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Mutations in Phospholamban Alter the Structure and Function of the Calcium ATPase Regulatory Complex

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This dissertation is dedicated to my daughter Nishka for being my strength.
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LIST OF ABBREVIATIONS

PLB    Phospholamban
SERCA Sarco(endo)plasmic reticulum calcium ATPase
Ca\(^{2+}\) Calcium
R9C    Arg9Cys
FRET   Fluorescence resonance energy transfer
PKA    Protein kinase A
CaMKII Calcium/calmodulin-dependent protein kinase II
DCM    Dilated cardiomyopathy
SSS    Cys36Ser/Cys41Ser/Cys46Ser
Cer    mCerulean
CFP    Cyan fluorescent protein
YFP    Yellow fluorescent protein
FRET   Fluorescence resonance energy transfer
H\(_2\)O\(_2\) Hydrogen peroxide
FRET\(_{\text{max}}\) Maximal FRET efficiency
K\(_D\)\(_1\) Apparent dissociation constant of the PLB oligomer
K\(_D\)\(_2\) Apparent dissociation constant of the SERCA-PLB regulatory complex
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<tr>
<td>R</td>
<td>Probe separation distance</td>
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<td>Å</td>
<td>Angstrom</td>
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<td>Iso</td>
<td>Isoproterenol</td>
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<td>WT</td>
<td>Wild-type</td>
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<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>DCM</td>
<td>Dilated cardiomyopathy</td>
</tr>
<tr>
<td>HCM</td>
<td>Hypertrophic cardiomyopathy</td>
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<tr>
<td>Ca^{2+}</td>
<td>Calcium</td>
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<tr>
<td>L39X</td>
<td>Leu-39stop</td>
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<tr>
<td>V49A</td>
<td>Val-49Ala</td>
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<tr>
<td>V49X</td>
<td>Val-49stop</td>
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<tr>
<td>TM</td>
<td>Transmembrane</td>
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<td>KL</td>
<td>Partition equilibrium constant estimated by localization</td>
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<tr>
<td>PI</td>
<td>Propidium iodide</td>
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<td>ND</td>
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<td>K_{Ca}</td>
<td>Calcium concentration at half-maximal SERCA activity</td>
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<td>V_{max}</td>
<td>Maximal SERCA activity</td>
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<tr>
<td>RMSD</td>
<td>Root-mean-square deviation</td>
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<td>AU</td>
<td>Arbitrary units</td>
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Phospholamban (PLB) is an integral membrane protein that plays an important role in regulation of cardiac calcium handling and contractility. PLB exists as a homopentamer in the membrane, which upon deoligomerization into active monomers reversibly inhibits sarco/endoplasmic reticulum calcium ATPase (SERCA). Mutations in PLB that change the PLB monomer-pentamer equilibrium result in dysregulation of SERCA. To determine the structural and regulatory role of the C-terminal residues of PLB in the membranes of living cells, we fused fluorescent protein tags to PLB and SERCA. We then studied the effect of C-terminal alanine substitutions and truncation mutations on PLB oligomerization and SERCA regulation by fluorescence resonance energy transfer (FRET) measurements in live cells. In addition, we also studied the structural and functional consequences of two naturally-occurring missense mutations of PLB that cause heart failure including L39stop (L39X) and Arg9Cys (R9C). Alanine substitution of PLB C-terminal residues significantly altered FRET from PLB to PLB and SERCA to PLB, suggesting a change in quaternary conformation of PLB pentamer and SERCA-PLB regulatory complex. We also quantified a decrease in PLB oligomerization affinity, and an increase in SERCA-PLB binding affinity for alanine mutants. Notably, truncation of only a few C-terminal residues resulted in significant loss of PLB membrane anchoring and
mislocalization to the cytoplasm and nucleus. C-terminal truncations including L39X resulted in progressive loss of PLB-PLB FRET, due to a decrease in the apparent affinity of PLB oligomerization. In addition, we quantified a decrease in the binding affinity of truncated PLB including L39X for SERCA, suggesting a change in quaternary conformation of the SERCA-PLB regulatory complex. Furthermore, FRET measurements revealed that R9C-PLB exhibited an increased propensity for oligomerization, and this was further increased by oxidative stress. The R9C also decreased PLB binding to SERCA, and altered the structure of the PLB-SERCA regulatory complex. In addition, we observed that acute expression of R9C-PLB exerts a positively inotropic and lusitropic effect in cardiomyocytes, in contrast to studies of chronic R9C-PLB expression in transgenic mice. Importantly, R9C-PLB exhibited blunted sensitivity to frequency potentiation and β-adrenergic stimulation, two major physiological mechanisms for the regulation of cardiac performance. We conclude that PLB C-terminal residues are critical for localization, oligomerization, and regulatory function. In particular, the PLB C-terminus is an important determinant of the quaternary structure of the SERCA-PLB regulatory complex. Furthermore, the heart failure mutants of PLB including L39X and R9C decrease SERCA inhibition by altering the structure and function of the SERCA-PLB regulatory complex.
CHAPTER I

INTRODUCTION

Cardiac Calcium Cycling and Contractile Function

The cardiac cycle constitutes the phases of contraction (systole) and relaxation (diastole) of the heart that together constitute the heart beat (Fukuta and Little 2008). During systole, the action potential causes membrane depolarization and induces a calcium (Ca\(^{2+}\)) influx through the L-type Ca\(^{2+}\) channels (Bers 2002). This influx triggers the Ca\(^{2+}\)-induced Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) via ryanodine receptors (RyR) (Bers 2002). The schematic representation of the key players involved in regulation of cardiac Ca\(^{2+}\) cycling and contractile function is shown in Fig. 1 (Bers 2002, 2008). The Ca\(^{2+}\) release from the SR via RyRs gives rise to the Ca\(^{2+}\) transient and the resultant rise in intracellular Ca\(^{2+}\) activates the myofilaments to produce cardiac contraction (inotropy). This is indicated by the rising phase of the Ca\(^{2+}\) transient, followed by the shortening of the sarcomere length during contraction of the myofilaments. During diastole, the RyRs close and the released Ca\(^{2+}\) is pumped back into the SR by sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) that results in cardiac relaxation (lusitropy). The resultant decrease in intracellular Ca\(^{2+}\) is indicated by the decay phase of the Ca\(^{2+}\) transient, which is followed by sarcomere relengthening during relaxation of the myofilaments. SERCA is under
Figure 1. Regulation of cardiac Ca\textsuperscript{2+} handling and contractile function of the heart. During systole, cardiac contraction or inotropy is initiated when ryanodine receptor (RyR) releases Ca\textsuperscript{2+} from the sarcoplasmic reticulum (SR) into the cytosol as indicated by rising phase of the Ca\textsuperscript{2+} transient, followed by sarcomere shortening. This is followed by cardiac relaxation or lusitropy during diastole, when Ca\textsuperscript{2+} is subsequently pumped back into the SR by SR Ca\textsuperscript{2+}-ATPase (SERCA), as indicated by decay phase of Ca\textsuperscript{2+} transient and sarcomere relengthening. PLB binds and inhibits SERCA, thereby directly regulating cardiac Ca\textsuperscript{2+} handling and contractile function of the heart.
the inhibitory control of a small inhibitory phosphoprotein, phospholamban (PLB) that decreases the Ca\textsuperscript{2+} affinity for SERCA (MacLennan and Kranias 2003). This process of cardiac Ca\textsuperscript{2+} cycling is highly regulated and is repeated for each beat of the heart to maintain normal Ca\textsuperscript{2+} homeostasis and cardiac function. Thus, SERCA-PLB regulatory complex plays a central role in regulation of cardiac Ca\textsuperscript{2+} cycling and contractile function (Kranias and Hajjar 2012). Dysregulation of SERCA-PLB complex results in pathological consequences, and targeting abnormal Ca\textsuperscript{2+} kinetics has emerged as a promising therapeutic target for treatment of human heart failure (Kranias and Hajjar 2012).

**SERCA**

SERCA is an ion-motive P-type ATPase that establishes intracellular Ca\textsuperscript{2+} stores needed for cell signaling (Lipskaia, Hulot, and Lompré 2009) and normal cardiac myocyte function. SERCA is a 110 kDa integral SR membrane protein, which utilizes ATP hydrolysis to transport Ca\textsuperscript{2+} into the SR (MacLennan and Kranias 2003). In human and rabbit ventricular cardiomyocytes, 70% of the cytoplasmic Ca\textsuperscript{2+} is removed by SERCA, 28% by the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, 1% by the sarcolemmal Ca\textsuperscript{2+}-ATPase and 1% by the mitochondrial Ca\textsuperscript{2+} uniporter (Bers 2002). During the enzymatic cycle, SERCA cycles between two primary conformations: the Ca\textsuperscript{2+}-bound state (E1) and the Ca\textsuperscript{2+}-unbound state (E2) (Toyoshima and Inesi 2004). The first high resolution (2.6 Å) crystal structure of SERCA with two Ca\textsuperscript{2+} ions bound in the E1 state was determined by Toyoshima and coworkers (Toyoshima et al. 2000). The same group later reported the crystal structure of SERCA in a Ca\textsuperscript{2+}-free E2 state at 3.1 Å resolution (Toyoshima
and Nomura 2002). The structure of SERCA constitutes four functional domains: nucleotide-binding (N), phosphorylation (P), actuator (A) and transmembrane (TM) as shown in Fig. 2 (Toyoshima and Inesi 2004). The N-domain contains the ATP-binding site and is responsible for placement of ATP to facilitate autophosphorylation, the P-domain forms the catalytic core of the enzyme and contains the autophosphorylated residue (Asp351), the A domain is involved in the transmission of major conformational dynamics following Ca\(^{2+}\) binding and translocation, and the TM domain constitutes 10 helices (M1-M10) and two Ca\(^{2+}\) binding sites (Toyoshima and Inesi 2004).

In humans, three SERCA genes, SERCA 1, 2 and 3 encode up to 10 isoforms (SERCA1a-b, SERCA2a-c, SERCA3a-f) by developmental or tissue-specific alternative splicing (Periasamy and Kalyanasundaram 2007). SERCA1 is mainly expressed in fast-twitch skeletal muscle and has two isoforms, SERCA1a (adult) and SERCA1b (neonatal) (Brandl et al. 1987, Brandl et al. 1986, Peters et al. 1997). SERCA2 has three isoforms, SERCA2a (cardiac and slow-twitch skeletal muscle tissue), SERCA2b (ubiquitously expressed in all tissues) and SERCA2c (cardiac muscle) (Dally et al. 2006). SERCA3 has six isoforms, SERCA3a-f, which are expressed in multiple tissues and cell types (Dally et al. 2009, Wuytack et al. 1995). The domain structure of SERCA isoforms is highly conserved, but they have varying affinities for Ca\(^{2+}\) and transport velocities (Periasamy and Kalyanasundaram 2007).

The cardiac SERCA isoform (SERCA2a) plays a critical role in regulating cardiac contraction and relaxation (Frank et al. 2003). During the cardiac cycle,
Figure 2. Schematic representation of the structure of SERCA. The crystal structure of SERCA2a in E1 conformation with the four domains highlighted; nucleotide-binding (N), phosphorylation (P), actuator (A) and transmembrane (TM). Structural model of SERCA was generated using PyMOL (PDB: 1SU4).
sequestration of Ca$^{2+}$ into the SR at the expense of ATP hydrolysis by SERCA is the fundamental mechanism for initiation of cardiac muscle relaxation during diastole (Kranias and Hajjar 2012, MacLennan and Kranias 2003, Periasamy and Huke 2001). Moreover, the rate of SERCA Ca$^{2+}$ uptake is one of the main determinants of the size of the Ca$^{2+}$ store, so SERCA is also critical for regulating the strength of cardiac contraction during systole (Kranias and Hajjar 2012, MacLennan and Kranias 2003, Periasamy and Huke 2001). SERCA facilitates the storage and distribution of Ca$^{2+}$ ions in the SR by maintaining a 1000-fold Ca$^{2+}$ gradient across the SR membrane (Frank et al. 2003). By actively transporting Ca$^{2+}$ ions into the SR, SERCA regulates cytosolic Ca$^{2+}$ concentration, SR Ca$^{2+}$ load, and cardiac inotropy and lusitropy. Alteration of the activity and expression of SERCA contributes to the decreased SR Ca$^{2+}$ content, resulting in cardiac dysfunction during heart failure (Periasamy, Bhupathy, and Babu 2008).

**Phospholamban**

SERCA2a activity is closely governed by an inhibitory interaction with its regulatory partner phospholamban (PLB), a 52 residue single span transmembrane peptide (Kranias and Hajjar 2012, MacLennan and Kranias 2003, Simmerman and Jones 1998). PLB is predominantly expressed in cardiac muscle (Simmerman and Jones 1998) and in small amounts in smooth muscle (Raeymaekers and Jones 1986), slow-twitch skeletal muscle (Movsesian et al. 1992) and endothelial cells (Sutliff et al. 1999). PLB is a homopentameric,
integral SR membrane protein, which upon deoligomerization into active monomers reversibly inhibits SERCA (Simmerman and Jones 1998, Karim et al. 1998, Robia et al. 2007), thereby directly regulating cardiac Ca\textsuperscript{2+} kinetics and contractility (Park and Oh 2013, Kranias and Hajjar 2012, MacLennan and Kranias 2003). The primary sequence and structural domains of PLB monomer determined by solid-state NMR spectroscopy have been highlighted in Fig. 3. PLB has a 'helix-loop-helix' tertiary structure consisting of an N-terminal cytosolic domain IA (residues 1-16), flexible linker (residues 17-22), domain IB (residues 23-30), and the C-terminal transmembrane (TM) domain II (residues 31-52) (Verardi et al. 2011, Zamoon et al. 2003).

The C-terminal TM domain is hydrophobic, highly conserved among species (Simmerman and Jones 1998), and is important for PLB oligomerization (Simmerman et al. 1996, Fujii et al. 1989), and SERCA regulation (Kimura et al. 1996). The key residues in the TM domain of PLB that are critical for pentamer formation and stability have been identified by the alanine- and phenylalanine-scanning mutagenesis studies (Simmerman et al. 1996), and subsequently supported by the NMR studies (Verardi et al. 2011). It has been shown that the PLB homopentamer is formed and stabilized by a leucine-isoleucine zipper by the close packing of Leu37, Ile40, Leu44, Ile47, and Leu51 (Simmerman et al. 1996). In addition, mutagenesis studies have shown that three TM cysteines (Cys36, Cys41 and Cys46) contribute to PLB pentamer stability on account of their steric properties, but not intermolecular disulfide bonding (Karim et al. 2001).
Figure 3. Schematic representation of primary sequence and structure of PLB. (A) The amino acid sequence of PLB protomer showing N-terminal cytosolic domain ia (residues 1-16), flexible linker (residues 17-22), domain lb (residues 23-30), and the C-terminal transmembrane (TM) domain II (residues 31-52). (B) The solid-state NMR structure of PLB monomer highlighting the four domains. Structural model of PLB was generated using PyMOL (PDB: 2KYV).
The N-terminal cytoplasmic domain is hydrophilic, and undergoes conformational changes in the PLB oligomer and SERCA-PLB regulatory complex as a result of mutations, or phosphorylation (Gustavsson et al. 2013, Glaves et al. 2011, Hou, Kelly, and Robia 2008). The cytoplasmic domain of PLB can be phosphorylated at Ser16 by cAMP-dependent protein kinase A (PKA) and at Thr17 by Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) (MacLennan and Kranias 2003, Hagemann and Xiao 2002). Several hereditary mutations in PLB have been shown to cause dilated cardiomyopathy and heart failure (Medeiros et al. 2011). The mutations in the PLB cytoplasmic domain that result in diseases include Arg9-to-Cys (R9C) (Schmitt et al. 2003), deletion of Arg14 (R14del) (Haghighi et al. 2006, DeWitt et al. 2006, Posch et al. 2009, van Rijssingen et al. 2014), Arg9-to-Leu (R9L) and Arg9-to-His (R9H) (Medeiros et al. 2011). The truncation at Leu39 (L39X) is the only hereditary mutation in the TM domain of PLB that causes heart failure (Haghighi et al. 2003). The heart failure mutants of PLB that are the focus of this study are discussed in greater detail in the section “Mutations in human PLB gene linked to heart failure”.

**PLB Inhibition of SERCA**

In the SR membrane, PLB exists in a dynamic equilibrium between the homopentamer and the monomer (Fig. 4). Monomeric PLB is the active inhibitory species of SERCA, and the pentamer acts as the storage of inactive form of PLB (MacLennan and Kranias 2003, Kimura et al. 1997). \(K_D1\) and \(K_D2\) represent the dissociation constants for PLB pentamer and PLB-SERCA regulatory complex, respectively (Robia et al. 2007, MacLennan and Kranias 2003). Importantly, PLB
Figure 4. Dynamic equilibrium between PLB pentamer and monomer regulates SERCA. PLB homopentamer deoligomerizes into active monomers that bind and inhibit SERCA. Disrupting PLB monomer-pentamer equilibrium leads to SERCA dysregulation. (K₀₁, K₀₂: Dissociation constants for PLB pentamer and PLB-SERCA regulatory complex, respectively). Structural models of PLB and SERCA were generated using PyMOL (PDB: 2KYV, 1IWO).
monomer-pentamer equilibrium plays an important role in the inhibition of SERCA, and is required for optimal regulation of contractile function of the heart (MacLennan and Kranias 2003). Any changes in PLB monomer-pentamer equilibrium via mutations in PLB or phosphorylation of PLB result in dysregulation of SERCA (MacLennan and Kranias 2003).

During the cardiac cycle, the unphosphorylated PLB monomer interacts with SERCA at resting Ca^{2+} concentration, reducing SERCA’s apparent affinity for Ca^{2+} and decreasing its activity (MacLennan and Kranias 2003, Kranias and Hajjar 2012). Several models are proposed in the literature for the mechanism for relief of inhibition, which is still an active area of investigation. The PLB inhibition of SERCA is partially relieved upon β-adrenergic stimulation, when PKA phosphorylates PLB at Ser-16 (MacLennan and Kranias 2003, Hagemann and Xiao 2002), increasing SERCA activity to meet increased physiological demand.

A complementary mechanism for relief of inhibition is PLB phosphorylation at Thr-17 by CaMKII (Hagemann and Xiao 2002). This pathway is activated by the elevation of cytosolic Ca^{2+} that accompanies increased pacing frequency during exercise. A previous study in our lab has shown that phosphomimetic mutations at both PKA and CaMKII sites result in increased oligomerization of PLB to form pentamers as well as altered the structure of SERCA-PLB regulatory complex (Hou, Kelly, and Robia 2008). In addition, direct phosphorylation of SERCA at Ser-38 by CaMKII has also been reported to increase SERCA activity (Toyofuku, Curotto Kurzydlowski, et al. 1994, Narayanan and Xu 1997). Thus, SERCA activity and PLB regulation of that activity by phosphorylation or mutations may
allow the heart to responsively compensate for rest, stress, or diseased conditions.

Notably, it has been reported that PLB inhibition of SERCA is also relieved by elevated cytosolic Ca\textsuperscript{2+} concentration (Asahi et al. 2000). However, several mechanisms for this functional effect have been proposed. According to the ‘dissociation model’, monomeric PLB binds selectively to the Ca\textsuperscript{2+}-free “E2” conformation of SERCA during cardiac relaxation when cytoplasmic Ca\textsuperscript{2+} concentration is low (Akin, Chen, and Jones 2010, Chen, Akin, and Jones 2010, Chen et al. 2006). The relief of SERCA inhibition results in unbinding of PLB from the Ca\textsuperscript{2+}-bound E1 conformation during cardiac contraction when cytoplasmic Ca\textsuperscript{2+} concentration is high (Akin, Chen, and Jones 2010, Chen, Akin, and Jones 2010, Chen et al. 2006). According to the ‘subunit model’, PLB remains bound to SERCA throughout the catalytic cycle and acts as a subunit of the pump (Mueller et al. 2004). Alternatively, our lab has previously proposed a model according to which the relief of functional inhibition does not require dissociation of the SERCA-PLB regulatory complex, and PLB can bind SERCA at high Ca\textsuperscript{2+} concentration, although with a lower binding affinity (Bidwell et al. 2011). This study suggested multiple modes of PLB binding or presence of alternative binding sites on SERCA. The present study supports that mutations in PLB cause translocation of PLB TM domain from canonical binding site to an alternative binding site on SERCA, resulting in alteration of the structure and function of SERCA-PLB regulatory complex.
Mutagenesis studies have reported that the residues Leu31, Asn34, Phe35, Ile38, and Leu42 in the TM domain of PLB are essential for inhibition of SERCA (Kimura et al. 1997). In addition, cross-linking studies and modeling of PLB-SERCA regulatory interaction has shown that the TM domain of PLB is accommodated in a groove formed by helices M2, M4, M6 and M9 of SERCA (Toyoshima et al. 2003). Another study has shown that the sequence Lys–Asp–Asp–Lys–Pro–Val-402 in the N-domain of SERCA is important for PLB inhibitory interaction (Toyofuku, Kurzydlowski, et al. 1994). Cryo-electron microscopy revealed a low-resolution structure of a co-crystal between SERCA and PLB, but it did not give any information about the binding site (Young, Jones, and Stokes 2001). A recent study reported the crystal structure of SERCA in complex with PLB at 2.8 Å resolution (Akin et al. 2013). This study revealed the interaction of SERCA with transmembrane domain of PLB, but it did not give information about the binding of cytoplasmic domain of PLB with SERCA. It is important to note that the structure and function of the SERCA-PLB regulatory complex may be altered by mutations in PLB or SERCA. Although these structural studies provide mechanistic information about the interaction between PLB and SERCA, they may not reveal the physiological interaction of the regulatory complex. In this study, FRET analysis provided insight into the effect of specific mutations on the alteration of quaternary conformation and function of PLB-SERCA regulatory complex in real time. The structural studies of SERCA-PLB regulatory complex interaction is still an active area of research.
Mutations in Human PLB Gene Linked to Heart Failure

Disordered Ca\textsuperscript{2+} transport or regulation may cause and result from cardiac diseases such as heart failure (Marks 2003, Asp et al. 2013, Luo and Anderson 2013). Heart failure is a leading cause of mortality, affecting an estimated 26 million people worldwide, and up to 6 million people in the US (Lopez-Sendon 2011). Dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM) are the leading causes of heart failure (Towbin and Bowles 2002, Kimura 2008). Both DCM and HCM can be caused by mutations in genes encoding cardiac Ca\textsuperscript{2+} handling proteins including PLB (Kimura 2008, Medeiros et al. 2011, Haghighi et al. 2003, Chiu et al. 2007, Landstrom et al. 2011, Schmitt et al. 2003, Haghighi et al. 2006, DeWitt et al. 2006, Posch et al. 2009, van Rijsingen et al. 2014). In particular, mutations (Schmitt et al. 2003, Haghighi et al. 2003, Medeiros et al. 2011, Landstrom et al. 2011) or deletions (Haghighi et al. 2006, DeWitt et al. 2006, Posch et al. 2009, van Rijsingen et al. 2014) of PLB give rise to human disease, underscoring the importance of this peptide and providing some insight into the molecular mechanisms of SERCA regulation by PLB.

Of particular interest is the human heart failure mutant R9C-PLB, which was identified in 2003 to be the first DCM causing human mutation of PLB (Schmitt et al. 2003). This mutation is caused by heterozygous substitution of Arg9 for Cys in the cytoplasmic domain of PLB. DCM is a leading cause of cardiovascular morbidity and mortality worldwide (Jefferies and Towbin 2010, Dellefave and McNally 2010, Parvari and Levitas 2012), so there is great interest in understanding how a discrete point mutation in PLB could induce pathological
dysfunction. The patients with R9C-PLB mutation had early symptom onset at the age of 20 to 30 years, and required cardiac transplantation depending on severity of the disease (Schmitt et al. 2003). The affected individuals suffered from progressive heart failure 5 to 10 years later and their average age at death was 25.1 ± 12.7 years (Schmitt et al. 2003). The proposed mechanisms for R9C pathology have been discussed in detail in Sections ‘Disordered structure/function mechanisms of R9C-PLB’ and ‘R9C Acts as a Phosphomimetic Mutation of PLB’ of Chapter V.

Another naturally-occurring missense mutation in PLB that leads to heart failure is caused by substitution of a stop codon for Leu39 (L39X) (Haghighi et al. 2003). This mutation results in truncation of the C-terminus of PLB midway through its TM domain (Haghighi et al. 2003). This mutation has been reported to cause dysregulation of SR Ca^{2+} cycling, DCM, HCM, heart failure and premature death in humans (Haghighi et al. 2003, Chiu et al. 2007, Landstrom et al. 2011). The patients heterozygous for this mutation exhibited hypertrophy without diminished contractile function (Haghighi et al. 2003). However, homozygous patients developed heart failure and early mortality, requiring cardiac transplantation between ages 16 and 27 (Haghighi et al. 2003). In humans, this mutant has been reported as a highly unstable, rapidly degraded and inactive form of PLB. On account of loss of myocardial PLB protein content and a resultant loss of PLB inhibitory function, the homozygous L39X individuals were described as PLB-null (Haghighi et al. 2003). In contrast, PLB ablation in mouse models results in chronically enhanced basal cardiac contractile function without
development of heart failure, even in advanced age (Luo et al. 1994). The critical
differences between cardiac phenotypes on account of PLB ablation in mouse
and humans emphasizes the need to better understand the pathophysiological
differences between the two species. While all other naturally-occurring human
PLB mutations have been identified in the cytoplasmic domain, L39X is unique to
the TM domain of PLB (Haghighi et al. 2003).

This shows that PLB is important for cardiac function and hereditary
mutations in PLB are a cause of heart failure in humans. In this study, we
investigated the role of the R9C-PLB mutation in determining the structure of
SERCA-PLB regulatory complex as well as the acute physiological
consequences of this mutation on SR Ca$^{2+}$ handling and contractile function.
Additionally, in order to understand the role of the TM domain in membrane
anchoring, localization, PLB oligomerization, and SERCA regulation, we
investigated the structural and functional consequences of subjecting the PLB
TM domain to alanine substitution mutations and truncation mutations including
L39X (Abrol et al. 2014). In addition to R9C (Schmitt et al. 2003) and L39X
(Haghighi et al. 2003), other genetic mutations in human PLB linked to heart
failure are R14del (Haghighi et al. 2006, DeWitt et al. 2006, Posch et al. 2009,
van Rijsingen et al. 2014), R9L and R9H (Medeiros et al. 2011). The
identification of additional human PLB mutants will further explain the functional
role of PLB in cardiac physiology and its effects on genetic predisposition to
cardiac diseases.
MATERIALS AND METHODS

Molecular Biology

mCerulean (Cer), cyan fluorescent protein (CFP), or enhanced yellow fluorescent protein (YFP) were each fused to the N-terminus of canine PLB or canine SERCA2a as described previously (Abrol et al. 2014, Bidwell et al. 2011, Hou and Robia 2010, Kelly et al. 2008, Robia et al. 2007). Fig. 5A represents the primary amino acid sequence of WT-PLB with the domains highlighted. The R9C-PLB mutant was generated by mutating the cytoplasmic Arg 9 residue to Cys (Fig. 5B). The SSS-PLB mutant was constructed by mutating the transmembrane Cys residues 36, 41, and 46 to Ser (Fig. 5B). As shown in Fig. 5C, a series of truncation mutants of PLB were constructed by introducing stop codons at residues 52, 51, 50, 49, 48, 39, 38, or 33 (Abrol et al. 2014). In addition, alanine (Ala) substitution mutants were generated by replacing the residues 52, 51, 50, or 49 by Ala (Fig. 5C) (Abrol et al. 2014). All the PLB mutants used in this study were generated using the QuikChange IIXL site-directed mutagenesis kit (Stratagene, La Jolla, CA) and custom oligonucleotide primers (Eurofins MWG Operon). The nucleotide sequences were verified by DNA sequencing (ACGT, Inc.). Adenoviral vectors of canine CFP-PLB or YFP-PLB were produced using AdEasy Adenoviral Vector System (Stratagene, CA).
Figure 5. Schematic representation of primary sequence of WT-PLB and the mutant PLB constructs. (A) The amino acid sequence of PLB protomer showing N-terminal cytosolic domain la (residues 1-16), flexible linker (residues 17-22), domain lb (residues 23-30), and the C-terminal transmembrane domain II (residues 31-52). Cer or YFP was fused to the N-terminus. (B) The structure of PLB monomer highlighting the R9C and SSS mutation sites. The thickness of the lipid bilayer is approximately 40 Å. (C) The structure of PLB monomer highlighting the C-terminal residues subjected to alanine substitution or truncation mutations.
Cell Culture

Left ventricular cardiomyocytes were enzymatically isolated from adult New Zealand White rabbits (Domeier, Blatter, and Zima 2009). All animal protocols including cardiomyocyte isolation was approved by the Loyola University Institutional Animal Care and Use Committee. The cardiomyocytes were washed with fresh PC-1 medium (Lonza, Basel, Switzerland) and plated onto laminin-coated glass coverslips that fit into 35 mm culture dishes. Cardiomyocytes were incubated at 37°C for 1 hour and CFP-PLB and/or YFP-PLB adenoviruses were added at a multiplicity of infection of 1000 as previously (Pallikkuth et al. 2013). Cardiomyocytes were then paced for 48 hours in culture using a C-Pace EP Pacer (IonOptix, Milton, MA) set to 10 volts with a frequency of 0.1 Hz, with 5 ms pulse duration (Bidwell et al. 2011, Pallikkuth et al. 2013).

AAV-293 cells were cultured in 60-mm tissue culture dishes in complete DMEM growth medium with 10% fetal bovine serum, 1% L-glutamine and incubated at 37°C under 5% CO₂. Transient transfection of cultured AAV-293 cells was performed by the calcium phosphate precipitation method using the MBS mammalian transfection kit (Stratagene, La Jolla, CA). Cells were co-transfected with plasmids encoding Cer-PLB and YFP-PLB or Cer-SERCA and YFP-PLB with a molar ratio of 1:5 or 1:20 respectively (Abrol et al. 2014, Bidwell et al. 2011). Following transfection, the cells were subjected to mild trypsinization, plated on poly-D-lysine-coated glass bottom dishes, and allowed to adhere for 2 hours before imaging, as described previously (Abrol et al. 2014, Bidwell et al. 2011).
Fluorescence Resonance Energy Transfer (FRET) Quantification

PLB oligomerization and interaction with SERCA was quantified in live cells using wide-field fluorescence microscopy as described previously (Abrol et al. 2014, Bidwell et al. 2011). MetaMorph software was used for acquisition of a montage of 48 images using a motorized stage (Prior, Rockland, MA). Focus was automatically maintained by an optical feedback system (Perfect Focus System, Nikon), and image acquisition was done using 40X objective with a numerical aperture of 0.75. The exposure time was 150 ms for each channel: Cer, YFP, and FRET (Cer excitation/YFP emission). Multi Wavelength Cell Scoring application module in MetaMorph was used for automated quantification of fluorescence intensity. The cells were selected by the software based on the criteria including minimum fluorescent area of 50 \( \mu \text{m}^2 \), diameter between 40 \( \mu \text{m} \) and 100 \( \mu \text{m} \), and an average intensity of 100 counts above background. The average intensities of each channel were then transferred to a spreadsheet for quantifying FRET efficiency. FRET quantification was done using acceptor sensitization (E-FRET) (Zal and Gascoigne 2004), as described previously (Abrol et al. 2014, Bidwell et al. 2011). After background subtraction, FRET efficiency was calculated according to the following formula: 

\[
E = \frac{[I_{DA} - a(I_{AA}) - d(I_{DD})]}{[I_{DA} - a(I_{AA}) + (G - d) (I_{DD})]};
\]

where \( I_{DD} \) is the intensity of fluorescence emission from the donor channel (472/30 nm) with excitation of 427/10 nm; \( I_{AA} \) is the intensity of fluorescence emission from the acceptor channel (542/27 nm) with excitation of 504/12 nm; and \( I_{DA} \) is the intensity of fluorescence emission detected in the FRET channel (542/27 nm) with excitation of 427/10 nm. The
constants a and d are cross-talk coefficients determined from acceptor-only or donor-only control samples, respectively, $a = \frac{I_{DA}}{I_{AA}}$, and $d = \frac{I_{DA}}{I_{DD}}$. $G$ represents the ratio of the sensitized emission to the corresponding amount of donor recovery. For the R9C-PLB FRET experiments, we used values of 0.082, 0.82 and 3.2 for $a$, $d$ and $G$ ratio respectively, as described previously (Bidwell et al. 2011). For the truncation mutations and alanine substitution FRET experiments, we obtained values of 0.083, 0.69 and 4.3 for $a$, $d$ and $G$ ratio respectively (Abrol et al. 2014).

For time-course experiments, the cells were imaged at 30 s time intervals for Cer, YFP, and FRET channels. After 5 min of image acquisition, 100 µM hydrogen peroxide ($H_2O_2$) was applied and the cells were imaged every 30 s for an additional 20 minutes. The FRET efficiency for individual cells at each time point was quantified using MetaMorph and the data from 3 independent experiments were averaged. The FRET ratio was calculated by dividing the intensity of FRET channel by the intensity of Cer channel. The calculated FRET ratios for all the cells at each time point were averaged after normalizing to the first time point. FRET images were acquired by dividing the image of FRET channel by Cer fluorescence using MetaMorph.

‘In-Cell’ Binding Assay

An ‘in-cell’ binding assay was performed to estimate the parameters related to structure and binding affinity (Abrol et al. 2014, Bidwell et al. 2011, Ha et al. 2011, Hou and Robia 2010, Hou, Kelly, and Robia 2008, Kelly et al. 2008). Briefly, the FRET efficiency of individual cells coexpressing Cer-PLB/YFP-PLB or
Cer-SERCA/YFP-PLB was plotted against relative protein concentration, which was quantified from the observed YFP fluorescence intensities. Fig. 6A shows AAV-293 cells expressing Cer-PLB and YFP-PLB. An example of PLB-PLB binding curve is shown in Fig. 6B. The concentration dependence of FRET was fit to a hyperbolic curve of the form $y = \frac{(\text{FRET}_{\text{max}})x}{(K_D + x)}$, with all parameters independently fit, where $y$ is the observed FRET efficiency, and $x$ is the protein concentration in the cell in arbitrary units (AU). FRET$_{\text{max}}$ is the intrinsic FRET of the protein complex and a measure of average distances between the binding partners, providing structural information. K$_D$ is the protein concentration that yields half FRET$_{\text{max}}$, and represents the dissociation constant of the protein complex, providing an estimate of the apparent binding affinity.

K$_D1$ is the apparent dissociation constant of the PLB oligomer and K$_D2$ is the apparent dissociation constant of the SERCA-PLB regulatory complex. The data are pooled from 3 to 4 independent experiments for each sample. Each binding curve was developed by using an average of approximately 2000 cells. The effect of mutations on PLB oligomerization affinity or SERCA-PLB binding affinity can be directly measured in real-time by estimating the changes in K$_D1$ or K$_D2$ respectively from the binding curve (Abrol et al. 2014, Bidwell et al. 2011, Ha et al. 2011, Hou and Robia 2010, Hou, Kelly, and Robia 2008, Kelly et al. 2008). As shown in Fig. 6B, the red and blue curves represent simulations of changes in affinity. A left-shifted binding curve (red) indicates a decrease in K$_D1$, and an increase in apparent binding affinity. A right-shifted curve (blue) indicates an increase in K$_D1$, and a resultant decrease in apparent binding affinity.
Figure 6. ‘In-cell’ binding assay (A) AAV-293 cells co-expressing Cer-WT-PLB and YFP-WT-PLB (B) The example of PLB-PLB binding curve is shown in black. FRET\textsubscript{max} and \(K_D\) are the two important parameters measured from the binding curve for the PLB pentamer. The red and blue curves represent simulations of increase and decrease in PLB-PLB oligomerization affinity respectively.
Probe separation distance \((R)\) for the SERCA-PLB regulatory complex was calculated using the Förster equation (Förster 1948), \(R = (R_o) [(1/E) - 1]^{1/6}\), where \(R_o\) is the Förster radius, and \(E\) is the measured FRET\(_{\text{max}}\). Intrapentameric probe separation distance was calculated from FRET\(_{\text{max}}\) using a MatLab application (Kelly et al. 2008), assuming a ring-shaped oligomer (Li et al. 1999, Robia, Flohr, and Thomas 2005, Runnels and Scarlata 1995), with a subunit number of 5 (pentamer). For the R9C-PLB FRET experiments, the acceptor molar fraction was 0.89 for the WT-PLB pentamer and 0.92 for the R9C-PLB pentamer. For the C-terminal mutants FRET experiments, the average acceptor molar fraction was calculated to be 0.92 ± 0.01 for WT-PLB, 0.89 ± 0.02 for the alanine substitution mutants and 0.88 ± 0.01 for the truncation mutants.

Previously, we estimated non-specific FRET to be 4\%, as determined from competition with unlabeled PLB or with a fluorescently-labeled PLB that is unable to participate in FRET (Kelly et al. 2008, Ha et al. 2011). We quantified non-specific FRET by calculating the apparent FRET efficiency for cells co-expressing Cer-WT-PLB and YFP-WT-PLB as shown in Fig. 7. The results indicated that the apparent FRET efficiency for cells expressing Cer-WT-PLB and YFP-WT-PLB was 48.8\% (Fig. 7, WT). In contrast, the control cells expressing non-interacting proteins Cer and YFP exhibited very low apparent FRET of 3.7\% (Fig. 7, Control). In addition, we performed another control experiment and estimated apparent FRET of 0.85\% for cells expressing Cer-PLB without any acceptor. For estimation of probe separation distances for both the pentamer and regulatory complex, the Förster radius of 49.8 Angstrom (Å) was used for the Cer
Figure 7. Quantification of non-specific FRET. The binding curves show apparent FRET efficiency for cells co-expressing Cer-WT-PLB and YFP-WT-PLB compared to control cells co-expressing non-interacting proteins Cer and YFP.
-YFP pair (Gadella 2009) and 4% non-specific FRET was subtracted from the measured FRET$_{\text{max}}$ values.

**IonOptix Data Acquisition and Analysis**

Adult rabbit left ventricular cardiomyocytes expressing YFP-WT-PLB or YFP-R9C-PLB were loaded with 10 μM Indo-1 AM Ca$_{\text{2+}}$ dye (Invitrogen Inc., France) for 20 minutes at room temperature, and washed with fresh Tyrode solution (135 mM NaCl, 4 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM d-Glc, 10 mM HEPES, pH 7.4). Following this, cardiomyocytes were electrically stimulated with 20V, 6 ms pulse duration, at increasing pacing frequencies of 0.3 Hz, 0.5 Hz, and 0.75 Hz. Ca$_{\text{2+}}$ transients and sarcomere shortening were recorded (IonOptix, Milton, MA) before and after 10 minute incubation with 100 nM isoproterenol (iso). Ca$_{\text{2+}}$ transient recordings were obtained by measuring fluorescence intensity at excitation and emission wavelengths of 340 and 405/485 nm respectively and analyzed using the IonOptix software.

**SDS PAGE and Western Blot Analysis**

Total cell lysates were obtained by washing AAV-293 cells expressing Cer-tagged PLB-WT and mutant constructs with phosphate-buffered saline (PBS; pH 7.4), and treating with Hunter’s buffer on ice (25 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM MgCl$_2$, 1 mM EGTA, 1% Na deoxycholate, 1% Triton X 100, 0.1% SDS, 10% glycerol, and complete protease inhibitor cocktail [Santa Cruz]). The lysates were sonicated and centrifuged at 14,000 rpm at 4°C for 30 min, and the supernatants were boiled in Laemmli buffer containing beta-mercaptoethanol prior to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).
and western blotting analysis. The proteins were transferred onto the PVDF membranes, which were blocked for 1 h with 5% milk in TBS-0.05% Tween 20 (TBST). The membrane was washed with TBST and incubated with anti-PLB mouse monoclonal primary antibody 2D12 (Abcam) at a dilution of 1:2000 at 4°C overnight. After washing with TBST, the membrane was incubated with fluorescent secondary antibody, Alexa Fluor 532 goat-anti-mouse IgG (Invitrogen) at a dilution of 1:10,000 at room temperature for 1 h. The membrane was then scanned using Typhoon Trio with the following settings of acquisition mode: Fluorescence; Emission Filter: 555 BP 20 R6G, HEX, AF532; Laser: Green-532; PMT: 425; Pixel size: 100 microns. The same samples were subjected to western blot analysis using anti-GFP antibody to detect mCer-PLB and anti-β-actin antibody to act as a loading control.

**Immunofluorescence Microscopy**

After a 48 hour period of adenoviral infection, cardiomyocytes in culture expressing CFP-R9C-PLB were fixed in 4% paraformaldehyde solution for 15 minutes, washed with PBS and permeabilized using 0.2% Triton-X-100 for 10 minutes at room temperature. The cells were blocked with 1% bovine serum albumin for 30 min, and then incubated with the anti-PLB mouse monoclonal 2D12 antibody at 1:500 dilution. The cells were incubated with Alexa Fluor 532 goat anti-mouse antibody at 1:1000 dilution for 1 hour at room temperature. The coverslips were washed with PBS and mounted on the microscopic slides using mounting media (Vector laboratories). After immunofluorescence staining, cells were subjected to confocal imaging using an inverted Leica TCS SP5 confocal
microscope with 63× water immersion objective. CFP and Alexa Fluor 532 were sequentially excited at 458 nm and 543 nm to detect the localization of exogenous CFP-R9C-PLB and endogenous WT-PLB respectively.

**Fluorescence Microscopy**

To quantify the membrane localization of PLB, cells were cotransfected with Cer-SERCA and YFP-PLB-truncation mutants at a 1:1 ratio and subjected to confocal imaging using an inverted Leica TCS SP5 confocal microscope with a 63× water immersion objective. Cer and YFP were sequentially excited at 458 nm and 514 nm respectively. The membrane partitioning of YFP-PLB-truncation mutants was evaluated by comparison with Cer-SERCA. The apparent membrane partition coefficient for PLB and all the truncation mutants of PLB was quantified as the ratio of YFP fluorescence intensity in the endoplasmic reticulum (ER) region (perinuclear) to nuclear fluorescence intensity. Solubilization of PLB was also quantified using widefield fluorescence microscopy to measure the loss of fluorescence from cells permeabilized with 100 μg/ml saponin. Cell impermeant nuclear stain propidium iodide (PI) was used to verify permeabilization. Cells expressing Cer-SERCA and YFP-PLB truncation mutants were preincubated with 2 μg/ml PI and imaged for Cer, YFP, and PI fluorescence during the course of saponin permeabilization.

**Statistical Analysis**

Errors are reported as standard error of the mean and statistical significance was evaluated using a standard unpaired two-tailed type 2 Student's T test, where p value < 0.05 was considered significant.
CHAPTER III
ACUTE INOTROPIC AND LUSITROPIC EFFECTS OF CARDIOMYOPATHIC R9C MUTATION OF PHOSPHOLAMBAN

In this study, we investigated the acute physiological consequences of R9C-PLB mutation on Ca\textsuperscript{2+} kinetics and contractility using adenoviral delivery of R9C-PLB to adult rabbit cardiomyocytes. Rabbit ventricular cardiomyocytes are particularly good models of human cardiac Ca\textsuperscript{2+} cycling (Hasenfuss 1998, Pattison et al. 2008, Bers 2002) compared to mice. We reasoned that acute expression might reveal new mechanistic information about R9C pathophysiology, complementing previous transgenesis studies that focused on long-term effects of R9C mutation. To specifically test the consequence of R9C mutation for PLB oligomerization, we modulated PLB oligomerization affinity with mutations of three transmembrane Cys residues to Ser (SSS) (Fig. 5B). This set of mutations has been proposed to abolish PLB oligomerization (Ceholski et al. 2012), and could isolate the effect of R9C from other determinants of PLB oligomerization.

**R9C-PLB Exerts a Positively Inotropic and Positively Lusitropic Effect in Cardiomyocytes**

We subjected AAV-293 cells expressing Cer-tagged PLB-WT and mutant constructs to SDS PAGE followed by western blot analysis. The results indicated that the commercially available PLB-2D12 antibody recognized both WT-PLB
and SSS-PLB, but not R9C-PLB or R9C+SSS-PLB (Fig. 8). This observation is likely due to the fact that the R9C mutation is within the epitope region for the PLB-2D12 antibody (between amino acid residues 9-17 of canine PLB). Since the R9C-PLB and R9C+SSS-PLB constructs were Cer-tagged, they could be easily detected by GFP antibody (Fig. 8). β-Actin served as a loading control (Fig. 8). Furthermore, adenoviral delivery of R9C-PLB tagged with CFP or YFP to enzymatically isolated adult rabbit cardiac myocytes yielded fluorescence detectable by confocal or widefield fluorescence microscopy after 48 hours in culture. We observed PLB localization in the perinuclear region and in longitudinal streaks and cross-striations (Fig. 9A) as previously observed for fluorescently labeled wild-type (WT)-PLB (Bidwell et al. 2011). The fluorescence pattern is consistent with localization in the sarcoplasmic reticulum (SR), and is similar to that previously observed for fluorescently labeled SERCA2a (Bidwell et al. 2011, Pallikkuth et al. 2013). In order to compare the localization of exogenous R9C-PLB with endogenous WT-PLB, we performed immunofluorescence microscopy using anti-PLB mouse monoclonal 2D12 antibody. We observed that the localization of endogenous WT-PLB labeled with Alexa Fluor 532 secondary antibody was similar to CFP-R9C-PLB (Fig. 9A), and demonstrates that exogenous PLB does not fully replace endogenous PLB. Thus, the present experimental system may be considered a model of heterozygous expression of R9C-PLB against a WT-PLB background. R9C-PLB and WT-PLB signals were not completely colocalized, as evidenced from subcellular regions with relatively more CFP or Alexa Fluor 532 signal (Fig. 9A).
Fig. 8. Both Cer-R9C-PLB and Cer-R9C+SSS-PLB are recognized by GFP, but not by PLB 2D12 antibody. AAV-293 cells expressing mCer-PLB WT and mutant constructs of PLB were subjected to SDS PAGE and western blot analysis. β-Actin was used as a loading control.
Figure 9. R9C-PLB expression in cardiomyocytes induces a positively inotropic and positively lusitropic effect. (A) Confocal images of an adult rabbit left ventricular myocyte expressing CFP-R9C-PLB (green) colocalized with endogenous WT-PLB (red). Scale bar = 10 μm. (B, C) Isolated cardiomyocytes expressing YFP-WT-PLB or YFP-R9C-PLB were used to record (B) averaged Ca\(^{2+}\) transients obtained from 8-10 events per cell; WT (n=17), R9C (n=16) and (C) averaged sarcomere shortening traces obtained from 8-10 events per cell; WT (n=9), R9C (n=7). Myocytes were electrically stimulated at increasing pacing frequencies of 0.3, 0.5, or 0.75 Hz.
This is likely due to non-uniform decoration of the cardiac myocytes by the primary or secondary antibodies used to visualize endogenous WT-PLB.

Chronic R9C-PLB expression in transgenic mouse models results in depressed Ca\(^{2+}\) handling and decreased myocyte contractility (Schmitt et al. 2009, Schmitt et al. 2003). To determine the physiological effect of acute R9C-PLB expression after adenoviral delivery, we compared Indo-1 Ca\(^{2+}\) transients and sarcomere shortening kinetics of myocytes expressing YFP-R9C-PLB or YFP-WT-PLB at increasing pacing frequencies of 0.3 Hz, 0.5 Hz, and 0.75 Hz (Fig. 9B, 9C). R9C-PLB-expressing myocytes showed markedly accelerated Ca\(^{2+}\) handling as evidenced from an elevated peak Ca\(^{2+}\) and decreased Ca\(^{2+}\) transient duration compared to WT-PLB-expressing myocytes (Fig. 9B). There was a corresponding increase in contractility for R9C-PLB-expressing myocytes as evidenced from an increased peak amplitude and decreased peak duration compared to WT-PLB-expressing myocytes (Fig. 9C). The baseline for Ca\(^{2+}\) transients and sarcomere length was not significantly different between WT-PLB- and R9C-PLB-expressing cells. We noted that the resting sarcomere length of the cultured myocytes was shorter than the 1.7-1.8 \(\mu m\) value observed for freshly isolated cardiac myocytes as a consequence of two days of maintenance in culture. Ca\(^{2+}\) transient parameters and sarcomere shortening data are summarized in Fig. 10 and Tables 1 and 2. R9C-PLB expressing cells were significantly hyperdynamic compared to those expressing WT-PLB, with a 28% decrease in the Ca\(^{2+}\) transient decay time (Fig. 10A), and a 20% decrease in the peak duration of the Ca\(^{2+}\) transient (Fig. 10C). The corresponding lusitropic effect
Figure 10. Quantification of inotropic and lusitropic effects of R9C-PLB. (A-F) Kinetics of intracellular Ca\textsuperscript{2+} transients and sarcomere shortening of isolated cardiomyocytes expressing YFP-WT-PLB or YFP-R9C-PLB. (A) WT expressing cells responded strongly to increased pacing frequency (black dotted line), whereas R9C expressing cells exhibited a blunted sensitivity to increased pacing frequency (red dotted line). *p < 0.05. Data are mean ± SE.
was evidenced by faster sarcomere relengthening, with a 23% decrease in the relaxation velocity time for R9C-PLB-expressing myocytes (Fig. 10B) and a 19% decrease in the peak duration of the sarcomere shortening transient compared to WT-PLB-expressing myocytes (Fig. 10D).

R9C-PLB Exhibits Blunted Sensitivity to Frequency Potentiation and β-Adrenergic Stimulation

Increasing the pacing frequency enhances cardiac contractility by increasing the rate of SR Ca$^{2+}$ release, also known as ‘force-frequency relationship’ (Endoh 2004, Janssen and Periasamy 2007, Janssen 2010). Frequency potentiation also accelerates cardiac relaxation by increasing the rate of SR Ca$^{2+}$ uptake, also described as ‘frequency-dependent acceleration of relaxation’ (Janssen and Periasamy 2007, Janssen 2010, Varian and Janssen 2007). Interestingly, while WT-PLB-expressing cells showed the expected increase in Ca$^{2+}$ uptake and sarcomere relaxation rate with rapid pacing, there was very little additional enhancement of Ca$^{2+}$ uptake or relaxation of R9C-PLB-expressing cells as pacing frequency increased from 0.3 to 0.75 Hz (Fig. 9B, 9C, Fig. 10). The data suggest that the R9C mutation of PLB maximally stimulates Ca$^{2+}$ uptake and cell relaxation, and additional stimulation by increased pacing frequency provides only a marginal additive effect. The blunted frequency response of the R9C-PLB-expressing cells is highlighted in Fig. 10A with a dashed red line, which may be compared to WT-PLB, dashed black line. We also observed a 52% increase in the amplitude of the peak of the Ca$^{2+}$ transient in R9C-PLB-expressing cells compared to WT-PLB (Fig. 10E), suggesting an
<table>
<thead>
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<tr>
<td><strong>Ca²⁺ transients parameters</strong></td>
<td><strong>WT</strong></td>
<td><strong>R9C</strong></td>
<td><strong>P value</strong></td>
</tr>
<tr>
<td>Baseline</td>
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<td>0.45 ± 0.006</td>
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<td>Departure velocity</td>
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<td>9.682 ± 1.011</td>
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<tr>
<td>Departure velocity time</td>
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<td>0.021 ± 9E-04</td>
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<td>Peak</td>
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<td>Peak amplitude</td>
<td>0.153 ± 0.009</td>
<td>0.233 ± 0.024</td>
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<tr>
<td>Fractional shortening</td>
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<td>51.62 ± 5.089</td>
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<td>Time to peak</td>
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<td>0.136 ± 0.006</td>
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<td>Return velocity</td>
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<td>Return velocity time</td>
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<td>0.478 ± 0.024</td>
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<td>1.141 ± 0.099</td>
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<tr>
<td>Transient decay time constant</td>
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<td>Peak duration</td>
<td>0.556 ± 0.018</td>
<td>0.447 ± 0.024</td>
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Table 1. Summary of quantitative Ca²⁺ transients data. Data are mean ± SE; WT (n=17), R9C (n=16).
<table>
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<td><strong>Sarcomere shortening parameters</strong></td>
<td><strong>WT</strong></td>
<td><strong>R9C</strong></td>
<td><strong>P value</strong></td>
</tr>
<tr>
<td>Baseline</td>
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<td>Contraction velocity</td>
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<td><strong>Peak</strong></td>
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<td>1.381 ± 0.033</td>
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<td>Peak amplitude</td>
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<td>0.165 ± 0.013</td>
<td>0.032</td>
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<tr>
<td>Fractional shortening</td>
<td>6.282 ± 1.174</td>
<td>10.72 ± 0.905</td>
<td>0.013</td>
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<tr>
<td>Time to peak</td>
<td>0.787 ± 0.04</td>
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<td>Relaxation velocity</td>
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<td>Time to 10.0% contraction</td>
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<td>Time to 50.0% contraction</td>
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<td>0.127 ± 0.013</td>
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<td>Time to 90.0% contraction</td>
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<td>Time to 50.0% relaxation</td>
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<td>0.002</td>
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<tr>
<td>Time to 90.0% relaxation</td>
<td>1.635 ± 0.054</td>
<td>1.355 ± 0.117</td>
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<tr>
<td>Relaxation time constant</td>
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<td>Peak duration</td>
<td>1.145 ± 0.046</td>
<td>0.926 ± 0.057</td>
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</table>

Table 2. Summary of quantitative sarcomere shortening data. Data are mean ± SE; WT (n=9), R9C (n=7).
increase in myocyte SR Ca\(^{2+}\) load. This increase in Ca\(^{2+}\) release resulted in positive inotropy, with a 71% increase in myocyte fractional shortening (Fig. 10F). Most of the Ca\(^{2+}\) handling and sarcomere shortening parameters quantified here and in Tables 1 and 2 showed the same pattern of a blunted frequency response, suggesting that R9C-PLB expressing cells were already maximally stimulated, with little additional capacity for frequency dependent lusitropy or inotropy.

Similarly, we observed decreased responsiveness of R9C-PLB expressing cells to β-adrenergic stimulation with isoproterenol (iso). While WT-PLB expressing cells showed a robust increase in peak Ca\(^{2+}\) and a faster Ca\(^{2+}\) transient decay in response to iso (Fig. 11A), R9C-PLB expressing cells were already hyperdynamic and iso caused no additional increase in Ca\(^{2+}\) handling kinetics (Fig. 11B). Instead, we observed a modest decrease in peak Ca\(^{2+}\), possibly as a result of Troponin I phosphorylation or increased Na\(^+\)-K\(^+\)-ATPase activity. Fig. 11C, 11D show the corresponding effects of iso stimulation on sarcomere shortening for WT-PLB and R9C-PLB expressing cells, respectively. Overall, the acute physiological effect of the R9C mutation of PLB is positively inotropic and lusitropic, consistent with a model of disinhibition of SERCA as a result of a loss of inhibitory function for R9C-PLB (Schmitt et al. 2003, Schmitt et al. 2009, Ceholski, Trieber, and Young 2012, Ha et al. 2011).

**R9C-PLB Increased Oligomerization and Decreased SERCA-Binding Despite Pentamer-Destabilization by SSS Mutation**

A previous study in our lab has attributed the loss-of-function character of
Figure 11. R9C-PLB exhibits lack of responsiveness to β-adrenergic stimulation. (A, B) Averaged Ca\textsuperscript{2+} transients (obtained from 8-10 events per cell) recorded from isolated cardiomyocytes expressing (A) YFP-WT-PLB, or (B) YFP-R9C-PLB in the presence [ WT (n=13), R9C (n=7) ] and absence [ WT (n=5), R9C (n=5) ] of 100 nM isoproterenol (iso). (C, D) Averaged sarcomere shortening traces (obtained from 8-10 events per cell) recorded from isolated cardiomyocytes expressing (C) YFP-WT-PLB or (D) YFP-R9C-PLB in the presence [ WT (n=9), R9C (n=4) ] and absence [ WT (n=5), R9C (n=4) ] of 100 nM iso.
R9C to increased PLB oligomerization secondary to oxidative crosslinking of the introduced cysteine in adjacent protomers of PLB pentamers (Ha et al. 2011). To investigate the change in PLB oligomerization energetics in more detail, we compared the relative effects of R9C mutation with substitution of transmembrane Cys residues 36, 41 and 46 with Ser (SSS) (Fig. 5B). The SSS mutation is known to destabilize the PLB pentamer (Fujii et al. 1989), and this mutant runs as a monomer in polyacrylamide gel electrophoresis (PAGE) (Ceholski et al. 2012). We performed E-FRET (Zal and Gascoigne 2004) measurements of large populations of AAV-293 cells (500-1500 cells per experiment). We observed a 12% increase in the average intrapentameric FRET for R9C-PLB compared to WT (Table 3), consistent with previous observations (Ha et al. 2011). SSS-PLB also exhibited FRET, suggesting that despite running as a monomer on SDS-PAGE (Ceholski et al. 2012), the SSS mutant can form pentamers in the membrane environment. However, SSS average FRET was 5% less than WT, suggesting that oligomerization was weakened by the mutations of the transmembrane domain (Table 3). To quantify PLB-PLB binding affinity, we used an in-cell binding assay described previously (Abrol et al. 2014, Bidwell et al. 2011, Ha et al. 2011, Hou and Robia 2010, Hou, Kelly, and Robia 2008, Kelly et al. 2008). The heterogeneous protein expression level of the transiently transfected population of cells provided insight into the dependence of FRET on protein concentration. FRET increased with protein expression up to a maximal level (Fig. 12) that reflected the intrinsic FRET of the pentamer (FRET$_{\text{max}}$). The concentration of protein that yielded half-maximal FRET is a measure of the
apparent dissociation constant ($K_{D1}$) of the PLB pentamer. Thus, the binding curve reveals the relative contributions of oligomerization and protein structure changes to the observed increase in intrapentameric FRET with mutations. As previously observed, R9C increased FRET$_{\text{max}}$, suggesting that the R9C-PLB pentamer had a more compact conformation (Fig. 12A, Table 3). This observation is consistent with a model in which disulfide crosslinking of introduced Cys residues on adjacent protomers brings the cytoplasmic domains and Cer/YFP fusion tags into closer proximity. The R9C mutation also increased the affinity of PLB oligomerization, as shown by a left shift of the binding curve of R9C (Fig. 12A), accounting for 55% decrease in $K_{D1}$ (Fig. 12B) compared to WT. In contrast, destabilization of the PLB pentamer by SSS right-shifted the binding curve compared to WT (Fig. 12A). Notably, addition of the R9C mutation increased the stability of the SSS-PLB pentamers, as shown by a left shift of the R9C+SSS-PLB binding curve (Fig. 12A), and a 29% decrease in $K_{D1}$ (Fig. 12B) relative to SSS. The data are summarized in Fig. 12B and Table 3. We conclude that R9C enhances PLB oligomerization both in WT and SSS background, indicating that R9C potentiates PLB oligomerization even for weakly oligomeric variants.

Increased oligomerization of R9C-PLB had the predicted consequence for binding of R9C-PLB to SERCA. Since SERCA is regulated by monomeric PLB (MacLennan and Kranias 2003, Kranias and Hajjar 2012), depletion of the monomer pool by increased oligomerization was expected to decrease PLB-SERCA binding. Indeed, we observed a right shift of the R9C-PLB-SERCA
<table>
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<tr>
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<th>SSS</th>
<th>R9C + SSS</th>
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<td>Average FRET (%)</td>
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<td>FRET&lt;sub&gt;max&lt;/sub&gt; (%)</td>
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<td>61.3 ± 1.1</td>
<td>55.7 ± 0.7 #</td>
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<td>K&lt;sub&gt;D1&lt;/sub&gt; (AU)</td>
<td>0.27 ± 0.04</td>
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<td>0.69 ± 0.05</td>
<td>0.49 ± 0.03 #</td>
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<td>R (Å)</td>
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<td>ND</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Average FRET (%)</td>
<td>14.2 ± 0.7</td>
<td>11.6 ± 0.5 *</td>
<td>20.8 ± 0.3</td>
<td>17.5 ± 0.5 #</td>
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<td>FRET&lt;sub&gt;max&lt;/sub&gt; (%)</td>
<td>27.2 ± 0.7</td>
<td>25.2 ± 0.8 *</td>
<td>45.1 ± 1.5</td>
<td>45.5 ± 1.5</td>
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<td>K&lt;sub&gt;D2&lt;/sub&gt; (AU)</td>
<td>11.4 ± 0.7</td>
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<td>17.0 ± 1.2</td>
<td>22.6 ± 1.4 #</td>
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<tr>
<td>R (Å)</td>
<td>60.8 ± 0.4</td>
<td>62.0 ± 0.1 *</td>
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Table 3. Summary of quantitative FRET data for the WT, R9C, SSS, and R9C+SSS mutant constructs of PLB. Data are mean ± SE of 4 independent experiments for PLB-PLB FRET and 3 independent experiments for SERCA-PLB FRET. *p < 0.05 vs. WT, #p < 0.05 vs. SSS, ND - not determined.
Figure 12. R9C-PLB causes increased oligomerization and decreased SERCA binding both in WT and SSS backgrounds. (A) In-cell intrapentameric FRET efficiency measurements for WT and mutant constructs of PLB. (B) R9C exhibited a decrease in oligomer dissociation constant ($K_D1$) compared to WT and R9C+SSS showed a decrease in $K_D1$ compared to SSS. Data are mean ± SE of 4 independent experiments; *p < 0.05. (C) In-cell SERCA:PLB FRET efficiency measurements for WT and mutant constructs of PLB. (D) R9C exhibited an increase in the dissociation constant of the SERCA:PLB complex ($K_D2$) compared to WT, and R9C+SSS exhibited an increase in $K_D2$ compared to SSS. Data are mean ± SE of 3 independent experiments; *p < 0.05, AU: arbitrary units.
binding curve (Fig. 12C), and a 29% increase in the apparent dissociation constant $K_D^2$ (Fig. 12D) compared to WT. The data are summarized in Fig. 12D and Table 3. The effect of the SSS mutation was less clear. We expected increased binding of SSS to SERCA vs. WT, but because the binding curve did not saturate, we could not accurately quantify FRET$_{max}$ and $K_D^2$ for this mutant. The failure to saturate may be due to increased non-specific FRET for the more monomeric PLB species (King et al. 2014). While the absolute value of $K_D^2$ is not certain, we did observe the expected relative change with the addition of R9C. Specifically, the combined mutant R9C-SSS showed a right-shifted binding curve relative to SSS (Fig. 12C), with an increase in $K_D^2$ relative to SSS (Fig. 12D). We also observed a decrease in FRET$_{max}$ for R9C relative to WT (Fig. 12C, Table 3). This parameter represents the intrinsic FRET efficiency of the bound PLB-SERCA regulatory complex. A decrease in FRET$_{max}$ suggests that a change in the conformation of the regulatory complex that moves the FRET acceptor (YFP) farther from the donor (Cer). The functional significance of this structure change is not clear, but it is reminiscent of the decrease in PLB-SERCA FRET$_{max}$ observed with phosphomimetic mutations of PLB (Hou, Kelly, and Robia 2008). Overall, we conclude that increased PLB oligomerization reduced SERCA regulation, accounting for the observed hyperdynamic Ca$^{2+}$ handling of the R9C-PLB expressing cardiac myocytes.

**R9C-PLB Exhibits Increased Sensitivity to Oxidative Stress**

To determine the role of Cys oxidation in the observed effect of R9C substitution on PLB oligomerization, cardiac myocytes were treated with 100 $\mu$M
H$_2$O$_2$ (Schroder and Eaton 2008) during observation by fluorescence imaging. Widefield fluorescence microscopy did not reveal a change in the localization of PLB (not shown), and we did not observe any aggregation of protein after treatment with H$_2$O$_2$. However, oxidation significantly increased in the relative emission of YFP/CFP (with CFP excitation). The observed 14% increase in FRET ratio is evident in Fig. 13A (+ H$_2$O$_2$) as a transition to warmer colors, and is quantified in Fig. 13B. There was no change in FRET ratio for cardiac myocytes expressing WT CFP/YFP-PLB (Fig. 13B), consistent with previous observations in AAV-293 cells (Ha et al. 2011). To differentiate the effect of oxidation of cytoplasmic domain Cys-9 from the transmembrane cysteines, we measured PLB-PLB FRET after mutating the three transmembrane cysteines to serine residues (SSS) (Fig. 5B). A quantitative comparison of average FRET efficiency of these mutants in AAV-293 cells showed that the R9C-PLB is already increasingly oligomeric before the addition of H$_2$O$_2$ (29% FRET vs. 26% for WT), and oxidation further increased FRET to a maximum of 35% (Fig. 13C, red). WT-PLB intrapentameric FRET does not increase with oxidation (Fig. 13C, black). SSS (Fig. 13C, green) is likewise unresponsive to H$_2$O$_2$, and average FRET is reduced compared to WT consistent with destabilization of SSS pentamers. Interestingly, the combination of R9C+SSS (Fig. 13C, blue) yields an intermediate level of FRET that is markedly increased by H$_2$O$_2$ oxidation, up to the same maximal 35% FRET efficiency observed for R9C-PLB. The data suggest that oxidation of R9C on the native transmembrane domain or the SSS
Figure 13. R9C-PLB exhibits increased sensitivity to oxidative stress. (A) Fluorescence microscopy images of live adult cardiomyocytes co-expressing CFP-R9C-PLB and YFP-R9C-PLB. Application of a 100 μM H₂O₂ increased FRET, as reflected by an increase in measured FRET ratio. (B) Quantification of (A). R9C-PLB expressed in cardiac myocytes showed a time-dependent increase in intrapentameric FRET after application of 100 μM H₂O₂, whereas the FRET ratio for WT was unaffected; WT (n=8), R9C (n=8). (C) Quantitative FRET for PLB expressed in HEK cells. 100 μM H₂O₂ increased FRET efficiency for both R9C and R9C+SSS, indicating that Cys-9 is the primary cause of oxidation-dependent R9C-PLB oligomerization. Arrows indicate the time of addition of 100 μM H₂O₂. Data are mean ± SE of 3 independent experiments; WT (n=17), R9C (n=15), R9C+SSS (n=11), SSS (n=12).
background results in maximal oligomerization of PLB regardless of the initial level of oligomerization.

We conclude that the primary mechanism of R9C pathology is a phosphomimetic effect of PLB cys-9 oxidation, manifested as increased oligomerization and a change in the structure of the PLB-SERCA regulatory complex. The resultant decrease in the availability of monomeric PLB reduces SERCA regulation, accounting for the inability to respond to frequency potentiation and β-adrenergic stimulation, and eventual heart failure.
CHAPTER IV

PHOSPHOLAMBAN C-TERMINAL RESIDUES ARE CRITICAL DETERMINANTS OF THE STRUCTURE AND FUNCTION OF THE CALCIUM ATPASE REGULATORY COMPLEX

We have previously shown that truncating the C-terminus of PLB midway through its TM domain by L39X mutation greatly reduced PLB oligomerization and SERCA binding (Kelly et al. 2008). In the present study, we investigated the role of C-terminal residues of PLB in membrane anchoring, localization, PLB oligomerization, and SERCA regulation. The results provide insight into the structural and functional consequences of mutating or truncating the TM domain, and reveal an unexpected role for PLB C-terminal residues in determining the quaternary conformation of the PLB-SERCA regulatory complex.

Functional Role of C-Terminal Residues in Regulation of SERCA Function

To investigate the role of C-terminal residues (Fig. 5C) in determining the inhibitory potency of PLB, we reconstituted SERCA with wild-type (WT) or with C-terminal Ala substitutions or truncations of PLB. Previous Ala substitution of individual residues in this region (Val49-Met-Leu-Leu52) revealed little change in inhibitory potency for L52A or M50A mutants and gain-of-function for the L51A and V49A mutants (Triebel, Afara, and Young 2009). C-terminal alanine substitution of all four residues 49-52 resulted in gain of PLB regulatory function and there appeared to be a nexus for gain-of-function at Val49 for both individual
and multiple alanine substitutions (Abrol et al. 2014). In contrast, deletion of these same residues, which shortens the PLB TM helix, resulted in a loss of regulatory function (Abrol et al. 2014). The data indicate that the C-terminal residues of PLB are important determinants for SERCA inhibition and that even small deletions have a particularly deleterious effect.

**Ala-Substitution Mutations of PLB C-Terminal Residues Alter PLB Pentamer Structure and Oligomerization Affinity**

To investigate the role of the C-terminal residues in determining the structure and affinity of the PLB pentamer, we quantified intraoligomeric FRET for mixed pentamers of Cer- and YFP-PLB. Replacement of C-terminal residues with a single Ala substitution generally decreased average FRET efficiency compared to WT ([Fig. 14A, Table 4](#)). To determine the relative contributions of pentamer structure change or a change in the degree of PLB oligomerization to the observed changes in average FRET, we performed an in-cell binding assay in which FRET is quantified from a heterogeneous population of transfected cells expressing a wide range of concentrations of PLB (Abrol et al. 2014, Bidwell et al. 2011, Ha et al. 2011, Hou and Robia 2010, Hou, Kelly, and Robia 2008, Kelly et al. 2008). **Fig. 14B** shows that cells expressing high concentrations of PLB exhibited higher FRET than cells with a low expression level. FRET increased with [protein] to a maximum level (FRET<sub>max</sub>), and this relationship was well-described by a hyperbolic fit. FRET<sub>max</sub> was modestly increased for L52A, the last residue of the helical TM domain of PLB (**Fig. 14C, red**). L51A also shows a small increase in FRET<sub>max</sub> (**Fig. 14C, blue**), but this value must be viewed with
Figure 14. C-terminal alanine substitutions alter the oligomerization affinity and structure of PLB pentamer. The effect of C-terminal alanine substitution mutants of PLB on (A) Average intrapentameric FRET efficiency (B) In cell intrapentameric FRET efficiency (C) FRET\textsubscript{max} for the pentamer (D) Oligomer dissociation constant (K\textsubscript{D}1). *p < 0.05, AU: arbitrary units.
## Table 4: Summary of quantitative FRET data for the alanine substitutions and truncation mutants of PLB

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<th>SERCA-PLB FRET</th>
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</thead>
<tbody>
<tr>
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<td>Average FRET (%)</td>
<td>FRET&lt;sub&gt;max&lt;/sub&gt; (%)</td>
</tr>
<tr>
<td>---------------------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>WT</td>
<td>35.4 ± 0.2</td>
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<tr>
<td>L52A</td>
<td>37.3 ± 0.4</td>
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</tr>
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<td>L51A</td>
<td>28.8 ± 0.4&lt;sup&gt;*&lt;/sup&gt;</td>
<td>46.2 ± 1.0&lt;sup&gt;*&lt;/sup&gt;</td>
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<tr>
<td>M50A</td>
<td>29.3 ± 0.3&lt;sup&gt;*&lt;/sup&gt;</td>
<td>39.6 ± 0.6</td>
</tr>
<tr>
<td>V49A</td>
<td>23.6 ± 0.3&lt;sup&gt;*&lt;/sup&gt;</td>
<td>32.0 ± 0.5&lt;sup&gt;*&lt;/sup&gt;</td>
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<table>
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<th>SERCA-PLB FRET</th>
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<td>Average FRET (%)</td>
<td>FRET&lt;sub&gt;max&lt;/sub&gt; (%)</td>
</tr>
<tr>
<td>------------------------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>WT</td>
<td>42.4 ± 0.3</td>
<td>50.8 ± 0.5</td>
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<td>53.3 ± 0.6</td>
</tr>
<tr>
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<td>54.2 ± 0.6</td>
</tr>
<tr>
<td>M50X</td>
<td>25.1 ± 0.4&lt;sup&gt;*&lt;/sup&gt;</td>
<td>60.9 ± 1.0</td>
</tr>
<tr>
<td>V49X</td>
<td>23.7 ± 0.4&lt;sup&gt;*&lt;/sup&gt;</td>
<td>56.6 ± 2.2</td>
</tr>
<tr>
<td>I48X</td>
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<td>19.9 ± 1.3&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
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<td>9.2 ± 0.8&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>I38X</td>
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<td>11.0 ± 0.4&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>I33X</td>
<td>9.0 ± 0.1&lt;sup&gt;*&lt;/sup&gt;</td>
<td>13.9 ± 0.4&lt;sup&gt;*&lt;/sup&gt;</td>
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**Table 4:** Summary of quantitative FRET data for the alanine substitutions and truncation mutants of PLB: Effect of C-terminal alanine substitutions and truncation mutations on PLB intrapentameric FRET efficiency and SERCA-PLB FRET efficiency. (FRET<sub>max</sub>: maximal FRET efficiency; K<sub>D1</sub>: oligomer dissociation constant; K<sub>D2</sub>: dissociation constant for the SERCA:PLB complex; AU: arbitrary units; R: distance between donor and acceptor fluorophores; ND: not determined). *p < 0.05.
caution as the FRET vs. [protein] relationship never achieves maximal FRET for this mutant (Fig. 14B). A failure to saturate is often observed for highly monomeric mutants of PLB as a result of increased non-specific FRET between non-interacting monomers. Thus, the fitted value of $FRET_{\text{max}}$ is compromised by this non-specific FRET contribution. Consistent with this non-saturating FRET relationship, we observed a right-shift of the L51A FRET vs. [protein] binding curve (Fig. 14B), suggesting a decreased oligomerization affinity. The PLB dissociation constant ($K_D1$) was significantly increased for all Ala mutants, indicating a decreased oligomerization affinity. We observed a >1.5-fold increase in $K_D1$ for L52A and a >4-fold increase in $K_D1$ for L51A compared to WT (Fig. 14D), as quantified from a hyperbolic fit of the data in Fig. 14B. Next, moving up the TM helix, we investigated the effect of an M50A substitution. This mutant showed no change in pentamer structure (Fig. 14C), but a 1.5-fold increase in $K_D1$ (Fig. 14D), as seen from the right-shifted binding curve of M50A (Fig. 14B, pink) relative to WT (Fig. 14B, black) without a change in maximal FRET. The data suggest that the mutant destabilizes oligomerization without altering the structure of the pentamer. Finally, V49A showed a very large decrease in $FRET_{\text{max}}$ (Fig. 14C) consistent with an increase in the average separation of N-terminal fluorescent tags of 3Å, and a 40% increase in the affinity of oligomerization for this mutant (Fig. 14D). The results are summarized in Table 4. The data demonstrate that the position of the fluorescent protein, fused to the N-terminus (on the cytoplasmic side of the bilayer), is altered by substitution of Leu or Val with Ala at remote sites in the C-terminus (on the luminal side of the
Mutation of PLB C-terminal Residues Alters Regulatory Complex Quaternary Structure and PLB-SERCA Binding Affinity

To determine how N-terminal residues affect the structure and affinity of the PLB-SERCA regulatory complex, we measured FRET from the N-terminal Cer tag on SERCA2a to YFP-PLB. Fig. 15A shows that average FRET was increased by L51A, and V49A mutants. In-cell binding assays revealed the relative changes in binding affinity and structure for these mutants (Fig. 15B). As observed in the oligomerization binding assay (Fig. 14B), L51A showed poor saturation (Fig. 15B) consistent with non-specific FRET from an increased population of monomers (increased K\textsubscript{D1}, Fig. 14D). Thus, the FRET\textsubscript{max} value for this mutant is not a clear representation of regulatory complex structure. The other mutant with a significant change in SERCA-binding was V49A which showed a 23% increase in FRET\textsubscript{max}, suggesting a very compact regulatory complex conformation, and a 73% decrease in dissociation constant K\textsubscript{D2} (Fig. 15D), indicating an increase in the apparent affinity for SERCA. The observed increase in SERCA binding is in harmony with previous reports that suggest increase inhibitory potency for this mutant (Chen et al. 2006, Akin et al. 2013).

C-terminal Residues are Critical for PLB Membrane Localization

A previous study in our lab observed solubilization of PLB by the human heart failure missense mutation L39X (Kelly et al. 2008), and we anticipated that smaller C-terminal deletions of the PLB TM domain could likewise disrupt anchoring of the protein in the membrane. To verify this, relative partitioning of
**Figure 15.** C-terminal alanine substitutions alter binding affinity and structure of SERCA-PLB regulatory complex. The effect of C-terminal alanine substitution mutants of PLB on (A) Average SERCA-PLB FRET efficiency (B) In cell SERCA-PLB FRET efficiency (C) FRET$_{\text{max}}$ for the SERCA-PLB complex (D) Dissociation constant for the SERCA:PLB complex (K$_D$). *p < 0.05, AU: arbitrary units.
PLB in the aqueous cytoplasm and the ER bilayer was assessed with confocal microscopy. While single Ala substitutions did not significantly alter the localization of PLB expressed in AAV-293 cells (not shown) we observed a significant degree of mislocalization of PLB with C-terminal truncations, as shown in Fig. 16. An overlay of images revealed co-localization of Cer-SERCA (Fig. 16A, cyan), and YFP-WT-PLB truncation mutants (Fig. 16A, yellow). Successive truncations caused mislocalization of PLB to the cytoplasm and nucleus (Fig. 16A), as quantified from the apparent membrane partition coefficient [ratio of ER (perinuclear) fluorescence to nuclear fluorescence)] (Fig. 16B). Interestingly, deleting only one C-terminal residue of PLB of the 22 TM domain residues was sufficient to cause partial loss of ER membrane localization. Deletion of more than 4 residues nearly abolished ER localization. Cer-SERCA localization was not changed (Fig. 16A).

To determine whether the observed mislocalization was due to solubilization of PLB, we selectively permeabilized the plasma membrane of cells using saponin (Kelly et al. 2008). Cer-SERCA ER localization was not changed during the course of saponin permeabilization, but truncated YFP-PLBL39X rapidly diffused out of the cell into the surrounding medium (Fig. 16C). As expected, the degree of PLB solubilization depended upon the severity of the PLB truncation. Longer PLB constructs left residual fluorescence in cells after permeabilization (Fig. 16D, 17A-17H). Loss of PLB truncation mutants after saponin permeabilization is summarized in Fig. 16D, and Fig. 17A-17H. We observed a progressive increase in solubilization of PLB upon subjecting the C-terminus of
Figure 16. Progressive truncation of the C-terminal residues of PLB resulted in increased mislocalization to the cytoplasm and nucleus. (A) Confocal microscopic images of AAV-293 cells expressing Cer-SERCA (top) cotransfected with YFP-PLB truncation mutants (middle). Overlay of Cer-SERCA and YFP-PLB truncation mutants (bottom). Cer-SERCA served as a marker for
ER membrane localization. Scale bar = 5 µm. (B) Successive truncations caused a progressive decrease in apparent membrane partition coefficient (ratio of ER fluorescence/nuclear fluorescence). Error bars represent cell-to-cell variability; an average of 40 to 50 cells were used for each mutant. (C) Plasma membrane permeabilization resulted in complete loss of YFP-PLB^{L39X}, no change in Cer-SERCA fluorescence, and increased propidium iodide staining. (D) Diffusion of YFP-PLB truncation mutants from cells after saponin permeabilization. (E) Successive truncations lead to decrease in partition equilibrium constants estimated either by localization ($K_L$) or permeabilization ($K_P$). AU: arbitrary units. (F) There was good agreement between $K_L$ and $K_P$. 
Figure 17. Truncation of the C-terminal residues of PLB increased solubilization of PLB. Fluorescence microscopic images of cells cotransfected with Cer-SERCA and YFP-PLB truncation constructs. Selective plasma membrane permeabilization resulted in no change in Cer-SERCA fluorescence, and increased propidium iodide staining for all the constructs. Note the changes in YFP-PLB fluorescence after saponin permeabilization. (A) WT, (B) L52X; (C) L51X, (D) M50X; (E) V49X, (F) L48X, (G) I38X, and (H) I33X.
PLB to truncation mutations (Fig. 17). Cells coexpressing Cer-SERCA and YFP-PLB WT or truncation constructs including L52X, L51X, M50X, V49X, L48X, I38X, or I33X were selectively permeabilized as shown with propidium iodide. Cer-SERCA fluorescence was unchanged, and propidium iodide staining for all the truncation constructs was increased indicating efficient permeabilization (Fig. 17A-17H). We observed that the more residues that were truncated from the C-terminus of PLB, the greater the degree of solubilization as indicated by progressive decrease in YFP-PLB fluorescence after saponin permeabilization (Fig. 17A-17H).

As expected, molecular dynamic simulation studies of the PLB pentamer structure revealed that the C-terminal domain of the WT protein was retained in the bilayer, while the C-terminal domain of the L39X mutant translocated out of the bilayer within a few ns and was fully solubilized by the end of the simulation (Abrol et al. 2014). Furthermore, we subjected mCer-PLB WT and truncation mutant protein samples to SDS PAGE followed by western blot analysis using anti-GFP antibody. The results of western blot analysis showed the expected mobilities for truncation mutants relative to full length PLB-WT (Fig. 18), indicating that the observed solubilization of PLB truncation mutants is not due to proteolysis. The dual bands were observed for the tagged monomer due to differential migration of folded and aggregated GFP fusion proteins. Additionally, the apparent membrane localization of PLB mutants was quantified by dividing the ER fluorescence (membrane-bound PLB) by the total fluorescence (nuclear + ER), or by dividing the residual PLB fluorescence after saponin permeabilization
Figure 18. The expressed PLB truncation mutants are not degraded. SDS PAGE and western blot analysis showed the expected mobilities for truncation mutants relative to full length PLB-WT, indicating that the observed solubilization of PLB truncation mutants is not due to proteolysis. Arrows indicate the presence of monomeric YFP-PLB (m) and pentameric species (p).
<table>
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<tr>
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<td>$0.39 \pm 0.01$</td>
<td>$0.04 \pm 0.01$</td>
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**Table 5: Summary of quantitative localization data:** Effect of deleting the C-terminal residues of PLB on partition equilibrium constants estimated either by localization ($K_L$) or permeabilization ($K_P$).
by total PLB fluorescence before permeabilization. Partition equilibrium constants estimated by the localization and permeabilization methods were referred to as $K_L$ and $K_P$, respectively. Truncations of PLB C-terminus decreased both $K_L$ and $K_P$ (Fig. 16E). The alternative methods were in good agreement with a linear relationship between $K_L$ and $K_P$ (Fig. 16F), though $K_L$ exhibited a non-zero offset due to the contribution of non-membrane bound PLB to the ER fluorescence signal. The data are summarized in Table 5.

**C-terminal Residues are Also Important for PLB Oligomerization**

Deletion of any of the PLB C-terminal residues greatly decreased PLB-PLB binding in live cells, as quantified by FRET between Cer and YFP fused to PLB N-termini. Fig. 19A shows that PLB average intrapentameric FRET efficiency was progressively decreased upon truncating the C-terminal residues of PLB. Truncation of 4 C-terminal residues of the 22 amino acid TM domain of PLB (V49X) resulted in a 44% decrease in the average FRET efficiency (Fig. 19A). FRET was largely abolished by truncation of more than 4 residues. Thus, the FRET data underscore the importance of Val-49 in determining PLB structure/function. In particular, we used the in-cell binding assay to quantify the relative contributions of altered binding and pentamer conformational change to the observed FRET change. Truncating the C-terminal residues of PLB resulted in a progressive decrease in PLB intrapentameric FRET efficiency as shown in Fig. 19B. $FRET_{\text{max}}$ was unchanged for L52X, L51X, M50X, or V49X compared to WT, indicating that PLB quaternary structure was not significantly affected upon truncating up to 4 C-terminal residues (Fig. 19C, Table 4). This is in contrast
Figure 19. C-terminal truncations of PLB result in progressive loss of PLB oligomerization (A) Progressive truncation of C-terminus of PLB resulted in decrease in average FRET efficiency. (B) In cell intrapentameric FRET efficiency measurements for WT and truncation mutant constructs of PLB. (C) Truncating more than 4 residues resulted in decrease in FRET<sub>max</sub>. (D) C-terminal truncations resulted in a progressive increase in oligomomer dissociation constant (K<sub>D1</sub>). *p < 0.05, AU: arbitrary units.
with Ala substitution mutants, several which profoundly affect PLB oligomer structure (Fig. 15C, Table 4). Truncating more than 4 residues of the 22 amino acid long TM domain significantly decreased FRET_{max}. We attribute this to loss of membrane anchoring, rather than a change in pentamer structure. C-terminal truncations also progressively increased the pentamer dissociation constant K_D1, indicating a decrease in the affinity of PLB oligomerization (Fig. 19D). Truncating only one C-terminal residue resulted in 2-fold increase in K_D1 compared to WT, and binding affinity worsened with each additional residue that was deleted. We quantified a 12-fold increase in K_D1 for V49X compared to WT indicating a decrease in oligomerization affinity. The affinity for the shortest truncation mutants was too low to measure. The data are summarized in Table 4. PLB-PLB binding is reduced by loss of C-terminal residues, and this loss of binding parallels the loss of bilayer anchoring. Predictably, colocalization of PLB protomers in the membrane is prerequisite to oligomerization.

**PLB C-terminal Residues are Critical for Regulatory Complex Structure and Function**

To determine how the C-terminal truncation of PLB affects its interaction with SERCA, we quantified FRET from Cer-SERCA to YFP-tagged truncation mutants of PLB. Surprisingly, truncation of up to 4 C-terminal residues (out of 22 residues in the TM domain) significantly increased SERCA-PLB FRET efficiency (Fig. 20A, 20B). This was not due to increased binding of PLB to SERCA since K_D2 predictably increased with deletion of C-terminal residues (decreased binding affinity) (Fig. 20D). Truncating only one residue resulted in a nearly 2-fold
Figure 20. C-terminal truncations of PLB alter binding affinity and structure of SERCA-PLB regulatory complex (A) Truncation of up to 4 C-terminal residues of PLB resulted in increased average FRET efficiency. (B) In cell SERCA-PLB FRET efficiency measurements for WT and truncation mutant constructs of PLB. Truncation of up to 4 C-terminal residues of PLB resulted in (C) increased FRET$_{\text{max}}$, and (D) increased dissociation constant for the SERCA-PLB complex ($K_\text{D}$). *p < 0.05, AU: arbitrary units.
increase in $K_D$2, truncating 4 residues (V49X) caused an approximately 2.5-fold increase in $K_D$2, and removing more than 4 residues resulted in a binding affinity that was too low to measure (Fig. 20D). It appears that despite the decrease in binding of truncated PLB for SERCA, the average FRET is still increased because the remaining regulatory complexes have a higher intrinsic FRET efficiency ($FRET_{\text{max}}$). Deleting only one C-terminal residue of PLB resulted in a 1.2-fold increase in $FRET_{\text{max}}$, and truncating 4 C-terminal residues (V49X) resulted in a greater than 2-fold increase in $FRET_{\text{max}}$ (Fig. 20C), consistent with an increase in the average separation of N-terminal fluorescent tags of 11.3 Å. The data suggest that that C-terminal truncations alter the structure of PLB:SERCA regulatory complex (Table 4). The structural effect of C-terminal deletions mimics the regulatory complex conformational change due to 49A (Fig. 15C), which also decreased the separation of fluorescent protein tags positioned at least 40 Å away on the other side of the bilayer.

We conclude that the luminal residues nearest the PLB C-terminus are critical for membrane anchoring and quaternary structure determination of both the PLB pentamer and the PLB-SERCA regulatory complex. The loss of membrane registration restraint by C-terminal residues (especially V49) causes displacement of PLB to an alternative position on SERCA. The data suggest multiple modes of binding for PLB on SERCA, and are compatible with a model in which PLB binds to the canonical inhibitory binding site and an additional novel site as discussed in Fig. 21, Chapter V.
CHAPTER V

DISCUSSION

In the cardiac muscle, PLB plays a predominant role in the regulation of Ca\textsuperscript{2+} homeostasis and contractility (MacLennan and Kranias 2003, Kranias and Hajjar 2012). The equilibrium between PLB homopentamer and monomer plays a critical role in regulating SERCA function and physiology of heart muscle (Robia et al. 2007, Kranias and Hajjar 2012) Notably, several hereditary mutations of PLB have been identified in humans to cause DCM and HCM, eventually resulting in heart failure (Medeiros et al. 2011). Using live cells, we studied the structural and functional consequences of two naturally-occurring human heart failure mutants of PLB including R9C and L39X. In addition, to identify the role of TM domain of PLB in membrane anchoring, PLB oligomerization, and SERCA regulation, we subjected the C-terminus of PLB to alanine substitutions and truncation mutations. In particular, we investigated the effect of these mutations on membrane localization, structure and oligomerization affinity of the PLB pentamer, structure and binding affinity of SERCA-PLB regulatory complex and function of SERCA-PLB regulatory complex.

PLB Mutations and Membrane Localization

PLB is an integral SR membrane protein that binds and inhibits cardiac Ca\textsuperscript{2+} pump, SERCA2a. We reasoned that localization of PLB in the membrane is
a prerequisite for PLB oligomerization. Two of the most common human mutations in PLB linked to heart failure involve a single residue deletion (Arg14-deletion) (DeWitt et al. 2006, Haghighi et al. 2006, Posch et al. 2009) and a multiple residue truncation (Leu39-stop) (Haghighi et al. 2003, Chiu et al. 2007, Landstrom et al. 2011). Since a large portion of the C-terminus of PLB is missing in this latter truncation variant, we wished to understand the comparative effects of mutations and truncations in this region. We studied the role of C-terminal residues in determining membrane localization of PLB by subjecting the C-terminus of PLB to alanine substitution and truncation mutations. While substitutions appeared to be benign for localization, we observed that C-terminal deletions including heart failure mutant L39X decreased PLB membrane anchoring (Fig. 21B, iv), releasing a fraction of the PLB into the cytoplasm, where it can no longer participate in regulatory interactions. Although SERCA coexpression has been shown to improve localization of sarcolipin truncation mutants (Gramolini et al. 2004), PLB localization was not improved by coexpression of SERCA in the present work or in previous studies (Butler et al. 2007, Stenoien et al. 2007). Previously, the C-terminal region of PLB region has been shown to be important for subcellular trafficking, as increasing the length of the TM domain of PLB by adding 4 extra leucine residues to the C-terminus resulted in mistargeting to the plasma membrane (Butler et al. 2007). Similarly, the C-terminal RSYQY sequence of the related SERCA regulator sarcolipin was shown to mediate its retention in the ER (Gramolini et al. 2004). The present results demonstrate an additional role for the PLB C-terminus in protein
localization. It is noteworthy that small deletions are so poorly tolerated. Although loss of only one C-terminal residue from the 22 amino acid TM domain of PLB resulted in significant disruption of membrane localization, deletion of 4 or more residues completely abolished localization. In accordance, deletion of 14 out of 22 TM residues in the heart failure mutant L39X completely abolished PLB membrane localization, which was further supported by molecular dynamic simulation studies (Abrol et al. 2014). This study highlights that despite the presence of many other hydrophobic residues in the PLB TM domain, luminal residues nearest the PLB C-terminus are critical for membrane anchoring.

**PLB Mutations Alter the Structure and Oligomerization Affinity of PLB Pentamer**

In the SR membrane, PLB homopentamer exists in dynamic equilibrium with the monomeric species that binds and inhibits SERCA. Maintenance of this dynamic equilibrium between PLB monomer and pentamer is critical for SERCA regulation, and thus, cardiac Ca\(^{2+}\) cycling and contractile function. We studied the effect of mutations in PLB on monomer-pentamer equilibrium and oligomerization affinity. Indeed, deletion and substitution mutations had distinct effects on PLB oligomerization. While deletions decreased PLB-PLB binding (Fig. 21B-i) without altering the pentamer structure, alanine substitutions increased or decreased PLB pentamer FRET\(_{\text{max}}\) consistent with a structural change (Fig. 21A-i) that alters the distance between FRET pairs. The structural details of this putative change in pentamer quaternary conformation are not clear. One possibility is that C-terminal substitutions alter PLB topology or perturb the
structural equilibrium of PLB between T and R states (Karim et al. 2006, Traaseth, Thomas, and Veglia 2006). It is noteworthy that the relatively conservative substitution of Val49-to-Ala exerts an effect over such a long distance, altering the position of fluorescent probes that are more than 40 Å away (Fig. 5). Focusing on the heart failure mutants, truncating 14 residues of the 22 amino acid long TM domain in the L39X mutant significantly decreased FRET$_{\text{max}}$, which can be attributed to loss of membrane anchoring, rather than a change in pentamer structure. In addition, the affinity of PLB oligomerization for the L39X mutant was too low to measure. Furthermore, substitution of Arg9 by Cys residue in the R9C mutant altered the PLB pentamer conformation (Fig. 22A), and increased PLB oligomerization affinity (Fig. 22B), shifting the equilibrium towards the pentamer. Thus, specific mutations in PLB alter the equilibrium between PLB monomer and pentamer. Our study highlights that mutations that cause a shift in the PLB-monomer:pentamer equilibrium result in a change in structure and oligomerization affinity of the PLB pentamer.

**PLB Mutations Alter the Structure and Binding Affinity of SERCA-PLB Regulatory Complex**

The monomer-pentamer equilibrium plays a direct role in regulation of SERCA and may affect the structure and binding affinity of SERCA-PLB regulatory complex. We investigated the structural consequences of alanine substitution and truncation mutations of PLB on SERCA-PLB regulatory complex. Although determining the true quantitative affinity of monomeric PLB for SERCA is complicated by oligomerization (Kelly et al. 2008) and differential protein
localization, it is clear that substitution and deletion mutations exert opposite effects on the apparent affinity of PLB for SERCA. V49A in particular shows a significant increase in SERCA binding (Fig. 21A, ii), while deletions of residues progressively decreases regulatory complex formation (Fig. 21B, ii). Both deletions and substitutions of C-terminal residues caused large changes in the regulatory complex structure (Fig. 21A and 21B, iii). Deleting only one C-terminal residue of PLB resulted in a 1.2-fold increase in FRET$_{\text{max}}$, which corresponds to a distance change from 61.9 Å to 58.9 Å (Table 4). Truncating 4 C-terminal residues (V49X) increased FRET$_{\text{max}}$ by more than 2-fold, which corresponds to a 11.3 Å decrease in the distance between donor and acceptor fluorophores (Table 4). Similarly, some Ala substitutions increased regulatory complex intrinsic FRET, with V49A showing a 23% increase in FRET$_{\text{max}}$, corresponding to a decrease in donor-acceptor distance of 3.3 Å. The magnitude of these changes in FRET distance may be appreciated by considering that functionally significant phosphomimetic mutations altered probe separation distance by only 4 Å (Hou, Kelly, and Robia 2008). One possible explanation for the observed structure change is that substitutions and deletions may cause misregistration of PLB in the inhibitory cleft (Fig. 21A and 21B, iii). Translocation of the TM domain could permit cytoplasmic domain repositioning, accounting for the observed increase in FRET from a donor fluorophore on the SERCA N-terminus. This study reveals that Val49 appears to be particularly important in setting the registration of the TM helix in the bilayer, affecting the disposition of the adjacent TM domain and the more distant PLB cytoplasmic domain
positioned on the other side of the bilayer, more than 40 Å away. As expected, the heart failure mutant L39X exhibited a profound decrease in SERCA-PLB FRET$_{max}$, and the affinity of SERCA-PLB regulatory complex for too low to measure, indicating lack of SERCA regulation. We also evaluated the effect of heart failure mutation, R9C on the regulatory complex structure. The R9C mutation also altered the structure of SERCA-PLB regulatory complex (Fig. 22D), and exhibited decreased binding of PLB to SERCA (Fig. 22C). However, this may be an indirect effect of decreased availability of monomeric PLB, rather than a change in the intrinsic affinity of PLB for SERCA. In conclusion, both the heart failure mutants of PLB including L39X and R9C exhibit decreased SERCA binding and alter the structure of the SERCA-PLB regulatory complex.

**PLB Mutations Alter the Function of SERCA-PLB Regulatory Complex**

To establish disruption of PLB monomer-pentamer equilibrium as the primary mechanism of cardiac Ca$^{2+}$ mishandling, it is important to evaluate the functional consequences of PLB mutations on SERCA activity. Given the extreme sensitivity of PLB pentamer and regulatory complex structure to mutations, we expected a robust alteration of PLB function and SERCA activity. Alanine substitution has been a common mutagenesis strategy for understanding how residues of PLB influence the functional regulation of SERCA (Kimura et al. 1996, Trieber, Afara, and Young 2009, Ceholski, Trieber, and Young 2012). This method effectively removes the side chain of individual residues, thereby providing information on their role in SERCA inhibition by PLB. In this study, we investigated the functional consequences of the structural changes induced by
alanine and truncation mutations. In measurements of SERCA ATPase activity in the absence and presence of PLB, individual or multiple Ala substitutions revealed a nexus for gain-of-function at Val49 of PLB (Abrol et al. 2014). The gain-of-function was an unexpected outcome, and it contrasted sharply with the deleterious effects of truncating these last few residues. Removal of just one residue severely reduced PLB function, and removal of two or more residues completely eliminated PLB function.

The most striking difference between the substitution and deletion mutants is the disparate effects on PLB inhibitory function. The loss-of-function character of the deletion mutants may be due in part to decreased membrane anchoring (Fig. 21B, iv) and decreased SERCA binding (Fig. 21B, ii). Alternatively, the putative misregistration of the PLB TM domain in the SERCA regulatory cleft may result in a non-inhibitory interaction. We and others have previously provided evidence that PLB and SERCA can interact in a non-inhibitory complex in the presence of Ca\(^{2+}\) (Bidwell et al. 2011, Mueller et al. 2004, Li, Bigelow, and Squier 2004) or after PLB phosphorylation (Pallikkuth et al. 2013). This physiological relief of inhibition also alters the affinity and structure of the PLB-SERCA complex (Pallikkuth et al. 2013, Bidwell et al. 2011, Mueller et al. 2004, Li, Bigelow, and Squier 2004). In contrast to deletion mutants or physiological regulation, the structure change induced by L51A or V49A mutation is strongly gain-of-function (Abrol et al. 2014). This observation is compatible with other groups’ previous observations. Although V49A was initially reported to be a loss-of-function mutation (Kimura et al. 1997, Minamisawa et al. 1999), subsequent
studies have demonstrated that V49A is a gain-of-function mutation (Chen et al. 2006). A possible mechanism for this was revealed by X-ray crystallography, which suggests that Val49 encounters steric hindrance from SERCA residue Val89 (Akin et al. 2013). The present data support the hypothesis that replacing PLB Val49 with a smaller residue can alleviate this hindrance, increasing the affinity and potency of the inhibitory interaction (Haghighi et al. 2001, Chen et al. 2006, Akin et al. 2013). It is noteworthy that deleting Val49 does not produce the same result, rather the affinity of the interaction is decreased, and even small deletions of the C-terminal residues result in loss of inhibitory function.

Next, we evaluated the functional consequences of the R9C mutation of PLB. Impaired regulation of SERCA by the R9C mutant results in blunted sensitivity to local regulation (frequency potentiation) and humoral (β-adrenergic) stimulation. This fundamental lack of responsiveness to physiological stress leads to pathological remodeling and heart failure caused by this mutant. The failing heart suffers from prevailing oxidative stress (Choudhary and Dudley 2002), exacerbating the oxidative modification of R9C-PLB that initiated the pathological pathway.

**Chronic vs. Acute Effects of R9C Mutation of PLB**

Previous studies of R9C-PLB showed that R9C-PLB is loss-of-function with respect to SERCA inhibition (Schmitt et al. 2003, Schmitt et al. 2009, Ha et al. 2011, Ceholski, Trieber, and Young 2012). Such *in vitro* observations lead to the expectation of enhanced SERCA activity and positive inotropy/lusitropy *in vivo*, but this is not observed in transgenic models or in human patients. Specifically,
R9C-PLB transgenic mouse myocytes show slower Ca\textsuperscript{2+} handling kinetics (Schmitt et al. 2003, Schmitt et al. 2009) compared to WT. We reasoned that chronic exposure to R9C-PLB may elicit compensatory changes and the long-term disease evolution may not reveal the fundamental mechanistic defect. To investigate the acute effect of R9C mutation of PLB, we introduced the mutated protein to rabbit cardiac myocytes using an adenoviral vector delivery. On a timescale of hours to days, the physiological effect of R9C-PLB is as predicted from its loss-of-function character, with YFP-labeled R9C-PLB expressing cardiac myocytes showing positive inotropy and lusitropy compared to YFP-WT-PLB expressing myocytes (Fig. 9, Fig. 10). Thus, the impaired hemodynamics of the mutant mouse may be long term consequences of secondary changes such as decreased SERCA2a mRNA and protein expression, progressive intracellular stress responses, cardiac remodeling, apoptotic signaling (Gramolini et al. 2008), or other changes in gene expression or cell/organ structure that evolve over weeks or months. We hypothesize that the fundamental pathological triggers of this disease progression is blunted sensitivity to frequency potentiation and β-adrenergic stimulation, such as is observed in the present acute physiological measurements (Fig. 11). These two mechanisms are the main physiological means of regulation of cardiac performance, and the lack of a functional response to stress is expected to induce cardiac remodeling and related decompensation processes. We conclude that transgenesis provides insight into the progression of R9C-PLB disease (and heart failure more generally), while acute delivery of mutant PLB provides insight into the fundamental Ca\textsuperscript{2+}-handling
Defects that initiate this disease process.

**Disordered Structure/Function Mechanisms of R9C-PLB**

The molecular basis of R9C-PLB pathology is of great interest and several possible mechanisms have been proposed. There is an emerging consensus that R9C-PLB is loss-of-function with respect to inhibition of SERCA (Schmitt et al. 2003, Schmitt et al. 2009, Ha et al. 2011, Ceholski, Trieber, and Young 2012), resulting in dysregulation of calcium cycling (Schmitt et al. 2003, Schmitt et al. 2009, Gramolini et al. 2008). There is also evidence for impaired phosphorylation of R9C-PLB by PKA (Schmitt et al. 2003, Schmitt et al. 2009, Ha et al. 2011, Ceholski et al. 2012). However, a cytoplasmic domain fragment of R9C-PLB was phosphorylated by PKA with normal kinetics (Ha et al. 2011), suggesting that there is not an intrinsic defect of PKA recognition of the R9C-PLB substrate. Schmidt et al. observed increased co-immunoprecipitation of R9C-PLB with PKA (Schmitt et al. 2003), suggesting the mutated PLB physically traps and inactivates the kinase, leading to impaired signaling and heart failure. While other studies have failed to detect PKA cross-linking to R9C-PLB (Ha et al. 2011), it is possible that PKA precipitation occurs secondary to other aggregation events, as discussed below. Other possible mechanisms for R9C-PLB pathology include decreased open probability of a putative PLB channel (Smeazetto et al. 2013), or disruption of hydrophobic balance of the PLB cytoplasmic domain leading to loss of inhibitory function (Ceholski, Trieber, and Young 2012). In addition, Gramolini et al. reported that the impaired Ca^{2+} handling by the R9C mouse may be a long term consequence of secondary changes such as decreased
SERCA2a mRNA and protein expression, or activation of the endoplasmic reticulum stress response, cytoskeletal remodeling, and apoptosis (Gramolini et al. 2008). In addition to these diverse pathological mechanisms, we have previously proposed that the introduced Cys residue may induce pathological intersubunit cross-linking, stabilizing the R9C-PLB pentamer (Ha et al. 2011). Evidence for this mechanism includes an increase in intrapentameric FRET for the R9C-PLB mutant (Fig. 12A, Fig. 13), which is further enhanced by oxidative stress (Fig. 13). In this regard, R9C-PLB crosslinking may mimic one of the functional effects of PLB phosphorylation, which also enhances PLB oligomerization (Simmerman and Jones 1998, Hou, Kelly, and Robia 2008, Wegener and Jones 1984). The expected functional consequence of increased PLB oligomerization is to reduce the availability of the inhibitory monomeric species, decreasing SERCA inhibition. The acute physiological result of decreased SERCA inhibition is apparent in the present experiments as a positively inotropic and lusitropic effect (Fig. 9, Fig. 10). The data are consistent with our previous observations in AAV-293 cells (Ha et al. 2011), and in the present study we extend that analysis using pentamer-destabilizing mutations of the transmembrane domain (Fujii et al. 1989) to more clearly isolate the effect of R9C in stabilizing PLB pentamers. SSS-PLB has been presumed to be a fully monomeric mutant based on SDS-PAGE mobility (Ceholski et al. 2012), but we demonstrate here that SSS-PLB can still form low affinity pentamers (Fig. 12A). Combining SSS and R9C mutations yielded an oxidation-sensitive mutant with a basal level of oligomerization that was intermediate between WT-PLB and SSS-
PLB (Fig. 13C). After oxidation with \( \text{H}_2\text{O}_2 \), SSS-R9C-PLB FRET increased dramatically, achieving the same final value as R9C-PLB with a wild-type transmembrane domain (Fig. 13C). The data suggest that the degree of oligomerization is dominated by cross-linking of cytoplasmic Cys-9 residue of the R9C-PLB pentamer.

**R9C Acts as a Phosphomimetic Mutation of PLB**

The transgenic expression of R9C-PLB in mouse hearts recapitulates many aspects of human DCM (Schmitt et al. 2003, Schmitt et al. 2009), but the fundamental molecular mechanism underlying the role of R9C-PLB in SERCA regulation is still unclear. Proposed mechanisms include trapping of PKA (Schmitt et al. 2003), disruption of PLB phosphorylation (Schmitt et al. 2003, Schmitt et al. 2009, Ha et al. 2011, Ceholski et al. 2012), loss of PLB inhibitory function (Schmitt et al. 2003, Schmitt et al. 2009, Ha et al. 2011, Ceholski et al. 2012, Ceholski, Trieber, and Young 2012). Notably, several studies have suggested that the R9C mutation mimics PLB phosphorylation by partial unfolding of the cytoplasmic helix resulting in decreased helical conformation (Paterlini and Thomas 2005), or detachment of PLB cytoplasmic domain from membrane surface (Yu and Lorigan 2013, 2014). Moreover, we have previously proposed that R9C mutation induces oxidation-dependent cross-linking of adjacent R9C-PLB protomers (Ha et al. 2011). This could also mimic the effect of PLB phosphorylation in increasing oligomerization (Wegener and Jones 1984, Simmerman and Jones 1998, Hou, Kelly, and Robia 2008), depleting the active inhibitory monomeric species and relieving SERCA inhibition. Another aspect of
R9C mutation that is reminiscent of PLB phosphorylation is the small decrease in FRET<sub>max</sub> for the R9C-PLB-SERCA regulatory complex (Fig. 12C, Table 3), a difference that did not achieve statistical significance in a previous study (Ha et al. 2011). The change in FRET<sub>max</sub> suggests a conformational change that increases the distance between the donor and acceptor fluorescent probes. This is similar to the structure change that results from phosphomimetic mutations of the PKA site (Ser-16) and CaMKII site (Thr-17) in the PLB cytoplasmic domain (Hou, Kelly, and Robia 2008). The R9C-dependent structure transition and the phosphorylation-dependent conformational change are similar in direction and magnitude (+1.2 Å and +4 Å, respectively). A regulatory complex structure change is the primary mechanism for relief of inhibition of SERCA by PLB phosphorylation (Dong and Thomas 2014, Gustavsson et al. 2013). How R9C mutation may imitate phosphorylation of PLB is not clear, but we speculate that there may be a modification of the Cys at position 9 to a negatively charged species that resembles phosphorylated residues of neighboring PKA/CaMKII sites on PLB. Likely modifications include Cys deprotonation to a negatively charged thiolate, or hyperoxidation to sulfinic, sulfinic, or sulfonic acid. By this mechanism, the functional consequence of oxidative crosslinking of PLB protomers in the pentamer would be reinforced by loss-of-function oxidative modifications of remaining monomeric PLB. The data suggest that the fundamental molecular mechanism underlying the R9C pathology is increased sensitivity to oxidative challenge, which occurs periodically in the healthy heart under conditions of physiological stress. Since these oxidative changes are
poorly reversible (Ha et al. 2011), damage from transient oxidative episodes may accumulate over time, leading to chronically impaired SERCA regulation, disordered Ca\(^{2+}\) handling, and eventual heart failure.

**Model for Structural and Functional Consequences of Alanine Substitution and Truncation Mutations of PLB**

The present observations are summarized in the schematic model of Fig. 21, with reversible equilibria indicated by black arrows and effects of mutations highlighted with red arrows. Based on our findings, we propose that the alanine substitution mutations of PLB C-terminus result in alteration of PLB pentamer structure and decreased oligomerization affinity (Fig. 21A, i). In addition, alanine substitution mutations also increased binding affinity of SERCA-PLB regulatory complex (Fig. 21A, ii), and altered quaternary conformation of the SERCA-PLB regulatory complex (Fig. 21A, iii). In comparison, C-terminal truncation mutants of PLB decreased oligomerization affinity of PLB pentamer (Fig. 21B, i), and decreased SERCA-PLB binding affinity (Fig. 21B, ii). C-terminal deletions also altered the quaternary conformation of the SERCA-PLB regulatory complex (Fig. 21B, iii), and decreased membrane anchoring (Fig. 21B, iv). In conclusion, both alanine and truncation mutants of PLB C-terminus resulted in alteration of structure and function of SERCA-PLB regulatory complex. We conclude that the C-terminal residues of PLB are important structural determinants, as mutations of the C-terminal amino acids have significant effects on protein-protein interactions and PLB quaternary structures. Despite the presence of many other hydrophobic residues in the PLB TM domain, luminal residues nearest the PLB C-terminus
**Figure 21.** Proposed model for the effect of PLB C-terminal substitution and deletion mutations on membrane localization, oligomerization and interaction with SERCA. The WT PLB protomers and SERCA are shown in gray and the alanine substitution (V49A) or the deletion mutant (V49X) PLB protomers are highlighted in red. The reversible equilibria are indicated by black arrows and effects of mutations are highlighted with red arrows. (A) We propose that C-terminal alanine substitution mutations of PLB result in (i) altered pentamer structure and decreased oligomerization affinity, (ii) increased SERCA-PLB binding affinity, and (iii) altered quaternary conformation of the SERCA-PLB regulatory complex. (B) In addition, C-terminal deletions of PLB result in (i) decreased oligomerization affinity, (ii) decreased SERCA-PLB binding affinity, (iii) altered quaternary conformation of the SERCA-PLB regulatory complex, and (iv) decreased membrane anchoring.
are critical for membrane anchoring and structure determination of both the PLB pentamer and the PLB-SERCA regulatory complex. It is noteworthy that both deletions and Ala substitutions of PLB C-terminal residues increase SERCA-PLB FRET, but have opposite effects on SERCA inhibition by PLB. Future detailed structural studies of the PLB-SERCA regulatory complex may reveal how substitutions and deletions at the C-terminal end of PLB can strongly alter the disposition of the cytoplasmic domain on the other side of the membrane.

**Model for Structural and Functional Consequences of PLB R9C Mutation**

The structural and functional consequences of the R9C-PLB mutation are summarized in Fig. 22. This scheme parallels our previous mechanistic model for the relief of SERCA inhibition by phosphorylation (Hou, Kelly, and Robia 2008). We propose that R9C mutation enhances transitions away from the inhibited SERCA-PLB complex (Fig. 22, high FRET), leaving disinhibited SERCA (Fig. 22, low FRET). R9C mutation altered the PLB pentamer conformation (Fig. 22A), increased oligomerization affinity (Fig. 22B), and altered the structure of regulatory complex (Fig. 22D). We also observed a decrease in binding of PLB to SERCA (Fig. 22C). However, this may be an indirect effect of decreased availability of monomeric PLB, rather than a change in the intrinsic affinity of PLB for SERCA. We propose that the predominant mechanism underlying the pathological effects of the R9C mutation is a phosphomimetic effect of PLB cys-9 oxidation. Focusing on the structural consequences of R9C mutation, we observed that R9C mutation leads to increased PLB oligomerization, decreased PLB-SERCA binding, and alteration of the structure of the regulatory complex.
Figure 22. Proposed model for the structural and functional consequences of R9C mutation in PLB. **Structural consequences:** R9C mutation alters (A) PLB pentamer structure, (B) oligomerization affinity, (C) PLB-SERCA binding (an indirect effect), and (D) PLB-SERCA regulatory complex structure. **Functional consequences:** We propose that R9C mutation leads to increased PLB oligomerization, decreased PLB-SERCA binding, and alteration of the structure of the regulatory complex. These effects result in decreased SERCA regulation, consistent with the observed increase in myocyte contractility observed in the present acute experiments. R9C-PLB is unable to regulate SERCA on a long-term basis, and thus exerts a pathological effect by lack of responsiveness to frequency potentiation and β-adrenergic stimulation, resulting in decreased cardiac performance and heart failure. Heart failure exhibits increased oxidative stress conditions, which further increases R9C-PLB oligomerization, thus exacerbating the cardiotoxic effects of the R9C mutation.
The functional consequences of the structural effects of R9C mutation were decreased SERCA regulation, consistent with the observed increase in myocyte contractility observed in the present acute experiments. The long-term inability of R9C-PLB to regulate SERCA exerts a pathological effect by blunting sensitivity to frequency potentiation and β-adrenergic stimulation, with consequent impaired cardiac performance and eventual heart failure. Heart failure exacerbates oxidative stress conditions, further enhancing R9C-PLB oligomerization, thus reinforcing the pathological effects of the R9C mutation.

Summary

Many hereditary mutations of PLB have been reported to cause heart failure in humans, underscoring the importance of PLB for calcium cycling and contractility of the cardiac muscle. The work in this dissertation offers new insights into the role of C-terminal residues of PLB as structural and functional determinants of SERCA-PLB regulatory complex. We propose that the transmembrane mutations alter the registration of PLB on SERCA. The C-terminal mutations may mimic translocation of PLB to a new binding site after relief of inhibition by PLB phosphorylation or Ca2+ binding to SERCA. This study confirms that in addition to the canonical binding site, there are multiple specific binding sites for PLB on SERCA. We determine that the primary function of C-terminal residues of PLB is membrane anchoring, which is critical for PLB oligomerization, and SERCA regulation. In addition, this study reports new information about the mechanistic effect of two naturally-occurring PLB mutations L39Stop and R9C, both of which have been shown to cause heart failure and
premature death in humans. We report that both L39X and R9C mutants exhibit alteration of PLB oligomerization and structure and function of the SERCA-PLB regulatory complex. In particular, this study highlights that the oxidative changes of the R9C-PLB protein mimic physiological phosphorylation, relieving inhibition of SERCA. We propose that the increased sensitivity of R9C-PLB to oxidative stress causes the observed hyperdynamic phenotype and reduced responsiveness to adrenergic signaling, leading to heart failure. We predict that any other truncation or loss of function mutations in the transmembrane domain of PLB, if found to occur naturally, would lead to cardiomyopathy due to alteration of PLB-SERCA structure-function mechanisms. Since PLB gene is prone to mutations, we propose to include PLB gene in genetic testing panels for patients suffering from cardiac diseases.
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VITA

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In August of 2008, Neha joined the Master of Science Program in the Department of Biological Sciences at Northern Illinois University, DeKalb, IL. Shortly thereafter, she joined the lab of Dr. Kenneth Gasser, where she worked on the role of purinergic receptors in pancreatic signal transduction. On account of excellence in academics and research, Neha was awarded with ‘Goerge L. Terwilliger Outstanding Graduate Student Award’ at 2010 Honors Convocation at Northern Illinois University DeKalb, IL.

During her academic career, Neha gained extensive work experience. She worked as a ‘Research Editor’ in the oncology division of ADI Biosolution Ltd, Punjab, India. Soon thereafter, she worked as a ‘Lecturer in Biotechnology’ at S.U.S. College of Engineering & Technology, Punjab, India. In addition, she worked as a ‘Graduate Teaching Assistant’ during her MS degree at Northern
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On July 17, 2011, Neha married Varun Nagpal, who is a PhD candidate at Northwestern University Chicago. On September 6, 2014, they were blessed with their first baby, Nishka. After completing her Ph.D., Neha will begin a post-doctoral position and will continue her research in the field of cardiac physiology.