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# BREAKTHROUGHS AND VIEWS

# Phospholamban: A Protein Coming of Age

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Sarcoplasmic reticulum (SR) is an intracellular membranous network, which plays a critical role in the maintenance of $Ca^{2+}$ homeostasis in muscle cells. During contraction, the SR functions as a $Ca^{2+}$ source while during relaxation, it serves as a $Ca^{2+}$ sink. The transport of $Ca^{2+}$ from the cytosol into the SR lumen is mediated by a $Ca^{2+}$-ATPase. This enzyme is under regulatory control by phospholamban, a 52 amino acid integral SR phosphoprotein (1,2,3). Phospholamban is mainly expressed in cardiac muscle, and in much lower levels in slow-twitch skeletal and smooth muscles. However, its regulatory effects on the SR $Ca^{2+}$-ATPase have best been documented in cardiac muscle. *In vitro* studies using purified cardiac SR membranes have shown that phospholamban can be phosphorylated at three distinct sites: namely serine 16 by cAMP-dependent protein kinase; threonine 17 by $Ca^{2+}$-calmodulin-dependent protein kinase; and serine 10 by $Ca^{2+}$-phospholipid-dependent protein kinase (4). Phosphorylation by either one of these three protein kinases results in stimulation of the SR $Ca^{2+}$-ATPase activity and thus, increased rate of $Ca^{2+}$ uptake into the cardiac SR (1-3,5-12). The mechanism of stimulation involves increases in the affinity of the SR $Ca^{2+}$-ATPase for $Ca^{2+}$ without any change in the maximum velocity ($V_{max}$) (10-12), although in some studies an increase in $V_{max}$ was also observed (13,14). The stimulatory effects of phospholamban phosphorylation can be reversed by a SR-associated phosphatase activity (15,16). Thus, the $Ca^{2+}$-ATPase in cardiac SR appears to be under reversible regulation by phosphorylation/dephosphorylation of phospholamban (Fig. 1). Actually, reconstitution studies of the purified SR $Ca^{2+}$-ATPase and phospholamban in lipid bilayers demonstrated that dephosphorylated phospholamban is an inhibitor of the SR $Ca^{2+}$-pump activity and phosphorylation relieves this inhibition (17,18).

## CLONING OF PHOSPHOLAMBAN

Phospholamban cDNA has been cloned from several cardiac muscles, such as canine (19), human (20), rabbit (20), rat (21) and mouse (22); from smooth muscles, such as rat aorta (23), pig stomach (24) and chicken gizzard (25); and from rabbit slow-twitch skeletal muscle (26). The deduced amino acid sequences for phospholamban from these cDNA's are highly conserved across all species and muscle types and there are currently no isoforms of phospholamban known. Northern blot analysis of total RNA isolated from various species and muscle types revealed the presence of multiple mRNA's, which are not due to alternative splicing or start sites, but rather to multiple polyadenylation signal sequences located in the 3′ untranslated region of the phospholamban cDNA's (22,25-27). Southern blot analysis of genomic DNA has shown that phospholamban is a single copy gene (22,25). Recently, the phospholamban gene has been characterized from chicken (25), rabbit (20) and rat (21). This gene is similar in structure among the various species, composed of two exons separated by a single intron. The coding region is located in exon 2, while exon 1 and most of exon 2 represent the 5′ and 3′ untranslated regions, respectively.

## STRUCTURE OF PHOSPHOLAMBAN AND MECHANISMS OF REGULATORY EFFECTS

Phospholamban contains 52 amino acids and the protein has been proposed to consist of two motifs: a hydrophilic, cytoplasmic domain (Domain I, amino acid residues 1-30) and a hydrophobic, membrane spanning domain (Domain II, amino acid residues 31-52) (4,19, 28-31). Domain I contains the three phosphorylation sites for the three protein kinases. This domain can be further divided into two sub motifs: domain Ia (amino acid residues 1-20) which contains the phosphorylation sites, has a higher preponderance of charged amino acids and has been suggested to exist in a helical conformation (32); and domain Ib (amino acid residues 21-30) which is highly polar and proposed to be unstructured. Domain II, the hydrophobic domain, has also been proposed to have a helical structure, which may



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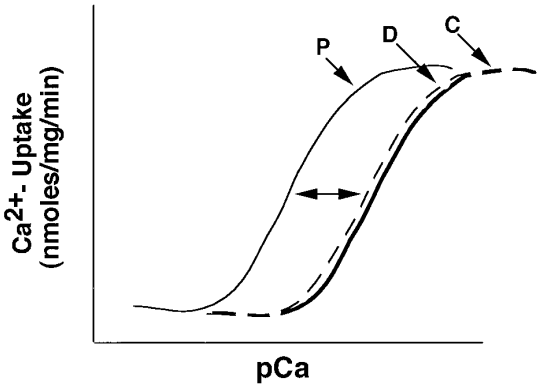


**FIG. 1.** Schematic representation of the effects of phospholamban on $Ca^{2+}$-transport in SR vesicles. Phosphorylation of phospholamban (P) is associated with increases in the affinity of SR $Ca^{2+}$-ATPase for $Ca^{2+}$ compared to nonphosphorylated or control SR vesicles (C). Dephosphorylation of phosphorylated phospholamban is associated with reversal of the stimulatory effects on the $Ca^{2+}$-affinity of the SR $Ca^{2+}$-ATPase (D).

be important in stabilizing the phospholamban pentameric association, detected in sodium dodecyl sulfate polyacrylamide gel electrophoresis (29-31,33-36)

Phospholamban has been proposed to exist as a pentamer in SR membranes (30,33,34,37,38). Evidence for pentameric assembly is based in part, on the migration pattern of phospholamban in sodium dodecyl sulfate polyacrylamide gels. Pentamer formation appears to be an intrinsic property of the protein, since expression studies in *Escherichia coli* showed that phospholamban formed pentamers even in this bacterial system (39). Low-angle laser light scattering techniques (37) and circular dichroism, transmission Fourier transform infrared and attenuated total reflection Fourier transform infrared spectroscopy (30) also indicate that phospholamban favors a pentameric assembly. Analysis of the immunolocalization of spin-labeled phospholipids by monomeric and pentameric phospholamban supports the evidence of pentameric phospholamban in membranes. Furthermore, mutagenesis has demonstrated that one face of the proposed helix in each phospholamban monomer is involved in pentamer formation (29-31,34-36,).

Dephosphorylated phospholamban is an inhibitor of the SR $Ca^{2+}$-ATPase activity and phosphorylation relieves this inhibition. Chemical crosslinking (40) presented the first evidence for protein-protein interaction between phospholamban domain I and a sequence upstream of the phosphorylation site in SR $Ca^{2+}$-ATPase. Site directed mutagenesis analyses (41,42) showed that specific residues among amino acids 2-18 in phospholamban interact with amino acids 336-412 in the SR $Ca^{2+}$-ATPase for functional modification (42). The direct interaction between phospholamban and the SR $Ca^{2+}$-ATPase (electrostatic) can be disrupted by high ionic strength (43), polyanions such as heparin (44), and neutralization of the highly basic charge (pI=11) upon phospholamban phosphorylation (45). Further evidence for such direct interaction was provided using time-resolved phosphorescence anisotropy, where dephosphorylated phospholamban was shown to mediate aggregation of the SR $Ca^{2+}$-ATPase molecules, resulting in a slow-down of the rate limiting step of the enzyme reaction cycle (46). While these cytoplasmic interactions are clearly involved in the regulatory effects of phospholamban, they do not appear sufficient to alter the apparent $Ca^{2+}$ affinity of the $Ca^{2+}$-ATPase (13). Coexpression studies of domain II with the SR $Ca^{2+}$-ATPase showed that this domain is capable of modifying the $Ca^{2+}$ affinity (47), suggesting that a regulatory circuit exists in the phospholamban : SR $Ca^{2+}$-ATPase inhibitor interaction, with cytoplasmic and transmembrane sites being connected through long range interactions.

Recently, Kimura et al (35), using alanine scanning mutagenesis of the transmembrane domain II in phospholamban, showed that mutations in one face of the transmembrane helix are associated with enhanced monomer formation and increased inhibitory function. Autry and Jones (48) also proposed that phospholamban monomers are more effective $Ca^{2+}$-pump inhibitors, based on their studies with the leucine 37 to alanine 37 phospholamban mutant. These findings suggest that the monomeric form of phospholamban is a more effective inhibitor than pentameric phospholamban. This model is further supported by electron paramagnetic spin resonance studies of phospholamban reconstituted in lipid bilayers, which indicated that an equilibrium exists between monomeric and pentameric states of phospholamban (Fig. 2) and that phosphorylation by protein kinase(s) shifts the equilibrium in favor of the pentameric form (38).

## REGULATORY EFFECTS OF PHOSPHOLAMBAN *IN VIVO*

In cardiac muscle, phospholamban has been shown to be phosphorylated *in vivo* during $\beta$-adrenergic stim-

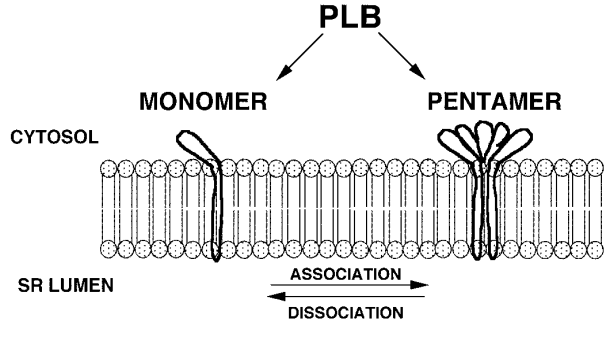


**FIG. 2.** A model depicting an equilibrium between the monomeric and pentameric states of phospholamban in SR membranes.

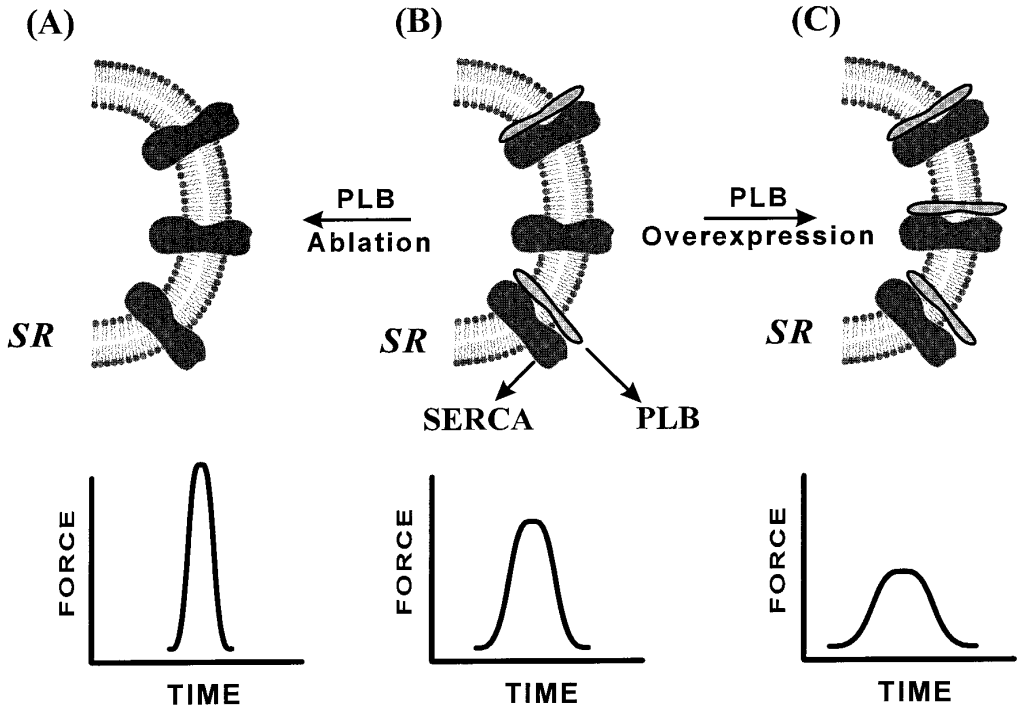


**FIG. 3.** Schematic representation of phospholamban (PLB) and SR $Ca^{2+}$-ATPase in native SR membranes from (A) phospholamban-deficient, (B) wild-type, and (C) phospholamban overexpression mice. Phospholamban ablation was associated with increased contractile parameters, while phospholamban overexpression was associated with decreased contractile parameters compared to wild-type mouse hearts.

ulation (49-52). Phosphorylation occurs on both serine 16, the cAMP-dependent protein kinase substrate site, and threonine 17, the $Ca^{2+}$-calmodulin-dependent protein kinase substrate site. Phosphorylation of phospholamban is associated with increases in the affinity of the SR $Ca^{2+}$-transport system for calcium and enhanced rates of myocardial relaxation. However, in addition to phospholamban, troponin I and C-protein in the myofibrils, and phospholemman in the sarcolemma are also phosphorylated during $\beta$-adrenergic stimulation. The relative contribution of these phosphoproteins to myocardial relaxation has recently been elucidated using genetically engineered mouse models with different levels of phospholamban expression (53-55). Reduction or ablation of phospholamban expression resulted in enhanced rates of relaxation and contraction in isolated cardiac myocytes, work performing hearts and whole animals (53,54,56,57). The enhanced rates of contraction and relaxation were associated with increases in the affinity of the SR $Ca^{2+}$-ATPase for $Ca^{2+}$ (Fig. 3). On the contrary, cardiac-specific overexpression of phospholamban led to depressed rates of contraction and relaxation in isolated cardiomyocytes, and this was associated with a decrease in the affinity of the SR $Ca^{2+}$-ATPase for $Ca^{2+}$ (Fig. 3). Interestingly, ablation of phospholamban was accompanied by attenuation of the $\beta$-adrenergic stimulatory effects, while the depressed contractile parameters in cardiomyocytes overexpressing phospholamban were alleviated by $\beta$-adrenergic stimulation. These results suggest that phospholamban is a repressor of basal myocardial contractility and is a key mediator of the effects of $\beta$-adrenergic agonists in the mammalian heart.

Besides cardiac muscle, phospholamban is also present in smooth and slow-twitch skeletal muscles. The physiological role of phospholamban in these muscles has been poorly defined, mainly due to the low expression levels of the protein in smooth and slow-twitch skeletal muscles. However, the phospholamban-deficient mouse offers an attractive model system to examine the functional significance of phospholamban in these muscles. In mouse aorta, phospholamban ablation was associated with increases in contraction rates, and decreases in sensitivity to both KCl and phenylephrine stimulation, compared to wild types (58). In mouse soleus muscle, phospholamban ablation was associated with significant increases in relaxation rates with no effects on contraction rates compared to wild types. Furthermore, isoproterenol stimulation had no effect on contraction rates of either wild type or phospholamban-deficient muscles, while it increased the rates of relaxation in wild type soleus to the basal levels observed in phospholamban-deficient muscles, which could not be further stimulated (59). These results suggest that phospholamban is a regulator of contractility in both smooth and slow-twitch skeletal muscles.

## SUMMARY

Phospholamban is a major regulator of the kinetics of cardiac contractility, through its ability to regulate the function of the cardiac SR $Ca^{2+}$-pump and thus the SR $Ca^{2+}$ load. *In vitro* expression studies have provided significant information on the structure/function of the phospholamban/$Ca^{2+}$-pump interaction. Furthermore, the generation of genetic animal models with altered phospholamban expression levels have permitted a through understanding of the physiological role of this regulatory phosphoprotein. Future studies aimed towards crystallization of phospholamban and the SR $Ca^{2+}$-ATPase in their native SR environment may provide clues to their tertiary and quaternary structures and may further elucidate the mechanisms underlying the phospholamban regulatory effects *in vivo*.

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