2012

An Investigation of the Phospholamban-Serca Regulatory Interaction with Fluorescence Resonance Energy Transfer

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LOYOLA UNIVERSITY CHICAGO

AN INVESTIGATION OF THE PHOSPHOLAMBAN-SERCA
REGULATORY INTERACTION WITH
FLUORESCENCE RESONANCE ENERGY TRANSFER

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM OF CELL AND MOLECULAR PHYSIOLOGY

BY

PHILIP A. BIDWELL

CHICAGO, IL

DECEMBER 2012
This dissertation is dedicated
to my wife Christy
for her continued love and support
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my mentor, Dr. Seth Robia. He has provided amazing assistance guidance throughout my graduate career. He has always had an open door and has helped me progress as a scientist, on and off the bench. I could not have progressed to this point without his support and belief in me.

I would also like to thank the whole Robia Lab for continued assistance and encouragement. In particular, I would like to thank Dr. Zhanjia Hou. He has been instrumental in helping develop many of the FRET protocols have utilized. Also, I would like to thank Dan Blackwell for developing the adenoviral vectors that I have utilized.

I would like to thank Aleksey Zima and his lab for assistance as well as provided the myocytes used for this work. I would also like to thank Dr. Greg Mignery and his lab for assistance in developing our in cell activity assay.

Lastly, for their time, effort, and guidance, I would like to thank my committee, Dr. Pieter de Tombe, Dr. Aleksey Zima, Dr. Edward Campbell, and Dr. J Michael O’Donnell.
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<tr>
<td>Å</td>
<td>angstrom</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>AKAP</td>
<td>A kinase anchoring protein</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>AU</td>
<td>arbitrary units</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>calcium</td>
</tr>
<tr>
<td>CamKII</td>
<td>calcium/calmodulin kinase II</td>
</tr>
<tr>
<td>CFP</td>
<td>cyan fluorescent protein</td>
</tr>
<tr>
<td>Da</td>
<td>dalton</td>
</tr>
<tr>
<td>E</td>
<td>FRET efficiency</td>
</tr>
<tr>
<td>EC(_{50})</td>
<td>half maximal effective concentration</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HAX-1</td>
<td>HS-1 associated protein X-1</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>HRC</td>
<td>histidine rich calcium binding protein</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>KD</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>NKA</td>
<td>Na, K-ATPase</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PLB</td>
<td>phospholamban</td>
</tr>
<tr>
<td>PLM</td>
<td>phospholemman</td>
</tr>
<tr>
<td>PP1</td>
<td>protein phosphatase-1</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarco/endoplasmic reticulum calcium ATPase</td>
</tr>
<tr>
<td>SLN</td>
<td>sarcolipin</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>Tg</td>
<td>thapsigargin</td>
</tr>
<tr>
<td>tmPLB</td>
<td>transmembrane phospholamban</td>
</tr>
<tr>
<td>V\textsubscript{max}</td>
<td>maximum rate</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
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</table>
ABSTRACT

With Fluorescence Resonance Energy Transfer (FRET), we are able to detect changes in the structure and affinity of the PLB-SERCA regulatory complex in live cells. Using this approach, we have detected a high level of PLB-SERCA interaction even at Ca\(^{2+}\) concentrations known to fully relieve PLB inhibition of SERCA, suggesting that dissociation is not required for relief of inhibition. We also detect no real-time change in PLB-SERCA binding over the course of a single Ca\(^{2+}\) transient in paced myocytes. The effect of Ca\(^{2+}\) on the PLB-SERCA interaction is best described as a reduced affinity with no change in the structure of the complex. Even though we do not observe a change in the structure of the PLB-SERCA complex with changes in calcium concentrations, we currently hypothesize that the reduced affinity is produced by a transition of PLB into an alternative binding site. We propose that the transmembrane domain of PLB could move independently of its cytosolic domain. Our fluorescent probe is attached to the cytosolic domain of PLB, which would explain our inability to detect a change in the position of the transmembrane domain. My current work attempts to address this by measuring the FRET between SERCA and a truncated PLB lacking the cytosolic domain.
CHAPTER I

INTRODUCTION

The sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) is a responsible for maintaining the Ca\(^{2+}\) gradient across the membrane of intracellular vesicles of all cells (MacLennan & Kranias, 2003). This process is of particular importance in muscle cells in which Ca\(^{2+}\) triggers contraction by binding troponin C, allowing myosin-actin crossbridge formation (Gordon, Homsher, & Regnier, 2000). SERCA is responsible for decreasing cytosolic Ca\(^{2+}\) concentrations necessary for muscle relaxation as well as loading the SR with Ca\(^{2+}\) necessary for further release events (Bers, 2008). SERCA is believed to function through a series of coordinated structure changes mediated through the binding of Ca\(^{2+}\) and ATP and subsequent hydrolysis of ATP (MacLennan, Toyofuku, & Lytton, 1992; Toyoshima & Inesi, 2004). The two primary generalized structural conformations of SERCA are described as the Ca\(^{2+}\) bound state, E1, and Ca\(^{2+}\) unbound state, E2. The continual beating of the heart requires almost constant SERCA activity, meaning proper function and regulation is of the utmost importance in the survival of the heart.

In cardiac tissue, phospholamban (PLB) is the primary regulator of SERCA activity, and has been shown to bind to and inhibit the activity of SERCA (MacLennan & Kranias, 2003). This inhibition is achieved by decreasing the apparent affinity of Ca\(^{2+}\) for
SERCA. In other words, higher concentrations of \( \text{Ca}^{2+} \) are required to the same levels of activity that occur in the absence of PLB. Therefore, \( \text{Ca}^{2+} \) can overcome in the inhibition of SERCA by PLB, but the mechanism for this relief of inhibition remains unclear. PLB is a key target of \( \beta \)-adrenergic signaling with phosphorylation partially relieving the inhibition of SERCA. Its name is derived from the Greek words for “phosphate” and “to receive.”

A general depiction of \( \text{Ca}^{2+} \) handling in cardiac myocytes is depicted in Fig. 1 (Bers, 2008). Membrane depolarization causes the opening of L-type \( \text{Ca}^{2+} \) channels, causing an influx of \( \text{Ca}^{2+} \) and resulting in a \( \text{Ca}^{2+} \)-induced \( \text{Ca}^{2+} \) release from the sarcoplasmic reticulum (SR) through the ryanodine receptor. \( \text{Ca}^{2+} \) accumulates in the cytosol, triggering muscle contraction. SERCA is responsible for pumping the accumulated \( \text{Ca}^{2+} \) back into the SR, reducing cytosolic concentrations and loading the SR with \( \text{Ca}^{2+} \) for future release. PLB can inhibit SERCA, modulating \( \text{Ca}^{2+} \) movement.

Two basic models have been used to explain the mechanism by which \( \text{Ca}^{2+} \) overcomes the inhibition by PLB. For the sake of discussion, I will refer to them as the “Dissociation Model” and the “Subunit Model.” The Dissociation Model suggests that PLB stabilizes a structural conformation of SERCA, thereby preventing its transition to an alternative conformation capable of binding \( \text{Ca}^{2+} \). \( \text{Ca}^{2+} \) essentially competes with PLB for binding to SERCA. Therefore, PLB must be displaced from SERCA for \( \text{Ca}^{2+} \) to bind and the pumping cycle to proceed. The Subunit Model suggests that PLB merely slows the
Figure 1: *Diagram of the basic calcium handling elements within cardiac myocytes.* Membrane depolarization opens L-Type calcium channels initiating a calcium induced calcium release by the ryanadine receptor (RyR), elevating cytosolic calcium concentrations. This cytosolic calcium is then pumped back into the SR against its concentration gradient via SERCA, the activity of which can be inhibited by PLB.
on-rate of $\text{Ca}^{2+}$, allowing the possibility that PLB can remain bound to SERCA throughout its reaction cycle. Both models appear to be incomplete and do not account all data in field. The primary goal of this dissertation is to address these inconsistencies and propose an alternative, more inclusive model which we have named the “Domain Displacement Model.”

In this dissertation, I will propose that PLB has an alternative binding site on SERCA and that the binding of $\text{Ca}^{2+}$ displaces PLB to this site rather than preventing any interaction. This model is supported through my research as well as evidence from the Na, K-ATPase and its regulator phospholemman, a protein system highly homologous to PLB-SERCA. This is also supported by previously published work by our lab showing that phosphorylated PLB can still bind SERCA. This model challenges the general concept that relief of inhibition requires dissociation, but is not in conflict with the previous research in the field.

Much of our understanding of SERCA function comes from steady state biochemical assays. However, the contracting heart is by no means a steady state environment, especially with respect to $\text{Ca}^{2+}$. $\text{Ca}^{2+}$ concentration is oscillating on a beat-to-beat basis facilitating contraction and relaxation. One of the primary questions of this dissertation is how PLB interacts with SERCA over the course of a single $\text{Ca}^{2+}$ transient. Can the elevated $\text{Ca}^{2+}$ from a single transient overcome the PLB inhibition of SERCA? Or can the effect of elevated $\text{Ca}^{2+}$ integrate over time?
The biochemical steps of SERCA activity have been well understood since the 1970’s. With the more recent detailed structural information coming from crystallographic studies, SERCA has provided a unique venue in the study of the structure-function relationship of complex proteins. In this dissertation, I will address how PLB is able to interact with various structural conformations of SERCA.

This dissertation will first start with a review of literature describing SERCA and PLB themselves as well our current understanding of the regulatory mechanism. I will then address the conflicting approaches used to assess the PLB-SERCA interaction. I will then discuss how disregulation of SERCA can be involved in disease to underscore the importance of this functional interaction.

In the next section I will discuss the materials and methods employed for this dissertation. This will be followed by a description of an in cell SERCA activity assay which we developed to test the functionality of our fusion proteins. The next section addresses the steady-state nature of the PLB-SERCA interaction addressing how PLB interacts with SERCA in various conformational steps. In chapter VI, I show my research that directly tests our newly proposed model of PLB-SERCA interaction. In chapter VII, I attempt to show the dynamic nature of the PLB-SERCA interaction by making real-time measurements. Finally, I will discuss the broader implications of my research and how my results integrate with the findings of others.
I would also like to note that the bulk of work shown in sections IV, V and VII was published this past October in the Journal of Biological Chemistry (Bidwell, Blackwell, Hou, Zima, & Robia, 2011).
CHAPTER II

REVIEW OF RELATED LITERATURE

SERCA

P-type Ion Pumps

SERCA belongs to the P-type pump family of ATPases (Kühlbrandt, 2004; Wuytack, Raeymaekers, & Missiaen, 2002). P-type pumps utilize ATP hydrolysis to undergo a series of structural transitions, producing a reversibly phosphorylated state, from which the family name is derived. Most family members have 10 transmembrane helices (M1-10), though the greatest variation between members occurs in the transmembrane region, which determines ion specificity.

The family also shares three cytosolic or headpiece domains, the most homologous region in the family (Kühlbrandt, 2004; Wuytack et al., 2002). They are commonly called the N, P, and A domains (Nucleotide, Phosphorylation, and Actuator). The ATP binding site is located on the N-domain. A conserved lysine is an integral component of the nucleotide binding site. The gamma-phosphate of that ATP is then transferred to an aspartate in a conserved motif, DKTGTLT, on the P-domain. The P-domain is also the most highly conserved domain within the family. The P-domain is also homologous to a class of bacterial enzymes demonstrating its general biochemical role and importance.
**SERCA Isoforms**

In vertebrates, 3 distinct genes encode for SERCA that produce at least 10 different isoforms through alternative splicing (Periasamy & Kalyanasundaram, 2007; Wuytack et al., 2002). SERCA2b is the “housekeeping” isoform found in all cells, at least at some low level. SERCA2a is the predominant isoform found in cardiac muscle and slow twitch skeletal muscle. SERCA1a is expressed in adult fast twitch skeletal muscle and has been the most highly studied isoform because of its abundance in and ease of collection from animal tissue. There are 6 SERCA3 isoforms (a-f) found in various tissues, with a, d, and f found at low levels in cardiac tissue. SERCA2a and SERCA1a are 84% identical with the SERCA3 isoforms being roughly 75% identical to either SERCA1a/2a. Because of the high degree of homology, all SERCA isoforms are believed to have almost identical structures. All isoforms are capable of being inhibited by thapsigargin. SERCA1a and SERCA2a have similar affinities for Ca$^{2+}$, but SERCA1a has a faster catalytic velocity. SERCA2b has a higher Ca$^{2+}$ affinity than either SERCA1a/2a, but lower catalytic rate.

**Reaction Scheme**

SERCA activity was first observed when isolated SR membrane was shown to have ATPase and Ca$^{2+}$ uptake ability from skeletal muscle (Ebashi & Lipmann, 1962), and later from cardiac myocytes (Hasselbach & Makinose, 1962). Evidence of a phosphorylated protein intermediate during the reaction process provides the first insight into the mechanism of SERCA function. In 1966, Jardetzkey postulated the
simple but still accepted model of ion transport where ions bind with differential affinity on opposing sides of the membrane (high on the binding or uptake side and low on the release or discharge side). This is coupled to a phosphorylation dependent conformational change closing the binding pocket on one side and opening it on the other (Jardetzky, 1966). Landgraf and Inesi were the first to demonstrate a conformational change of SR protein in response to ATP through EPR spin labeling (Landgraf & Inesi, 1969). Tanford further proposed that the change in binding pockets and affinities could occur simultaneously through a simple movement of membrane helices altering amino acid side chain interactions that facilitate ion interaction (Tanford, 1982).

In 1970, Inesi first described a hallmark of SERCA activity, the dependence of Ca\(^{2+}\) on the rate of ATPase activity. At low concentrations where little Ca\(^{2+}\) is available to be pumped across the membrane, SERCA activity is low. Increasing Ca\(^{2+}\) increases SERCA activity up to maximal level. Along with Inesi, many other groups begin to characterize the stepwise reaction scheme describing the SERCA reaction cycle (Carvalho, de Souza, & de Meis, 1976; Coan & Inesi, 1976; Hasselbach, 1978; Kanazawa & Boyer, 1973; de Meis & Vianna, 1979), on which a general consensus is reached by 1979, as depicted in Fig. 2. All steps were shown to be reversible, providing the possibility of ATP production. These concepts are still accepted today. A Ca\(^{2+}\) to ATP
Figure 2: Simplified SERCA reaction diagram. One ATP and two Ca\(^{2+}\) ions bind on the cytosolic face of the E1 conformation of SERCA, which initiates ATP hydrolysis and phosphate transfer. The phosphorylated SERCA then readily transitions to the E2 conformation, changing the Ca\(^{2+}\) binding sites from high affinity and cytosolic facing to low affinity and lumen facing. Ca\(^{2+}\) is then released to the ER/SR lumen, along with the release of ADP and PI, producing the free E2 conformation.
stoichiometry of 2:1 was first proposed in 1963 (Hasselbach & Makinose, 1963), but not clearly described until 1980 (Inesi, Kurzmack, Coan, & Lewis, 1980). In the same study, Inesi et al. also demonstrate the cooperative binding of Ca\(^{2+}\). Spin labeling detected a structure change after binding of the first Ca\(^{2+}\) ion which is presumed to increase the binding affinity of the second ion.

**SERCA Structure**

SERCA was first isolated from rabbit skeletal muscle in 1970 (MacLennan, 1970). Structural information first came from proteolysis analyses where trypsin digestion of SERCA produced various fragments and subfragments. ATP and Ca\(^{2+}\) binding could be associated with these specific fragments. In 1980, partial amino acid sequences were determined for the cytosolic fragments (Allen, Trinnaman, & Green, 1980). In 1985, SERCA1a was cloned for the first complete sequence (MacLennan, Brandl, Korczak, & Green, 1985). The following year SERCA2a was cloned and sequenced (Brandl, Green, Korczak, & MacLennan, 1986). From these sequences, the MacLennan group deduced the topology and secondary structure of SERCA, predicting the helical transmembrane domains and the globular cytosolic domains.

SERCA was first visualized with electron microscopy identifying 40 Å diameter surface particles on skeletal muscle microsomes (Martonosi, 1968), and later identified by freeze fracture (Baskin & Deamer, 1969; Deamer & Baskin, 1969). The first low resolution crystal structure from X-ray diffraction (14 Å) showed a large cytosolic headpiece attached to a narrow transmembrane region (Toyoshima, Sasabe, & Stokes,
With enhancement of the resolution to 8 Å, the 10 transmembrane helices could be visualized and could be associated to the appropriate primary structure sequence (Zhang, Toyoshima, Yonekura, Green, & Stokes, 1998). After developing conditions sufficient to grow crystals suitable for X-ray diffraction, a high resolution (2.6 Å) structure was determined capable of resolving the 3 cytosolic domains (Toyoshima, Nakasako, Nomura, & Ogawa, 2000). High resolution structures have subsequently been determined under a variety of conditions and have been associated with intermediate conformations of SERCA (Toyoshima & Inesi, 2004; Toyoshima & Nomura, 2002). Fig. 3 depicts a few of the proposed intermediate conformations showing potential large movements of the cytosolic headpiece.

**Structure Function Relationship**

Integrating the enzymatic and structural data has been a fundamental goal within the field. After cloning SERCA, the MacLennan lab performed extensive mutagenesis studies investigating the role of individual residues (MacLennan, 1990). Many loss-of-function mutants retained the capability of undergoing partial reactions, enabling investigation into the particular steps affected by alteration of these residues. This approach clearly identified Asp$^{351}$ as the site of phosphoryl transfer (Maruyama et al., 1989). This approach also lead to the preliminary identification of the Ca$^{2+}$ binding sites in the transmembrane region (Clarke et al., 1989; Vilsen, Andersen, Clarke, & MacLennan, 1989) and of the ATP binding site (Clarke, Loo, & MacLennan, 1990). These binding sites have been subsequently confirmed by high resolution X-ray crystallography
Figure 3: Proposed SERCA structural transitions. SERCA is believed to undergo conformational transitions upon binding of calcium and ATP, potentially producing large movements of the cytosolic headpiece. Images represent various crystal structures produced under conditions thought to be associated with reaction step intermediates.
These studies demonstrate that ATP binding and hydrolysis occur in the cytosolic headpiece far from the Ca\(^{2+}\) binding sites in the transmembrane region. Therefore the transmembrane reorientation necessary for Ca\(^{2+}\) transport is linked to the required chemical energy through long-range protein interactions.

The various high resolution crystal structures have shown many distinct conformations of SERCA (Toyoshima & Inesi, 2004; Toyoshima & Nomura, 2002; Toyoshima et al., 2000). The conditions under which these crystals were produced can be associated with endpoints of the enzymatic half reactions. Therefore, these conformations are thought to correspond to the various enzymatic states of the SERCA reaction cycle (Fig. 3). Initial results suggested that the stepwise binding of Ca\(^{2+}\) and ATP induce large scale conformational changes in the cytosolic headpiece domains. These conformational shifts in the headpiece were thought to produce the mechanical force necessary to rearrange the transmembrane helices shifting the orientation of the Ca\(^{2+}\) binding sites. However, more recent crystal structures suggest that the structural changes are much smaller and more subtle than previously believed (Jensen, Sørensen, Olesen, Møller, & Nissen, 2006; Sørensen, Møller, & Nissen, 2004). This concept of smaller structural movements is also supported by FRET distance measurements between probes attached to different cytosolic domains (Winters, Autry, Svensson, & Thomas, 2008).
**Phospholamban**

*Discovery*

The existence of PLB was first observed with the PKA dependent stimulation of the ATPase dependent Ca\(^{2+}\) uptake in cardiac SR vesicles (Kirchberger, Tada, Repke, & Katz, 1972; Tada, Kirchberger, Repke, & Katz, 1974). These vesicles were then shown to be radiolabeled by P\(^{32}\) upon stimulation by PKA (Kirchberger, Tada, & Katz, 1974). This radiolabeling was found to be targeted to a 22 kDa protein which was shown to be a modulator of SERCA (Tada, Kirchberger, & Katz, 1975). This phosphoprotein was subsequently termed *phospholamban* coming from the Greek terms “to receive” and “phosphate” (Kirchberger, Tada, & Katz, 1975).

This 22 kDa protein was shown to be capable of dissociating into lower molecular weight components upon boiling or treatment with Triton-X (Jones, Besch, Fleming, & Mcconnaughey, 1979; Lamers & Stinis, 1980; Le Peuch, Haiech, & Demaille, 1979; Will, Levchenko, Levitsky, Smirnov, & Wollenberger, 1978). Freezing of the sample was shown to cause reassociation of the lower-molecular weight forms back to the 22 kDa. These observations suggested that phospholamban could exist as an oligomer. The lower molecular form was eventually determined to be a 6 kDa peptide that could be independently phosphorylated by PKA (Bidlack & Shamoo, 1980; Bidlack, Ambudkar, & Shamoo, 1982). After improved purification techniques, the high molecular weight form was shown to be a 30 kDa pentamer of composed of indistinguishable monomers which could each be independently phosphorylated (Fujii,
Kadoma, Tada, Toda, & Sakiyama, 1986; Wegener & Jones, 1984; Wegener, Simmerman, Liepnieks, & Jones, 1986). The full sequence of PLB was determined after it was cloned by the Tada lab (Fujii et al., 1987).

**Structure**

PLB is a 52 amino acid integral membrane protein that is comprised of 3 domains, Ia (residues 1-20), Ib (residues 21-30), and II (residues 31-52) (Bhupathy, Babu, & Periasamy, 2007). Proteolysis studies first suggested the existence of 2 domains, a protease sensitive cytosolic domain (1a/b) and protease insensitive transmembrane domain (II) (Wegener et al., 1986). Circular dichroism experiments further suggested the existence of a cytosolic (Ia) and transmembrane (II) alpha helices separated by and unstructured linker or hinge domain (1b) (Simmerman, Lovelace, & Jones, 1989; Terzi, Poteur, & Trifilieff, 1992). These observations were consistent with sequence analysis proposing likely alpha helical and hydrophobic/hydrophilic regions (Fujii et al., 1986, 1987). These observations are also consistent with NMR structures of PLB (Traaseth et al., 2009) and molecular dynamics simulations (Paterlini & Thomas, 2005).

A fundamental aspect of phospholamban is that it can exist in a monomer as well as a pentamer (MacLennan & Kranias, 2003; Simmerman & Jones, 1998). The alpha helical structure of the PLB transmembrane region generates heptad repeats. Because this pattern, every 3 to 4 residues of the helix align, organizing leucines and isoleucines along one side or face PLB. Mutagenesis targeting these residues destabilized pentamer formation whereas mutations along the opposite face of PLB did not alter pentamer
stability. This suggests that the leucine zipper formation found within the PLB structure is critical for pentamer assembly (Simmerman, Kobayashi, Autry, & Jones, 1996). This conclusion has been supported by subsequent NMR studies (Verardi, Shi, Traaseth, Walsh, & Veglia, 2011). Mutations at 3 cysteines within the transmembrane domain (residues 36, 41, and 46) have been shown to destabilize PLB pentamer formation (Fujii, Maruyama, Tada, & MacLennan, 1989), but disulfide linkages were not observed and are believed be unnecessary for pentamer stability (C. B. Karim, Stamm, Karim, Jones, & Thomas, 1998; Simmerman, Collins, Theibert, Wegener, & Jones, 1986). Replacement of cysteines with an isosteric amino acid (α-amin-n-butyric) was shown to have no affect on pentamer stability (C. B. Karim et al., 2001). This demonstrates that the steric properties of the cysteines, not their reactivity, contribute pentamer stability.

Because PLB can exist as a pentamer, monomer, and monomer bound to SERCA, PLB exists in a coupled equilibrium depicted in Fig. 4. This concept was first demonstrated when mutations that make PLB more monomeric were shown have enhanced inhibitory potential and are considered “superinhibitors” (Y Kimura, Asahi, Kurzydlowski, Tada, & MacLennan, 1998; Y Kimura, Kurzydlowski, Tada, & MacLennan, 1997). These observations are also consistent with work in our lab showing that a superinhibitory PLB mutant enhances FRET between PLB and SERCA while diminishing PLB pentamer FRET to an equivalent degree (Kelly, Hou, Bossuyt, Bers, & Robia, 2008). These studies suggest that the monomer is the active inhibitor of the pump, with the pentamer being an inactive reservoir, incapable of interacting with SERCA. Through
**Figure 4:** Coupled equilibrium of PLB binding. PLB can exist as a pentamer, free monomer, and monomer bound to SERCA. PLB readily transitions between pentamer and free monomer (KD1) and between free monomer and monomer bound to SERCA (KD2). Therefore changes in pentamer binding will alter the binding of PLB to SERCA.
mass action, changes in pentamer binding will alter binding between PLB and SERCA, thereby altering the ability of PLB to inhibit SERCA.

X-ray crystal studies have suggested the possibility that the pentamer could directly interact with SERCA (Stokes, Pomfret, Rice, Glaves, & Young, 2006). However, this inconsistent with studies from our lab showing a 1:1 stoichiometry in PLB-SERCA FRET (Kelly et al., 2008). Because this interaction could be an artifact of crystallization, further study is needed for its validation.

**Phosphorylation**

PLB is the target of β-adrenergic signaling through PKA phosphorylation of Ser\(^{16}\) and CamKII phosphorylation of Thr\(^{17}\) (Fujii et al., 1989; Simmerman et al., 1986). Thr\(^{17}\) has also been shown be phosphorylated independently of Ser16 in a frequency dependent manner likely through enhanced CamKII activity (Hagemann et al., 2000; Mundiña-Weilenmann, Vittone, Ortale, de Cingolani, & Mattiazzi, 1996). Phosphorylation of Ser\(^{16}\) and Thr\(^{17}\) can occur independently *in vitro* (Davis, Schwartz, Samaha, & Kranias, 1983; Jackson & Colyer, 1996; Kranias, 1985). However, during *in vivo* studies, phosphorylation of Ser\(^{16}\) appears to be a prerequisite for phosphorylation of Thr\(^{17}\) through the β-adrenergic pathway (Chu et al., 2000; Luo et al., 1998). Protein phosphatase-1 (PP1) has been shown to dephosphorylate PLB at both sites which decreases SERCA activity (Kranias & Di Salvo, 1986; MacDougall, Jones, & Cohen, 1991; Nicolaou, Hajjar, & Kranias, 2009).
These phosphorylations can independently relieve PLB inhibition of SERCA thereby enhancing its activity by restoring the Ca\textsuperscript{2+} sensitivity of SERCA (MacLennan & Kranias, 2003). In some early studies, it was suggested that the Ser\textsuperscript{16} and Thr\textsuperscript{17} phosphorylations have an additive effect on SERCA activity (Kranias, 1985; Le Peuch et al., 1979; Raeymaekers, Hofmann, & Casteels, 1988), but these studies do not directly assess phosphorylation efficiency of their experimental conditions. Further studies demonstrating full phosphorylation show no additive effect suggesting that the previously observed additive effect may have been due to incomplete phosphorylation of PLB (Colyer & Wang, 1991; Jackson & Colyer, 1996).

**Mechanism of PLB Inhibition of SERCA**

SERCA activity increases with Ca\textsuperscript{2+} concentration up to a maximum level with a Hill function relationship (Fig. 5, black). PLB is known to reduce the apparent Ca\textsuperscript{2+} affinity of SERCA, producing a rightward shift in the SERCA activity curve (Fig. 5, red). Higher Ca\textsuperscript{2+} concentrations are then needed to achieve the same levels of activity observed in the absence of PLB. In other words, increases in Ca\textsuperscript{2+} concentration can overcome the inhibition by PLB. Phosphorylation of PLB relieves the inhibition on SERCA by partially restoring Ca\textsuperscript{2+} sensitivity, represented as a leftward shift in the SERCA activity curve (Fig. 5, blue). The process of this relief of inhibition is not fully understood and is the focus of this dissertation.

SERCA is believed to exist in various conformational states. Early evidence comes from protein digestion studies, where changes in tertiary structure block or allow
Figure 5: Simulated example of SERCA activity assay. SERCA activity, which can be measured by ATPase activity or calcium uptake, increases with increasing cytosolic calcium concentration in a sigmoidal manner up to a maximal level (black). The presence of PLB causes a decreased apparent calcium affinity represented by a rightward shift in the activity curve (red). Higher cytosolic calcium concentrations are needed to achieve the same SERCA activity. Phosphorylation of PLB shifts this curve back to left (blue).
access to cut sites. This approach identified 2 primary SERCA conformations: the E1 
Ca$^{2+}$ bound state and the E2 Ca$^{2+}$ unbound state. More recent X-ray structures have 
been determined for SERCA under various conditions, corresponding to more 
conformational states. These conformational states are believed to correspond to 
reaction cycle intermediates.

**Dissociation Model**

The prevailing view favors a model where PLB binds the E2 Ca$^{2+}$ free state of 
SERCA. Interaction with PLB stabilizes this conformation thereby blocking transition to 
the E1 conformation by increasing the free energy barrier. Inability to transition to a 
conformation capable of binding Ca$^{2+}$ terminates the reaction cycle of SERCA. PLB must 
then completely dissociate from SERCA for transition to occur and activity to be 
restored. In this model, binding of PLB and Ca$^{2+}$ to SERCA is mutually exclusive. I will 
refer to this model as the “Dissociation Model” which is depicted in Fig. 6A.

The Dissociation Model is well supported by chemical crosslinking studies from 
Jones group. In an initial study, a mutant which introduced a Cys at residue 30 of PLB 
(N30C) was coexpressed in insect vesicles (Jones, Cornea, & Chen, 2002). Addition of a 
homobifunctional thiol probe, bismaleimidoxyhexane, was shown to chemically crosslink 
the introduced Cys of N30C to Cys$^{318}$ of canine SERCA2a. This demonstrated that 
residue 30 of PLB was in close proximity to residue 318 of SERCA. This chemical 
crosslinkage could be disrupted through increases in Ca$^{2+}$ concentration, exhibiting an 
EC$_{50}$ of 130 nM. At high Ca$^{2+}$ concentrations, crosslinking was completely abolished.
Figure 6: Models of PLB inhibition of SERCA. (A) The Dissociation Model suggests that PLB selectively binds the E2 calcium unbound state of SERCA preventing transition to the E1 conformation capable of binding calcium. PLB must then completely dissociate from SERCA for function to be restored. (B) The Subunit Model suggests that PLB binds all conformations of SERCA, and merely slows the binding of calcium and transition to the E1 conformation. Evidence for both models stems from assessment of the ability to bind the E1 conformation (orange boxes), produced by high calcium conditions, with different methods favoring different models.
Further studies using a similar approach showed that residue 27 of PLB was in close proximity to residue 328 of SERCA, and that residues 45-52 of PLB were in close proximity to residue 89 of SERCA. Crosslinking at these sites was also completely abolished by increases in Ca\textsuperscript{2+} concentration, with EC\textsubscript{50} values ranging from 100 to 900 nM (Chen, Akin, Stokes, & Jones, 2006; Chen, Stokes, & Jones, 2005).

In a recent study, the Jones group has generated a method to crosslink endogenous PLB and SERCA2a from isolated human cardiac tissue, generating results consistent with their previous studies (Akin, Jones, 2012). This is an advancement over previous studies since measurements are made with endogenously expressed proteins in the most physiologically relevant membrane environment. Any accessory and/or anchoring proteins would also be intact within this system. Also, neither protein has crosslinking sites introduced through a mutation that could alter proper function and interaction.

These studies show the disruption of PLB-SERCA binding at multiple points of contact by elevated Ca\textsuperscript{2+} concentrations. While some residual level of crosslinking (5% of control) can be observed even at saturating concentrations, the Jones group states Ca\textsuperscript{2+} completely abolishes crosslinking. The Jones group then concludes that PLB is able to compete with Ca\textsuperscript{2+} for its binding site on SERCA, and that complete dissociation of PLB and SERCA is required for restoration of SERCA activity.

Their approach and conclusions are supported by additional studies exploring the effects of PLB phosphorylation on PLB-SERCA crosslinking. Much like elevated Ca\textsuperscript{2+}
concentration, phosphorylation of PLB is known to overcome SERCA inhibition by PLB. Also like elevated Ca\(^{2+}\), they demonstrated that PKA phosphorylation of PLB prevented crosslinking between PLB and SERCA (Chen, Akin, & Jones, 2007). This further supports their previous conclusion that relief of PLB inhibition is accomplished through the unbinding of PLB from SERCA.

The *Dissociation Model* is also supported by immunoprecipitation studies by the MacLennan group. They first demonstrated that inhibitory ability of various mutants (loss and gain of function) correlates with their ability to co-immunoprecipitate with SERCA (Asahi, Kimura, Kurzydlowski, Tada, & MacLennan, 1999). Subsequently, they demonstrated that Ca\(^{2+}\) can also disrupt the ability of PLB to co-immunoprecipitate with SERCA (Asahi, McKenna, Kurzydlowski, Tada, & MacLennan, 2000). These studies demonstrate that relief of inhibition correlates to loss of PLB-SERCA interaction, in support of the Jones group conclusions.

Despite the clear and well characterized results of the crosslinking and immunoprecipitation assays, the approaches and subsequent conclusions have limitations. While the crosslinking assay is highly sensitive and specific, the close proximity of targeted residues on PLB and SERCA are only assessed. Even subtle changes in the position and/or orientation of PLB in relation to SERCA could prevent the crosslinking. Small rotations in the transmembrane helices of SERCA could prevent access of the chemical crosslinkers to the target residues. These factors demonstrate how crosslinking could be abolished without significant dissociation of the PLB-SERCA
complex. Furthermore, even at saturating Ca\textsuperscript{2+} concentrations, a low level of crosslinking is observed in many cases. This observation is not in agreement with complete dissociation.

Despite agreement between crosslinking and immunoprecipitation on the effect of Ca\textsuperscript{2+} on the PLB-SERCA interaction, the observations from these approaches differ under other conditions. A SERCA ligand, thapsigargin, as well as phosphorylation of PLB have been shown to block the PLB-SERCA crosslinking (Chen et al., 2006, 2005), but neither affects PLB-SERCA co-immunoprecipitation (Asahi et al., 2000).

The largest complication with immunoprecipitation is that the membrane is perturbed by detergents prior to the detection of the interaction between PLB and SERCA. Immunoprecipitation between membrane bound proteins like PLB and SERCA will be highly susceptible to variations in experimental conditions. Furthermore, antibodies targeted to PLB, such as those used to pull down the PLB-SERCA complex in the co-immunoprecipitation experiments, have themselves been shown to block PLB-SERCA crosslinking (Chen et al., 2007) and relieve PLB inhibition of SERCA (Cantilina, Sagara, Inesi, & Jones, 1993; Y Kimura et al., 1991; Morris, Cheng, Colyer, & Wang, 1991; Sham, Jones, & Morad, 1991). These factors may explain the inconsistent results between the two approaches. These factors also argue why immunoprecipitation may not be an effective strategy to investigate PLB-SERCA binding and therefore may not lend the crosslinking studies sufficient corroborating support.
The *Dissociation Model* is described by the competitive binding of PLB and Ca\(^{2+}\) for SERCA. In competitive inhibition, Ca\(^{2+}\) binding (activity) can always be overcome by further increases in the concentration of inhibitor. However, this is inconsistent with studies measuring the dose response of PLB. The maximal inhibition of SERCA, indicated by decreased apparent Ca\(^{2+}\) affinity, is achieved by a 2.6 fold increase of PLB expression (Brittsan, Carr, Schmidt, & Kranias, 2000). Further overexpression of PLB does not enhance the inhibition of SERCA, as would be predicted if PLB competed with Ca\(^{2+}\). Furthermore, crosslinking is never completely abolished even at saturating concentrations inconsistent with the Jones group conclusions and the concept of competitive inhibition.

**Subunit Model**

In contrast to the *Dissociation Model*, the *Subunit Model* has been proposed suggesting that PLB can remain bound to SERCA throughout its reaction cycle as depicted in Fig. 6B. The decrease in apparent Ca\(^{2+}\) affinity is achieved by slowing a rate limiting step of the reaction cycle, likely the on-rate of Ca\(^{2+}\). This is consistent with the observation that high Ca\(^{2+}\) can overcome inhibition by PLB.

Support for this model first comes from a study showing that a PLB antibody enhances Ca\(^{2+}\) transport by SERCA in isolated cardiac vesicles (Cantilina et al., 1993). This antibody produces an increased formation of the autophosphorylated SERCA intermediate (E1-P) as would be expected with increased activity and turnover of the pump. However, this antibody was unable to alter the Ca\(^{2+}\) binding in the absence of
ATP at equilibrium. This suggests that SERCA inhibition by PLB is not achieved by lowering the actual affinity of Ca\textsuperscript{2+} for the pump. The decrease in apparent Ca\textsuperscript{2+} affinity in the presence of PLB is then achieved by a decreased turnover of the SERCA pump. Decreased turnover is explained by a slowed transition from the E2 to E1 conformations produced by a slower Ca\textsuperscript{2+} on-rate.

These equilibrium binding experiments were performed in the absence of ATP, which has been viewed as a flaw. Since ATP is a critical and necessary for SERCA function, it could then alter the Ca\textsuperscript{2+} affinity and control how PLB might affect this equilibrium. The conclusion that PLB decreases the on-rate of Ca\textsuperscript{2+} has then been disputed on this assertion (Jones et al., 2002). However, no study has described equilibrium Ca\textsuperscript{2+} binding in the presence of ATP in the nearly 20 years since Cantilina et al was published. This is likely due to the complexity of this seemingly simple experiment. The presence both Ca\textsuperscript{2+} and ATP will induce SERCA cycling and Ca\textsuperscript{2+} transport, thereby preventing equilibrium from being achieved.

Equilibrium Ca\textsuperscript{2+} binding experiments have also been performed in using purified proteins in a reconstituted membrane (Hughes, Starling, Sharma, East, & Lee, 1996). Consistent with Cantilina et al, the results show no effect of PLB on Ca\textsuperscript{2+} binding at equilibrium. However, in contrast to Cantilina et al, this study shows no change in the rate of the SERCA auto-phosphorylation, and suggests that PLB disrupts the off-rate of Ca\textsuperscript{2+}. 
The *Subunit Model* is also supported by a study measuring FRET between PLB and SERCA (Mueller, Karim, Negrashov, Kutchai, & Thomas, 2004). High levels of energy transfer were detected between purified, fluorescently-labeled PLB and SERCA in a reconstituted membrane, even at saturating Ca\(^{2+}\) concentrations. A small but significant increase in the distance between the fluorescent probes was detected by the elevation of Ca\(^{2+}\) concentration. This suggests that relief of inhibition occurs through a structural rearrangement in the PLB-SERCA complex, rather than from complete dissociation of PLB from SERCA.

One criticism of this study was the use of purified protein in a reconstituted membrane. Being in such a non-physiological system could disrupt proper interaction between PLB and SERCA, thereby producing anomalous results. The membrane composition and high relative concentration of protein could produce nonspecific binding. Also, the fluorescent probes themselves could induce aggregation of PLB and SERCA. However, this argument neglects that the distance estimations between PLB and SERCA are consistent with the predicted binding location. Furthermore, activity assays demonstrate that elevated Ca\(^{2+}\) can overcome PLB inhibition in this system. Since functional regulation occurs, it suggests that the PLB-SERCA binding detected are specific and not anomalous. Also, if the fluorescent probes were inducing nonspecific aggregation, this would reduce the ability of PLB to regulate SERCA, which is not observed. Labeled and unlabeled PLB inhibit SERCA activity to the same degree.
**Structure-Function Relationship of PLB inhibition**

PLB is only expressed in cells expressing SERCA2a and not in cells expressing SERCA1a (MacLennan & Kranias, 2003; Tada & Toyofuku, 1998). However, the interaction between PLB and SERCA1a has been extensively studied despite not being physiological binding partners. This interaction has been studied due to the wealth of structural information of SERCA1a and the suspected high degree of structural and functional similarities between the two isoforms (Asahi et al., 1999; Kühlbrandt, 2004; Morth et al., 2007). Furthermore, PLB has been shown to inhibit SERCA1a and SERCA2a to the same extent (Toyofuku, Kurzydlowski, Tada, & MacLennan, 1993). PLB also shows the ability to crosslink both isoforms in a similar manner (Chen et al., 2006, 2005; Jones et al., 2002; Toyoshima et al., 2003). The study of the PLB-SERCA1a therefore aids in the better modeling of the PLB-SERCA interaction. Despite these extensive similarities, our lab has shown that PLB has a higher affinity for SERCA1a compared to SERCA2a (Hou & Robia, 2010). While this observation does not discount the other similarities, it is an important consideration when interpreting the results from SERCA1a.

The hallmark of PLB inhibition of SERCA is a reduced apparent affinity of Ca$^{2+}$ (increased $K_{Ca}$ as in Fig. 5) which is generally thought to occur through transmembrane interactions of the two proteins (MacLennan & Kranias, 2003). This concept is supported by studies showing that a truncated PLB lacking the cytosolic domain was capable of reducing Ca$^{2+}$ sensitivity of SERCA to the same degree as full length PLB (Hughes et al., 1996; Y Kimura, Kurzydlowski, Tada, & MacLennan, 1996). Evidence for
the role of transmembrane interactions also comes from a homolog of PLB, sarcolipin (SLN). The transmembrane regions of PLB and SLN have a high degree of similarity, but SLN lacks a comparable cytosolic domain (Odermatt et al., 1997). Despite the lack of cytosolic domain, SLN displays the ability to reduce the apparent affinity for Ca\(^{2+}\) of SERCA like PLB (MacLennan, Asahi, & Tupling, 2003; Odermatt et al., 1998).

While the role of the transmembrane region in the inhibition of SERCA is well described, the role of the cytosolic domain of PLB is less understood. PLB can inhibit both SERCA1a and SERCA2a, but is unable to inhibit SERCA3 (Kimura et al., 1996). However, a truncated PLB lacking the cytosolic domain is able to inhibit SERCA3 indicating a critical role for the cytosolic domain. A region in the nucleotide binding domain of SERCA3 was identified that prevented regulation by PLB (Toyofuku et al., 1993; Toyofuku, Kurzydlowski, Tada, & MacLennan, 1994). Specifically, when the SERCA3 sequence of Gln-Gly-Glu-Gln-Leu\(^{401}\) was converted to the corresponding SERCA2a sequence of Lys–Asp–Asp–Lys–Pro\(^{401}\), SERCA3 gained the ability to be regulated by PLB. However, a specific role for this sequence has not been determined, but these findings point to at least some regulatory role of the PLB cytosolic domain that does not directly confer inhibition.

A direct inhibitory function of the cytosolic domain has been suggested and investigated, though the findings have not been consistent. Many studies have examined the potential effect in isolated cardiac vesicles. Early studies suggested that phosphorylation of the cytosolic domain of PLB could enhance the maximal catalytic
rate ($V_{\text{max}}$) of SERCA in isolated cardiac SR vesicles (Adunyah, Jones, & Dean, 1988). However, later studies disputed these findings showing that phosphorylation of PLB had no effect on $V_{\text{max}}$ in isolated cardiac vesicles (a Mattiazzi, Hove-Madsen, & Bers, 1994; M A Movsesian, 1992; Matthew A Movsesian, Colyer, Wang, & Krall, 1990). An isolated peptide corresponding to the PLB cytosolic domain (residues 1-31) was shown to decrease the $V_{\text{max}}$ of SERCA with no impact on Ca$^{2+}$ affinity (Sasaki, Inui, Kimura, Kuzuya, & Tada, 1992). These results were supported by studies showing that the removal of the PLB cytosolic domain through tryptic digestion (Lu & Kirchberger, 1994; Lu, Xu, & Kirchberger, 1993) caused an increase in the $V_{\text{max}}$ of SERCA in isolated cardiac vesicles. Exposure of an antibody targeted to the cytosolic domain of PLB to isolated vesicles was shown to decrease $V_{\text{max}}$ in one study (Antipenko, Spielman, Sassaroli, & Kirchberger, 1997) while having no effect on $V_{\text{max}}$ in another (Cantilina et al., 1993). Variations in vesicle preparation were shown to impact detection of a $V_{\text{max}}$ change potentially explaining the differing results (Antipenko, Spielman, & Kirchberger, 1999).

Rather than using isolated vesicles, many labs studied the role of the cytosolic domain by expressing PLB and SERCA in heterologous cells or reconstituted them in a purified membrane. When SERCA was reconstituted into a membrane with and without PLB, the expected change in $K_{\text{Ca}}$ was observed with no change in the $V_{\text{max}}$ parameter (Reddy et al., 1995; Reddy, Jones, Pace, & Stokes, 1996). These studies also observed no change in SERCA activity in response to the isolated peptide corresponding to residues 1-31 of PLB. These studies were also supported by an analogous study expressing PLB
and SERCA in HEK cells also showing no change in the $V_{\text{max}}$ parameter (A. Odermatt, Kurzydlowski, & MacLennan, 1996). These studies have been disputed by another group showing that the isolated PLB cytosolic domain can decrease $V_{\text{max}}$ in a reconstituted system and that a truncation of PLB removing the cytosolic domain increases Vmax (Hughes, East, & Lee, 1994; Hughes et al., 1996; A. P. Starling, Hughes, Sharma, East, & Lee, 1995). They also demonstrate that experimental conditions used to reconstitute PLB and SERCA have an effect on the observation of a $V_{\text{max}}$ change (A. P. Starling et al., 1995), much like Antipenko et al describes with vesicle preparations (Antipenko et al., 1999).

While phosphorylation of PLB is known to favor pentamer formation, mass action equilibrium binding changes may not fully explain the relief of PLB inhibition through phosphorylation. Phosphorylation of PLB has been shown to prevent chemical crosslinking to SERCA (Chen et al., 2007), which is in agreement with the idea of mass action changes. However, our lab has shown using FRET that phosphomimetic mutants of PLB can interact with SERCA, though with a diminished affinity and altered structure compared to wild-type PLB (Hou, Kelly, & Robia, 2008). This structure change in the regulatory complex might also be linked to relief of inhibition. EPR amd NMR studies have been used to show that phosphorylation of PLB introduces disorder into the alpha-helix of the cytosolic domain (Metcalfe, Traaseth, & Veglia, 2005) and that this phosphorylation does not alter the affinity of the transmembrane domain for SERCA (Traaseth, Thomas, & Veglia, 2006). To explain the PLB-SERCA inhibitory mechanism as
well as the effect of phosphorylation on this mechanism, the Veglia group proposes an allosteric model of PLB interaction (Traaseth et al., 2006; Zamoon, Nitu, Karim, Thomas, & Veglia, 2005). They propose that PLB is in equilibrium between two states, an ordered T state and a disordered R state. In both states, the transmembrane domain of PLB is capable of interacting with SERCA, but only the T state can inhibit SERCA. Unphosphorylated PLB favors the order T state and is therefore inhibitory, while phosphorylated PLB favors the disordered R state and is unable to inhibit.

**Predicted PLB Binding Location**

A structure of a co-crystal between SERCA1a and PLB monomer has been determined using cryo-electron microscopy, though not to sufficient resolution to determine the binding location of PLB (H. S. Young, Jones, & Stokes, 2001). Scanning alanine mutagenesis of SERCA1a indentified several residues on helix M6 critical for PLB inhibition suggesting that this helix is a likely point of contact (M Asahi et al., 1999). This observation was consistent with later molecular modeling positioning the transmembrane domain of PLB in close proximity to helix M6 (Hutter et al., 2002). Using distance constraints from PLB-SERCA crosslinking studies, subsequent modeling predicts that the transmembrane domain of PLB is positioned in a groove comprised of helices M2, M4, and M6 of SERCA (Fig. 7A) (Toyoshima et al., 2003). This study utilized crosslinking results from both SERCA1 and SERCA2a since the distance constraints were compatible. The consistency in the crosslinking studies also demonstrates that PLB likely interacts with SERCA1a and SERCA2a in a similar manner.
Figure 7: Predicted regulator binding locations. (A) Crosslinking studies predict PLB (red) to interact with SERCA in a binding pocket described by helices M2, M4, and M6 (blue). (B) Co-crystalization has provided evidence of that PLM (red) interacts with NKA with a more exterior helix M9 (green).
While the positioning of the transmembrane domain of PLB in relation to SERCA is well supported, less is known about the binding location of the cytosolic domain. Initial crosslinking studies predicted that Lys$^3$ of PLB interacts with either Lys$^{397}$ or Lys$^{400}$ of SERCA (James, Inui, Tada, Chiesi, & Carafoli, 1989). This observation is supported by a study showing that the sequence Lys–Asp–Asp–Lys–Pro–Val$^{402}$ in SERCA is critical for PLB regulation (Toyofuku et al., 1994). These results have been used to model the position of the cytosolic domain. However, subsequent crosslinking studies have been unable to provide corroborating evidence calling the initial study into question (Chen, Stokes, Rice, & Jones, 2003).

Insights into the PLB–SERCA regulatory complex structure may also come from homologous proteins, Na, K-ATPase (NKA) and phospholemman (PLM). NKA is a P-type ion pump like SERCA and is thought to function in a similar manner (Kühlbrandt, 2004; Wuytack et al., 2002). The X-ray crystal structure of NKA shares a high degree of resemblance to that of SERCA (Morth et al., 2007) further supporting their potential functional similarities. PLB and PLM are structurally homologous having a single transmembrane domain and a cytosolic domain capable of being phosphorylated. Much like PLB and SERCA, PLM inhibits NKA by reducing ion affinity (Bers & Despa, 2009). Taken together, these observations suggest a high likelihood that PLB and PLM interact with their partners in a similar way. Unlike PLB and SERCA, a high resolution crystal structure has been determined for PLM and NKA (Shinoda, Ogawa, Cornelius, & Toyoshima, 2009). Rather than showing interaction in the M2/M4/M6 binding groove
predicted for PLB, PLM interacts with the more exterior helix M9 of NKA (Fig. 7B). Due
to the similarities between the proteins, this suggests the possibility of an alternative
binding site on SERCA for PLB. However, this observation has not been supported
through alternative approaches like crosslinking or mutagenesis, so it could be an
artifact of crystallization. While unlikely due to the high degree of similarity, it is also
possible that PLB and PLM confer inhibition through alternative means and binding
sites.

**Potential Interacting Proteins**

While PLB and SERCA are a clear functional unit, one must also consider the
possibility that additional proteins may interact with PLB and SERCA in a larger protein
complex. Therefore, additional proteins may alter SERCA function and the ability of PLB
to inhibit in a physiological setting. This has not been a heavily studied area, but there
are several proteins of interest that have been identified. As with many proteins that
can be phosphorylated by PKA, PLB has been found in complex with an A kinase
anchoring protein (AKAP) and has been shown to affect the phosphorylation status of
PLB *in vivo* (Lygren et al., 2007; Manni, Mauban, Ward, & Bond, 2008). The anti-
apoptotic HS-1 associated protein X-1 (HAX-1) has been shown to interact with SERCA
and regulate its expression (Vafiadaki et al., 2009). HAX-1 overexpression has been
shown to reduce contractility of cardiac myocytes, and this effect was abolished in the
PLB knockout mouse (Zhao et al., 2009). HAX-1 was identified as a binding partner of
PLB using the yeast two-hybrid method (Vafiadaki et al., 2007) and was shown to
destabilize PLB pentamer formation (Vafiadaki et al., 2009). Histidine rich Ca\textsuperscript{2+} binding protein (HRC) is an SR Ca\textsuperscript{2+} buffering protein that has been shown to have a regulatory role in Ca\textsuperscript{2+} release via triadin and the ryanadine receptor. Additionally, HRC has been shown to interact with SERCA and alter Ca\textsuperscript{2+} sequestration (D. a Arvanitis et al., 2007; Pritchard & Kranias, 2009). This dual role for HRC suggests a potential crosstalk role between Ca\textsuperscript{2+} uptake and release.

**Disease and Therapy**

*Physiological Principles of Disease*

SERCA activity maintains SR Ca\textsuperscript{2+} load which is a primary determinant of Ca\textsuperscript{2+} release and contractility (Bers, 2008). Decreases in SERCA activity would then seemingly be associated with reduced contractility, a general characteristic of heart failure. In many observed cases, decreased SERCA activity has indeed been related with heart failure (Hasenfuss, 1998; Phillips et al., 1998; Wickenden et al., 1998). In most but not all cases, decreased SR Ca\textsuperscript{2+} content has been observed with heart failure. In many instances, this decreased SR Ca\textsuperscript{2+} content has been connected to depressed SERCA function. However, increased activity of the Na, Ca-exchanger has also been implicated in reduced SR content when no change in SERCA function was observed (Bers, 2008; Hobai & Rourke, 2000; Houser, Piacentino, Mattiello, Weisser, & Gaughan, 2000; Pogwizd, 2000). Depressed SERCA function associated with heart failure can be attributed to decreased expression of SERCA2a or enhanced inhibition from SERCA.
There are no mutations in SERCA2a known to cause a predisposition of heart failure (Kranias & Bers, 2007). In the only screen of SERCA2a mutations in heart failure patients, only 4 point mutations were found, all of which were conserved mutations resulting in no change in the amino acid sequence (Schmidt et al., 2003). This suggests that genetic polymorphisms are not likely to account for differences in SERCA activity in heart failure patients. Darier’s disease is characterized as a haploinsufficiency of the SERCA2 gene resulting in a skin disorder (Brini & Carafoli, 2009; Dally, Corvazier, Bredoux, Bobe, & Enouf, 2010). Interestingly however, adult Darier patients are not predisposed to heart failure suggesting a robust compensatory mechanism for cardiac SERCA expression or sufficient expression from one allele. It has been speculated that the lack of genetic variation within SERCA2a suggests that even minor changes to SERCA2a activity cannot be tolerated and are incompatible with life (Kranias & Bers, 2007).

While there are no known SERCA mutations associated with heart failure, there have been 3 important mutations identified within PLB associated with dilated cardiomyopathy. A substitution of Arg9 for Cys (R9C) has been shown to impair phosphorylation of PLB, thereby enhancing inhibition of SERCA (Brini & Carafoli, 2009; Ha et al., 2011; Schmitt et al., 2003). A premature stop codon at Leu39 (L39stop) has been shown prevent proper insertion of PLB into the SR membrane thereby preventing its ability to regulate SERCA (Kranias & Bers, 2007). The deletion of Arg14 (R14del) is
thought to enhance the interaction with SERCA, thereby increasing the inhibition of SERCA (Haghighi et al., 2006).

**Therapeutic Targets**

Since decreased contractility is a component of heart failure, enhancing SR load through increased SERCA activity is a logical therapeutic path. This concept is supported by studies showing that increasing SERCA2a expression levels through gene transfer in a failing mouse heart model has the ability to restore contractility (Del Monte, Hajjar, Harding, & Inesi, 2001). In fact, this approach is currently in human clinical trials. Decreasing the ability of PLB to inhibit SERCA is also a potential target for therapy. A phosphomimetic PLB mutant (S16E) which has a decreased ability to inhibit SERCA has been shown to be capable of relieving SERCA inhibition in heart failure (Hoshijima et al., 2002). Also, ablation of PLB through antisense DNA transfer has been effective in restoring contractility in a rat failing heart model (Tsuji et al., 2009). While PLB ablation or replacement with non-inhibitory mutants might provide an effective means of restoring contractility, deleterious effects might arise from an inability of SERCA function to respond to β-adrenergic stimulation. Therefore, development of designer PLB mutants that have a reduced ability to inhibit SERCA while retaining the ability to respond to phosphorylation would be a significant improvement of this approach.
CHAPTER III
MATERIALS AND METHODS

Overview of Fluorescence Resonance Energy Transfer

Fluorescence Resonance Energy Transfer (FRET) is a highly effective means of detecting protein-protein interactions and is the predominant methodology I have utilized for this work. A thorough description of FRET can be found in *Principles of Fluorescence Spectroscopy 3rd Edition* (Lakowicz, 2006), but will a brief description of FRET will be provided here.

FRET utilizes the biophysical property of appropriately paired fluorescent molecules (donor and acceptor) that causes the transfer of light energy when in close proximity (<100 Å). This study uses Cerulean (CFP derivative) and YFP as the donor and acceptor fluorophores, respectively. When Cerulean is excited, the energy can be emitted from that fluorophore as a photon of blue light or transferred to YFP (exciting YFP) causing the emission of a photon of yellow light. The percent of the donor excitation energy that is transferred to the acceptor is known as a FRET Efficiency ($E$). $E$ is commonly determined through quantification of the donor and acceptor fluorescence intensities. This work uses the acceptor-photobleaching and E-FRET methods to measure FRET and both are discussed in detail below. When these fluorophores are attached to proteins that interact (*e.g.*, SERCA and PLB), the fluorophores can be (but
not always) in close enough proximity to undergo energy transfer. Observed energy transfer therefore provides an indicator of interaction of the target molecules. $E$ is highly sensitive to and can measure the distance between the fluorophores (inversely related to the distance to the power of 6). Therefore $E$ is more than just an indicator of interaction since it provides some information about the orientation of the interacting proteins by producing a distance constraint.

**Acceptor Photobleaching**

Acceptor photobleaching experiments were performed with an inverted microscope equipped with a 60X 1.49 NA objective, and a back-thinned CCD camera (iXon 887, Andor Technology, Belfast, Northern Ireland). The detector was cooled to -100 °C, using a recirculating liquid coolant system (Koolance, Inc., Auburn, WA). Image acquisition and acceptor photobleaching was automated with custom software macros in MetaMorph (Molecular Devices Corp, Downingtown, PA) that controlled motorized excitation/emission filter wheels (Sutter Instrument Co., Novato, CA) with filters for CFP/YFP/mCherry (Semrock, Rochester NY). The progressive photobleaching protocol was as follows: 100 ms acquisition of CFP image, 40 ms acquisition of YFP image, followed by 10 s exposure to YFP-selective photobleaching (504/12 nm excitation. FRET efficiency was calculated from the fluorescence intensity of the CFP donor before and after acceptor-selective photobleaching, according to the relationship: 

$$E = 1 - \frac{F_{\text{Prebleach}}}{F_{\text{Postbleach}}}.$$  

Representative pre and post bleach images are shown in Fig. 8A.
Figure 8: Acceptor Photobleaching FRET. (A) CFP and YFP images pre and post YFP photobleaching. After photobleaching, the YFP fluorescence is abolished while CFP fluorescence has been enhanced. (B) Average normalized fluorescence intensity of CFP (blue) and YFP (green) during the course of YFP photobleaching. CFP intensity increases with decreasing YFP intensity.
displaying enhanced CFP fluorescence corresponding to abolished YFP fluorescence. Fig. 8B shows the time course of average fluorescent intensity of CFP increasing as average fluorescent intensity of YFP decreases.

**E-FRET**

I have also utilized E-FRET or “3-cube” FRET (Hou & Robia, 2010; Zal & Gascoigne, 2004) rather than acceptor-photobleaching to improve on the speed to data collection. Measurements are consistent with the well established acceptor photobleaching method that our lab has previously used (Hou & Robia, 2010; Hou et al., 2008; Kelly et al., 2008). For these measurements, we used a similar microscope setup described above for acceptor photobleaching. For each sample, automated acquisition of a field of 48 images was performed using a motorized stage (Prior, Rockland, MA) controlled by MetaMorph software. Focus was maintained by an optical feedback system (Perfect Focus System, Nikon). Images were obtained with a 40X 0.75 N.A. objective with 100 ms exposure for each channel: Cer, YFP, and “FRET” (Cer excitation/YFP emission). Fluorescence intensity was automatically quantified with a multi-wavelength cell scoring application in MetaMorph. Cell selection criteria included having a diameter of 35-75 pixels and having an average intensity of 100 counts above background. The data were transferred automatically to a spreadsheet for analysis. E-FRET is a ratiometric FRET estimation calculated from

\[
E = \frac{I_{DA} - a(l_{AA}) - d(l_{DO})}{I_{DA} - a(l_{AA}) + (G-d)(l_{DD})}
\]
where \( I_{AA} \) is the intensity of fluorescence emission detected in the donor channel (472/30 nm) with excitation of 427/10 nm; \( I_{AA} \) is acceptor channel (542/27 nm) emission with excitation of 504/12 nm; \( I_{DA} \) is the “FRET” channel, with 542/27 nm emission and excitation of 427/10 nm; \( a \) and \( d \) are cross-talk coefficients determined from acceptor-only or donor-only samples, respectively. We obtained values for \( d \) of 0.82 for mCerulean, and a value for \( a \) of 0.082 for YFP. \( G \) is the ratio of the sensitized emission to the corresponding amount of donor recovery, which was 3.2 for this setup.

**Binding Curves and Fitting Analysis**

In addition the distance between the fluorescent probes, the percentage of targets engaged in energy transfer determines the FRET efficiency of an individual cell. Expression and affinity of the targets will dictate this percentage, causing FRET efficiency to vary within a population of cells with heterogeneous protein expression. When the FRET efficiency of individual cells is plotted against PLB protein concentration estimated by the YFP-PLB fluorescence intensity, FRET efficiency increases with protein expression up to a maximal level and can be well described by a hyperbolic function, \( y = \frac{(\text{FRET}_{\max})x}{(K_D^2 + x)} \) An example scatter plot is shown in Fig. 9A along with its corresponding hyperbolic fit in black. 300-800 cells are typically used to develop a single binding curve. \( \text{FRET}_{\max} \) describes the intrinsic FRET between SERCA and PLB where FRET is limited only by the distance between the probes, providing structural information (Fig. 9A, blue). \( K_D \) is the protein concentration at half maximal FRET which represents the dissociation constant of the regulatory complex providing an affinity index (Fig. 9A, red).
Figure 9: Quantitative analysis of protein concentration dependence of FRET. FRET efficiency varies within a population of cells, and can be related to protein concentration with a hyperbolic function. A representative scatter plot of individual cell values is shown along with corresponding fitting curve (A, B, black). (A) The FRET efficiency that the fitted curve is approaching is known as FRET$_{\text{max}}$ (blue dashed line), the intrinsic FRET value that limited only by the distance between the fluorescent probes. The protein concentration at half maximal FRET efficiency is the K$_D$ value (red dashed lines). (B) The blue line corresponds to a sample with a decreased FRET$_{\text{max}}$ or increased distance. The red line corresponds to a sample with a reduced affinity.
The absolute values of FRET obtained through our methodology include some level of uncertainty. Due to their unstructured linkage with PLB and SERCA, the fluorescent proteins likely retain a high degree of flexibility with respect to the target proteins. The fluorescent proteins are also relatively large. In combination, these two factors limit the ability of our method to make precise structural conclusions. Presently, we are unable to determine specific concentrations from these fluorescence measurements. Therefore our concentration parameter is presented in arbitrary units (AU) rather than molar units. Therefore, changes or shifts in these $FRET_{\text{max}}$ and $K_D$ parameters provide more significant information than do their absolute values. The red and blue curves in Fig. 9B represent simulations of changes in affinity and structure, respectively. The right-shifted curve in red represents where data corresponding to reduced affinity should fall. The down-shifted curve in blue represents a distance increase corresponding to a structure change.

Non-specific FRET, or energy transfer between close but non-interacting targets, is a concern with FRET measurement, especially if the targets are constrained to the membrane. While we presently have no true negative control for PLB-SERCA FRET, we have estimated non-specific FRET through a competition assay. Addition of increasing amounts of unlabeled protein decreases FRET efficiency in our system to 4% (Hou & Robia, 2010). Therefore, even at the highest expression levels, only 4% of the measured FRET efficiency is accounted for by non-specific FRET.
Confocal Microscopy

Confocal Images

To verify proper expression of YFP-PLB and Cer-SERCA2a, high resolution confocal images were taken of infected myocytes. To visualize sarcolemma and t-tubules, cells were also stained with FM 4-64 (Invitrogen) according to manufactures instructions. Briefly, dye at a stock concentration of 1 mM was diluted to a concentration of 1 µM in PC-1 medium was used to bathe cells for approximately 10 min, after which cells were washed in additional PC-1 to remove excess dye. Images were obtained using an inverted Leica TCS SP5 confocal microscope with a 63x water immersion objective and an 8000 Hz resonant scanner. Cer, YFP, and FM 4-64 were sequentially excited using an argon laser with wavelengths of 458, 514, and 543 nm, respectively.

Confocal Scanning

Confocal scanning microscopy was used obtain simultaneous and high temporal resolution of FRET and Ca^{2+} concentration measurements in paced adult ventricular myocytes. As an index of FRET, the ratio of YFP/Cer fluorescence intensities was obtained with 458 excitation and detection of YFP (525-560 nm) and Cer (470-515 nm) using the Leica system described above. Cells were loaded with X-Rhod-1 AM as an indicator of Ca^{2+} concentration which was detected with 543 nm HeNe laser excitation and an emission range of 570-650 nm. Myocytes were perfused with PC-1 medium
during data collection. Experiments were conducted in x-t mode to study individual Ca\textsuperscript{2+} transients and x-y-t mode to study alternating periods of pacing and rest.

**Tissue Culture**

*Heterologous Cell Culture and Transient Transfection*

AAV-293 cells were cultured in complete DMEM growth medium (HyClone, Waltham, MA) with 10% fetal bovine serum (heat inactivated) (HyClone, Waltham, MA), 2mM L-glutamine (CellGro, Manassas, VA). Cells were split every few days once a confluency of 90-95% had been reached. Cells at approximately 70-80% confluency were transfected according to manufacturer’s instructions, using MBS Mammalian Transfection Kit (Stratagene, La Jolla, CA). 6% modified bovine serum was used instead of the 10% suggested by the manufacturer during the three hour incubation of cells with the CaPO\textsubscript{4}-DNA precipitate. Two days after transfection, cells were trypsinized and plated onto poly-D-lysine coated glass bottom dishes and incubated at 37 C for approximately 2 hours to permit cell attachment before data acquisition.

For Tg exposure, cells were washed 2 times with PBS, and then bathed in PBS containing the appropriate concentration of Tg, 10 pM – 100 uM. For Ca\textsuperscript{2+} experiments, cells were permeabilized with 10 µg/ml saponin for 1 min. and then washed in bath solution of composition 100 mM KCl, 5 mM NaCl, 2 mM MgCl\textsubscript{2}, 20 mM imidazole, 5 mM EGTA, and 2 mM ATP (pH 7.0), with varying free Ca\textsuperscript{2+}. Ca\textsuperscript{2+} concentration was calculated with Maxchelator (Schoenmakers, Visser, Flik, & Theuvenet, 1992) and validated with a Ca\textsuperscript{2+}-sensitive electrode (Thermo Scientific, Waltham, MA).
**Myocyte Culture and Adenoviral Infection**

The Ca\(^{2+}\) phosphate transfection system used to express our fusion proteins in AAV-293 is insufficient for expression in adult myocytes. We therefore developed adenoviral vectors of canine Cer-SERCA2a and canine YFP-PLB using the AdEasy system (Stratagene, La Jolla, CA). Cardiac ventricular myocytes were isolated from adult New Zealand White rabbits by a collaborating lab as previously described (Domeier, Blatter, & Zima, 2009). All protocols were approved by the Loyola University Institutional Animal Care and Use Committee. Myocytes were transferred to culture tubes and washed with PC-1 medium (Lonza, Basel, Switzerland) containing penicillin and streptomycin (Invitrogen).

Myocytes were cultured in dishes containing laminin coated glass coverslips and adenoviruses were added at multiplicity of infection of 100. Sufficient expression of our constructs required approximately 48 hours of culture, however viability of the myocytes declines rapidly. To improve viability, myocytes were paced in culture using a C-Pace EP pacer (IonOptix, Milton, MA) set to 10 volts with a frequency of 10 Hz and 5 ms pulse duration.

**Calcium Indicator**

Experiments required both AAV-293 and adult myocytes to be loaded with a red-shifted fluorescent Ca\(^{2+}\) indicator, X-Rhod-1 AM (Invitrogen). The dye was resuspended in Pluronic® F-127 *20% solution in DMSO (Invitrogen) to a concentration of 1 mM, and sonicated briefly to aid in solubilization. This stock solution was diluted to 1 µM in
either PBS or PC-1, which was used to bathe the cells for 2 min. Cells were then washed gently 3x in either PBS or PC-1 and equilibrated for 20 min prior to imaging.
CHAPTER IV

LIVE CELL CALCIUM UPTAKE ASSAY

Rationale for Development of Assay

Much of the work done for this dissertation and other work in our lab utilizes fluorescent protein fusions. These fusion proteins enable the detection of protein-protein interactions with FRET in live cells, in real-time. While this methodology provides significant advantages, it is not without drawbacks. How the attachment of relatively large fluorescent proteins will affect our target proteins is a valid concern. Therefore, assessing the functionality of our fusion proteins is a necessary step in validating our overall approach.

Traditional SERCA activity assays such as ATP consumption and Ca\(^{2+}\) uptake are needed to quantify the activity of SERCA and the ability of PLB to inhibit this activity. These assays are necessary to directly compare the activities of wild type SERCA and PLB to our fusion proteins. However, these assays can be difficult and time consuming. Having an alternative method to rapid screen for function is therefore highly beneficial for our lab. To complement more direct quantification of our fusion protein activity that our lab has undertaken, we developed an indirect live cell Ca\(^{2+}\) uptake assay, the particulars of which are discussed below.
Protocol

AAV-293 cells are transfected with various constructs as discussed previously. 24 hours post-transfection, cells are plated on poly-D-lysine coated glass slides, and allowed to attach for 2 hours also discussed previously. Ca^{2+} indicator loading is described in the Materials and Methods section. Briefly, cells are washed 2 times with PBS containing 2 mM Ca^{2+} and loaded with a red-shifted Ca^{2+} indicator, X-Rhod-1, AM. Cells are washed an additional 3 times to remove excess dye, and cells are bathed in PBS containing 2 mM Ca^{2+} for 20 min.

First, a 100 ms CFP or YFP is taken to assess transfection status of cells in a given field of view. Then a mCherry or red channel image is then taken every 5 secs for approximately 8 min (100 images). At the 1 min time point, a sufficient baseline X-Rhod signal has been obtained, at which 100 µM ATP is added to the cells. 4 min later, 10 mM thapsigargin is added, and data collection continues for approximately 3 more minutes. The mean X-Rhod and std. error of 30-60 cells is plotted against time.

With typical transfection efficiency around 50-60%, there generally is a heterogeneous population of transfected and untransfected cells in any field of view. This is depicted in Fig. 10A showing CFP, YFP, and red channel images along with the corresponding overlay. This field contains both transfected (T) and untransfected (UT) cells. This allows simultaneous data collection from expressing and non-expressing cells, thereby providing an internal control within the experiment.
Principle of Assay and Results

Activation of cell surface purinergic receptors through ATP stimulation induces an IP₃ mediated Ca²⁺ release in many cell types, including AAV-293 (Ralevic & Burnstock, 1998). Endogenous SERCA Ca²⁺ uptake is initially overwhelmed by this release event, causing Ca²⁺ to accumulate in the cytosol. Desensitization of purinergic receptors and IP₃ degradation halts Ca²⁺ release, preventing further accumulation, and allowing the endogenous SERCA to lower cytosolic Ca²⁺ to basal concentrations. If cells are loaded with a fluorescent Ca²⁺ dye, a spike in fluorescence intensity following ATP stimulation will be observed (Fig. 10B, black). In cells overexpressing our Cer-SERCA, we observed that this spike was nearly abolished (Fig. 10B, red). We believe that enhanced SERCA activity due to overexpression of our fusion protein is able to keep pace with the Ca²⁺ release, thereby preventing Ca²⁺ accumulation.

Because we do not directly measure Ca²⁺ uptake activity, abolishing Ca²⁺ accumulation alone is not sufficient to demonstrate SERCA function. Overexpression of our fusion proteins (Cer-SERCA, YFP-PLB) could deleteriously affect the cell, perhaps causing a depletion of the ER Ca²⁺ stores that would prevent a Ca²⁺ release and accumulation. There is a constant leak of Ca²⁺ from the ER, but basal SERCA Ca²⁺ uptake maintains low cytosolic Ca²⁺ concentrations. Blocking SERCA activity will then cause accumulation of cytosolic Ca²⁺. By applying the SERCA inhibitor thapsigargin (Tg) following ATP stimulation, we are also able to assess ER Ca²⁺ content of these cells.
Figure 10: Ca\(^{2+}\) uptake in live cells. (A) Wide-field fluorescence images of AAV-293 cells transfected with Cer-SERCA and YFP-PLB, loaded with Ca\(^{2+}\) indicator X-rhod-1. This field contains examples of transfected (T) and untransfected (UT) cells. (B) Ca\(^{2+}\) transients were observed after the addition of extracellular ATP in untransfected cells (black), but not in cells expressing Cer-SERCA (red), suggesting Ca\(^{2+}\) uptake activity of Cer-SERCA. Ca\(^{2+}\) transients were partially restored in cells expressing both Cer-SERCA and YFP-PLB (blue). When compared with untransfected controls, Tg-releasable ER Ca\(^{2+}\) content was increased by Cer-SERCA. Cells expressing YFP-PLB (green) had reduced Ca\(^{2+}\) transients and reduced Ca\(^{2+}\) load, suggesting that exogenous PLB inhibited endogenous SERCA.
In both untransfected (Fig. 10B, black) and cells expressing exogenous SERCA (Fig. 10B, red), we observed this Tg-mediated Ca\textsuperscript{2+} accumulation, demonstrating that expression of our fusion protein does not deplete Ca\textsuperscript{2+} stores. In fact, cells overexpressing Cer-SERCA show a greater Tg-mediated Ca\textsuperscript{2+} accumulation compared to untransfected cells, suggesting a greater ER Ca\textsuperscript{2+} content. This provides further evidence of enhanced Ca\textsuperscript{2+} uptake upon overexpression of our SERCA fusion protein.

Co-expression of Cer-SERCA with YFP-PLB partially restores the ATP-mediated Ca\textsuperscript{2+} accumulation to control levels suggesting that YFP-PLB can functionally inhibit Cer-SERCA (Fig. 10B, blue). Also, this further demonstrates the activity of Cer-SERCA, since its known inhibitor can diminish its observed effect. Expression of YFP-PLB alone (Fig. 10B, green) showed smaller ATP and Tg-mediated Ca\textsuperscript{2+} accumulation compared to untransfected cells, indicating YFP-PLB can regulate endogenous SERCA.

Summary

This assay provides a rapid and clear qualitative assessment of our fusion protein activity. We observed that Cer-SERCA1a is active, which can be functionally regulated by YFP-PLB. We also observed that Cer-SERCA2a is also active and can be functionally regulated by YFP-PLB (not shown). While not used in this work, we have also observed that a SERCA tagged with two fluorescent proteins (one of which is inserted in the middle of SERCA) is also active and can be regulated by YFP-PLB (not shown). This demonstrates that robustness of our system, wherein a complex containing 3 fluorescent proteins can remain functional.
CHAPTER V

STEADY-STATE DETECTION OF PLB-SERCA BINDING

Rationale

As discussed in the Literature Review, multiple models of PLB regulation have been proposed, but no clear consensus has been achieved despite extensive research on this interaction. Chemical crosslinking, immunoprecipitation, and FRET studies have been used to investigate the steady-state PLB-SERCA interaction under various conditions that favor particular conformations of SERCA. In addition to their strengths, previous methods have significant limitations. This necessitates additional points of view from new and alternative approaches. We have used FRET in intact cells to quantitatively study the PLB-SERCA interaction, a method that has its own strengths and weaknesses. By using this complementary method, we have sought to tease out some nuances of the PLB-SERCA interaction and integrate the present data in the field into a cohesive model.

Mean FRET

Thapsigargin (Tg) is a cell permeable SERCA inhibitor commonly used to block the Ca^{2+} uptake of intact cells (Michelangeli & East, 2011). Tg has also been shown to completely block chemical crosslinking between PLB and SERCA in isolate vesicles (Chen et al., 2006, 2005; Jones et al., 2002). We therefore initially hypothesized that Tg would
completely displace PLB from SERCA, and therefore completely abolish PLB-SERCA FRET in intact AAV-293 cells. To test this hypothesis we exposed the cells transiently expressing YFP-PLB and CFP-SERCA1a to either 1 µM Tg or vehicle control in PBS for 10 min. Using the acceptor photo-bleaching method, we then measured the average FRET efficiency. We observed that the Tg decreased average FRET efficiency from 13.4 ± 1.7% to 5.2 ± 1.1% (Fig. 11A). FRET between PLB and SERCA was decreased, but not abolished.

In a parallel experiment, we examined the effect of Ca^{2+} on the average PLB-SERCA FRET. As with Tg, we hypothesized that Ca^{2+} would completely displace PLB from SERCA based upon chemical crosslinking studies, and therefore completely block PLB-SERCA FRET in AAV-293 cells. Cells expressing YFP-PLB and CFP-SERCA1a were bathed in 10 µM ionomycin, a Ca^{2+} ionophore, for 10 min in PBS with or without 1.8 mM Ca^{2+}. FRET was then measured in these cells as before. FRET was not significantly decreased by the presence of high Ca^{2+} (21.4 ± 2.2% at low Ca^{2+} and 18.8 ± 2.3% at high Ca^{2+}) (Fig. 11C).

 Antibodies targeting PLB have been previously shown to block the ability of PLB to inhibit SERCA (Cantilina et al., 1993) as well as block PLB-SERCA crosslinking (Chen et al., 2007). In an additional experiment, we examined the effect of rabbit anti-PLB (2D12) on PLB-SERCA FRET in AAV-293 cells. Cells expressing YFP-PLB and CFP-SERCA1a were permeabilized using 20 mg/ml saponin and then bathed in PBS with or without
Figure 11: Mean PLB-SERCA FRET. The mean PLB-SERCA FRET is decreased by (A) Tg and (B) PLB antibody (2D12), but not by (C) elevated calcium concentration.
2D12 antibody. FRET was then measured in these cells using the acceptor-photobleaching method. Average FRET was reduced by PLB antibody to $3.9 \pm 1.2\%$ from a control of $9.7 \pm 1.1\%$ (Fig. 11B). FRET again was not completely abolished as was initially hypothesized.

These average FRET results were inconsistent with the published crosslinking studies which predicted we would observe a complete blockage of FRET with Ca$^{2+}$, Tg, or PLB antibody (Chen et al., 2007, 2006, 2005). We observed no significant reduction in FRET with high Ca$^{2+}$ concentrations, and only partially reduced FRET in the presence of Tg and PLB antibody. The lack of the predicted effects could be due to several factors. Our experimental conditions could have insufficiently saturated our system (incomplete binding of Ca$^{2+}$ and Tg to SERCA or 2D12 to PLB) thereby preventing the full dissociation of PLB from SERCA. However, our initial hypothesis that PLB would completely dissociated PLB from SERCA may have also been incorrect.

**Protein Dependence of FRET**

Using average FRET efficiency from a population of cells as an indicator of protein-protein interaction is simple and straightforward, but is also very limiting. We can detect binding and changes in binding, but cannot fully describe these changes. A decrease in average FRET efficiency could be caused by unbinding or dissociation of a portion of the proteins undergoing FRET indicating a decrease in affinity. Also, there could be an increase in the distance between our probes attached to our target proteins indicating a structure change. We decided further experiments should be analyzed
using a standard lab protocol showing the dependence of FRET efficiency of a cell on its protein expression level. This approach has been shown to distinguish between changes in affinity ($K_d2$) and structure ($FRET_{\text{max}}$) of the PLB-SERCA regulatory complex (Ha et al., 2011; Hou & Robia, 2010; Hou et al., 2008; Kelly et al., 2008) as well as the Na, K-ATPase-phospholemman interaction (Song, Pallikkuth, Bossuyt, Bers, & Robia, 2011).

We are able to observe a clear trend when we plot FRET efficiency of many cells against their protein concentration, estimated by YFP fluorescence intensity (Fig. 9). In control cells, FRET increases with protein concentration up to a maximal level and is described by a hyperbolic function of the form $y = \frac{FRET_{\text{max}}}{K_d2 + x}$. $FRET_{\text{max}}$ and $K_d2$ values are consistent with previously published work from this lab. Addition of 10 μM Tg, as described above, causes a rightward shift of the fitted curve (Fig. 12A). $K_d2$ is increased from 7.0 to 11.9 AU. We observed no change in the $FRET_{\text{max}}$ parameter. These two observations suggest that the decrease in average FRET was due to a decrease in the amount of PLB interacting with SERCA and not an increase in the distance between the two proteins. While there is reduced binding, there is not a complete abolishment in binding that we initially hypothesized based on the crosslinking experiments.

Using this same approach, we further analyzed the Ca$^{2+}$ effect on PLB-SERCA binding. Unlike the mean FRET experiment, we utilized PBS containing 1 mM EGTA, along with 10 μM ionomycin, to further lower Ca$^{2+}$ concentration. This condition produced a typical curve approaching a $FRET_{\text{max}}$ of 30.6%. Addition of 2 mM Ca$^{2+}$ caused
Figure 12: Protein dependence on FRET, acceptor photobleaching. Both (A) Tg and (B) elevated calcium cause a rightward shift in the dependence curves, describing a reduced PLB-SERCA binding affinity.
a slight rightward shift of the data (Fig. 12B). The control and high Ca\(^{2+}\) datasets overlap, but are clearly resolvable with an increase of K\(_D\)2 from 4.5 to 6.0 AU. As with Tg, the Ca\(^{2+}\) effect on PLB-SERCA binding can be described as a decreased affinity, partially reducing binding, but not completely abolishing it.

This is unlike the mean FRET result shown previously where no significant Ca\(^{2+}\) effect was observed. Several factors could explain this discrepancy. Use of EGTA could have partially lowered the control K\(_D\)2 value thereby producing a change that was not present in the mean FRET experiment. Also, with the high degree of overlap in the datasets and a relatively small change in K\(_D\)2, about a 30% increase, the change may have been too subtle to observe with the mean FRET approach. This extra dimension provides an advantage of this binding curve FRET analysis of discerning very subtle changes.

**E-FRET rationale**

Although we observed clear results from the acceptor photobleaching approach, we opted for the E-FRET method to improve the rate of data collection. A two condition plot such as Fig. 12 would require a full day of data collection, whereas a comparable E-FRET plot would take only a few minutes. Therefore it is much easier to compare data from many different conditions. With acceptor photobleaching, the time of Tg/ Ca\(^{2+}\) exposure would vary between 30-120 minutes. Due to the shorter time frame of data collection, Tg or Ca\(^{2+}\) exposure could be more directly controlled, thereby reducing variability within samples. Additionally, a typical E-FRET plot would have a much larger
sample size as an added benefit. Fewer coverslips and transfected cells are needed also improving cost effectiveness of the assay as well.

E-FRET requires calculations with more variables and assumptions, thereby producing more uncertainty in the absolute FRET efficiencies measured. However, we observed consistent results with acceptor photobleaching and E-FRET for both the Tg and Ca\(^{2+}\) effects. The FRET\(_{\text{max}}\) parameters were similar between the two approaches. Because of the different microscope setups used, the K\(_D\) values of the fitted curves, measured in AU, are not the same between the approaches, but the magnitude of the K\(_D\) shifts are similar. Because my conclusions are based more on the relative changes of these curves rather than the specific values, E-FRET was the most effective option for further experimentation.

**Calcium Dose Effect**

Rather than using ionomycin, we opted to saponin-permeabilize our cells to introduce internal solutions with EGTA buffered Ca\(^{2+}\) to more directly control Ca\(^{2+}\) concentration. Cells expressing Cer-SERCA1a and YFP-PLB were permeabilized with 10 µg/ml saponin for 1 min. and then washed in bath solution of composition 100 mM KCl, 5 mM NaCl, 2 mM MgCl\(_2\), 20 mM imidazole, 5 mM EGTA, and 2 mM ATP (pH 7.0), with varying free Ca\(^{2+}\). Free Ca\(^{2+}\) concentration was calculated with Maxchelator.

After 2-3 min in bath solution, the automatic E-FRET acquisition protocol was performed for each Ca\(^{2+}\) concentration. The fluorescence intensities of cells were quantified using the Metamorph multiwavelength application, from which FRET
efficiencies were generated as previously described. Five independent experiments were performed.

Initially, all datasets were fit by a hyperbolic function of the form $y = \frac{\text{FRET}_{\text{max}}x}{(K_{D2} + x)}$, with all parameters independently fit. Because mean $\text{FRET}_{\text{max}}$ was not significantly different for low and high concentrations of $\text{Ca}^{2+}$, we concluded that $\text{FRET}_{\text{max}}$ did not change in response to changes in $\text{Ca}^{2+}$. Therefore, this parameter was shared in a subsequent global analysis, yielding a single $\text{FRET}_{\text{max}}$ fit with independent $K_{D2}$ values for each condition.

A typical plot of is shown in Fig. 13A for low (100 pM) and high (1 mM) $\text{Ca}^{2+}$ concentrations with their corresponding fitted curves. The data points are pooled for visualization, showing average data with error bars corresponding to standard error. A similar $\text{Ca}^{2+}$ effect was observed with cell permeabilization compared to ionomycin treatment used previously producing an approximately 40% increase in $K_{D2}$ value. The $K_{D2}$ values generated from all data sets were then plotted against $\text{Ca}^{2+}$ concentration. Fig. 13B shows the dependence of $K_{D2}$ on increasing $\text{Ca}^{2+}$ concentration. A modified Hill function shows a 41% increase in $K_{D2}$ with $\text{Ca}^{2+}$, with an $EC_{50}$ of 410 nM and a Hill coefficient of 1.1.

**Calcium Electrode**

Since the Maxchelator predicted free $\text{Ca}^{2+}$ concentrations are just theoretical estimations, they require further validation. Fig. 14A depicts the dose response of $\text{Ca}^{2+}$ using solutions containing 1 mM EGTA, corresponding to the predicted Maxchelator
Figure 13: Calcium effect on PLB-SERCA FRET, E-FRET. (A) As with acceptor photobleaching, elevated calcium (red) causes a right-shifted curve compared to control (black) corresponding to a decreased PLB-SERCA affinity with no change in the FRET$_{\text{max}}$ parameter. (B) The calculated $K_D2$ values from the example fitted curves in A are shown plotted against calcium concentration. $K_D2$ appears to transition between two affinity states upon elevation of calcium.
**Figure 14:** *Calibration of calcium buffered solutions.*  (A) The initial calcium response curve with an EGTA of 1 mM with the calcium concentration values measured estimated by MaxChelator. (B) The calcium calibration curve of our prepared solutions with 1 mM EGTA (red) compared to standard solutions (black). While in agreement at high calcium concentrations, our solutions deviated from the known standard solutions at lower concentrations. (C) The dose curve in A with calcium concentration adjusted using the voltage curve in B showing that the low calcium solutions were all clustered around the same concentration. The red curve shows the original fitted curve in A, and the blue and green curves show other potential curves that could also fit the data set. An estimate of the high affinity (low KD) state was therefore unable to verified using only 1 mM EGTA. (D) The calcium calibration curve of our prepared solutions with 5 mM EGTA (red) compared to standard solutions (black) showing agreement of the full range of calcium concentrations.
values. Using a Ca\textsuperscript{2+} sensitive electrode (Thermo Scientific), we compared the free Ca\textsuperscript{2+} concentration of our solutions, to a set of standard Ca\textsuperscript{2+} solutions (World Precision Instruments). As shown in Fig. 14B, we observed good agreement with the standards at concentrations above 100 nM, but deviation from the standards at lower concentrations.

Our initial Ca\textsuperscript{2+} dependence plot is shown in Fig. 14C after correction with the standard concentration curve. With the low Ca\textsuperscript{2+} data points clustered around 100nM, we could not conclude that we were accurately quantifying the lowest possible K\textsubscript{D2} value. The blue and green curves represent simulations that could also fit the data, showing large differences in the potential low K\textsubscript{D} value. By increasing EGTA concentration from 1 mM to 5 mM, our prepared solution were in good agreement with the standards over the full range of concentrations (Fig. 14D). The experiments were then independently repeated 5 times with the newly determined solutions. With the increased EGTA concentration, a slightly larger increase in the K\textsubscript{D2} change was observed, but the overall trend remained the same. While it did not alter our final conclusions, we further validated our methodology.

**Thapsigargin Dose Effect**

Using the same high-throughput E-FRET described for Ca\textsuperscript{2+}, we quantified the Tg effect over a range of Ca\textsuperscript{2+} concentrations, 10 pM to 100 uM, in AAV-293 cells expressing YFP-PLB and Cer-SERCA1a (Fig. 15A). This dose response was repeated with 4 independent experiments. As with acceptor photobleaching, we observed a rightward
shift to the concentration dependence of PLB-SERCA FRET signifying an increased $K_D$ value, or a decreased affinity.

Initially, all datasets were fit by a hyperbolic function of the form $y = \frac{(FRET_{\text{max}})x}{(K_D + x)}$, with all parameters independently fit. Because mean $FRET_{\text{max}}$ was not significantly different for low and high concentrations of Tg, we concluded that $FRET_{\text{max}}$ did not change in response to changes in Tg. Therefore, this parameter was shared in a subsequent global analysis, yielding a single $FRET_{\text{max}}$ fit with independent $K_D$ values for each condition.

A typical plot is shown in Fig. 15A for low (10 pM) and high (100 uM) Tg concentrations with their corresponding fitted curves. Mean $K_D$ (+SE) values generated from all data sets were then plotted against Tg concentration (Fig. 15B). A modified Hill function shows a 78% increase in $K_D$ with Ca$^{2+}$, with an EC50 of 350 nM and a Hill coefficient of 0.7.

**PLB Antibody Effect**

In a continuation of the mean FRET experiments, we also quantified the protein concentration dependence of FRET in the presence of the PLB antibody 2D12. AAV-293 cells expressing Cer-SERCA1a and YFP-PLB were permeabilized with 50 µg/ml saponin for 1 min. and then washed in bath solution of composition 100 mM KCl, 5 mM NaCl, 2 mM MgCl2, 20 mM imidazole, 1 mM EGTA, and 2 mM ATP (pH 7.0), containing 10 µg/ml 2D12 PLB antibody. The E-FRET protocol was performed at 5, 30, and 120 minutes on the same cells in the presence or absence of antibody, to determine the protein
Figure 15: Tg effect on PLB-SERCA FRET, E-FRET. (A) As with acceptor photobleaching, elevated Tg (red) causes a right-shifted curve compared to control (black) corresponding to a decreased PLB-SERCA affinity with no change in the FRET$_{max}$ parameter (B) The calculated $K_D2$ values from the example fitted curves in A are shown plotted against TG concentration. $K_D2$ appears to transition between two affinity states upon elevation of Tg.
dependence on FRET. At the 5 min. time point, no change in FRET was observed from exposure to PLB antibody (not shown). PLB antibody greatly reduced PLB-SERCA FRET at 30 min (Fig. 16A), and to a greater extent at 120 min (Fig. 16B). At both time points, there appears to be a reduction in the FRET_{\text{max}} parameter without a change in the K_{\text{D2}} value. More attempts are needed to determine significance.

Additionally, increased incubation time, higher antibody concentrations, and more robust permeabilization could conceivably produce a larger effect by the PLB antibody. Further tests are also needed to verify that a maximal effect has been achieved before more explicit conclusions can be made. Attempts to generate a longer time point (overnight) failed to produce usable data because of sample degradation. Any future experiments should also include nonspecific IgG as control rather than no antibody. However, this may suggest that the antibody may not fully dissociate PLB from SERCA. The reduction in FRET_{\text{max}} would suggest an altered structure of the PLB-SERCA complex.

**PLB-PLB FRET**

To assess PLB pentamer formation, we also quantified FRET in cells co-expressing Cer-PLB and YFP-PLB using the E-FRET method. Cells were either exposed to 10 mM Tg or had Ca^{2+} controlled to low (1 nM) and high (1 mM) concentrations with EGTA buffered solutions as in PLB-SERCA FRET experiments described earlier. Neither Tg exposure or changes in Ca^{2+} concentrations affected the FRET_{\text{max}} or K_{\text{D1}} parameters, (Bidwell et al., 2011) and FRET_{\text{max}} value of 54.2 ± 0.7 is consistent with previously
Figure 16: PLB antibody (2D12) effect on PLB-SERCA FRET. The protein dependence of FRET in the absence (black) and presence (red) of PLB antibody after (A) 30 min and (B) 120 min incubation times.
measured values (Hou et al., 2008; Kelly et al., 2008). This demonstrates that neither our exposure of Tg or changes in Ca$^{2+}$ concentrations alter pentamer structure or affinity, which was expected since neither Tg or Ca$^{2+}$ have been predicted to directly interact with the PLB pentamer.

Since PLB exists in a coupled equilibrium (Fig. 4), this demonstrates that our observed decreases in PLB-SERCA affinity by Ca$^{2+}$ (Fig. 13) and Tg (Fig. 15) are not caused by increases pentamer affinity. With no change in PLB-PLB FRET observed, this demonstrates that our methods of Tg exposure and control of Ca$^{2+}$ concentration do not have an effect on fluorophore stability or membrane dynamics that could globally decrease FRET efficiency or membrane protein affinity. Therefore, the absence of PLB-PLB FRET changes provides a good control experiment validating our methodology and demonstrating that our PLB-SERCA effects are specific.

**Summary**

We observed a decrease in PLB-SERCA FRET in response to increases in Ca$^{2+}$ and Tg concentration. This decrease can be attributed to a reduced affinity, as observed by an increased $K_{D2}$ value. With no change in the FRET$_{\text{max}}$ parameter, no change in the distance between the fluorescent probes was observed. However, some structural rearrangement decreasing the strength of the intermolecular contacts must account for the decrease in affinity. This structure change is likely just not accompanied by a change in the position of the fluorescent probes.
We observed $K_D$ increases in response to increases in $Ca^{2+}$ and Tg concentrations up to a maximal level corresponding to a transition in the affinity of the PLB-SERCA regulatory complex. We therefore believe that the $Ca^{2+}$ dose response (Fig. 13B) shows PLB transitioning from binding the E2 ($Ca^{2+}$ unbound) and the E1 ($Ca^{2+}$ bound) conformations of SERCA. So even at saturating $Ca^{2+}$ concentrations known to fully relieve PLB inhibition of SERCA, significant PLB-SERCA binding is still observed. This suggests that unbinding of PLB is not required for relief of PLB-SERCA inhibition.
CHAPTER VI

SERCA INTERACTION WITH TRUNCATED PHOSPHOLAMBAN

Rationale

To account for our data in the previous chapter as well as other data in the field, we propose the \textit{Domain Displacement Model} where high $\text{Ca}^{2+}$ concentrations displace the transmembrane domain of PLB to a second binding site on SERCA with no change in the position of the cytosolic domain. To directly test this model, we need to assess changes in position of the transmembrane which could not be accomplished in my earlier studies. Since YFP is N-terminally linked to PLB in previous studies, we are only able to confirm that the position of the cytosolic domain does not change in response to $\text{Ca}^{2+}$. If the transmembrane domain is displaced independently of the cytosolic domain, the position of YFP would not change in response changes in $\text{Ca}^{2+}$ concentration.

To take this factor into account, we have sought to quantify FRET between SERCA and a truncated PLB which lacks the cytosolic domain. An N-terminally linked YFP would then be contiguous with transmembrane domain. Therefore, if the transmembrane domain is displaced to an alternative binding site, we expect to see a change in our $\text{FRET}_{\text{max}}$ parameter unlike with full-length PLB.

A concern with truncating PLB is that it may not then properly interact with SERCA; therefore assessing its interaction would not be sufficient to test our hypothesis.
Previous studies have shown that truncated PLB corresponding to residues 25-52 (Hughes et al., 1996) and 28-52 (Y Kimura et al., 1996) are capable of fully inhibiting SERCA activity. These results provide some feasibility of our approach.

**Vector Construction**

The sequence corresponding to residues 28-52 of canine PLB was amplified with PCR, the product of which included BglIII and HindIII restriction sites. A double digestion and ligation were performed inserting amplified region of PLB into the eYFP-C1 vector (Clontech) at the multiple cloning site, generating YFP-tmPLB. A complementary vector, Cer-tmPLB, was produced by performing a double digest and ligation with YFP-tmPLB and pmCerulean-C1 (Clontech) using the BglIII and HindIII restriction sites. Both vectors have a 7 residue spacer between the N-terminus of residues 28-52 of PLB and the fluorescent protein, coming from the remaining sequence in the multiple cloning site.

**Simulation of tmPLB FRET**

Using Pymol, we estimated the anticipated distance changes produced by YFP-tmPLB, compared to full-length PLB, using the PDB structures represented in Fig. 17A. Since the positions of the fluorescent probes are unknown, some uncertainty in the distance measurements, the estimations were made by applying distance changes to the observed distance calculated from the FRET$_{\text{max}}$ value generated by the steady-state experiments. I first measured the distance between the N-terminus and residue 28 of
Figure 17: FRET between YFP-tmPLB and Cer-SERCA. Simulated binding curves based on PDB structure measurements (A) and experimentally observed binding curves (B) with wtPLB shown shaded and dashed and tmPLB shown in solid. (A) At low calcium concentration (black), our model predicts that decrease in FRETmax with tmPLB with a further decrease at high calcium (red). (B) At low calcium, we observe the expected decrease in FRETmax (black), but do not observe the expected additional decrease at high calcium concentration (red).
PLB to account for the truncation. I then measured the distance between the predicted binding pocket of helices M2/M4/M6 and the exterior position of helix M9.

**Calcium Effect**

FRET was quantified by the E-FRET method while Ca\(^{2+}\) concentration was controlled as previously described in cells expressing Cer-SERCA1a and either YFP-PLB (full-length) or YFP-tmPLB. As seen previously, saturating Ca\(^{2+}\) (1 mM) caused a rightward shift in the curve describing protein dependence of FRET compared to low Ca\(^{2+}\) (< 1 nM) for YFP-PLB expressing cells, shown in Fig. 17B with the shaded, dashed lines. At low Ca\(^{2+}\) concentration in cells expressing YFP-tmPLB, the anticipated decrease in FRET\(_{\text{max}}\) to 20.1% was observed (Fig 17B, black). However, saturating Ca\(^{2+}\) concentrations had no effect on the protein dependence of FRET in these cells (Fig. 17B, red). Neither the anticipated increase in K\(_D\) or further decrease FRET\(_{\text{max}}\) was observed, suggesting that no change in the binding of YFP-tmPLB and Cer-SERCA1a occurred.

**YFP-tmPLB Activity**

To fully characterize the YFP-tmPLB that was used in the above experiment, we assessed its ability to inhibit SERCA activity using the *Live Cell Calcium Uptake Assay* described previously. Expression of Cer-SERCA1a alone greatly reduces the ATP mediated Ca\(^{2+}\) accumulation observed in untransfected cells. However, co-expression of YFP-tmPLB does not attenuate the SERCA effect (Fig. 18B) as previously observed with YFP-PLB (full-length) (Fig. 10). Interesting this suggests that YFP-tmPLB does not functionally regulate Cer-SERCA1a despite being able to interact.
Figure 18: Characterization of tmPLB. (A) tmPLB pentamer FRET has an increased FRETmax compared to wtPLB consistent with truncation of the cytosolic domain. The $K_{D1}$ parameter is the same for both tmPLB and wtPLB. (B) tmPLB does not restore the ATP dependent calcium accumulation in our in cell activity assay when co-expressed with Cer-SERCA.
**Pentamer FRET**

To further characterize YFP-tmPLB, we also assessed its ability to bind to itself by measuring FRET between YFP-tmPLB and Cer-tmPLB using the E-FRET method described previously. In cells expressing full length PLB tagged with YFP and Cer, we observed a FRET$_{\text{max}}$ of 53.1% (Fig. 18A, **black**), consistent with values obtained previously (Bidwell et al., 2011). In cells expressing YFP-tmPLB and Cer-tmPLB, we observed an increased FRET$_{\text{max}}$ to 60.9% (Fig. 18A, **red**) consistent a decreased distance produced with removal of the cytosolic domain placing the fluorescent closer together. The same K$_D$1 value was observed for both full length and truncated PLB, demonstrating that truncation of PLB in YFP-tmPLB does not alter pentamer affinity.

**Summary**

A change in the FRET$_{\text{max}}$ parameter was not observed when saturating Ca$^{2+}$ was added to cells expressing YFP-tmPLB and Cer-SERCA1a. Therefore, the movement of the transmembrane domain to an alternative binding site was not observed as predicted. YFP-tmPLB-Cer-SERCA FRET is completely unresponsive to changes in Ca$^{2+}$ concentration with neither the FRET$_{\text{max}}$ nor K$_D$2 parameter changing upon addition of saturated Ca$^{2+}$. Also, YFP-tmPLB does not appear to functionally regulate SERCA activity. Taken together, these observations suggest that YFP-tmPLB does not associate with SERCA as does full-length PLB.

However, YFP-tmPLB does interact with SERCA since it expresses correctly in the ER and undergoes FRET with Cer-SERCA1a. The FRET$_{\text{max}}$ parameter is decreased to
20.1% compared to 28.2% in full length expressing cells. This decrease is consistent with a change in the position of YFP attached to a truncated PLB, as predicted by distance measurements of the PDB structure. Also, the observed $K_D$ parameter (pentamer affinity) is the same for both full-length and truncated PLB. These observations suggest that YFP-tmPLB retains some binding characteristics of full-length PLB despite inability to regulate SERCA and unresponsiveness to changes of $Ca^{2+}$ concentration.

Presently, we hypothesize that steric hindrance of the fluorescent protein in YFP-tmPLB prohibits functional interaction with SERCA, thereby preventing its ability to regulate SERCA and to respond to $Ca^{2+}$. Changes to the YFP-tmPLB construct may then allow proper interaction. The planned changes and further characterization of the current YFP-tmPLB construct are reviewed in detail in the Discussion section.
CHAPTER VII
REAL-TIME PLB-SERCA FRET

Rationale

To this point, all investigation of the PLB-SERCA interaction has utilized steady-state biochemical approaches. Because of the limitations of these studies, little is understood about the kinetics and dynamics of this interaction. A fundamental question that remains is how this interaction changes over-time in response to changes in Ca$^{2+}$ concentration. This question is all the more important considering Ca$^{2+}$ concentrations change rapidly on a millisecond timescale in cardiac and slow twitch skeletal muscle, the only PLB expressing tissues. In the case of cardiac muscle, these changes are occurring continuously, on a beat to beat basis of the heart.

The range of Ca$^{2+}$ concentration over which PLB alters the interaction with and activity of SERCA corresponds to concentrations of a Ca$^{2+}$ transient of cardiac myocyte (100 nM – 1 µM). In other words, PLB interaction and inhibition of SERCA could theoretically vary over the course of a single Ca$^{2+}$ transient. That is if changes in binding and inhibition occur on a fast enough timescale, which is undetermined at the present time due to limitations of past approaches.
Our FRET system describes a clear change in PLB-SERCA FRET in response to elevated Ca\(^{2+}\). This change occurs in the same range of Ca\(^{2+}\) response to assays predicting complete dissociation, suggesting that the approaches are compatible and detect the same change in binding. The variations in the approaches produce a different observable endpoint, favoring different mechanistic models. A significant benefit of our system over all other methods is that we can detect changes in PLB-SERCA binding in real time within intact cells. Therefore, our methodology has the potential to investigate the time domain of this interaction, no matter the explicit model of PLB-SERCA interaction.

Evidence of the timescale of PLB-SERCA binding comes from earlier work from our lab (Robia et al., 2007). Using a FRET recovery after photobleaching approach, rapid exchange between PLB and SERCA was observed with a time constant of 1.4 s. With this result in combination with our steady-state experiments where Ca\(^{2+}\) decreases PLB-SERCA FRET, we initially hypothesized that a single Ca\(^{2+}\) transient would cause a small but detectable decrease in PLB-SERCA FRET. To test this hypothesis, we expressed our fluorescent PLB and SERCA proteins in paced adult ventricular myocytes and simultaneously measured FRET and Ca\(^{2+}\) concentration.

**Characterization Myocyte FRET Detection**

Myocyte culture and adenoviral infection are discussed earlier in the *Materials and Methods* section. Localization of YFP-PLB and Cer-SERCA2a was verified with confocal microscopy. Images show adult ventricular myocytes expressing Cer-SERCA2a
Figure 19: Adenoviral expression of Cer-SERCA and YFP-PLB in cardiac myocytes. Confocal images of an isolated adult rabbit ventricular myocyte expressing Cer-SERCA and YFP-PLB. Both proteins were distributed in longitudinal streaks as well as striations. The striations were due to localization of both proteins at the Z-line, as indicated by counterstaining with the membrane dye FM 4-64. The overlay image shows the relative localization of Cer-SERCA, YFP-PLB, and FM4-64. Scale bar = 10 μM.
and YFP-PLB displaying a striated pattern of fluorescence as well as longitudinal streaks (Fig. 19), suggesting localization in the junctional and transverse SR, respectively. Counterstaining with the membrane dye FM 4-64 demonstrate that the t-tubule system was largely intact after 2 days of culture. The overlay image shows that the striated pattern of SERCA and PLB corresponds to the Z-lines of the sarcomeres. By measuring fluorescence recovery after photobleaching, our lab has also shown that both PLB and SERCA can diffuse across multiple sarcomeres during the course of several minutes (Bidwell et al., 2011).

The protein dependence of FRET between PLB and SERCA in these cells was also quantified using the E-FRET method used in the earlier steady-state experiments in AAV-293 cells and described in the Materials and Methods section. FRET increased with protein concentration towards a maximal value, consistent with the measurements discussed previously in AAV-293 cells (Bidwell et al., 2011).

**Single Calcium Transient FRET Measurements**

Myocytes were infected with Cer-SERCA2a and YFP-PLB and loaded with Ca^{2+} indicator dye as discussed in Materials and Methods and then perfused with PC-1 medium and stimulated at a rate of .25 Hz. Confocal time course imaging was performed in x-t mode (linescanning) also described in Materials and Methods. Fig. 20A shows the average of 21 con- transients obtained by line-scan mode measurements of the indicator X-rhod-1 (black trace) normalized to diastolic Ca^{2+}. We obtained simultaneous measurements of YFP and Cer fluorescence with 458 nm excitation. We
Figure 20: Quantification of calcium and FRET changes in single transients. (A) An average of 21 consecutive calcium transients quantified by X-rhod-1 fluorescence (black) and the corresponding PLB-SERCA FRET ratio (red) in a paced ventricular myocyte. (B) 5 µM isoproterenol increased the magnitude and decreased the width of the calcium transient.
did not detect a systole-to-diastole change in the normalized YFP/Cer ratio (Fig. 20A, red trace), suggesting that FRET from Cer-SERCA to YFP-PLB is not abolished during systole. The β-adrenergic agonist isoproterenol (5 µM) increased the amplitude and decreased the duration of the Ca\(^{2+}\) transient (Fig. 20B), but FRET was still unaffected.

**FRET Response to Pacing**

Since individual Ca\(^{2+}\) transients were insufficient to produce an observable change in PLB-SERCA FRET we hypothesized that FRET would be abolished by a larger prolonged elevation of cytosolic Ca\(^{2+}\). To do so, we alternated between intervals of rest (no stimulation) with intervals of 1-Hz pacing producing a rapid and progressive increase in average Ca\(^{2+}\). While not naturally occurring, this protocol shows that our detection method is capable of observing real-time changes in PLB-SERCA FRET in response to rapid changes in physiological Ca\(^{2+}\) concentration. Essentially, we use the cellular machinery of the myocytes rapidly alter Ca\(^{2+}\) concentration.

In response to Ca\(^{2+}\) changes of this protocol, we observed a modest reduction in the normalized YFP/Cer ratio with rapid pacing apparent in the absence (Fig. 21A) or presence (Fig. 21B) of 5 µM isoproterenol. Fig. 21C/D (black) shows the mean cytosolic Ca\(^{2+}\) elevation for control (n = 6 cells) and after the addition of isoproterenol (n = 4 cells) normalized to resting Ca\(^{2+}\), and the corresponding mean YFP/Cer ratio is shown in red. During pacing intervals, the mean cytosolic Ca\(^{2+}\) was significantly elevated when compared with rest, both before \((p < 0.01)\) and after \((p < 0.01)\) isoproterenol. FRET was also significantly decreased when compared with rest, both before \((p < 0.05)\) and after
Figure 21: Quantification of calcium and FRET changes by alternating pacing. (A) Prolonged elevations of Ca\(^{2+}\) (black) were achieved with intervals of rapid (1 Hz) pacing after periods of rest (no stimulation). A modest decrease in FRET (red) was observed during rapid pacing. The blue lines represent the Ca\(^{2+}\) and FRET traces smoothed by 5 second adjacent averaging. (B) As in A, after 5 \(\mu M\) isoproterenol, a small decrease in FRET (red) was observed during rapid pacing. (C) Averaging multiple experiments showed that rapid pacing increased mean [Ca\(^{2+}\)], with a larger increase observed after addition of 5 \(\mu M\) isoproterenol. (D) Averaging multiple experiments showed that rapid pacing decreased the mean YFP/Cer ratio, and this decrease was larger after addition of 5 \(\mu M\) isoproterenol. The first, second, and third pacing intervals are marked “a, b, c.”
(p < 0.01) isoproterenol. Overall, the data are consistent with a small decrease in FRET during intervals of rapid pacing.

**FRET Response to Calcium Changes in Heterologous Cells**

With the time resolution of Fig. 21, we can estimate that the change in FRET occurs within 2 seconds of elevation of the Ca$^{2+}$. However, this is a much longer than the time course of a single Ca$^{2+}$ transient. We therefore cannot conclude if the rest/pace protocol produced a change in FRET due to a higher Ca$^{2+}$ concentration, a more prolonged Ca$^{2+}$ elevation, or both.

To test these possibilities, we set out to measure the time response of PLB-SERCA FRET to elevations of Ca$^{2+}$ in AAV-293. Cells expressing Cer-SERCA and YFP-PLB were loaded with X-Rhod-1 as in the myocytes, and then bathed in PBS containing 1 mM EGTA and ionomycin. Image acquisition of E-FRET and Ca$^{2+}$ concentration every 10 s was started at this low concentration. After 1 min of acquisition to achieve baseline signals, PBS containing 2 mM Ca$^{2+}$ added and acquisition continued. Fig. 22 shows that increases in Ca$^{2+}$ concentration appear to directly coincide with decreases in FRET signal, suggesting a rapid response. However, time resolution of this approach is insufficient to clearly determine the time course of the Ca$^{2+}$ action. Further experimentation with faster time resolution is needed, but indicates that this methodology clearly detects changes in FRET in response to Ca$^{2+}$ over time.
Figure 22: Time course of calcium effect on PLB-SERCA FRET. In AAV-293 cells bathed in 10 µM ionophore, addition of 1 mM Ca$^{2+}$ to the bath solution increases intracellular Ca$^{2+}$ concentrations estimated by average X-Rhod-1 fluorescence (black). Average PLB-SERCA FRET (red) decreases rapidly in response to the elevated Ca$^{2+}$. 
Summary

We observed no change in PLB-SERCA FRET in response to the individual Ca\(^{2+}\) transients of paced cardiac myocytes. Even with the enhancement of these transients from the exposure to β-adrenergic stimulation, no change could be observed. However, we did observe a small (2%) decrease in FRET in response to intervals of prolonged rapid pacing and rest, which produced relatively higher and more prolonged elevations of cytosolic Ca\(^{2+}\) compared to individual transients. While not a physiologic pacing pattern, this protocol demonstrates the capability of our approach in detecting real-time changes in FRET in response to physiological changes in Ca\(^{2+}\) concentration. We must now attempt to determine the specific time domain of Ca\(^{2+}\) action on PLB-SERCA FRET.
CHAPTER VIII

DISCUSSION

Global versus Single Molecule Effects

One of the ultimate goals of this research is to understand the mechanism of PLB inhibition of SERCA with respect to how individual molecules interact and function. However, our work, as well as the studies that have preceded it, is attempting to do so by making global assessments of a large population of molecules. While we attempt to favor the existence of a particular conformation of SERCA in our steady-state measurements, we ultimately only achieve an equilibrium shift in a distribution of several conformations. In our real-time FRET measurements, we are not observing synchronous changes in all PLB-SERCA interactions, so at any given point in time we are observing several interacting states. Therefore, any given measurement of the PLB-SERCA interaction is a composite of many interacting molecules from which we attempt to deduce information on the interaction of individual molecules. This is not a critique of our approach given the inherent difficulty of making single molecule measurements, but merely an important consideration necessary to put our results, and the results of others, in the proper perspective.
Steady State FRET

We have used FRET to measure the interaction between fluorescently tagged PLB and SERCA in cells under conditions that favor particular structural conformations of SERCA. At low Ca\(^{2+}\) concentrations that favor the E2 SERCA state, we have observed that PLB interacts with SERCA in a protein dependent manner consistent with our previous use of this approach (Ha et al., 2011; Hou & Robia, 2010; Hou et al., 2008; Kelly et al., 2008; Song et al., 2011). This observation is also consistent with both the Dissociation and Subunit Models of PLB-SERCA regulation, since both predict that PLB interacts with the E2 conformation with high affinity.

When Ca\(^{2+}\) was elevated to saturating concentrations favoring the E1 conformation, we observed that PLB-SERCA FRET was decreased, but not completely abolished (Fig. 13). The decreased FRET can be explained as a reduced affinity between PLB and SERCA (increased K\(_D\)) without a change in the structure of the regulatory complex (no FRET\(_{max}\) change). While binding is decreased at any given protein concentration, significant levels of binding are detected at saturating concentrations of Ca\(^{2+}\). This is inconsistent with the Dissociation Model where FRET would be predicted to be completely abolished. In theoretical terms, the mutually exclusive binding described by the Dissociation Model predicts a K\(_D\) value that approaches infinity with increasing Ca\(^{2+}\) concentrations. K\(_D\) certainly would not achieve a maximal level as we observed. Under physiological constraints, an increase in the K\(_D\) value by a few orders of magnitude at millimolar Ca\(^{2+}\) would effectively correspond to the Dissociation Model.
However, the much lower 40% increase in $K_d$ would not be consistent with complete
dissociation of PLB and SERCA at physiological conditions $Ca^{2+}$ concentrations.

One might argue detection of PLB-SERCA binding in the presence of $Ca^{2+}$ is an
artifact of our approach. The high concentrations of proteins in the membrane will
place non-interacting proteins in close enough proximity to undergo energy transfer. It
is certainly possible that unbound proteins will undergo FRET. However, in a
competition assay using unlabeled PLB, we currently estimate FRET between unbound
membrane proteins or “non-specific FRET” around at most 4%. This is clearly much
lower than the 28% FRET observed with our assay. Furthermore, we have shown that
the estimated distance between the probes can be specifically altered and that this
distance is consistent with known structure of SERCA (Hou & Robia, 2010; Kelly et al.,
2008; Song et al., 2011). These observations suggest that FRET is not likely caused by
aggregation of the fluorescent probes which has been suggested to skew FRET
measurements (Chen et al., 2006).

These results are more in line with the Subunit Model and the previous FRET
studies where high levels of PLB-SERCA binding are detected even in the presence of
$Ca^{2+}$ (Mueller et al., 2004). Mueller et al observed only a minimal decrease in FRET in
the presence of $Ca^{2+}$ at the highest protein concentrations rather over the full range of
concentration as was observed with our approach. This decrease in was consistent with
a small structure change of the regulatory complex corresponding to approximately a 1
Å movement that was not detected with our approach. Unlike our results, Mueller et al
did not observe a change in PLB-SERCA affinity, but it is possible that their method did not assess interaction at sufficiently low protein concentrations where the affinity effect would be observed. Although we observed no change in our distance measurements, a 1 Å movement is within the error of our method. The larger size and rotational mobility of the fluorescent proteins used in our method may mask the subtle change Mueller et al observed using small, covalently attached fluorophores.

Just like Ca$^{2+}$, the presence of the SERCA inhibitor Tg has been shown to completely abolish PLB-SERCA crosslinking (Chen et al., 2006, 2005) but only slightly diminish FRET in our system. Like Ca$^{2+}$, we observed that Tg reduced PLB-SERCA affinity (increased $K_D$) with no change in the structure of the complex (no change in $FRET_{max}$) (Fig. 15). While assessing the ability of PLB and SERCA to interact in the presence of Tg does not assist in the modeling the PLB-SERCA mechanism, it does aid in the validation of our FRET approach. Because Tg is membrane permeable, its application is a more straight forward protocol as compared to Ca$^{2+}$ which requires saponin permeabilization to introduce artificial cytosolic solutions. Our observation of a lack of complete PLB-SERCA dissociation at elevated Ca$^{2+}$ concentrations is likely not due to insufficient permeabilization or inappropriate solutions since Tg produced a comparable effect.

Early proteolysis studies displayed no difference in the fragmentation patterns of SERCA at low Ca$^{2+}$ concentrations and in the presence of Tg (Sagara & Inesi, 1991). This observation was thought to indicate that SERCA bound by Tg was comparable to the E2 Ca$^{2+}$ free conformation. Tg was then used to stabilize SERCA in what was assumed to be
the E2 conformation for some of the first high resolution crystallographic studies (Danko, Yamasaki, Daiho, Suzuki, & Toyoshima, 2001; Toyoshima & Nomura, 2002). However, current consensus suggests that SERCA bound by Tg is not representative of the E2 conformation, and alternative crystal structures of the E2 conformation have been developed in the absence of Tg. Our Tg dose response curve (Fig. 15B), showing two PLB affinity states, also suggests that SERCA bound by Tg is a structural conformation distinct from the E2 Ca\(^{2+}\) free conformation. We have named this conformation “E2-Tg” for its similarity to, yet distinct from, the E2 Ca\(^{2+}\) free conformation. The E2 and E2-Tg conformations likely have similar cytosolic orientation exposure the same cleavage sites, yielding the same banding pattern. They however differ greatly in the transmembrane region where the strength of the PLB-SERCA interaction is likely dictated.

Both the Ca\(^{2+}\) and Tg effects on our FRET measurements occur in the same range of concentrations as the crosslinking approach. This similarity suggests that both approaches are likely detecting the same change in PLB-SERCA binding, but are just detected by a different signal. This consistency helps validates our overall FRET approach showing that the changes we observe are not unique to our system.

Because of the predicted large structure changes in the cytosolic headpiece between the E1 and E2 conformations of SERCA, we thought it likely that this could generate a large structural change in cytosolic region of the PLB regulatory complex. However, we did not observe a change in the FRET\(_{\text{max}}\) parameter, indicating no change
in the position of the fluorescent probes attached to the N-terminus of both PLB and SERCA, loci that would be thought to move apart. During the E2 to E1 transition, there could be a coordinated movement of both fluorescent probes that does not change the distance between them. On the other hand several studies have suggested that the movement of the SERCA headpiece is much smaller than initially predicted (Jensen et al., 2006; Sørensen et al., 2004; Winters et al., 2008). These more subtle changes could also explain the lack of a FRET$_{\text{max}}$ change in our experiments.

Under no conditions were we able to detect complete loss of PLB-SERCA FRET. In particular, we observed a high degree of FRET between PLB and SERCA under conditions that favor particular conformation (E1 and E2-Tg) that have been shown to block chemical crosslinking. These crosslinking observations are the backbone of the Dissociation Model. In contrast, our evidence suggests the high likelihood that PLB can bind all conformations of SERCA, although with varying affinities. This conclusion is summarized in Fig. 23 showing the coupled equilibrium between PLB pentamer, monomer, and monomer bound to various conformations of SERCA with independent dissociation constants.

While we have not performed an exhaustive investigation into all possible conformations, we have observed a high level of FRET even at saturating Ca$^{2+}$ concentrations known to fully relieve PLB inhibition of SERCA. This suggests that PLB can remain bound throughout the reaction cycle SERCA, implying ability to bind all conformations. We therefore conclude that unbinding of PLB is not required for relief
Figure 23: Summary of PLB binding. PLB monomer can bind to itself forming a pentamer or bind to SERCA in any conformation, however doing so with varying affinity.
of PLB-SERCA inhibition. This is not without precedence since it phosphorylation of PLB has been suggested to relieve inhibition without causing dissociation of the regulatory complex.

**Alternative Binding Site**

In our Ca$^{2+}$ dose curve, we detect two different modes PLB-SERCA binding. The change in binding states could describe a simple structural reorientation merely decreasing the strength of the intermolecular contacts of PLB at the same location on SERCA. On the other hand, the detected change could be described by displacement of PLB to an alternative binding site. Crosslinking by the Jones lab (Chen et al., 2007, 2006, 2005; Jones et al., 2002) and mutagenesis studies by the MacLennan lab (Y Kimura et al., 1996; MacLennan, Kimura, & Toyofuku, 1998; Toyofuku et al., 1993) provide good evidence for a site of interaction that also likely facilitates inhibition. To this point, there has been no direct evidence of an alternative binding site for PLB on SERCA.

However, some evidence for an alternative site comes from X-ray crystallography studies predicting the binding of homologous proteins, NKA and PLM, at a more exterior location on helix M9 (Fig. 7) (Shinoda et al., 2009). While this provides sufficient evidence to hypothesize the existence of two distinct PLB binding sites on SERCA, it certainly does not confirm it. This exterior binding site could be unique to the PLM-NKA complex and could be the sole site of interaction. However, it seems unlikely that proteins with such similarities would have vastly different interactions and inhibitory mechanisms. The more likely explanation is that this detected interaction is
just an artifact of crystallization and does not describe the actual functional site of
interaction between NKA and PLM.

One could argue that interaction at the \( \text{Ca}^{2+} \) binding site is the only important
interaction. The displacement of PLB to an alternative binding site and the complete
displacement of PLB from SERCA are not significantly different, since both describe a
movement out of the functional site. However, this difference would greatly alter the
dynamics of the system. Binding of PLB to an alternative site would enhance the on-rate
of PLB by keeping it in close proximity to the functional site. In other words, its effective
concentration would be much higher.

The Jones group has questioned our proposal of an alternative binding site for
PLB since their crosslinking studies have found no evidence of a second site (Akin &
Jones, 2012). However, they have yet to look for enhancement of crosslinking at an
exterior site by \( \text{Ca}^{2+} \). Their evidence is the use of crosslinkers varying in distance from 0-
15 Å which have not shown differential binding. However, movement of PLB by 20 Å
places it just outside the predicted binding groove and our exterior site corresponds to a
movement of 40 Å.

The role of this potential binding site is unclear and could be merely serve as a
place holder to enhance on and off rates of PLB. The importance as a place holder
should not be underestimated, because variations in the ability to bind the alternative
site will likely have a tremendous impact on how PLB interacts with the functional site.
Changes at the alternative site would alter the equilibrium binding producing a different
stead-state interaction and inhibition. Also, there would be a significant impact on the dynamics of inhibition since the on and off rates at the functional site could be altered. Besides affecting how PLB interacts at the primary functional site, the secondary site itself may have the ability to functionally regulate SERCA. Whatever the purpose of the secondary site, the next step of the investigation is determining whether it exists, followed by a characterization of the site. We could then attempt to alter the interaction at the secondary site and then determine how the ability of PLB to regulate SERCA is changed. If this secondary site is confirmed to exist, an intriguing question that results is how binding at one site excludes binding at the other site, since a strict PLB:SERCA stoichiometry of 1:1 is observed.

**Proposed Model of PLB Inhibition**

We have attempted to reconcile the findings of others to account for the limitations of all approaches and develop a model that is consistent with all data in the field. We propose that high Ca$^{2+}$ displaces the transmembrane domain of PLB to an alternative, lower affinity binding site on SERCA, with no change in the position of the cytosolic domain. We have termed this the “Domain Displacement Model.” In other words, binding to the E1 and E2 conformations of SERCA differs only in the position and affinity of the transmembrane domain of PLB. This accounts for our data showing that Ca$^{2+}$ reduces the affinity of PLB for SERCA. This also explains why our data and FRET measurements from Mueller et al detect no or only a small change in the position of the cytosolic domain where the fluorophores are attached. Movement of the
transmembrane domain to a more exterior site would also explain why Jones sees no
crosslinking at high Ca$^{2+}$. The differences between the models are summarized in Fig.
24.

We started to directly test this model by measuring FRET between SERCA and a
truncated PLB lacking the cytosolic domain which would assess the position of the
transmembrane domain of PLB in association with SERCA. Our YFP-tmPLB construct
expresses normally and interacts with itself and SERCA at low Ca$^{2+}$ concentrations as
anticipated. However, FRET between Cer-SERCA and YFP-tmPLB is unresponsive to Ca$^{2+}$
and does not appear to regulate SERCA. So while YFP-tmPLB retains some of the
binding characteristics of full-length PLB, clear differences are apparent. We suspect
that steric barriers created by the membrane and SERCA may be preventing proper
localization, and therefore function, of YFP-tmPLB. Further experimentation is needed
to address these results including additional characterization of the YFP-tmPLB
construct as well as development of alternative constructs. While this construct did not
function as initially hypothesized, it does provide evidence for a non-functional PLB-
SERCA interaction. Assessment of how this interaction occurs without inhibition of
SERCA could also be critical to our understanding of the PLB-SERCA mechanism.

Earlier studies showing the ability of truncated PLB to inhibit SERCA were
performed at a high excess of PLB peptide (Hughes et al., 1996; Y Kimura et al., 1996),
with no assessment of its interaction or affinity. The conclusions from these studies that
Figure 24: Models of PLB-SERCA binding. The previously discussed Dissociation and Subunit Models are shown in comparison to our proposed Domain Displacement Model. We propose that elevated calcium concentration displaces the transmembrane domain of PLB to alternative SERCA binding site of lower affinity, with no change in the binding location of the cytosolic domain.
fully inhibit SERCA may have been incorrect, and even if the suspected steric issues with YFP-tmPLB are corrected, full inhibition may not be achieved.

**Effects of Pacing on Myocyte FRET**

Irrespective of the mechanistic model of inhibition, it is critical to understand how the PLB-SERCA interaction changes over time. Because the ability of PLB to inhibit SERCA varies with respect to Ca\(^{2+}\) concentration, the level of PLB inhibition will not be constant in cardiac myocytes. Not only is Ca\(^{2+}\) concentration perpetually changing over the course of individual Ca\(^{2+}\) transients; basal concentrations can also be altered by various stimuli. To date however, all measurements of the ability of PLB to bind to and regulate SERCA have been taken at equilibrium. So we have no comprehension of how rapidly the PLB-SERCA binding changes in response to changes in Ca\(^{2+}\) concentration. Since Ca\(^{2+}\) concentration changes rapidly and constantly in the physiological environment of PLB and SERCA, we must assess the dynamics of the PLB-SERCA interaction to fully understand its physiologic function. Unlike previous studies, our FRET approach is capable of assessing PLB-SERCA binding in real time. We are therefore uniquely poised to address this critical question.

We detected no change in PLB-SERCA FRET over the course of a single Ca\(^{2+}\) transient. Only by using a non-physiological rest/pace protocol that produced higher and more sustained Ca\(^{2+}\) increases were we able to observe changes in FRET. These observations were somewhat expected since our steady-state experiments predict at most a small 3% change in FRET in response to increases in Ca\(^{2+}\) concentration. These
observations could be explained in a few ways. It might indicate that there are two significant pools of SERCA, PLB unbound (active) and PLB bound (inactive). This PLB unbound and active pool of SERCA is sufficient to move the Ca\textsuperscript{2+} of a typical single transient back into the SR. Only when Ca\textsuperscript{2+} concentrations are elevated during our rest/pace protocol, and additional SERCA activity is required, is PLB inhibition relieved and a decrease in FRET observed. However, these distinct bound and unbound pools are inconsistent with several studies that have suggested that SERCA is nearly 100% bound by PLB under basal conditions in larger mammals (Waggoner et al., 2009). Our observed pacing effects are more consistent with the scenario where dissociation of PLB from SERCA is not required for the relief of inhibition. The SERCA activity necessary over the course of a Ca\textsuperscript{2+} transient could then be achieved without a significant change in PLB-SERCA interaction. Only when Ca\textsuperscript{2+} concentrations are elevated during our rest/pace protocol is the PLB-SERCA affinity shifted sufficiently to generate a decrease in FRET.

Lack of an observable change in FRET does not indicate an absence of change in PLB-SERCA binding over the course of a single Ca\textsuperscript{2+} transient. Certainly, a change in binding can occur that would not significantly alter FRET. As discussed previously concerning an alternative binding site on SERCA, the position of the fluorescent protein at the N-terminus of PLB does not allow detection of a change in the position of the transmembrane domain. If applying our proposed Domain Displacement Model of PLB interaction (Fig. 24), one could envision that the transmembrane domain of PLB
transitions rapidly between the two binding sites in response to changes in Ca\(^{2+}\), with no change in the position of the cytosolic domain. A YFP-tmPLB that could successfully produce the anticipated response to Ca\(^{2+}\) would be a useful tool to investigate potential changes in the position of the transmembrane domain in response to Ca\(^{2+}\) transients. However, if successful, this type of experiment should be viewed with caution since the cytosolic domain could facilitate any transition. Its absence could certainly then affect the rates of transition.

**Future Directions**

While our proposed model of PLB inhibition of SERCA is a plausible explanation that could integrate our data with previously published research, we lack sufficient support for this model. We must first determine whether the secondary site exists and whether Ca\(^{2+}\) can cause transition to this binding site. The FRET experiments with the truncated PLB could be capable of showing both, but the initial experiments have not produced the anticipated results. We suspect that steric hindrance of the fluorescent protein may be preventing proper localization of tmPLB. If correct, tmPLB lacking YFP should then be able to properly interact and inhibit SERCA. This could be tested by developing an unlabeled peptide consisting of only residues 28-52 of PLB probing the ability of this peptide to inhibit SERCA. If unlabeled tmPLB is not functional, this would directly challenge previous observations that the transmembrane domain in isolation is fully capable of inhibiting SERCA (Hughes et al., 1996; Y Kimura et al., 1996). Though this seems unlikely since an analogous protein sarcolipin, which lacks a comparable
cytosolic domain, is able to inhibit SERCA activity (Bhupathy et al., 2007; Periasamy, Bhupathy, & Babu, 2008).

Also, if FRET between YFP-tmPLB and Cer-SERCA is diminished by expression of unlabeled PLB or tmPLB (if functional), this would demonstrate binding competition. This would indicate that the YFP-tmPLB interaction with SERCA is not anomalous and not an artifact of our approach. Furthermore, it could indicate that YFP-tmPLB preferentially interacts at the secondary, non-inhibitory site.

If steric hindrance of YFP is the contributing factor preventing ability of YFP-tmPLB to inhibit SERCA, modifications to the construct could relieve the steric block. Specifically, the 7 amino acid linker between tmPLB and YFP could be lengthened so that YFP could be positioned further away from the membrane. Use of the shortest linker that is able to allow proper inhibition of SERCA would be ideal. This would alleviate some potential variability in FRET measurements produced flexibility and movement of the fluorescent protein. In order to be versatile, the additional linker sequence should be comprised of simple amino acids such as glycines. Use of prolines could also be considered since they may provide some stability to the linker, thereby reducing flexibility and potential variability of FRET measurements.

Rather than developing PLB variants to investigate the potential secondary site, we could also make modified SERCA proteins. We could make targeted mutations of helix M9, in an attempt to disrupt binding at the proposed secondary site. Diminished binding at the secondary site would be observed as a further lowering of affinity at high
Ca\textsuperscript{2+} concentrations compared to non-mutated SERCA. Furthermore, if PLB exists in equilibrium between the two sites, decreased affinity at the secondary site could enhance affinity at the primary functional site presenting as increased affinity at low Ca\textsuperscript{2+} concentrations and enhanced inhibition by PLB.

SERCA3 could provide for an interesting model for the investigation of the PLB-SERCA mechanism. While PLB has been clearly shown to have no effect on the activity of SERCA3 in functional assays (Y Kimura et al., 1996; Toyofuku et al., 1994), the ability of PLB to bind SERCA3 has not been determined. Measuring FRET between YFP-PLB and Cer-SERCA3 might provide a unique perspective, potentially showing a non-inhibitory binding interaction. Perhaps this interaction would be unresponsive to Ca\textsuperscript{2+} suggesting PLB can only bind the secondary, non-inhibitory site.

Our FRET results and proposed model would greatly benefit from support of alternative approaches. While crosslinking results to date have directly contrasted our findings, we believe this approach could be modified to show binding at a secondary site. Jones et al have not found evidence of a secondary binding site, but this could be attributed to the fact that they have yet to directly test the existence of an alternative binding site. All studies have targeted residues in the suspected binding pocket of M2/M4/M6 on SERCA. Binding at a more exterior position such as M9 would go undetected. Therefore, use of untargeted reagents such as benzophenone and glutaraldehyde which bind carbon and amine groups may successfully crosslink PLB to SERCA at a position outside the proposed binding pocket under saturating Ca\textsuperscript{2+}
conditions. Additionally, SERCA mutants could be generated that introduce cysteines or lysines on helix M9 to perform so that the site directed crosslinking protocols of the Jones lab could be used.

**Concluding Remarks**

In this dissertation, I have shown our evidence that suggests that dissociation of PLB from SERCA is not required for relief of inhibition. To account for this observation as well as previous data from other labs, I have also described the Domain Displacement Model of regulation. Furthermore, I have also demonstrated the ability to detect PLB-SERCA binding in real-time in response to physiological changes of Ca$^{2+}$ concentration. Doing so, I have shown evidence that single Ca$^{2+}$ transients of paced cardiac myocytes do not produce an observable change in PLB-SERCA binding. The results and methodology presented here have added to our understanding of PLB-SERCA regulation and continuation of this research is poised to answer critical questions in the field.
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