PHOSPHOLAMBAN: A CRUCIAL REGULATOR OF CARDIAC CONTRACTILITY

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Heart failure is a major cause of death and disability. Impairments in blood circulation that accompany heart failure can be traced, in part, to alterations in the activity of the sarcoplasmic reticulum Ca\(^{2+}\) pump that are induced by its interactions with phospholamban, a reversible inhibitor. If phospholamban becomes superinhibitory or chronically inhibitory, contractility is diminished, inducing dilated cardiomyopathy in mice and humans. In mice, phospholamban seems to encumber an otherwise healthy heart, but humans with a phospholamban-null genotype develop early-onset dilated cardiomyopathy.

Cardiac function is regulated on a beat-to-beat basis through the sympathetic nervous system. When the demand arises, the heart can respond to stress and increase blood flow to peripheral tissues within seconds. This is thanks to the large CARDIAC RESERVE in humans; the slow heart beat rate and submaximal contractility at rest are increased markedly after the release of adrenaline into the blood\(^1\). Adrenaline and other \(\beta\)-AGONISTS initiate an important signal-transduction pathway in the heart by binding to and activating \(\beta\)-ADRENERGIC RECEPTORS in the cell membrane (FIG. 1). The signal proceeds through G\(_s\) proteins to stimulate the formation of cyclic AMP by adenylyl cyclase\(^2\). Elevations in cAMP concentration activate cAMP-dependent protein kinase (PKA), which then phosphorylates and alters the function of a few cardiac proteins that have key effects on the overall cardiac function. Prominent among these proteins is phospholamban (PLN), a small, reversibly phosphorylated, transmembrane protein that is located in the cardiac SARCOPLASMIC RETICULUM (SR), which, depending on its phosphorylation state, binds to and regulates the activity of a Ca\(^{2+}\) pump, the SARCOENDOPLASMIC RETICULUM Ca\(^{2+}\)-ATPASE SERCA2a.

The trigger for cardiac contraction is the elevation of the Ca\(^{2+}\) concentration in the cytoplasm of the muscle cell (BOX 1), which is mediated by Ca\(^{2+}\)-release channels (RYANODINE RECEPTORS; RyRs) that tap the Ca\(^{2+}\) store in the lumen of the SR, or plasma-membrane Ca\(^{2+}\) channels (DIHYDROPYRIDINE RECEPTORS; DHPRs) that tap the high concentrations of Ca\(^{2+}\) in the extracellular space. The trigger for relaxation is the lowering of the cytosolic Ca\(^{2+}\) concentration by the combined activity of SERCA2a, PLASMA-MEMBRANE Ca\(^{2+}\)-ATPASES (PMCA) and Na\(^+\)/Ca\(^{2+}\) EXCHANGERS (NCXs) that replenish the SR and extracellular Ca\(^{2+}\) stores\(^1\). In humans, the activity of SERCA2a determines the rate of removal of \(>70\%\) of cytosolic Ca\(^{2+}\), thereby determining the rate of relaxation of the heart, and influences cardiac contractility by determining the size of the lumenal Ca\(^{2+}\) store that is available for release in the next beat. In its dephosphorylated state, PLN binds to SERCA2a at resting Ca\(^{2+}\) concentrations and inhibits Ca\(^{2+}\) pump activity; phosphorylation of PLN alters the PLN–SERCA2a interaction, relieving Ca\(^{2+}\)-pump inhibition and enhancing relaxation rates (INOTROPIC effects) and contractility (INOTROPIC effects)\(^4\).

In this review, we discuss advances in the understanding of the molecular aspects of the interaction between PLN and SERCA2a that have been derived from extensive mutagenesis and structural modelling studies\(^5\). We describe gain- and loss-of-inhibitory-function PLN mutants, which led to the investigation...
Beta-adrenergic stimulation

The ligand- or agonist-dependent activation of beta-adrenergic receptors and subsequent signalling events.

Sarcoplasmic reticulum (SR). An organellar membrane system that encases each myofibril within a muscle cell. Its essential components are a Ca2+-ATPase (pmCa pump), luminal Ca2+-sequestering proteins and a Ca2+-release channel.

Sarcoplasmic reticulum Ca2+-ATPase (SERCA). A pump that is located in sarcoplasmic or endoplasmic reticulum membranes that couples ATP hydrolysis to the transport of Ca2+ from cytosolic to luminal spaces.

Ryanodine receptor (RyR). A Ca2+-release channel that is located in the membrane of the sarcoplasmic and the endoplasmic reticulum that is regulated by protein–protein interactions with the dihydropyridine receptor and by a series of ligands, including Ca2+ itself.

Dihydropyridine receptor (DHPR). A slow, or L-type, voltage-dependent Ca2+-entry channel that is located in the plasma membrane, DHPRs require a membrane potential that is greater than ~30 mV for activation, and they are commonly found in neurons, neuroendocrine cells and muscle cells.

Plasma-membrane Ca2+-ATPase (PMCA). A plasma-membrane pump that couples ATP hydrolysis to the transport of Ca2+ from cytosolic to extracellular spaces.

Na+/Ca2+ exchanger (NCX). A plasma-membrane enzyme that exchanges three moles of Na+ for one mole of Ca2+ either inward or outward, depending on ionic gradients across the membrane.

Inotropic

Affecting cardiac relaxation.

Lusitropic

Affecting the force of cardiac contractions.

Cardiomyopathy

A disease of the heart muscle.

V_{max}
The maximal rate of enzymatic activity of mutations in the PLN gene as a potential cause of cardiomyopathy. We also discuss studies that were carried out in transgenic mice that overexpress superinhibitory forms of PLN and that have confirmed this potential. The creation and analysis of PLN-null mice of mice that overexpress PLN have enabled the elucidation of the remarkable role of PLN in the regulation of cardiac contractility. The fact that ablation of PLN or suppression of the inhibitory function of PLN can intervene to prevent the progression of dilated cardiomyopathy in well-characterized animal models opens the door to the investigation of the diverse pathways that lead to end-stage heart failure and to potential therapeutic interventions. Finally, we discuss very recent data, which show not only that mutations in PLN can cause dilated cardiomyopathy in humans, but also introduce new principles.

Figure 1 | Interactions between cardiac signalling pathways. The heart provides an example of how two signalling pathways that are involved in elevating the levels of two intracellular second messengers, cyclic AMP (cAMP) and Ca2+, can interact physiologically (the two pathways are shown by the blue and red arrows, respectively). In response to depolarization, Ca2+ enters the cytoplasm through Ca2+ channels in the plasma membrane (dihydropyridine receptors; DHPRs). This ‘trigger’ Ca2+ then binds to the Ca2+-release channels (ryanodine receptors; RyRs) to stimulate Ca2+ release from the sarcoplasmic reticulum (SR). After activating muscle contraction by binding to troponin C in the thin filament, Ca2+ is removed from the myoplasm by plasma-membrane Ca2+-ATPases (PMCA2a) or Na+/Ca2+ exchangers (NCX2), which are located in the plasma membrane, or by the sarco(endoplasmic reticulum Ca2+-ATPase SERCA2a, which is located in the SR. As SERCA2a activity accounts for the removal of >70% of myoplasmic Ca2+ in humans, it determines both the rate of Ca2+ removal (and, consequently, the rate of cardiac muscle relaxation) and the size of the Ca2+ store (which affects cardiac contractility in the subsequent beat). SERCA2a activity is regulated by its interaction with phospholamban (PLN), which is a target for phosphorylation by protein kinase A (PKA) through the second signalling pathway — the beta-adrenergic-receptor pathway (see main text). In its dephosphorylated form, PLN is an inhibitor of SERCA2a, but, when phosphorylated by PKA (or Ca2+/CaM kinase), PLN dissociates from SERCA2a, activating this Ca2+ pump. As a result, the rate of cardiac relaxation is increased and, on subsequent beats, contractility is increased in proportion to the elevation in the size of the SR Ca2+ store and the resulting increase in Ca2+ release from the SR. PLN is dephosphorylated by a protein phosphatase (PP1), which terminates the stimulation phase. AKAP, A-kinase anchoring protein; [Ca2+]i, Ca2+ concentration; RgI, regulatory binding subunit A.
REVIEWS

During the evolution of phosphate- and organic-acid-based metabolic processes, it became important that cytosolic Ca²⁺ concentrations be maintained below ~10 µM; and so the need for Ca²⁺ pumps that lower resting Ca²⁺ concentrations to <1 nM and establish a 10,000-fold gradient across the cell membrane. The gradient provides ideal conditions for the use of Ca²⁺ as a cellular signalling molecule. Brief openings of plasma-membrane or organelar Ca²⁺ channels create rapid elevation of cytosolic Ca²⁺ concentrations to 1–10 µM, a range in which Ca²⁺ binds to and activates specific proteins (the ‘on’ mechanism). Ca²⁺ pumps are among the first proteins activated to eject Ca²⁺ (the ‘off’ mechanism), ensuring that the elevation of Ca²⁺ concentration will be transient. For cardiac and skeletal muscle, the key Ca²⁺-binding protein is troponin C and the crucial event is a Ca²⁺-triggered conformational change in troponin C, which removes a steric block to the interaction between actin in thin filaments and myosin in thick filaments, allowing contraction. The steric block is reinstated when myoplasmic Ca²⁺ levels are reduced by the action of Ca²⁺ pumps, so that Ca²⁺ dissociates from troponin C.

As transient elevations of Ca²⁺ have such an important signalling role in muscle contraction, it is not surprising that Ca²⁺ dysregulation is important in human muscle disease. Mutations in Ca²⁺ release channels (RYR1) cause malignant hyperthermia, a toxic response to anaesthetics, and central core disease, which is a muscle myopathy. Mutations in the cardiac Ca²⁺-release channel (RYR2) cause arrhythmias and sudden death. Mutations in SERCA1a (ATP2A1), the ‘housekeeping’ isoform of the Ca²⁺ pump, induce separation of keratinized and non-keratinized layers of the skin, leading to Darier disease.

Mutations in the voltage-dependent, L-type Ca²⁺ channel (CACNAIS), which interacts with RyR1, also cause malignant hyperthermia. Mutations in the cardiac Ca²⁺-release channel (RYR2) cause arrhythmias and sudden death. Mutations in SERCA1a (ATP2A1) and SERCA2a are also important in human muscle disease, with mutations in SERCA2a causing malignant hyperthermia, central core disease, cardiomyopathy (see main text for more details), and mutations in SERCA2b (ATP2A2), the ‘housekeeping’ isoform of the Ca²⁺ pump, induce separation of keratinized and non-keratinized layers of the skin, leading to Darier disease.

In early studies, the addition of cAMP and PKA increased both the rate and the extent of Ca²⁺ uptake in cardiac SR vesicles. This stimulatory effect was mediated by the phosphorylation of a 22-kDa protein, PLN, which colocalized with SERCA2a in the cardiac SR membrane. Kinetic analysis showed that the principal effect of the association of dephosphorylated PLN with SERCA2a is to diminish the apparent affinity of SERCA2a for Ca²⁺, with little or no effect on Vₘₜₗₐ at saturating Ca²⁺ and ATP concentrations. Equilibrium measurements of Ca²⁺ binding to the ATPase, in the absence of ATP, are unchanged by PLN. This might indicate that the binding of Ca²⁺ and PLN to SERCA is not competitive, so that PLN can bind to the Ca²⁺-bound form of SERCA. However, this seems unlikely in light of the structural considerations that are described below. In fact, the effect of PLN is to reduce the bidirectional rate constants of a rate-limiting, Ca²⁺-dependent conformational change. Owing to the second-order kinetics of that step, the effect of PLN is reflected as a replacement of the Ca²⁺ concentration curve that is required for ATPase activation.

In the physiologically relevant Ca²⁺ concentration range of 100 nM to 1 µM, decreased Ca²⁺ affinity diminishes SERCA2a activity. However, SERCA2a activity is increased two- to threefold by phosphorylation of PLN by either PKA or Ca²⁺/calmodulin-dependent protein kinase (Ca²⁺/CaM kinase). In vivo, PLN is phosphorylated by both cAMP-dependent and Ca²⁺/CaM-dependent protein kinases during β-adrenergic stimulation. The relief of the inhibitory effects of PLN on SERCA2a is the principal contributor to the positive inotropic and lusitropic effects of β agonists.

The cloning of PLN revealed that it is a 52-amino-acid protein of 6.1 kDa that forms a homopentamer, which accounts for the original observation that phosphorylated PLN has an apparent mass of 22 kDa. From analysis of the amino-acid sequence, it was suggested that the protein is organized in three domains: cytosolic domain Ia (amino acids 1–20) contains serine (Ser) 16, the site of phosphorylation by PKA, and threonine (Thr) 17, the site of phosphorylation by Ca²⁺/CaM kinase; cytosolic domain Ib (amino acids 21–30) is rich in amidated amino acids; and domain II (amino acids 31–52), which traverses the membrane (Box 2). A homologue of PLN is sarcolipin (SLN). Sequences are not conserved in the amino and carboxyl termini of the two proteins, but their transmembrane sequences are well conserved (Box 2), which indicates the importance of the transmembrane domain as an inhibitory site. NMR structures show that PLN in chloroform/methanol is composed of two helices, spanning amino acids 4–16 and 21–49, that are connected by a short β-turn, whereas sarcolipin has a single transmembrane helix.

James et al. provided the first clear evidence for a physical interaction between PLN and SERCA2a by crosslinking lysine (Lys) 3 in PLN domain Ia to Lys397 and Lys400 in the cytosolic nucleotide-binding domain of SERCA2a. The interaction was disrupted by an elevated Ca²⁺ concentration or by phosphorylation of PLN. In the cycle of Ca²⁺ transport that is catalysed by SERCA2a (Box 3), several interconvertible phosphorylated and unphosphorylated conformations of SERCA2a have been defined: Ca²⁺ and ATP bind independently, forming E₁P·(Ca²⁺)2, the high-energy phosphoenzyme conformation, is formed in the phosphorylation (occlusion) stage; E₁P·(Ca²⁺), the low-energy phosphoenzyme, is formed during the stage of Ca²⁺ release to the lumen; and E₂P is formed after hydrolysis of the acylphosphate and release of inorganic phosphate. PLN asserts its inhibitory effect by binding to SERCA2a in its E₁P·(Ca²⁺) conformation (Fig. 2) and prolonging the time that SERCA2a spends in this conformation. Either the phosphorylation of PLN or the conformational changes in SERCA2a that accompany Ca²⁺ binding and progression from the E₁P·(Ca²⁺) state to the E₂P·(Ca²⁺) state dissociate the inhibited PLN–SERCA2a heterodimer (Fig. 3).
measure the effects of mutations in either SERCA or PLN molecules on Ca\(^{2+}\) affinity (functional interaction), whereas co-immunoprecipitation of PLN and SERCA2a as a complex has been used as a measure of the effects of mutations on physical interactions. The loss of functional or physical interaction has identified potential interacting amino acids. Such studies confirmed that there is a cytosolic interaction site in SERCA2a between amino acids Lys397 and valine (Val) 402 (REF 41; FIG. 2), and showed that both hydrophilic and hydrophobic amino acids in PLN domain Ia can alter its functional interactions with SERCA2a (REF 42).

The inhibitory function of PLN was retained in PLN molecules in which the cytosolic domains were largely deleted or substituted nonspecifically, showing that PLN–SERCA2a transmembrane interactions have a key inhibitory role41. The first scanning mutagenesis of the PLN transmembrane sequence was carried out to investigate the interesting phenomenon that PLN forms a homopentamer that is resistant to depolymerization by the detergent sodium dodecyl sulphate (SDS) at ambient temperature44,45. So, in SDS–PAGE, PLN is observed to be 80–90% pentameric and 10–20% monomeric. Mutagenesis revealed that the transmembrane domain was helical, as predicted, and that the homopentamer structure was stabilized by hydrophobic interactions involving a series of leucine (Leu) or isoleucine (Ile) residues that are located along one side, or ‘face’, of each transmembrane helix44,45.

When scanning mutagenesis was used to analyse the inhibitory function of PLN, two helical faces could be distinguished47. Mutation of one face of the helix had little effect on pentamer formation, but diminished the ability of PLN to interact with and inhibit SERCA. Mutation of the other face disrupted the ability of PLN to interact and inhibit SERCA. Two helical faces could be distinguished47 and on the modelling of PLN in association with sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase SERCA1a, and the folding of sarcoplasm (SLN) is based on an NMR structure109. The homology of the two proteins is most clearly seen in their transmembrane helices (see figure).

SLN has emerged as an interesting homologue of PLN108. In humans, SLN expression is high in fast-twitch skeletal muscle and low in the heart; in rat and mouse hearts, SLN expression is atrial specific and closer to the levels seen in skeletal muscle. Overexpression of SLN in soleus muscle, like overexpression of PLN in the heart, impairs contraction, with powerful effects on the \(V_{\text{max}}\) of Ca\(^{2+}\) uptake111. In heterologous cell culture, SLN co-expressed with PLN becomes superinhibitory112. SLN has a higher affinity for PLN than PLN itself, so that it can depolymerize PLN pentamers. At equal concentrations and at equilibrium, the predominant species seems to be a PLN–SLN heterodimer. A ternary PLN–SLN–SERCA complex can be immunoprecipitated by antibodies against either PLN or SLN, indicating that the ternary complex, rather than any of the binary complexes, is the most stable complex when all three proteins are expressed together. Modelling shows that SLN can fit into the PLN-binding groove in the E2 conformation of SERCA1a (FIG. 3; REF 113): modelling of the ternary complex shows that the PLN–SLN heterodimer can also fit into this groove. The tighter fit creates a higher affinity, whereas the unique carboxy-terminal sequence of SLN adds to the stability by binding to aromatic residues in the loop connecting the M1 and M2 transmembrane helices of SERCA1a. Although a PLN–SLN complex fits snugly into this site, a PLN–PLN complex would not. These studies hint at another level of regulation of SERCA2a by a PLN–SLN complex. It will be of great interest to watch this story unfold.

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<thead>
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<td>P-serine; P-threonine</td>
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**Box 2 | Sarcolipin is a phospholamban homologue**

The folding of phospholamban (PLN) is based on an NMR structure109 and on the modelling of PLN in association with sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase SERCA1a, and the folding of sarcoplasm (SLN) is based on an NMR structure109. The homology of the two proteins is most clearly seen in their transmembrane helices (see figure).

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**FasT-Twitch Skeletal Muscle**

A rapidly contracting and relaxing muscle, such as the extensor digitorum longus, which is primarily involved in bodily movement.
The discovery of superinhibitory forms of PLN indicates that these residues were crucial for the interaction with PLN. Transmembrane helices M4 and M6 also contribute strongly to Ca\textsuperscript{2+} binding\textsuperscript{49}\textsuperscript{50}, but the faces that are involved in Ca\textsuperscript{2+} binding are different from those involved in forming interactions with PLN. The formation of direct disulphide crosslinks by the PLN/SERCA\textsubscript{1a} double mutants Asn27Cys/Leu321Cys and Val49Cys/Val89Cys (where Cys is cysteine) established two points in the transmembrane helices of PLN and SERCA\textsubscript{1a} at which amino acids were directly apposed and likely to interact. These amino acids provided membrane-surface reference points from which the structural modelling of the PLN–SERCA\textsubscript{1a} complex could proceed\textsuperscript{5}. In the model (FIG. 2), PLN fits into a groove (formed by M2, M4, M6 and M9 helices) on the lipid-facing surface of SERCA\textsubscript{1a} in the E\textsubscript{1} conformation. When SERCA\textsubscript{1a} moves to the E\textsubscript{2},Ca\textsuperscript{2+} conformation\textsuperscript{3,5,6,10}, the groove becomes much narrower because of a large movement of helix M2, forcing PLN out of this inhibitory site.

The PLN transmembrane helix must unwind near the carboxy-terminal end of domain Ib to avoid collision with the extension of M4 in SERCA\textsubscript{1a}. In this model, unwinding extends the length of the cytosolic domain of PLN, so that it can fit into another groove in the cytosolic domain of SERCA\textsubscript{1a}, accounting for hydrophobic interactions between the helix of PLN domain Ib and SERCA\textsubscript{1a}. This groove, a salt bridge forms between glutamic acid (Glu) 2 in PLN and Lys397 in SERCA\textsubscript{1a}.

Although there is no clear evidence, it seems likely that the sites of phosphorylation in PLN domain la fit into the active sites of PKA or Ca\textsuperscript{2+}/CaM kinase when PLN is bound to SERCA, as phosphorylation disrupts the inhibitory PLN–SERCA interaction. In the model (FIG. 2), there is a space between SERCA\textsubscript{1a} and much of domain Ib and part of domain la where kinases could fit. The crystal structure of PKA bound to a 20-amino-acid inhibitory peptide\textsuperscript{62} is informative as to how PLN domain la must interact with PKA. By analogy, the domain la helix would have to unwind between Thr8 and Glu19 to fit into the active site of PKA.

An alternative model of PLN–SERCA interactions\textsuperscript{62} is based on the premise that domain Ib and domain II of PLN form a continuous \(\alpha\)-helix, as they do in an NMR structure\textsuperscript{63}. This model is not very satisfactory because the distance constraints that are introduced by adherence to the NMR structure left the hydrophobic portion of PLN suspended partially in and partially out of the membrane, in contrast to the seamless boundaries between water-accessible and water-inaccessible domains that characterize the model of Toyoshima et al.\textsuperscript{3}.

The structural view of PLN splayed across one side of SERCA\textsubscript{1a}, tugging transmembrane helices together and extending into the cytosol to immobilize the nucleotide-binding domain of SERCA, provides a visual illustration of the mechanism by which PLN inhibits SERCA in its E\textsubscript{1} conformation. As the top of M2 winds...
Box 3 | \textbf{Ca}^{2+} \text{ transport by Ca}^{2+}\text{-ATPases}

The Ca\(^2+\) ATPase family has two principal branches: one group of Ca\(^{2+}\) pumps is located in the sarcoplasmic reticulum (SERCAs), and the other in the plasma membrane (PMCs). Biochemical analyses\(^{60,114,115}\) and crystal structures of SERCA1a in two different conformations\(^{36,35}\) serve as a model for the structure–function relationships that drive Ca\(^{2+}\) transport in all family members. These proteins have cytoplasmic nucleotide-binding (N), phosphorylation (P) and actuator (A) domains, a stalk domain and ten transmembrane helices, of which four form the Ca\(^{2+}\)-binding sites (FIG. 2). Massive conformational changes are powered overall by ATP hydrolysis, but are induced as discrete steps by Ca\(^{2+}\) binding (see figure, part a), phosphoenzymes formation (see figure, part b), Ca\(^{2+}\) release (see figure, part c) and dephosphorylation (see figure, part d). In the \(E_2\) conformation of SERCA1a (FIG. 2), Ca\(^{2+}\) has access to the centre of the membrane, but the binding sites are not yet formed and there is no pathway for Ca\(^{2+}\) entry into the lumen. In this conformation, the cytosolic domains are clustered together. In the \(E_2\, Ca^{2+}\) conformation (FIG. 2), the cytosolic domains are dispersed, but the transmembrane domain is ordered so that two Ca\(^{2+}\) ions are bound side-by-side in sites that are formed by the precise juxtaposition of four transmembrane helices. The cytosolic access pathway is now closed, but the outline of a luminal access pathway emerges. So, Ca\(^{2+}\) transport, energized by the formation of a phosphoenzymes from ATP, involves: first, access of cytosolic Ca\(^{2+}\) to potential Ca\(^{2+}\)-binding sites in the transmembrane domain, while access to the lumen is prevented; second, formation of high-affinity binding sites under conditions in which access to the lumen is prevented; and third, disruption of the high-affinity binding sites under conditions in which access to the lumen is gained, while access from the cytosol is prevented.

As Ca\(^{2+}\) transport requires such extensive movements of both cytosolic and transmembrane domains, it is easy to understand how phospholamban (PLN) can inhibit Ca\(^{2+}\) transport. In the transmembrane domains, PLN binds to amino acids in both transmembrane helices M4 and M6, which are key helices in Ca\(^{2+}\) binding. If they cannot move, they cannot bind Ca\(^{2+}\). In addition, cytosolic domains of PLN bind to the SERCA nucleotide-binding domain, presumably blocking its ability to participate in the clustering and dispersion of the SERCA cytosolic domains, which power conformational changes in the transmembrane domain.

### Role of PLN in basal cardiac function

**Targeting of PLN.** Much of our recent insight into the role of PLN in the regulation of cardiac function has been established through targeting the PLN gene in embryonic stem cells and generating heterozygous and homozygous PLN-targeted mice, in which PLN protein levels are reduced by 60% and 100%, respectively\(^{30,34}\). The reduction in PLN levels is associated with a linear increase in the affinity of SERCA2a for Ca\(^{2+}\), and with a linear increase in such measures of contractility as the extent of myocyte cell shortening, and the rates of myocyte contraction and lengthening\(^{34}\) (FIG. 4). Changes in cardiac myocyte contractility reflect increases in the amplitude and rates of the rising and falling phases of the Ca\(^{2+}\) transient, which, in turn, reflect the fact that the size of the Ca\(^{2+}\) store is increased in PLN-null animals\(^{36,38}\). This is an especially important indication that the size of the SR Ca\(^{2+}\) store is a principal determinant of cardiac contractility. A linear correlation was also observed between PLN levels and the rates of contraction and relaxation in isolated heart preparations\(^{36}\) or intact mice\(^{37}\). The enhanced basal contractile parameters in PLN-null hearts could be stimulated only minimally by \(\beta\)-agonists, such as isoproterenol, which would normally stimulate phosphorylation of PLN, relieving its inhibitory effects on SERCA2a. This was because contraction and relaxation rates in PLN-null hearts were already very close to the maximally stimulated contraction and relaxation rates of wild-type hearts\(^{36,38}\). The attenuated responses are believed to be induced through the loss of PLN, as they could not be ascribed to alterations in other potential players in the \(\beta\)-agonist pathway\(^{36}\).

PLN ablation and the resultant hyperdynamic cardiac function were not associated with compensatory alterations in the levels of contractile proteins or of other Ca\(^{2+}\)-cycling proteins, with the exception of the Ca\(^{2+}\)-release channel (RyR). RyR levels were decreased by 25% — a compensatory mechanism which could dampen an excessive increase in Ca\(^{2+}\) release from the SR that would be expected to result from an increase in the size of the SR Ca\(^{2+}\) store\(^{36,39}\). A second compensatory mechanism was an increase in the inactivation kinetics of the L-type Ca\(^{2+}\)-channel current of the DHPR, which supplies Ca\(^{2+}\) from the extracellular space during each heart beat\(^{36}\). This would decrease Ca\(^{2+}\) entry from extracellular spaces together with the increased Ca\(^{2+}\) cycling through the SR in PLN-null cardiac myocytes. Finally, metabolic adaptations established a new energetic steady state\(^{41}\), such that the increased ATP demands in the hyperdynamic PLN-null hearts could be met.

PLN ablation did not diminish cardiac performance during maximal cardiovascular stress that was provided by graded treadmill exercise\(^{36}\). The hyperdynamic cardiac function of the PLN-null hearts persisted throughout the ageing process, without any alterations in cardiac cell shape, and PLN ablation did not shorten life span\(^{36}\). Furthermore, the incidence of heart failure was similar between PLN-null and wild-type animals in which blood flow to the heart was reduced by constricting or ‘banding’ the transverse section of the aorta\(^{46}\). However, PLN ablation increased cardiac susceptibility to ischaemic injury, with females being less susceptible than males; this protection seemed to be mediated by the ability of females to produce more nitric oxide\(^{46}\), which is a potent vasodilator.

Collectively, these studies of PLN-targeted animals indicate that PLN is a crucial regulator of basal cardiac Ca\(^{2+}\) cycling and contractile parameters, and that PLN is an important determinant of \(\beta\)-adrenergic-agonist stimulatory responses in vivo (FIG. 4).
The role of PLN in smooth and skeletal muscles. PLN is also expressed at very low levels in smooth muscle and SLOW-TWITCH SKELETAL MUSCLE. In the PLN-deficient tonic aorta and bladder, isometric force, which was already high, was less responsive to added agonists, compared with wild-type muscle. PLN is also present in vascular endothelium and it seems to modulate the endothelium-dependent relaxation of the aorta. In SOLEUS muscles, ablation of PLN was associated with significant increases in relaxation rates, without any effects on contraction rates. So, PLN is a regulator of contractility in smooth and slow-twitch skeletal muscles.

Overexpression of PLN. In contrast to PLN-null mice, transgenic mice that express twofold and fourfold higher levels of PLN in the heart, compared with their wild-type littermates, showed decreased shortening fractions and rates of contraction and relaxation in isolated, unloaded cardiac myocytes (FIG. 4). These changes were reflected in decreases in the amplitude of the Ca²⁺ signal and a prolonged time for the decay of the Ca²⁺ transient. These inhibitory properties were abolished by stimulation with the β-agonist, isoproterenol, which stimulated phosphorylation of PLN, relieving its inhibitory effects on SERCA2a. Mice with twofold PLN overexpression did not show any phenotypic alterations throughout the ageing process, but, as a compensatory adaptation, mice with fourfold PLN overexpression had elevated levels of adrenaline and noradrenaline, which would enhance the phosphorylation of PLN to relieve its inhibitory effects on cardiac function. This compensatory response became maladaptive in the long term, as adrenaline induced changes in transcription, which led to cardiac remodelling and progression to heart failure and early mortality. Early mortality was gender dependent and seemed to be regulated by the levels of p38 and the MKP-1 phosphatase.

An important question in evaluating PLN function is the functional stoichiometry between PLN and SERCA2a. The measured PLN:SERCA2a molar ratio in canine hearts lies between 2.1 and 2.5:1 (REF. 72). As there are probably varying ratios of active monomeric and inactive pentameric forms of PLN, the question of functional stoichiometry can never be answered simply. One approach to this has been to ask whether the magnitude of inhibition of contractility could be correlated with PLN levels across the range of PLN-null and PLN-overexpressing animals, but no clear answer could be obtained because of elevated catecholamine levels, which attenuated the PLN inhibitory effects. However, transgenic mice that overexpress Ser16Ala (where Ala is alanine) or Thr17Ala mutant forms of PLN, which could not be phosphorylated, provided a better model. The apparent affinity of SERCA2a for Ca²⁺ was maximally inhibited in this model when PLN expression levels were increased 2.6 fold over wild-type PLN levels, indicating that approximately 40% of the SR Ca²⁺ pumps are functionally regulated by PLN under normal conditions in mouse hearts. On the basis that PLN normally exceeds SERCA2a by 2.5 fold and that only 40% of SERCA2a is normally inhibited, for every PLN monomer that interacts with SERCA2a about 5 monomers do not. These monomers are likely to form into a homopentamer.

The phenotypes of mice that express no PLN, normal and twofold normal PLN levels show that several physiological responses change in a linear fashion over a wide range of PLN expression levels, as outlined above. This has provided a strong experimental basis for our present insight into the physiological role of PLN. PLN is a key component at the intersection of two signal-transduction pathways in the heart: the β-adrenergic signalling pathway leading to protein phosphorylation and the Ca²⁺ signalling pathway leading to muscle contraction. In its phosphorylated form, PLN steps out of its role as an inhibitor of the Ca²⁺ pump and allows the use of the cardiac reserve, as defined at the beginning of this review. In this way, PLN is a principal regulator of the kinetics of cardiac contractility.

Superinhibitory PLN mutants. The question of whether superinhibitory mutations in PLN might impair cardiac function was tested in a series of transgenic mice. Overexpression of the superinhibitory PLN mutants...
PLN ablation has stimulatory effects on Ca\textsuperscript{2+} transport that are similar to PLN phosphorylation. Although not shown in this figure, PLN is present in both monomeric and pentameric forms, resulting in higher inhibition. PLN is also less phosphorylated in heart failure, becoming more inhibitory overall. This was reflected by a decrease in fractional shortening of cardiac myocytes and in their rates of shortening and relengthening.

The depression of myocyte Ca\textsuperscript{2+} kinetics and mechanics, observed with the monomeric Leu37Ala and Ile40Ala PLN mutants, was reversed by isoproterenol, in line with the view that superinhibition with these mutants is mainly due to mass action resulting from increases in the concentration of the active PLN monomer. This hypothesis is supported by the observation that several-fold overexpression of wild-type PLN, presumably elevating the basal level of monomeric PLN, can also lead to superinhibition of SERCA2a activity\textsuperscript{11,70}. Periodic reversal of the superinhibitory effects of the Leu37Ala and Ile40Ala mutants by the elevation of endogenous \( \beta \)-agonists probably allows the mice to survive. Although life span did not seem to be shortened for these animals, significant left-ventricular hypertrophy was detected by echocardiography, and female mice often died with greatly enlarged hearts during the delivery of their second or third litters.

At present, Asn27Ala is the strongest superinhibitory mutant yet noted in heterologous cell culture\textsuperscript{79}. However, neither Asn27Ala nor another superinhibitory mutant, Val49Gly, alter the monomer:pentamer ratio. These observations indicate that their efficacy must be due to an enhanced affinity for SERCA and this is confirmed by structural modeling studies\textsuperscript{79}. The interactions between Asn27 in PLN and Leu321 in SERCA1a and between Val49 in PLN and Val89 in SERCA1a have been shown to be very close, and mutation of any of these residues has significant effects on the PLN–SERCA affinity\textsuperscript{79}. In vivo, both Asn27Ala and Val49Gly mutants inhibited cardiac contractile parameters to a greater extent than similar levels of wild-type PLN. Furthermore, the impaired functions could not be reversed fully with the experimental application of isoproterenol. So, PLN becomes a chronic inhibitor and SERCA2a no longer has the potential to be fully functional. Mice that overexpress the Asn27Ala mutation on the PLN-null background survive for less than one year and die of dilated cardiomyopathy\textsuperscript{44}, whereas transgenic males that overexpress Val49Gly die of dilated cardiomyopathy when they are six months old\textsuperscript{44}.

Analysis of the small group of PLN mutants described above indicates that PLN can be viewed as an unnecessary encumbrance to a healthy functional heart, as mice live happily without it. It becomes a problem, however, if it is overexpressed or mutated to a superinhibitory form. If the inhibitory function of PLN can be reversed by endogenous \( \beta \)-agonists, life is not threatened, but dilated cardiomyopathy occurs if the mutation is highly inhibitory and is not reversible by endogenous \( \beta \)-agonists.

**Physiological role of PLN in \( \beta \)-adrenergic stimulation.** The functional significance and interplay of dual-site phosphorylation of PLN at Ser16 and Thr17 was determined through the generation and characterization of transgenic mice that express phosphorylation-site-specific PLN mutants in the null background. The Ser16Ala mutant hearts showed a depressed response to isoproterenol and a lack of Thr17 phosphorylation. The Thr17Ala mutant hearts showed Ser16 phosphorylation and a response to isoproterenol that was similar to that observed with wild-type hearts\textsuperscript{79,76}. These findings indicated, first, that Ser16 phosphorylation might be a prerequisite for Thr17 phosphorylation; and, second, that Ser16 phosphorylation might be sufficient to mediate the contractile responses of the heart to \( \beta \)-agonist stimulation. However, Thr17 phosphorylation has been shown to be independent of Ser16 phosphorylation in the myocardium of the transgenic mice, indicating that this site is not necessary for the \( \beta \)-agonist response.
Phospholamban in heart failure

The abnormal Ca\(^{2+}\) cycling in animal models of heart failure and human failing hearts has been suggested to reflect, at least in part, impaired SR Ca\(^{2+}\) uptake that results in diminished SR Ca\(^{2+}\) stores\(^{79-81}\). Most findings now indicate that the levels of PLN protein remain unchanged, whereas the levels of SERCA2a protein decrease in human heart failure\(^{1,2,3}\). A decrease in the level of SERCA relative to PLN would be expected to lead to an increased functional stoichiometry of PLN to SERCA, increased inhibition of the Ca\(^{2+}\) affinity of SERCA2a and prolonged relaxation time. In addition, the phosphorylation status of PLN at Ser16 and Thr17 is decreased\(^{71,86}\), indicating that there is an increased inhibitory function by PLN. Indeed, SR Ca\(^{2+}\) uptake in donor and failing hearts indicates that there are decreases in both V\(_{\text{max}}\) and Ca\(^{2+}\) affinity\(^{71}\). So, alterations in the PLN:SERCA2a ratio and the degree of PLN phosphorylation might contribute to depressed SR Ca\(^{2+}\) uptake, leading to increased diastolic Ca\(^{2+}\) levels. Consequently, the SR Ca\(^{2+}\) store decreases and less Ca\(^{2+}\) is available for subsequent contractions (FIG. 4).

The strategy of altering SERCA2a and/or PLN levels or activity to restore perturbed Ca\(^{2+}\) uptake into the SR might hold therapeutic promise in the treatment of heart failure. Potential therapeutic strategies are to increase SERCA2a levels or to attenuate the PLN inhibitory effect on SERCA2a. Overexpression of SERCA2a, using recombinant adenovirus-mediated gene transfer, led to enhanced contractility in isolated myocardium\(^{87,88}\) and improved cardiac function, metabolism and survival in a rat model of heart failure\(^{89,90}\). Transgenic overexpression of SERCA2a was also associated with increases in both SR Ca\(^{2+}\) uptake and contractility\(^{91-95}\). These studies support the idea that an enhanced expression of SERCA2a restores disturbed intracellular Ca\(^{2+}\) handling by decreasing the relative ratio of PLN to SERCA2a.

Other potential approaches to influence the PLN–SERCA2a complex might use techniques such as expression of PLN antisense RNA or expression of non-functional PLN mutants to decrease PLN levels or activity. Studies of decreased PLN expression, carried out using recombinant adenoviral-mediated gene transfer in isolated cardiac myocytes\(^{96,97}\) and improved cardiac function, metabolism and survival in a rat model of heart failure\(^{89,90}\). Transgenic overexpression of SERCA2a was also associated with increases in both SR Ca\(^{2+}\) uptake and contractility\(^{91-95}\). These studies support the idea that an enhanced expression of SERCA2a restores disturbed intracellular Ca\(^{2+}\) handling by decreasing the relative ratio of PLN to SERCA2a.

Human PLN mutations and cardiomyopathy

Dilated cardiomyopathy segregates in some human families. Recent searches in some of these families have identified causal mutations in the PLN gene. The inheritance of the PLN mutation encoding Arg9Cys was linked to the dominant inheritance of dilated cardiomyopathy in a large American family with a probability of 10,000 to 1 that the mutation was causal of the disease (LOD score >4 at Θ=0.00)\(^{96}\).

The effects of the PLN Arg9Cys mutation were characterized by expression in heterologous cell culture, by the creation of a transgenic mouse and by analysis of cardiac tissue obtained from an explant. In all cases, the level of PLN phosphorylation was reduced markedly. In heterologous cell culture, the Arg9Cys mutant PLN had reduced inhibitory properties in the homozygous state, but, when expressed in the heterozygous state, did not function in a dominant fashion to prevent the inhibition of SERCA2a by wild-type PLN. The key effect of the mutation was enhancement of the affinity of Arg9Cys mutant PLN for PKA. In attempting to phosphorylate mutant PLN, PKA becomes trapped in a stabilized mutant PLN–PKA complex and can no longer dissociate to phosphorylate wild-type PLN molecules. The effect seems to be local and restricted to the SR, perhaps because a specific fraction of PKA is associated with...
So, how can this discrepancy between the cardiac phenotypes in mice and humans be explained? In contrast to humans, mice have relatively little cardiac reserve and differ in the balance of myocyte Ca\textsuperscript{2+} fluxes.

**Conclusion**

PLN is an important regulator of the kinetics of cardiac Ca\textsuperscript{2+} transients and of contractility, and is a key determinant of \(\beta\)-adrenergic stimulation of the heart. Significant advances have been made in understanding the structure and dynamics of the interaction of PLN with SERCA2a. And the generation of mouse models with altered PLN expression levels or activity has allowed a thorough understanding of the ‘physiological brake’ that is provided by PLN in vivo. Although the mouse and human myocardia differ in excitation–contraction coupling and in the isoforms of contractile proteins that they express in different developmental stages, studies of mouse form the basis for comparative studies in humans. They also form the foundation for potential therapeutic approaches in humans through disruption of PLN inhibition of SERCA2a.

Depressed SR Ca\textsuperscript{2+} cycling is a common feature of depressed contractility that is seen in hypertrophied and failing myocardia, so that interference...
with the PLN–SERCA2a interaction through a molecule ‘mole’ trap might rescue contracture dysfunction and prevent transcription-based remodelling of the heart, thereby preventing progression to heart failure. It is evident, however, that elimination of PLN activity might not benefit all forms of heart failure, especially those in which depressed SR Ca²⁺ cycling is not a primary event.

Further structural studies of the PLN–SERCA2a complex might provide further clues as to the mechanisms that underlie PLN regulation and provide new insights into ways to design drugs that would disrupt this interaction selectively. The characterization of other deleterious PLN mutants in the human population will further elucidate the functional role of PLN in cardiac physiology and its effects on predisposition to heart disease.
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Acknowledgements

We are grateful to D. Bers, A. Gran Milano, K. Highgirl, G. Inest and C. Toyoohira for their helpful comments on this manuscript. The original studies described in this review were supported by grants to D.H.M. from the Heart and Stroke Foundation of Ontario, the Canadian Institutes for Health Research and the Canadian Genetic Diseases Network of Centres of Excellence, and to E.G.K. by grants from the National Institutes of Health (USA).