utilizing sarcoplasmic reticulum vesicles (116, 174, 209). Under physiological conditions, inhibition of the major phosphatase that dephosphorylates PLB, the type 1 phosphatase (PP1) associated with cardiac sarcoplasmic reticulum (147), is apparently required to realize significant phosphorylation of PLB at threonine-17. Inhibition of PP1 activity associated with sarcoplasmic reticulum by cAMP-elevating agents occurs as a consequence of release of the catalytic subunit of the phosphatase from the membrane after phosphorylation of the regulatory subunit by cAMP-dependent protein kinase (1, 69), and also as a result of phosphorylation of phosphatase inhibitor 1 by cAMP-dependent protein kinase, which in the phosphorylated state is a potent inhibitor of PP1 catalytic activity (170). Supporting this mechanism, dephosphorylation of PLB in cardiac myocytes is greatly stimulated by inactivation of cAMP-dependent protein kinase (252). For realization of the maximal contractile effect of PLB phosphorylation in intact myocardium, it thus appears that phosphorylation of both serine-16 and threonine-17 is required and that this requirement is only fulfilled by cAMP-dependent mechanisms involving an interplay between protein kinases and phosphatases (164).

From studies of the types described above, it was fairly certain that PLB was a major phosphoprotein participant in the regulation of cardiac contractility. However, because of the myriad of proteins phosphorylated in intact heart in response to β-adrenergic stimulation, it was always impossible to be absolutely certain that phosphorylation of PLB, by and of itself, was sufficient to alter myocardial contractile properties (97). In more recent work, the prime importance of PLB phosphorylation has been unambiguously demonstrated by two different approaches. In the first, ventricular myocytes were dialyzed with a monoclonal antibody to PLB that stimulates calcium uptake into sarcoplasmic reticulum at low ionized calcium concentration, and intracellular calcium transients were measured with the calcium-sensitive dye fura 2 (190). Dialysis of myocytes with the antibody significantly increased the amplitude and decreased the duration of the calcium transient, and effects of β-agonist stimulation by isoproterenol on the calcium transient were virtually eliminated by the antibody (190). Thus removal of the inhibitory effect of PLB on the calcium pump of sarcoplasmic reticulum was proposed to be sufficient to elicit most of the effects of β-adrenergic stimulation on the intracellular calcium transient in intact cardiac myocytes (190). Consistent with this conclusion, it was recently shown that overexpression of PLB in cultured cardiac myocytes by infection of cells with PLB-encoding adenovirus decreased the amplitude and increased the duration of the calcium transient (60b).

In the second approach, a PLB gene knockout strategy was implemented by Kranias and co-workers (144) to
create a transgenic mouse lineage completely devoid of the protein. The profound influence of PLB on contractility was then demonstrated in an elegant series of experiments using this model (reviewed in Refs. 95 and 114). Perfused hearts from PLB knockout mice had dramatic contractile changes in comparison with hearts from littermate control mice. Both rates of tension development and rates of myocardial relaxation were increased and, moreover, β-agonist stimulation of these transgenic hearts by isoproterenol was sharply attenuated, due to the fact that the basal state of contractility in the transgenic hearts was already close to the maximal level. Similar changes were shown in live PLB knockout mice by use of echocardiography (66). The results obtained from transgenic mouse hearts completely lacking the PLB protein (66, 144) are consistent with results from normal cardiomyocytes dialyzed with the highly specific anti-PLB monoclonal antibody (190), demonstrating that PLB is a key suppressor of basal myocardial contractility and that phosphorylation of PLB can account for most of the contractile effects of β1-adrenergic stimulation on the heart, including both positive inotropic effects and enhanced relaxation (95, 114). The positive inotropic effect associated with β-adrenergic stimulation of the heart arises from increased calcium loading by the sarcoplasmic reticulum, which makes more calcium available for calcium release. The augmented calcium release promotes more rapid calcium binding to troponin, stimulating tension development. The increased rate of muscle relaxation is a direct consequence of the accelerated rate of calcium removal from the cytoplasm, allowing calcium to be rapidly unbound from troponin (114, 210). Consistent with this idea, targeted overexpression of PLB to mouse ventricle (95a) or mouse atrium (168) in transgenic animals prolongs contraction time and depresses contractility in both tissues, whereas β-adrenergic effects are amplified. Although β-adrenergic-mediated contractile effects are strongly attenuated in PLB knockout mice, some contractile response to catecholamines does persist both in live animals (66) and in isolated myocytes (245a), suggesting that phosphorylation of proteins other than PLB (troponin I, ryanodine receptor, phospholemman, and dihydropyridine-sensitive calcium channel) may also play a role in regulating contraction-relaxation dynamics of heart cells (245a).

The PLB knockout mouse model has also proven to be useful in confirming the mechanism of action of PLB in inhibiting the Ca2+-ATPase of sarcoplasmic reticulum membranes. Calcium transport into sarcoplasmic reticulum vesicles was compared between homogenates from transgenic mouse hearts lacking PLB and homogenates from littermate controls containing normal amounts of the protein (144). Calcium uptake into sarcoplasmic reticulum vesicles from PLB-deficient transgenic mouse hearts was augmented, but only at low ionized calcium concentration, reflecting an increase in the apparent affinity of the calcium pump for calcium, with no change in the $V_{\text{max}}$ of the enzyme detected (144). In another study, the control and transgenic mouse hearts were found to contain identical levels of the calcium pump protein (29a). Thus, recent in vitro and in vivo studies using state-of-the-art technologies produce consistent results demonstrating the leading role of PLB in transmitting β1-adrenergic effects by modulating the calcium sensitivity, but not the $V_{\text{max}}$ of the calcium pump.

**VII. PHOSPHOLAMBAN EXPRESSION IN HUMAN HEART FAILURE**

Despite the importance of PLB to normal cardiac function, its role in the pathophysiology of human heart disease remains unknown. Two of the physiological hallmarks of human heart failure are extended calcium transients and prolonged myocardial relaxation (158), yet in one study, the magnitude of the PLB effect on calcium uptake was observed to be the same in normal versus failing heart sarcoplasmic reticulum vesicles from patients with idiopathic dilated cardiomyopathy (161). Movsesian et al. (161) showed that a monoclonal antibody to PLB stimulated calcium uptake equally well at low ionized calcium concentration with microsomes isolated from normal human hearts or with microsomes from patients with dilated cardiomyopathy. Consistent with these results, five other studies found no change in protein expression levels of either PLB or the Ca2+-ATPase in hearts from patients with end-stage ischemic or dilated cardiomyopathy (17, 46, 132, 162, 188). Earlier studies detected a decrease in PLB mRNA levels in samples from failing human hearts (6, 43), but this decrease in PLB mRNA is not associated with a decrease in PLB protein (46, 132). However, one study found that the PLB protein level was decreased slightly (18%) in hearts from patients with dilated cardiomyopathy (153), and two studies (64, 153) suggested that the protein level of the calcium pump may be decreased more substantially (36–41%). In two studies, calcium uptake (64, 188) and Ca2+-ATPase (188) activities were decreased in tissue preparations from heart failure patients, but coupling of the calcium pump to PLB was not analyzed, and there was disagreement on whether or not PLB and SERCA2a protein levels were changed. Some of the differences between laboratories are probably a consequence of the difficulties involved in obtaining adequately preserved myocardial samples from human patients. Nevertheless, all groups seem to agree that in failed human myocardium, the protein expression level of PLB is unchanged or only negligibly affected (17, 46, 132, 153, 162, 168), even though in certain animal models of heart failure the level of PLB can be downregulated substantially (184). Although the intrinsic mechanism of PLB coupling to the Ca2+-ATPase is unaltered in normal and failed
human hearts (161), it remains likely that regulation of PLB phosphorylation by cAMP-dependent mechanisms (17) or other second messenger pathways (169) is perturbed, which may account partially for the diastolic dysfunction and prolonged calcium transients associated with this disease. Although much remains to be learned about the possible role of PLB in human heart failure, selective disruption of the phospholamban/calcium pump interaction is a potentially powerful target for pharmaceutical intervention to improve contractility.

VIII. CONCLUDING REMARKS

Phospholamban, a small protein of only 52 amino acids, has proven to be an intriguing and surprising molecule, the characterization of which traverses many fields, including membrane protein biochemistry, protein kinases and phosphatases, protein structure-function and protein-protein interactions, ion transport/ion channels, second messenger regulation of contractility and tissue excitability, and pathophysiological mechanisms involved in cardiovascular disease. The small size of PLB, its easy genetic manipulation, and its large-scale purification and chemical synthesis, coupled with the paramount physiological importance of the protein, ensure that it will be the subject of intense scrutiny across diverse fields for years to come. Phospholamban is a key mediator of β-adrenergic mechanisms regulating contractility in mammalian heart. As such, understanding the structure-function relationships of PLB and the protein-protein interactions with which it participates will be necessary to exploit its activities for therapeutically useful purposes.

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