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Cardiac Calcium Atpase Dimerization Measured by Fluorescence Resonance Energy Transfer and Chemical Cross-Linking

Daniel Blackwell

Loyola University Chicago

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LOYOLA UNIVERSITY CHICAGO

CARDIAC CALCIUM ATPASE DIMERIZATION MEASURED BY FLUORESCENCE RESONANCE ENERGY TRANSFER AND CHEMICAL CROSS-LINKING

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

PROGRAM IN CELL AND MOLECULAR PHYSIOLOGY

BY

DANIEL J. BLACKWELL

CHICAGO, ILLINOIS

AUGUST 2016
To my parents and my wife

for their love and support
ACKNOWLEDGEMENTS

This work could not have been done without the outstanding mentorship of Dr. Seth Robia. He dedicated a truly staggering amount of time to my education and I am fortunate to have been trained by him. It is difficult to overestimate his contributions to my instruction, goals, development, and direction. He possesses all the qualities of an exceptional mentor and I am grateful for his help.

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>viii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>x</td>
</tr>
<tr>
<td>CHAPTER ONE: CARDIAC CALCIUM ATPASE AND THE HEART</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER TWO: REVIEW OF RELATED LITERATURE</td>
<td>7</td>
</tr>
<tr>
<td>CHAPTER THREE: METHODS AND MATERIALS</td>
<td>26</td>
</tr>
<tr>
<td>CHAPTER FOUR: RESULTS</td>
<td>41</td>
</tr>
<tr>
<td>CHAPTER FIVE: DISCUSSION</td>
<td>78</td>
</tr>
<tr>
<td>REFERENCE LIST</td>
<td>90</td>
</tr>
<tr>
<td>VITA</td>
<td>107</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Figure 1</td>
<td>Cardiac calcium handling</td>
</tr>
<tr>
<td>Figure 2</td>
<td>SERCA ATPase activity</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Post-Albers Scheme</td>
</tr>
<tr>
<td>Figure 4</td>
<td>FRET distance dependence</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Acceptor photobleaching images</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Enzyme-linked assay schematic</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Co-immunoprecipitation and chemical cross-linking</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Dodecylphosphocholine titration</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Brightness analysis</td>
</tr>
<tr>
<td>Figure 10</td>
<td>ATPase assay</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Labeling strategy for FRET measurements</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Sensitized emission FRET</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Effect of PLB, calcium, and ATP on SERCA oligomerization</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Effect of inhibitors on SERCA oligomerization</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Effect of HAX-1 on SERCA oligomerization</td>
</tr>
<tr>
<td>Figure 16</td>
<td>FRET competition assay</td>
</tr>
<tr>
<td>Figure 17</td>
<td>Antibody titration</td>
</tr>
<tr>
<td>Figure 18</td>
<td>Acceptor photobleaching</td>
</tr>
</tbody>
</table>
Figure 19. Acceptor photobleaching standards 63
Figure 20. SERCA/PLB acceptor-sensitized FRET and photobleaching measurements 65
Figure 21. Labeling strategy and fluorescence lifetime decays 67
Figure 22. Fluorescence lifetime analysis 68
Figure 23. Residual plots 70
Figure 24. Two-component lifetime analysis 72
Figure 25. Effect of PLB phosphorylation on lifetime measurements 74
Figure 26. ATPase measurements 76
Figure 27. A model for SERCA/PLB interaction 83
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Å</td>
<td>angstrom(s)</td>
</tr>
<tr>
<td>A.U.</td>
<td>arbitrary units</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>calcium</td>
</tr>
<tr>
<td>CaMKII</td>
<td>calcium/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>Cer</td>
<td>mCerulean</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>FRET$_{\text{max}}$</td>
<td>maximum FRET</td>
</tr>
<tr>
<td>GFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>HAX-1</td>
<td>HS-1 associated protein X-1</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton(s)</td>
</tr>
<tr>
<td>K$_d$</td>
<td>apparent dissociation constant</td>
</tr>
<tr>
<td>LV</td>
<td>left ventricular</td>
</tr>
<tr>
<td>$n_H$</td>
<td>Hill coefficient</td>
</tr>
<tr>
<td>NKA</td>
<td>sodium potassium ATPase</td>
</tr>
<tr>
<td>pCa</td>
<td>a measure of calcium concentration equal to $-\log_{10}[\text{Ca}^{2+}]$</td>
</tr>
<tr>
<td>pCa$_{50}$</td>
<td>calcium concentration at half maximal rate</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarco/endoplasmic reticulum calcium ATPase</td>
</tr>
<tr>
<td>SLN</td>
<td>sarcoldipin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>PKA</td>
<td>protein kinase A (cyclic AMP-dependent protein kinase)</td>
</tr>
<tr>
<td>PLB</td>
<td>phospholamban</td>
</tr>
<tr>
<td>PP1</td>
<td>protein phosphatase 1</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TG</td>
<td>thapsigargin</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>maximum rate of reaction</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
</tr>
<tr>
<td>YFP</td>
<td>enhanced yellow fluorescent protein</td>
</tr>
</tbody>
</table>
ABSTRACT

The cardiac sarco/endoplasmic reticulum calcium ATPase (SERCA) establishes the intracellular calcium gradient across the sarcoplasmic reticulum membrane. It has been proposed that SERCA forms homo-oligomers that increase the catalytic rate of calcium transport. We investigated SERCA oligomerization in rabbit left ventricular myocytes using a photoactivatable cross-linker. Western blotting of cross-linked SERCA revealed higher molecular weight species consistent with SERCA oligomerization. Fluorescence resonance energy transfer (FRET) measurements in cells transiently transfected with fluorescently-labeled SERCA2a revealed that SERCA readily forms homo-dimers. These dimers formed in the absence or presence of the SERCA regulatory partner, phospholamban (PLB) and were unaltered by PLB phosphorylation or changes in calcium or ATP. Fluorescence lifetime data are compatible with a model in which PLB interacts with a SERCA homo-dimer in a stoichiometry of 1:2. Functional data show that homo-dimerization of SERCA is an important component of SERCA regulation. Together, these results suggest that SERCA forms constitutive homo-dimers in live cells that and dimer formation is not modulated by SERCA conformational poise, PLB binding, or PLB phosphorylation.
CHAPTER ONE

CARDIAC CALCIUM ATPASE AND THE HEART

The heart is essential for providing adequate blood flow to meet the demands of the body; blood carries oxygen and nutrients to cells and removes carbon dioxide and metabolites. Heart failure (HF) arises when the heart is unable to meet the demand for oxygen, and is one of the leading causes of morbidity and mortality worldwide (Mozaffarian, Benjamin et al. 2016). Clinical treatment of cardiovascular diseases, such as HF, continues to be a leading health care cost and places a substantial financial burden on healthcare (Mozaffarian, Benjamin et al. 2016). Advances in HF treatment from the period 1950-1969 to 1990-1999 reduced one-year mortality rates in men from 30% to 28% and in women from 28% to 24% (Levy, Kenchaiah et al. 2002). Despite these incremental advances, the mortality rates remain unacceptably high with five-year mortality greater than 50% (Roger, Weston et al. 2004). There is currently no cure for HF.

Sarco/endoplasmic reticulum calcium ATPase (SERCA) is responsible for transporting calcium from the cytoplasm/sarcoplasm to the luminal side of the endoplasmic/sarcoplasmic reticulum (ER/SR). It is also the primary means by which calcium is removed from the sarcoplasm. This process sequesters calcium in the SR lumen. In the heart this process is essential for relaxation of the myocardium, as calcium is a necessary signaling molecule that enables actin and myosin interactions to drive
muscle contraction (Bers 2002). In addition to altering relaxation of the heart, SERCA also affects contraction as the sequestered SR calcium load influences the strength of subsequent contractions.

Figure 1 shows the general process of calcium handling for cardiac myocyte contraction and relaxation. Depolarization across the sarcolemma membrane opens L-type calcium channels (LTCC), causing an influx of calcium into the sarcoplasm. This induces calcium release from the SR lumen through the ryanodine receptor (RyR) in a process termed calcium-induced calcium release (CICR). Sarcoplasmic calcium binds to troponin C on the myofilaments and a series of coordinated events exposes binding sites on actin, allowing myosin and actin to contract. Relaxation is produced by removal of calcium from the sarcoplasm though SERCA, plasma membrane calcium ATPase (PMCA), or the sodium/calcium exchanger (NCX). SERCA accounts for approximately 70% of calcium removal and readies the myocyte for subsequent contractions.

Previous studies have demonstrated that calcium handling is altered in HF (Gwathmey, Copelas et al. 1987). End-diastolic cytosolic calcium levels are found to be elevated in human failing myocardium (Gwathmey, Warren et al. 1991, Beuckelmann, Nabauer et al. 1992). Multiple studies showed that SR calcium stores are decreased in human failing myocardium (Lindner, Erdmann et al. 1998, Piacentino, Weber et al. 2003) alongside a reduction in calcium uptake into the SR (Pieske, Maier et al. 1999). These changes are characterized by a blunted calcium transient with elevated sarcoplasmic baseline calcium, slower calcium release from the SR stores, reduced peak amplitude of release, and slower calcium reuptake rates.
Figure 1. Schematic representation of calcium handling in the cardiac myocyte. Depolarization of the sarcolemmal membrane opens L-type calcium channels (LTCC), allowing calcium into the cell. The increase in intracellular calcium opens ryanodine receptors (RyR), releasing calcium from the SR stores and increasing the sarcoplasmic calcium concentrations further. The elevated intracellular calcium binds to myofilaments and activates contraction through the action of myosin. Relaxation is produced by removal of calcium through the plasma membrane calcium ATPase (PMCA), the sodium calcium exchanger (NCX), or sarco/endoplasmic calcium ATPase (SERCA). SERCA is inhibited by phospholamban (PLB), but this inhibition can be alleviated by phosphorylation of PLB through protein kinase A (PKA) or calcium/calmodulin-dependent protein kinase II (CaMKII).
This results in weaker muscle contractions, longer time to relax, and reduced cardiac output. As the chief constituent of calcium handling, SERCA presents an attractive target for correcting disordered calcium handling as a possible treatment for HF.

In the heart, SERCA is predominantly regulated through a 52 amino acid inhibitory peptide called phospholamban (PLB, Figure 1). PLB inhibits SERCA by reducing the apparent affinity for calcium, thus decreasing the SERCA calcium transport rate over the physiological range of calcium concentrations. Relief of inhibition by PLB can be overcome by phosphorylation of PLB at serine 16 by protein kinase A (PKA) or by calcium calmodulin protein kinase II (CaMKII) at threonine 17 (Tada, Kirchberger et al. 1975, Plank, Pifl et al. 1983, Simmerman, Collins et al. 1986). SERCA is a primary target of beta-adrenergic signaling through PLB phosphorylation by PKA, and an important component for increasing both inotropy and lusitropy in response to demand for higher cardiac output. In addition to altering calcium sensitivity of the SERCA pump, some evidence suggests that PLB also increases the maximal rate of transport ($V_{\text{max}}$). Studies show that when phosphorylated PLB is present $V_{\text{max}}$ is increased beyond what can be achieved in the absence of PLB (Antipenko, Spielman et al. 1997, Reddy, Cornea et al. 2003). This may be an additional regulatory mechanism for enhanced calcium transport that uniquely relies on the presence of phosphorylated PLB.

Evidence suggests that, in addition to regulation by PLB, SERCA activity may also be regulated by assembly into homo-oligomers. Data show that this could be an inhibitory mechanism where SERCA protomers form aggregates in the membrane that
have reduced ATPase activity (Voss, Jones et al. 1994, Mersol, Kutchai et al. 1995). Other experiments show that SERCA protomers form specific oligomers that may enhance calcium transport through cooperative mechanisms. It has also been suggested that phosphorylated PLB may be a requirement for this cooperative action (Negash, Chen et al. 1996). However, these models have not been widely adopted, in part because detergent-solubilized SERCA has been shown to couple hydrolysis of ATP with calcium transport (Dean and Tanford 1978, Vilsen and Andersen 1987). Whether these solubilization conditions produce only monomeric SERCA and whether homo-oligomerization is a necessary component of activity is still not clear. This necessitates a greater understanding of calcium transport across the SR membrane.

The SERCA/PLB regulatory complex may include additional partners that regulate SERCA function. Previous studies have shown that a mitochondrial anti-apoptotic protein HS-1 associated protein X-1 (HAX-1) interacts with PLB (Vafiadaki, Sanoudou et al. 2007). Data suggest that HAX-1 regulates cardiac performance by promoting formation of PLB monomers which readily inhibit SERCA. Further evidence supports cell-survival mediated by the interaction between PLB and HAX-1. The exact role of this protein in the regulatory complex has not been fully fleshed out.

An area of study that remains unresolved is how phosphorylation of the cytosolic domain of PLB causes relief of SERCA inhibition. Our previous work has examined two models that explain how PLB inhibition is relieved. The first model proposes that phosphorylation of PLB causes unbinding from SERCA; this frees up SERCA to actively pump calcium (James, Inui et al. 1989, Kimura, Kurzydlowski et al. 1997, Chen, Akin et al. 1997).
2006). Our own studies and studies by other groups have supported a second model where phosphorylated PLB remains bound to SERCA (Negash, Yao et al. 2000, Bidwell, Blackwell et al. 2011). Regardless of the mechanism for relief of inhibition, neither model provides a complete understanding for the role of PLB phosphorylation on altered SERCA calcium sensitivity. Previous studies by our group have shown that there is a small structural change upon phosphorylation (Hou, Kelly et al. 2008).

We set out to examine the regulatory complex in more detail and investigate the role of homo-oligomerization on SERCA function.
SERCA is a member of the ion-motive P-type ATPase family, which includes transporters such as the Na+/K+-ATPase and the H+/K+-ATPase, and H+-ATPase (Apell 2004). P-type ATPases establish and maintain important electrochemical gradients that drive many essential cellular processes. ATPases pump ions across membranes, usually against their concentration gradients, by utilizing energy from ATP. The P-type ATPase family is highly homologous and characterized by four important domains: the nucleotide binding domain (N), the actuator domain (A), the phosphorylation domain (P), and the transmembrane domain (TM). The soluble A-, N-, and P-domains sit within the cytosolic lumen, while the TM-domain anchors the ATPase in the appropriate membrane. Most members of the P-type ATPase family contain 10 transmembrane helices, which dictate ion specificity (Clarke, Loo et al. 1989, Toyoshima, Nakasako et al. 2000, Bublitz, Poulsen et al. 2010).

**Discovery and characterization of SERCA**

Identification of an SR membrane calcium pump first came from ATP-dependent calcium uptake experiments in crude SR muscle preparations (Hasselbach and Makinose 1961, Ebashi and Ebashi 1962). These preparations sequestered calcium in SR fractions and produced relaxation similar to that of application of a calcium chelator. Spin-labeling of SR vesicles showed that SR membrane protein underwent conformational
changes in response to ATP (Landgraf and Inesi 1969, Coan and Inesi 1976). A defining feature of SERCA calcium transport was first described Inesi, that of calcium dependence on ATPase activity. At low calcium concentrations there is little ATPase activity. As calcium concentrations increase, the activity of SERCA increases until it reaches a maximal rate of transport at saturating calcium. An example of this is shown in figure 2 (black). Refinement of ATPase measurements later demonstrated cooperativity of the calcium binding process; the first calcium binds with low affinity and the second with high affinity (Inesi, Kurzmack et al. 1980). The range of calcium over which ATPase activity is sensitive occurs from about 30 nM to 2 μM, the approximate physiological range of calcium in muscle tissue between relaxation (low calcium) and contraction (high calcium). The calcium threshold for activation of SERCA essentially sets the resting sarcoplasmic calcium concentration.

In 1970 David MacLennan isolated and purified SERCA from rabbit skeletal muscle. The purified protein possessed all the hallmarks of what was then known about SERCA: the requirement for Mg2+ and Ca2+, inhibition by the addition of EGTA, and inhibition by mersalyl. Additionally, ATPase activity was found to be dependent on an intact phospholipid membrane, indicating that this protein is a membrane protein (MacLennan 1970). The molecular weight of purified SERCA was determined to be 102 kDa and partial trypsin digestions helped identify the site of ATP binding and other features of SERCA structure (Stewart and MacLennan 1976, Reithmeier and MacLennan 1981). In 1985 the cDNA sequence was determined for rabbit skeletal muscle (MacLennan, Brandl et al. 1985). Later work identified a total of 11 different isoforms:
Figure 2. A representative ATPase activity curve for SERCA in the absence (black) or presence of PLB (red). As calcium concentrations increase, the ATPase activity increases up to a maximal level. PLB produces a characteristic rightward shift in this relationship.
SERCA1a/b, SERCA2a/b/c, and SERCA3a-f (Brandl, Green et al. 1986, Burk, Lytton et al. 1989, Lytton, Zarain-Herzberg et al. 1989, Gelebart, Martin et al. 2003, Bobe, Bredoux et al. 2004). SERCA1a is the predominant fast skeletal isoform and is the most studied isoform due to its abundance in skeletal muscle and ease of purification. SERCA2b is ubiquitously expressed in all cell types. SERCA2a is the cardiac isoform; however, it is also expressed in slow skeletal muscle.

**SERCA transport mechanism**

SERCA ion transport is broadly characterized by transitions between the calcium-bound E1 state and the calcium-free E2 state. However, there exist many catalytic transitions between these two simplified steps. The mechanism of P-type ATPase ion translocation, termed Post-Albers cycle, was first described in the Na+/K+-ATPase (Albers 1967, Post, Hegyvary et al. 1972). Briefly, ions bind to the TM-domain followed by binding of ATP in the N-domain. This is followed by phosphoryl transfer from ATP that forms a phosphoanhydride intermediate with the P-type ATPase at a highly conserved aspartic acid residue located in the P-domain. Ion translocation is achieved through a series of concerted motions that come from the hydrolysis of ATP. These motions drive the ions through the TM-domain to the opposite side of the membrane where they are released. SERCA transports two calcium ions from the cytosol to the ER/SR lumen for every ATP hydrolyzed (Inesi, Kurzmack et al. 1978). In addition, SERCA countertransports 2 or 3 H+ ions (Yu, Carroll et al. 1993, Stokes and Green 2003). Figure 3 illustrates a simplified Post-Albers scheme for SERCA. Many of these transitions have
**Figure 3.** A Post-Albers schematic representation of SERCA calcium cycling. SERCA in the E1 state (high calcium affinity) binds ATP and calcium on the cytosolic side. Hydrolysis of ATP produces a phosphoenzyme intermediate followed by transition to the low calcium affinity E2 state. Calcium is released into the SR lumen followed by loss of the phosphate group and a structural transition back to the E1 state.
been characterized, yielding different rate constants for each step. Importantly, some of these transitions are faster than others, indicating a rate-limiting step.

**SERCA in disease**

Only two diseases have been identified specifically originating from mutations to SERCA. A number of mutations in skeletal SERCA1 isoform have been shown to cause Brody disease (Karpati, Charuk et al. 1986, Odermatt, Taschner et al. 1996). Brody disease is characterized by impaired muscle relaxation, cramping, and stiffness.

Mutations in the SERCA2 gene have been linked to Darier’s disease, an autosomal dominant skin disorder (Dhitavat, Macfarlane et al. 2003). Despite mutations in the SERCA2 gene, these individuals do not display increased rates of cardiovascular disease or HF. It is important to note that knockout of cardiac SERCA is embryonic lethal, indicating that mutations that significantly alter SERCA function would not be tolerated (Periasamy, Reed et al. 1999).

Alterations to cardiac SERCA expression and regulation during HF have been observed across a wide range of studies, underscoring the importance of this protein. SERCA protein and RNA expression have been observed to decrease in HF in guinea pigs (Kiss, Ball et al. 1995), rats (Komuro, Kurabayashi et al. 1989, de la Bastie, Levitsky et al. 1990), rabbits (Nagai, Zarain-Herzberg et al. 1989), and even dogs (O’Rourke, Kass et al. 1999). Whether changes in SERCA activity and expression are a cause of progression to HF or whether these changes are simply correlated with HF is a matter of some debate. However, when SERCA was overexpressed in various animal models of HF, cardiac function was improved, demonstrating the importance of this protein in cardiac
Evidence suggests that the alterations in SERCA expression seen in various animal models might not be present in human cardiomyopathies. Some studies of human myocardium have shown similar reductions in both SERCA mRNA levels (Mercadier, Lompre et al. 1990, Arai, Alpert et al. 1993) and protein expression (Hasenfuss, Reinecke et al. 1994). However, other studies have shown that, while mRNA levels are decreased, protein levels remain the same (Schmidt, Hajjar et al. 1999, Munch, Bolck et al. 2002). Patients with HF had similar levels of SERCA protein compared to those without HF; however SERCA activity was significantly decreased (Schwinger, Bohm et al. 1995). Additional animal studies corroborate this observation; Zarain-Herzberg et al. found that SERCA activity was reduced more than expected for the level of decrease in protein expression (Zarain-Herzberg, Afzal et al. 1996), suggesting that reduced SERCA function is not entirely due to decreased expression. A recent clinical trial utilized overexpression of SERCA to treat patients with HF but did not improve clinical outcomes, indicating that our understanding of SERCA function is incomplete. Development of such future therapies will require a better understanding of function and regulation to address the role of depressed SERCA activity in disease.

Discovery and characterization of phospholamban

Evidence for cyclic AMP-dependent kinase targeting the SR membrane was first described in the early 1970’s by multiple groups (Wollenberger 1972, Wray, Gray et al. 1973, La Raia and Morkin 1974). A potential modulator of SERCA activity was discovered
when Arnold Katz’s group showed that cardiac SR preparations had increased rates of calcium uptake following phosphorylation by cyclic AMP-dependent protein kinase (Kirchberger, Tada et al. 1972). They later identified this phosphorylation as targeting a 22 kDa SR membrane protein (Tada, Kirchberger et al. 1975). Arnold Katz’s wife suggested naming this protein phospholamban after the greek “phospho” (phosphate) and “lambano” (to receive) (Katz 1998). Later experiments utilized boiling and detergent to show that the phospholamban monomer is approximately 5-6 kDa (Will, Levchenko et al. 1978, Bidlack and Shamoo 1980). The original identification of the 22 kDa species was more accurately determined to be a stable homo-pentamer with a molecular weight of 25-30 kDa (Fujii, Kadoma et al. 1986, Wegener, Simmerman et al. 1986). PLB has also been observed as dimers, trimers, and tetramers on gels, however the monomer and pentamer are the predominant forms (Thomas, Reddy et al. 1998). It is thought that the pentamer is an inactive reserve pool of PLB, although some studies have suggested that the pentamer may form an ion pore or functional channel (Kovacs, Nelson et al. 1988, Oxenoid and Chou 2005). Förster transfer recovery experiments have shown that the pentamer is a highly stable complex that exists in dynamic equilibrium with the monomeric form of PLB, which binds to and inhibits SERCA (Kimura, Asahi et al. 1998, Robia, Campbell et al. 2007).

PLB is composed of an amino-terminal cytosolic alpha helix connected with a hinge region to a single-pass carboxy-terminal transmembrane alpha helix (Simmerman, Collins et al. 1986, Simmerman, Lovelace et al. 1989). Crystal structures show that the transmembrane region of PLB binds to a groove in SERCA formed by the M2, M4, M6,
and M9 helices of the TM-domain of SERCA (Akin, Hurley et al. 2013). This groove was also observed to be the site of binding by a PLB homologue, sarcolipin, in skeletal isoform SERCA1a (Toyoshima, Iwasawa et al. 2013, Winther, Bublitz et al. 2013). The cytosolic domain of PLB has not been resolved in crystal structures. NMR experiments have shown that this region possibly exists in equilibrium between different structural substrates (Verardi, Shi et al. 2011).

**Stoichiometry of the SERCA/PLB regulatory complex**

Extensive cross-linking studies and X-ray crystallography have shown that a single PLB interacts with SERCA (James, Inui et al. 1989, Akin, Hurley et al. 2013, Toyoshima, Iwasawa et al. 2013, Winther, Bublitz et al. 2013). However, some evidence suggests that more than one PLB may interact with SERCA. Two-dimensional co-crystallization of SERCA and PLB showed that the PLB pentamer can interact with SERCA (Stokes, Pomfret et al. 2006). Further studies showed that mutations promoting pentamer formation retained inhibitory function, indicating that this might be the active inhibitory state (Glaves, Trieber et al. 2011). Published co-crystallization studies also suggested a PLB:SERCA stoichiometry of 1:2 (Young, Jones et al. 2001). Co-immunoprecipitation studies demonstrated that both PLB and SLN can simultaneously bind SERCA, possibly generating a superinhibitory complex (Asahi, Kurzydlowski et al. 2002, Asahi, Sugita et al. 2003). These studies suggest a secondary binding site and a possible PLB:SERCA stoichiometry of 2:1. However, most studies support a model of a single PLB bound to SERCA in a 1:1 stoichiometry (Mueller, Karim et al. 2004). Mutations that enhance the monomeric form of PLB show an improved ability to inhibit SERCA,
suggesting that an oligomeric form of PLB is not required for inhibition (Kimura, Kurzydlowski et al. 1997). Previous measurements of fluorescently-labeled PLB and SERCA support the model of a single PLB in the regulatory complex (Robia, Campbell et al. 2007). Many of the studies examining the stoichiometry of the complex have employed unnatural experimental conditions, thus it may be beneficial to examine this stoichiometry in a native environment.

**Inhibition of SERCA by PLB**

PLB inhibits SERCA through physical interaction with SERCA. Inhibition of SERCA is relieved by PKA phosphorylation of PLB at serine 16 or CaMKII phosphorylation at threonine 17 (Le Peuch, Haiech et al. 1979, Simmerman, Collins et al. 1986). It is not clear whether this mechanism works by physical dissociation of PLB from SERCA (Chen, Akin et al. 2006, Akin, Chen et al. 2010) or whether the two proteins remain bound during phosphorylation of PLB (Li, Bigelow et al. 2004, Mueller, Karim et al. 2004, Bidwell, Blackwell et al. 2011). Previous work has shown that stimulation of the beta-adrenergic cascade produces a robust phosphorylation of PLB at serine 16 (Tada, Kirchberger et al. 1975) that increases SERCA activity (Kranias, Garvey et al. 1985). Phosphorylation makes SERCA a predominant target of beta-adrenergic signaling in the heart and comprises an important response to demand for increased contractility. SERCA inhibition can also be relieved by phosphorylation of PLB at threonine 17, although this probably does not occur independently of PKA serine 16 phosphorylation in the heart (Plank, Pifl et al. 1983, Lindemann and Watanabe 1985, Simmerman, Collins et al. 1986, Mundina-Weilenmann, Vittone et al. 1996). Dephosphorylation of both sites
has been shown to be due to protein phosphatase-1 activity (Kranias and Di Salvo 1986, Steenaart, Ganim et al. 1992).

As previously shown in figure 2, SERCA activity increases in response to increasing calcium concentration. PLB inhibits SERCA by decreasing the calcium sensitivity, resulting in a rightward shift in this relationship (red trace). In fact, the first experiment on calcium uptake in the cardiac SR took advantage of “half-maximal” calcium, thus being poised to observe the greatest possible difference between unphosphorylated and phosphorylated PLB (Kirchberger, Tada et al. 1972). As seen in figure 2, at calcium concentrations below the maximum ATPase rate, SERCA activity is reduced when PLB is present. Phosphorylation of PLB will reverse this effect such that the calcium sensitivity is shifted back to the left.

The maximal calcium ATPase activity may also be altered by PLB. Studies have identified distinct roles for serine 16 and threonine 17 in regulating SERCA (Mattiazzi, Hove-Madsen et al. 1994). Maximal calcium uptake rate ($V_{\text{max}}$) increased in the presence of CaMKII phosphorylation (Mattiazzi, Hove-Madsen et al. 1994, Mundina-Weilenmann, Vittone et al. 1996). One of these studies suggested that $V_{\text{max}}$ changes were specific to CaMKII, while PKA phosphorylation only altered calcium sensitivity and not $V_{\text{max}}$ (Mattiazzi, Hove-Madsen et al. 1994). Other groups have observed $V_{\text{max}}$ increases in systems phosphorylated by PKA. Antipenko et al. reported a 33 – 44% increase in $V_{\text{max}}$ upon stimulation with PKA in purified SR (Antipenko, Spielman et al. 1997). This was also observed by Reddy et al. in a reconstituted system and by Lu et al. in cardiac SR with nonionic detergent (Lu and Kirchberger 1994, Reddy, Cornea et al. 2003). Trieber et al.
provided evidence that interaction of PLB with SERCA enhances $V_{\text{max}}$ even in the absence of phosphorylation and $V_{\text{max}}$ was not further altered by phosphorylation (Trieber, Douglas et al. 2005). ATPase measurements in the presence of synthetic peptides provided a potential mechanism of SERCA inhibition by PLB. Expression of the cytosolic region of PLB (amino acids 1 – 31) only decreased the $V_{\text{max}}$ of SERCA while expression of the transmembrane region of PLB (amino acids 28 – 47) only decreased the calcium affinity, differentiating the role of these domains in calcium transport regulation (Sasaki, Inui et al. 1992). Tryptic digestion of the cytosolic domain of PLB enhanced $V_{\text{max}}$, corroborating the functional role of the cytosolic domain in calcium transport (Lu, Xu et al. 1993). A variety of experiments have indicated a possible functional effect of PLB by alteration of $V_{\text{max}}$ although the exact mechanism by which PLB would alter $V_{\text{max}}$ has not been established.

Careful examination of the maximum rate of calcium transport has shown evidence for increases in $V_{\text{max}}$ with PLB. This may serve as an additional mechanism for increased calcium transport in the presence of phosphorylated PLB. However, not all groups have observed changes in $V_{\text{max}}$ despite attempts to examine this effect. Morris et al. did not detect a change in $V_{\text{max}}$ following phosphorylation with PKA or with incubation of two antibodies that mimic the effects of PLB (Morris, Cheng et al. 1991). These results were corroborated in SR preparations from human myocardium, again showing the failure of an anti-PLB antibody to alter $V_{\text{max}}$ (Movsesian, Colyer et al. 1990, Movsesian 1992). Earlier work from Reddy et al. found that PLB had no effect on $V_{\text{max}}$ in skeletal SR preparations co-expressed with recombinant PLB. They observed that
expression of the recombinant cytosolic domain of PLB (residues 1 – 31) had no apparent effect even at molar ratios as high as 300:1 (Reddy, Jones et al. 1995). Other studies also reported similar failure of PKA- or CaMKII-mediated phosphorylation to increase $V_{\text{max}}$ (Odermatt, Kurzydlowski et al. 1996, Schwinger, Munch et al. 1999). The inconsistent results obtained from measurements of ATPase activity remains an unresolved problem in the field and necessitates further examination of this effect.

**Phospholamban in disease**

A few mutations to PLB have been observed to cause cardiovascular disease. A mutation of arginine 9 to cysteine results in dilated cardiomyopathy and early death (Schmitt, Kamisago et al. 2003). Our group has previously characterized this mutant as being particularly susceptible to oxidative stress, decreasing regulation of SERCA (Abrol, de Tombe et al. 2015). Another mutation, L39stop, leads to heart failure (Haghighi, Kolokathis et al. 2003). Previous work has shown that this results in mislocalization of PLB to the sarcoplasm, reducing physical interaction with SERCA (Abrol, Smolin et al. 2014). Deletion of arginine 14 was found in a family with hereditary HF (Haghighi, Kolokathis et al. 2006). More recently mutation of arginine 25 to cysteine was shown in a familial pedigree with dilated cardiomyopathy (Liu, Morales et al. 2015). These mutations to PLB underscore the importance of proper SERCA regulation and highlight the role of SERCA in cardiac pathogenesis.

Evidence suggests that PLB protein expression does not change during the progression to HF (Munch, Bolck et al. 1998, Huang, Wang et al. 1999). However, studies suggest that phosphorylation of PLB is decreased in HF (Huang, Wang et al. 1999,
Schwinger, Munch et al. 1999). Measurements of protein expression in rats with congestive HF found that SERCA:PLB and pentamer:monomer ratios were preserved while phosphorylation of PLB at serine 16, but not threonine 17, was decreased (Sande, Sjaastad et al. 2002). These results also showed no change in SERCA expression.

The decrease in PLB phosphorylation alone could explain the reduced SERCA activity observed in HF, however, multiple studies show a decreased responsiveness to stimulation by either PKA or CaMKII in failing myocardium. Schmidt et al. observed a decreased activation of SERCA in response to either PKA or CaMKII, suggesting that phosphorylation alone could not account for the decreased activity (Schmidt, Hajjar et al. 1999). Schwinger et al. found that PKA phosphorylation of failing human myocardium corrected the differences in calcium sensitivity, but a significant decrease in $V_{\text{max}}$ persisted (Schwinger, Munch et al. 1999). Blockade of phosphatase activity with okadaic acid reversed the loss of PLB phosphorylation but did not fully restore calcium transient amplitudes and rates of relaxation (Huang, Wang et al. 1999). The results seen in these studies show that the reduced PLB phosphorylation seen in HF does not fully explain the decrease in SERCA activity, suggesting that an additional mechanism may be involved.

**SERCA homo-oligomerization**

Multiple studies have provided evidence for the assembly of SERCA into homo-oligomers. Early work on the skeletal SERCA isoform suggested that SERCA naturally forms oligomers in muscle tissue preparations. Electron microscopy of reconstituted purified SERCA in vesicles showed formation of tetramers (Vanderkooi, Ierokomas et al. 1977). In 1984, Yamamoto et al. found that gel filtration of skeletal muscle SR
preparations produced two distinct fractions containing SERCA, the first at a molecular weight of approximately 150 kDa and the second at a molecular weight of approximately 360 kDa, suggesting formation of SERCA homo-dimers (Yamamoto, Yantorno et al. 1984). Careful analysis of the kinetic properties of calcium transport provided additional evidence for homo-dimerization (Mahaney, Albers et al. 2005, Chen, Yao et al. 2009). However, other kinetic studies detailing phosphoenzyme formation and decay support a model for SERCA homo-trimerization (Mahaney, Thomas et al. 2008).

Higher order oligomerization has also been seen in time-resolved optical anisotropy measurements revealing the formation of SERCA aggregates in the presence of thapsigargin or melittin (Voss, Birmachu et al. 1991, Mersol, Kutchai et al. 1995).

Many of the studies investigating oligomerization of SERCA have suggested that this may be an important regulator of SERCA function. Some studies have correlated oligomerization/aggregation with decreased SERCA catalytic activity. This observed inhibition by self-association could be relieved by phosphorylation, which is proposed to disrupt SERCA self association through charge reversal in the cytosolic region of PLB (Voss, Jones et al. 1994, Thomas, Reddy et al. 1998). Inhibitory molecules such as thapsigargin or melittin produced irreversible self association of SERCA (Voss, Birmachu et al. 1991, Mersol, Kutchai et al. 1995). However, other data suggest that SERCA oligomerization may be a mechanism for increased transport activity after relief of inhibition by adrenergic stimulation. In particular, saturation transfer electron paramagnetic resonance experiments indicated that phosphorylation of PLB reduced the rotational mobility of SERCA2a consistent with SERCA homo-oligomerization.
This interpretation is also supported by kinetic measurements of calcium transport that indicated SERCA2a dimerizes in the presence of phosphorylated PLB (Mahaney, Albers et al. 2005). Earlier work also demonstrated that SERCA-SERCA interactions altered responsiveness to ATP (Moller, Lind et al. 1980) or magnesium (Yamamoto, Yantorno et al. 1984, Vilsen and Andersen 1987). Significantly, the kinetic data suggested conformational coupling of SERCA protomers into a single functional unit to increase the rate of calcium transport. In this model energetically unfavorable structural transitions of one SERCA protomer are assisted by coupling them to energetically favorable conformational changes of another protomer. Examination of different kinetic transitions of calcium transport revealed that some steps were enhanced beyond the rate constants of a single protomer (Mahaney, Albers et al. 2005). By reducing the barrier of the slow step the overall rate of calcium transport is increased. Radiation inactivation of SERCA showed a reduction in SERCA activity that was greater than the inactivated fraction of SERCA, suggesting that the activity of SERCA is greater than the sum of its parts (Chamberlain, Berenski et al. 1983). An analogous experiment was performed by Chen et al., showing the ATPase activity was reduced more than would be expected from the molar ratio of WT to inactive SERCA (Chen, Yao et al. 2009). This process of conformational coupling enables SERCA to overcome catalytically unfavorable steps by coupling multiple SERCA protomers in a cooperative manner. These data may provide insight into the discrepancy between unchanged SERCA expression and depressed function. SERCA homo-oligomerization may be a key regulatory component of SERCA activity in addition to regulation by PLB.
Further precedence for SERCA homo-oligomerization comes from studies of other P-type ATPases. Early cross-linking of the Na+/K+-ATPase with copper and o-phenanthroline yielded a tetramer. Interesting, only half of the ATPases were phosphorylated, suggesting that protomers may act cooperatively through alternating steps of the catalytic cycle (Askari and Huang 1980). Later work by Kunihiro Mimura et al. identified tetramer, dimer, and monomer fractions of this enzyme. The tetramer had half the ATPase activity of the monomer or dimer, suggesting a possible regulatory mechanism for self-association (Mimura, Tahara et al. 2008). Irradiation experiments of the gastric H+/K+-ATPase showed that dimerization was necessary for enzyme activity (Morii, Hayata et al. 1996). Despite the evidence for P-type ATPase oligomerization, these models have not been widely adopted as the monomeric forms are shown to possess ATP-dependent ion transport (Goormaghtigh, Chadwick et al. 1986, Andersen 1989).

**Interaction of Hax-1 with the SERCA/PLB regulatory complex**

HAX-1 was originally identified in a yeast two-hybrid screen as a binding partner of HS1, a regulator of cell proliferation and death in lymphoid cells (Suzuki, Demoliere et al. 1997). Immunohistochemistry showed strong mitochondrial localization with some HAX-1 present in the ER membrane and nuclear envelope. Recent work has provided evidence for association of HAX-1 with the cytosolic domain of PLB (Vafiadaki, Sanoudou et al. 2007). Interestingly, western blotting of mouse ventricular myocytes from HAX-1 overexpressing mice showed reduced SERCA protein levels (Vafiadaki, Arvanitis et al. 2009). These studies found higher levels of HAX-1 when PLB was present in the heart.
than when it was absent in a knockout mouse model. In addition, SERCA activity was more inhibited than the wild type mice, suggesting a regulatory mechanism for HAX-1 that went beyond altering protein expression. In fact, phosphorylation of PLB abrogated interaction with HAX-1 as determined from co-immunoprecipitation experiments. Of note, HAX-1 was shown to interact with residues 16-22 on PLB. Fluorescence resonance energy transfer (FRET) experiments suggested that HAX-1 decreases PLB pentamer stability, increasing the pool of PLB monomers available to inhibit SERCA. This model was compatible with FRET measurements of the SERCA/PLB regulatory complex showing increased binding of PLB to SERCA (Vafiadaki, Arvanitis et al. 2009). The exact mechanism by which HAX-1 alters SERCA activity has not been resolved. It is possible that HAX-1 regulates SERCA by stabilizing or destabilizing SERCA homo-oligomers, thus altering the functional cooperativity. It would be prudent to determine whether this protein has a role in SERCA homo-oligomerization.

**Importance of SERCA**

The role of calcium handling in cardiovascular disease is evident. In addition to the data presented above, SERCA has been implicated in other diseases as well. Artemisinins are potent anti-malarial drugs used in treating multi-drug resistant malaria. Recently, they were determined to target PfATP6, a SERCA orthologue found in parasites that transmit malaria (Eckstein-Ludwig, Webb et al. 2003). Calcium is an important part of cell survival and growth and has been implicated in many signaling pathways (Yano, Tokumitsu et al. 1998, Apati, Janossy et al. 2003). Consequently, SERCA has been implicated in some forms of cancer and is being considered as a therapeutic
target (Denmeade, Jakobsen et al. 2003, Dubois, Vanden Abeele et al. 2013, Roti, Carlton et al. 2013). A greater understanding of the SERCA/PLB regulatory complex and mechanisms of activity may provide new interventions for disease.

In the present study we set out to examine SERCA homo-oligomerization and regulation. We used photoactivatable cross-linking to investigate whether SERCA is oligomeric in cardiac myocytes. This work was extended to AAV-293 cells in order to quantify important parameters of structure and regulation. Using spectroscopic approaches, we quantified the stoichiometry of the putative oligomer and determined whether oligomerization was regulated by mechanisms known to be important determinants of SERCA activity. Various approaches were employed to examine how oligomerization can be pharmacologically or physically altered within the live membrane. Lifetime measurements allowed us to resolve discrete populations within the cell and to examine the interaction of PLB with SERCA and regulation in response to phosphorylation of PLB. Finally, functional measurements of SERCA activity helped provide insight into the physiological role of homo-oligomerization.
CHAPTER THREE
MATERIALS AND METHODS

A major enabling technology of the present study was the use of fluorescence resonance energy transfer (FRET). FRET provides many distinct advantages over other methods aimed at examining protein-protein interactions. It can be used in live cells so that the full physiological environment is preserved. Live cells also provide easy manipulation of physiological pathways and effectors. Beyond that, FRET techniques provide information about protein-protein affinity, structure, concentration, stoichiometry, quantitative measurements of distance, and identification of multiple populations of species.

FRET is a process that describes the biophysical property of nonradiative energy transfer between two chromophores. This requires that the emission spectrum of the donor chromophore overlaps with the excitation spectrum of the acceptor chromophore (Tsien 1998). If the two labels are in close proximity the excited donor will transfer energy to the acceptor. FRET is very sensitive to distance and is most effective when the distance between donor and acceptor fluorescent proteins is between 25 and 100 Å; the ideal range varies depending on the physical properties of the particular donor and acceptor selected. Figure 4 illustrates the FRET signal in response to distance changes for a CFP donor and YFP acceptor. At distances shorter than 25 Å the FRET signal is saturated and changes in distance will not alter the apparent FRET signal. At
Figure 4. A simulation of the dependence of FRET efficiency on fluorescent protein separation distance (R) for cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). The Förster distance ($R_0$) is the distance at which FRET efficiency = 0.5.
distances greater than 100 Å energy transfer cannot take place. This has the distinct advantage of generally limiting measurements to short distances where protein-protein interactions likely occur. There are many ways to measure FRET, some of which are outlined below. Each method has its own advantages and disadvantages, but, used together, these methods provide a substantial amount of information about the protein-protein interaction being studied.

**Molecular biology and cell culture**

Canine SERCA2a was labeled with mCerulean (Cer), enhanced green fluorescent protein (GFP), or enhanced yellow fluorescent protein (YFP). Labels were positioned at the N-terminus, between residues 508 and 509, or at the C-terminus. Canine PLB was labeled at the N-terminus with YFP or mCherry. Mutations were made to the PKA-dependent phosphorylation site serine 16 of PLB to produce the nonphosphorylatable (S16A) and phosphomimetic (S16E) variants. FRET standards were used as positive controls in which Cer is fused to Venus fluorescent protein with a 5, 17, or 32 amino acid linker (C5V, C17V, or C32V) or Cer is fused between two Venus fluorescent proteins with linker sizes of five amino acids (VCV). All constructs were validated with nucleotide sequencing. Constructs were expressed by transient transfection of AAV-293 cells using the MBS mammalian transfection kit (Agilent Technologies). After 24 hours, the cells were trypsinized for one minute and plated on glass-bottom chambered slides (Matek) coated with poly-D-lysine. Cells were allowed to adhere to the slide for two hours before carrying out FRET measurements.
**Progressive acceptor photobleaching**

Acceptor photobleaching is a technique that utilizes irreversible selective destruction of the acceptor chromophore through prolonged excitation. Once destroyed, the acceptor is no longer capable of quenching the donor and, if the donor and acceptor are at a distance capable of FRET, the donor intensity will increase. The stoichiometry of a molecular complex can be determined from the relationship between the donor and acceptor intensities over time. If this relationship is linear, then it indicates the donor- and acceptor-labeled complex exists as a dimer (N = 2). However, if this relationship is curved, then the complex exists as a higher order oligomer (N > 2) (Kelly, Hou et al. 2008).

Acceptor photobleaching was carried out on an inverted microscope (Nikon) equipped with a 60X oil immersion objective with a numerical aperture of 1.49. A Lumen 200 metal halide lamp (Prior) and excitation and emission filters (Semrock) were used to selectively excite the Cer and YFP channels and collect the emission signal. Images were collected using a back-thinned iXon 887 CCD camera (Andor Technology). Selection of the appropriate excitation and emission filters was managed by motorized filter wheels (Sutter Instruments). Cells were imaged every 10 seconds for 500 seconds and YFP was selectively photobleached by exposure to YFP excitation (504/12 nm) in the time between each image. The photobleaching power at the sample was approximately 1.59 nW/μm². Images were analyzed in the MetaMorph software program by manual selection of the cell fluorescence area. The average intensity values of the fluorescent area of the cell over time were normalized to the prebleach intensity using the
equation: $\frac{F}{F_0} = \frac{(F - F_{bg})}{(F_0 - F_{0bg})}$, where $F$ is the fluorescence intensity, $F_0$ is the prebleach fluorescence intensity, and $F_{bg}$ and $F_{0bg}$ are the background fluorescence signals. Figure 5 shows a representative field of cells expressing Cer-SERCA and YFP-PLB before and after the photobleaching process.

**Acceptor-sensitized emission FRET**

We utilized acceptor-sensitized emission FRET as a way of measuring protein-protein affinity and structure. This method allows FRET to be calculated from images of the donor and acceptor intensities with their respective excitation as well as a third image that captures the acceptor emission with donor excitation. It does not require long collection times such as needed with acceptor photobleaching. This allows a large number of cells to be quantified in a relatively short time period. Previous studies have shown that acceptor-sensitized FRET is consistent with other methods (Hou, Kelly et al. 2008, Kelly, Hou et al. 2008, Hou and Robia 2010).

Acceptor-sensitized FRET was performed on a Nikon inverted microscope equipped with a 40X NA dry objective. A motorized stage surveyed 120 individual locations and collected 150 ms exposures of the intensity of Cer emission following Cer excitation (Cer), YFP emission following YFP excitation (YFP), and YFP emission following Cer excitation (FRET). The 120 images for each condition were stitched together into a single montage image. Fluorescence intensity was measured by a multi-wavelength cell scoring application (MetaMorph). The background threshold was set to 110 counts. Cell size criteria were limited to 9 – 19 μm in diameter. FRET efficiency was calculated using the equation $E = \frac{G}{(G+4.6 \times F_{Cer})}$ where $G$ is an instrumental correction factor defined by
Figure 5. Representative images of AAV-293 cells expressing Cer-SERCA and YFP-PLB before photobleaching (pre-bleach) and after photobleaching for 10 minutes (post-bleach). Note the decrease in YFP intensity and concomitant increase in Cer intensity as the donor and acceptor are close enough for FRET.
the equation \( G = \text{F}_{\text{FRET}} - a \times \text{F}_{\text{YFP}} - d \times \text{F}_{\text{Cer}} \). \( \text{F}_{\text{FRET}}, \text{F}_{\text{YFP}}, \) and \( \text{F}_{\text{Cer}} \) are the cell average fluorescence intensities for the FRET, YFP, and Cer channels, respectively, and \( G \) is the FRET intensity corrected for bleed through of the donor (\( d \)) and acceptor (\( a \)) channels.

The donor bleed-through constant was calculated by imaging cells that only expressed the donor and dividing the resulting FRET image by the donor image. The acceptor bleed-through was similarly calculated from cells that expressed only the acceptor and dividing the resulting FRET image by the acceptor image. The bleed through constants \( a \) and \( d \) were determined to be 0.085 and 0.737, respectively on our experimental setup.

Each cell that expressed both Cer and YFP at a level above background threshold was plotted as percent FRET over the range of expressed protein concentration, as determined by the fluorescence intensity of the YFP channel (expressed in arbitrary units). The plotted data were fit by a hyperbola using the equation: \( \text{FRET}=\text{FRET}_{\text{max}} \times \frac{X}{(K_{d} + X)} \) where \( X \) is the measured YFP intensity, \( \text{FRET}_{\text{max}} \) is the maximum FRET, and \( K_{d} \) is the apparent dissociation constant of the donor- and acceptor-labeled proteins for each other.

**Fluorescence lifetime measurements**

Fluorescence lifetime decays were collected using a Nikon Eclipse Ti inverted microscope equipped with a 60X NA water immersion objective. GFP was excited using a supercontinuum laser pulsed at a rate of 20 MHz (Fianium) and a 482/18 nm bandpass filter (Semrock Inc.). The fluorescence emission was passed through a dual band bandpass filter (523/610) and dichroic filter in the microscope (488/561 nm) and a 50 \( \mu \)m confocal pinhole. The emission was then directed through a 525/50 nm bandpass
filter to an avalanche photodiode (Micro Photon Devices) using a dichroic beamsplitter (565 nm, Chroma).

Fluorescence lifetime measurements were obtained from AAV-293 cells expressing GFP-SERCA alone or GFP-SERCA with mCherry-PLB. The level of expression of fluorescently labeled proteins in each cell was determined by using a 500 mm focal length planoconvex lens in a flip mount to defocus the excitation beam (560/14 nm) of the supercontinuum laser in order to excite the entire cell. The area of excitation at the Z-position of the cell was calculated to be 30 μm in diameter (approximately the size of one cell). The emitted fluorescence of mCherry-PLB was detected using a 640/50 nm bandpass filter and a CCD camera (CoolSNAP K4, Photometrics). The average fluorescence intensity of the cell (expressed in arbitrary units) was used as an index of the protein concentration. After each image was obtained, the lens was removed from the path and time correlated single photon counting was performed using single point excitation and confocal detection using the avalanche photodiode. Photons were collected until the peak of the decay reached a minimum of $10^4$ photons.

Fluorescence decays obtained from each cell were analyzed independently by tail fitting with a one- or two-component exponential decay from 3.5 ns using the SymPhoTime 64 software (Picoquant Photonics). The amplitude parameter was constrained to values between 0% and 100%. The tau parameter was compared to the average fluorescence intensity of mCherry-PLB for each cell measured with the CCD camera to determine the dependence of fluorescence lifetime on protein expression. Two-component analysis of GFP-SERCA fluorescence decays was performed by fixing
one lifetime to the donor-only tau in order to limit the number of floating parameters. FRET efficiency ($E$) was calculated using the equation: $E = 100 \times \left(1 - \frac{\tau_{DA}}{\tau_D}\right)$, where $\tau_{DA}$ is the lifetime of the donor in the presence of acceptor and $\tau_D$ is the lifetime of the donor-only sample. Distance measurements were calculated from the relationship $R = R_0(E^{-1} - 1)^{1/6}$, where $R_0$ is the Förster distance for the GFP/mCherry pair (5.31 nm) and $E$ is the average FRET efficiency from the lifetime measurements, assuming a random orientation factor ($\kappa^2 = 2/3$).

**Photon counting histogram (PCH) analysis**

AAV-293 cells expressing GFP-SERCA were cross-linked and separated out using SDS-PAGE as detailed below. Monomeric SERCA (135 kDa) and cross-linked SERCA (301 kDa) were excised from the polyacrylamide gel and soaked in PBS for three hours at room temperature. Fluorescence correlation spectroscopy (FCS) was used to determine the concentration of GFP-SERCA of the gel eluants and the samples were diluted in order to compare equal concentrations of the eluted species in the subsequent PCH analysis. Photon counting was performed with a PicoHarp 300 TCSPC module (PicoQuant Photonics). FCS data were collected until the peak of the fluorescence decay had reached $10^4$ photons. Histograms were generated in the SymPhoTime 64 software using a bin width of 0.2 ms and exported to Globals Software for Spectroscopy and Images (LFD, University of California, Irvine). PCH brightness analysis was performed using a one-photon, three dimensional Gaussian-Lorentzian model with one- or two-species minimization.
Co-immunoprecipitation

Co-immunoprecipitation was performed in cells expressing GFP-SERCA2a and cMyc-SERCA2a at a molar plasmid ratio of 1:1. The cells were detached with a cell scraper in PBS and centrifuged at 500 G for five minutes. The supernatant was aspirated and the pellet was resuspended in cell lysis buffer containing 150 mM NaCl, 50 mM Tris, 1% Nonidet-P-40, and EDTA-free protease inhibitor cocktail at pH = 8.0. The suspended pellet was incubated at 4 °C on a shaker for 20 minutes and then centrifuged at 13,000 G for 10 minutes at 4 °C. The supernatant was transferred to a new centrifuge tube and incubated overnight at 4 °C with mouse anti-cMyc antibody (Clontech Laboratories, Cat. #631206) or mouse non-specific IgG antibody (Sigma-Aldrich, Cat. # I5381). The resulting immunocomplex was transferred to a new microcentrifuge tube containing protein-G magnetic Dynabeads. The sample was incubated for one hour at room temperature and then washed four times with cell lysis buffer. The final solution containing the beads was incubated at 55 °C for 10 minutes in 1% SDS with beta-mercaptoethanol to elute the bound protein. The resulting protein was separated out using SDS-PAGE and western blotting.

Photoactivatable chemical cross-linking

Left ventricular cardiomyocytes were isolated from adult New Zealand white rabbits by a core facility as previously described (Domeier, Blatter et al. 2009). All animal protocols were approved by the Loyola University Institutional Animal Care and Use Committee. Myocytes were permeabilized for two minutes with 100 μg/mL saponin (Sigma-Aldrich) in a potassium-free relaxing solution containing: 100 mM NaCl, 5 mM
MgCl₂, 2 mM EGTA, 10 mM Imidazole, 4 mM ATP, and 50 mM tris(2-carboxyethyl)phosphine at pH = 7.0. A complete protease inhibitor cocktail (Santa Cruz Biotechnology) was added prior to the start of the experiment. The permeabilized myocytes were centrifuged for 5 minutes at 500 G at 4 °C. The supernatant was removed and replaced with potassium-free relaxing solution containing the photoactivatable cross-linker benzophenone-4-maleimide (BPM) (Sigma-Aldrich). BPM is a heterobifunctional cross-linker which contains a sulfhydryl-specific group and a photoactivatable group. The pellet was resuspended and incubated for one hour at room temperature. The suspension was then placed on ice and irradiated for 20 minutes by a long wave ultraviolet lamp set at 365 nm (UVP). A 2X cell lysis solution (300 mM NaCl, 100 mM Tris, 2% SDS, pH = 8.0) was added in equal volume to the suspension in preparation for western blotting.

**Protein quantification**

Protein concentrations were determined from a bicinchoninic acid protein assay (Pierce). 20 μL of sample and water were loaded in triplicate on a 96-well plate. A standard curve was generated by using 2 mg/mL bovine serum albumin. Reagent 1 and 2 were combined as per the provided protocol and 200 μL were added to each well. The plate was incubated at 37 °C for 30 minutes and then placed in an Epoch microplate spectrophotometer (BioTek Epoch) to measure the absorbance at 562 nm.

**Western blotting**

Samples were separated on a 4-15% polyacrylamide precast gradient gel (Bio-Rad). The separated protein was transferred to polyvinylidene difluoride membrane at
100 V for two hours at 4 °C. The membrane was blocked in milk for one hour at room
temperature followed by incubation of the primary antibody rabbit anti-GFP (1:2000; Life Technology), mouse anti-SERCA2 (1:2000, AbCam, Cat. # ab2817), or mouse anti-
cMyc (1:2000, Clontech Laboratories, Cat. # 631206) overnight at 4 °C. Blots were
incubated with anti-mouse or -rabbit secondary antibodies conjugated to horseradish
peroxidase for two hours at room temperature (1:20,000 dilution). The blots were
developed using an electrochemiluminescence substrate (Perkin Elmer) and imaged
using a ChemiDoc XRS+ (Bio-Rad).

ATPase measurements

SERCA enzymatic activity was quantified in cell homogenates by
spectrophotometric measurement of the rate of NADH consumption in an enzyme
coupled activity assay. AAV-293 cells expressing SERCA and/or PLB were detached using
cold PBS and cell scraper. The cells were pelleted for 5 minutes at 4 °C and 500 G. The
supernatant was aspirated and the cell pellet was resuspended in homogenization
buffer (0.5 mM MgCl2, 10 mM Tris-HCl at pH = 7.5, and a complete protease inhibitor
cocktail). The suspension was left on ice for 5 minutes and then homogenized with 30
strokes of a Potter-Elvehjem homogenizer. An equal amount of 2X sucrose buffer (100
mM MOPS at pH = 7.0, 500 mM sucrose, and 1 Complete protease inhibitor cocktail)
was added followed by DNAse equal to 20% of the volume.

A 5X buffer was made from 0.25 M MOPS, 0.5 M KCl, 25 mM MgCl2, 5.0 mM
EGTA, pH = 7.0. This was used to make a 2X assay mix buffer consisting of 20% 5X buffer
(by volume), 0.4 mM NADH, 1.0 mM PEP, 5.0 mM ATP, 10 IU/mL LDH, 10 IU/mL PK, and
0.00712 mg/mL calcium ionophore. A 2X sucrose buffer was made containing: 40 mM MOPS (pH = 7.0), 0.6 M sucrose, and 2 mM NaN3 at pH = 7.0 at room temperature. A range of 20X calcium solutions were made by combining 20 mM CaCl2 with ddH2O as detailed in Table 1. This 20X calcium solution was diluted in water and 2X sucrose buffer to yield a 4X calcium solution at 1X sucrose. The homogenate, 4X calcium solution, and 2X assay mix were heated to 37 °C and then combined. The absorbance at 340 nm was read on a plate reader at 37 °C. The enzymatic reaction scheme for this protocol is detailed in figure 6. Purified rabbit fast-twitch skeletal SR preparations were obtained as described by Mueller et al. (Mueller, Karim et al. 2004). Purified pig cardiac SR membrane vesicles were obtained as described by Fruen et al. (Fruen, Bardy et al. 2000). Both the skeletal and cardiac SR preparations were a gift from David D. Thomas at the University of Minnesota.

The rate of the decrease in NADH absorbance was plotted against the calcium concentration. The data were fit with a Hill equation of the form:

\[ y = y_0 + \left( V_{\text{max}} - y_0 \right) \frac{x^n}{\left( k^n + x^n \right)} \]

where \( y_0 \) is the y offset, \( V_{\text{max}} \) is the maximum rate of calcium transport, \( k \) is the calcium concentration at half maximal activity, and \( n \) is the Hill coefficient.
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**Table 1.** The necessary volumes of 20 mM CaCl$_2$ and ddH$_2$O to produce a 20X calcium solution at 37 °C.
Figure 6. A schematic representation of the enzyme-linked ATPase assay. SERCA consumes ATP which is regenerated with pyruvate kinase and phosphoenolpyruvate (PEP). Pyruvate is turned into lactic acid with lactate dehydrogenase, oxidizing NADH in the process. The rate of decrease in absorbance at 340 nm is taken as an index of the rate of ATP consumption.
CHAPTER FOUR

RESULTS

SERCA homo-oligomerization

Previous studies have shown that SERCA is capable of forming homo-oligomers in reconstituted lipid bilayers. To determine whether SERCA is oligomeric in live cells we performed co-immunoprecipitation in AAV-293 cells transiently co-transfected with GFP-SERCA and cMyc-SERCA. We used a buffer containing Nonidet P-40, which has been previously shown to preserve ER membrane protein interactions with SERCA and other regulatory partners (Han, Cai et al. 2011). Immunoprecipitation with anti-cMyc pulled down GFP-SERCA (Figure 7A). A control IgG antibody did not pull down either cMyc- or GFP-labeled SERCA, suggesting that SERCA protomers interact with each other.

As an alternative approach, we investigated the interaction of SERCA protomers in the biosynthetic membranes of ventricular cells. We treated isolated rabbit cardiac left ventricular (LV) myocytes with saponin to selectively permeabilize the sarcolemma and performed photoactivatable cross-linking with BPM. BPM is a heterobifunctional cross-linker with a thiol-specific maleimide group and a photoactivatable benzophenone group capable of reacting with alpha carbons. This cross-linker is advantageous because the maleimide chemistry enables targeting of SERCA, which is estimated to contain more than 50% of the reactive thiols in the SR membrane by content (Viner, Williams et al. 1999). Furthermore, this benzophenone group does not depend on prior
Figure 7. Oligomerization of SERCA probed with co-immunoprecipitation (co-IP) and cross-linking. A) Co-IP of GFP-SERCA with Myc-SERCA. B) Western blot analysis of rabbit LV myocytes. Increasing concentrations of benzophenone-4-maleimide (BPM) decreased the amount of monomeric SERCA (band 1) and gave rise to two additional bands (Band 2 and Band 3). C) Pretreatment of myocytes with 50 nM isoproterenol (iso) did not appreciably alter the SERCA electrophoretic pattern. D-F) Quantification of Bands 1, 2, and 3 relative to total SERCA. Values are mean ± SD from N = 3 independent blots.
identification of the binding interface or mutation of specific residues for reactivity and successful cross-linking.

Western blots of the cross-linked LV myocytes were probed with a SERCA2 primary antibody, revealing a single band at approximately 99 kDa (Figure 7B, Band 1) in keeping with the molecular weight of the SERCA monomer. Increasing concentrations of BPM caused a decrease in the relative amount of the SERCA monomer (Figure 7D) and yielded a new prominent band at 230 kDa (Figure 7B, Band 2). At the highest BPM concentrations (>100 μM) we observed a third band at 315 kDa (Figure 7B, Band 3). The contributions of these cross-linked species are quantified in figures 7D-F.

Previous studies have suggested that phosphorylation of PLB at serine 16 may play an essential role in SERCA homo-oligomer formation. However, we did not observe a significant change in cross-linking after treatment of myocytes with 50 nM beta-adrenergic agonist isoproterenol (iso) (Figure 7C). The data suggest that PLB phosphorylation does not alter BPM cross-linking of SERCA. The relative amount of the SERCA monomer increased with pre-incubation of increasing amounts of the detergent n-dodecylphosphocholine (DPC) (Figure 8A & B). It was noteworthy that high molecular weight species were still produced even after with pre-solubilization of apparent SERCA dimers with 1% DPC, suggesting a robust oligomeric interaction was present before the addition and activation of cross-linker. This may also indicate that previous studies using detergent-solubilized SERCA may not have contained 100% monomers.

We attempted to identify the reactive cysteine on SERCA that may be responsible for cross-linking. Canine SERCA contains 26 cysteines. 10 of these are
Figure 8. Detergent solubilization of SERCA A) Pre-incubation with the detergent n-dodecylphosphocholine (DPC) in 300 μM BPM increased the relative amount of monomeric SERCA (Band 1). B) Quantification of A.
located in the TM domain and are likely inaccessible. Additionally, some of the
cysteines positioned in the cytosolic domain form internal disulfide bridges and would
not be reactive (Sharov, Dremina et al. 2006). We selected four reactive cysteines for
mutation: C364, C498, C636, and C674. Individual mutations of these residues did not
abrogate the cross-linking reaction, suggesting that the cross-linked species observed by
western blotting are not a result of BPM interaction with these residues. This indicates
that a different cysteine residue may be responsible for the cross-link or the cross-link
may form from modification of another binding partner.

To determine whether the high molecular weight bands seen by cross-linking
contained multiple SERCA protomers or a single SERCA protomer cross-linked to another
binding partner (Arvanitis, Vafiadaki et al. 2007, Vafiadaki, Sanoudou et al. 2007, Zhao,
Li et al. 2015), we performed cross-linking of GFP-SERCA heterologously expressed in
AAV-293 cells. Western blotting of the non cross-linked control sample showed GFP-
SERCA monomer at a molecular weight of 135 kDa (figure 9A). This +35 kDa shift in
mobility compared to the unlabeled SERCA seen in figure 7B is consistent with the
increase in molecular weight corresponding to attachment of a single GFP of
approximately 30 kDa (Prendergast and Mann 1978). The cross-linked GFP-SERCA
produced a second band at 301 kDa. We noted that this band was shifted by a value of
+71 kDa, approximately equal to the molecular weight of two GFP molecules. The third
band was not evident by western blot and is possibly indistinguishable from the second
band or perhaps too large to enter the gel.
Figure 9. Brightness analysis. A) A representative western blot of GFP-SERCA cross-linked in AAV-293 cells. The areas in blue demarcate the molecular weight range where samples were extracted from polyacrylamide gels. B) Photon counting histograms (PCH) for the two extracted fractions. Fits (shown in gray) were generated from the Globals for Spectroscopy software. C) Brightness measurements from PCH analysis of GFP-SERCA isolated from low molecular weight (black) and high molecular weight (red) fractions revealed an increased molecular brightness (ε) for the high molecular weight species. Molecular brightness values are mean ± SD from N = 3 individual gel slices (unpaired t-test, p < 0.01). D) Soluble GFP brightness measurements in sodium dodecyl sulfate (mean ± SD).
The two GFP-SERCA SDS-PAGE bands were excised and eluted from polyacrylamide gels by incubation in PBS. The concentration of GFP-labeled SERCA was quantified by fluctuation correlation spectroscopy (FCS). FCS was used to generate an autocorrelation function for GFP-excited sample. The Y-axis intercept of the function \( G(0) \) is directly dependent on concentration of the sample. The \( G(0) \) values were then used to dilute the two GFP-SERCA samples down to equal concentrations. A photon counting histogram (PCH) was generated from the FCS data (Figure 9B) and used to calculate brightness. Brightness measurements revealed that the 135 kDa section of the gel containing monomeric GFP-SERCA had a molecular brightness of 2338 ± 76 counts per second per molecule (cpsm) while the cross-linked species (301 kDa) had a molecular brightness of 2832 ± 35 cpsm (mean ± SD) (figure 9C).

Notably, the apparent molecular brightness of the cross-linked band was not increased 2-fold. Many factors may contribute to the apparently decreased brightness of the cross-linked species: A) BPM cross-linking may covalently modify some GFP and decrease or eliminate its fluorescence. B) UV exposure (for BPM activation) photobleaches some GFP molecules. C) Competition from endogenous SERCA creates some complexes where only a single SERCA has a GFP tag. D) Background fluorescence adds to the apparent molecular brightness of both the high and low molecular weight samples. E) SDS detergent presumably denatures a large number of the GFP molecules. All of these factors are expected to reduce the apparent difference between the low and the high molecular weight bands. Qualitatively, the data are taken to indicate
increased molecular brightness of the high molecular weight species consistent with an oligomeric complex of several GFP-SERCA protomers.

To confirm that SDS itself does not alter GFP brightness, we performed brightness measurements on purified soluble GFP protein. Brightness slightly decreased with increasing concentrations of SDS, but was not significant (Figure 9D). The values obtained compared favorably to the brightness measurements from GFP-SERCA. Previous studies have shown that soluble GFP may have a slightly greater brightness than GFP attached to proteins, as the latter may be quenched in part by the bound protein (Nils 2010).

**Oligomeric structure and regulation**

We next sought to examine the quaternary structure and regulation of SERCA homo-oligomers in live cells using FRET techniques. To determine if attachment of fluorescent proteins alters SERCA function we performed ATPase assays on microsomes expressing SERCA. Cer-SERCA in the absence of PLB displayed calcium-dependent ATPase activity with sensitivity similar to skeletal SERCA1a (figure 10). Co-expression with YFP-PLB caused a rightward shift in this relationship; the magnitude of this change was similar to previous reports (Mueller, Karim et al. 2004). We also noted that expression of fluorescently-labeled SERCA and PLB targeted and localized to the ER/SR membrane correctly.

We made FRET measurements from Cer-SERCA to SERCA labeled with YFP at the N-terminus ("N"), inserted before residue 509 ("509"), or fused to the C-terminus ("C") as diagrammed in figure 11A of a SERCA crystal structure: PDB 4KYT
Figure 10. Calcium ATPase measurements for skeletal SR microsomes (black) prepared from rabbit fast twitch muscle and microsomes prepared from AAV-293 cells expressing Cer-SERCA (red) or Cer-SERCA and YFP-PLB (blue).
Figure 11. FRET labeling strategy. A) FRET was measured from the N-terminus of Cer-SERCA to another SERCA labeled with YFP at either the N-terminus, between amino acids 508 and 509, or at the C-terminus. B) All three acceptor-labeled positions produced FRET. The control FRET was measured using Cer-SERCA and non-fusion YFP.
We observed FRET for all labeled positions (figure 11B); however, these values are averaged from many cells and so we could not infer relative positions of the different labeling sites. We selected the N-terminal labeling strategy for a more detailed analysis of the SERCA-SERCA interaction. Figure 12 shows the FRET values obtained from individual cells (dark gray) plotted as a function of whole-cell average fluorescence intensity, taken as an index of protein concentration. We used co-transfection of Cer-SERCA and untethered YFP as a negative control (light gray). For clarity, the data were pooled and the values are superimposed with error bars representing SEM. FRET efficiency increased with increasing protein concentration and this relationship was well-described by a hyperbolic fit (black line), yielding two important parameters, the apparent dissociation constant ($K_d$) and maximum FRET ($FRET_{max}$). These two parameters are quantified as indices of the Cer-YFP separation distance and the complex binding affinity, respectively.

$FRET_{max}$ and $K_d$ were not significantly different in the absence or presence of PLB (figure 13). Activation of the PKA pathway by application of 100 μM forskolin (an activator of adenylate cyclase) did not alter $FRET_{max}$ or $K_d$, even with blockade of phosphatase activity with 100 μM 3-isobutyl-1-methylxanthine ("+PLB+F+I"), consistent with our chemical cross-linking measurements made in myocytes following stimulation with isoproterenol (figure 7C). We employed a monomeric mutant super-inhibitory phospholamban containing a triple cysteine to serine mutation (C36S/C41S/C46S) in order to test whether PLB oligomeric state altered SERCA homo-dimerization. We detected no significant effect on SERCA-SERCA $FRET_{max}$ or $K_d$ even with activation of the
Figure 12. Protein concentration dependence of FRET from Cer-SERCA2a to YFP-SERCA2a (N) (black) or negative control Cer-SERCA2a to non-fusion YFP (red). Individual cells are shown in gray, with pooled data (mean ±SEM) in red or black. Data were modeled with a hyperbolic fit (lines). Maximum FRET (FRET$_{\text{max}}$) and the apparent dissociation constant (K$_d$) are calculated from the fit.
Figure 13. Apparent dissociation constants ($K_d$) and maximum FRET ($FRET_{\text{max}}$) values calculated from the hyperbolic fits of FRET data in the absence (-PLB) or presence of phospholamban (+PLBwt). Application of 100 μM forskolin and 100 μM 3-isobutyl-1-methylxanthine (+PLB+F+I) did not alter the apparent affinity ($K_d$) of the dimer. A more monomeric form of PLB with three cysteines mutated to serine (SSS) did not alter the SERCA oligomer, even with application of forskolin and IBMX. Coexpression with non-phosphorylatable (+PLB-S16A) or phosphomimetic (+PLB-S16E) PLB mutants had no appreciable effect. Alterations to calcium (2 mM) or ATP (4 mM) did not elicit any changes. Mean ± SEM, N = 3 independent experiments (no statistically significant differences between groups by one-way ANOVA).
PKA pathway. Mutations to PLB at serine 16 to mimic (S16E) or prevent (S16A) phosphorylation also had no effect on these two parameters. Thus, the present data are not consistent with previous studies that indicated that PLB serine 16 phosphorylation altered SERCA homo-oligomerization (Negash, Chen et al. 1996).

Previous studies have suggested that oligomerization may occur only during specific catalytic transitions in the ATPase cycle (Andersen and Vilsen 1985, Akin and Jones 2012). This may be a mechanism driven by binding and unbinding of homo-oligomers. To investigate whether oligomerization is affected by SERCA conformational poise we measured FRET in cells permeabilized with saponin and bathed in solutions of defined composition. We did not observe any significant changes to FRET\textsubscript{max} or K\textsubscript{d} when calcium and ATP were removed or when calcium (2 mM) and/or ATP (4 mM) were present (figure 13). These results suggest that SERCA conformational poise does not alter SERCA homo-oligomeric structure or affinity, such as during active cycling.

Previous studies have indicated that oligomerization may be enhanced by inhibitors such as thapsigargin (TG) and melittin as part of a regulatory mechanism (Mersol, Kutchai et al. 1995, Voss, Mahaney et al. 1995). We used a 2-color SERCA construct comprised of a donor fluorescent protein attached to the N-terminus (A-domain) and an acceptor fluorescent protein positioned between amino acids 508 and 509 (N-domain) (figure 14A) to determine if TG induces structural changes in the pump. Titration of TG reduced FRET by approximately 10%, indicating separation of the A- and N-domain and a more “open” conformation following binding by TG (figure 14B). This was in agreement with previous data from our laboratory.
Figure 14. Effects of inhibitors on SERCA oligomerization. A) The labeling strategy for “2-color” SERCA consisting of a Cer donor attached to the N-terminus (A-domain) and a YFP acceptor inserted between amino acids 508 and 509 (N-domain). Generated from the SERCA crystal structure 1IWO (Toyoshima and Nomura 2002). B) Titration of thapsigargin decreased the FRET between the N- and A-domain, indicating an increase in separation distance (Hill fit shown in red). C) Application of 10 μM thapsigargin to AAV-293 cells expressing Cer-SERCA and YFP-SERCA did not alter FRET\textsubscript{max} or K\textsubscript{D}. D) Treatment with 50 μg/mL saponin and/or 250 μM melittin did not alter FRET (mean ± SD).
(Hou, Hu et al. 2012, Pallikkuth, Blackwell et al. 2013). Despite this large conformational change, we did not detect any alterations to the SERCA homo-dimer \( \text{FRET}_{\text{max}} \) or \( K_d \) (figure 14C). We also examined the effect of melittin (an active component in bee venom) on homo-dimerization. We found that melittin disrupted the cell membrane integrity; however the average FRET was not significantly altered by application of 250 \( \mu \text{M} \) melittin (figure 14D).

Previous work has identified HAX-1 as a regulator of the SERCA/PLB regulatory complex through stabilization of the PLB monomer. FRET experiments showed that SERCA/PLB FRET is increased in the presence of HAX-1 while PLB pentamer FRET is decreased (Vafiadaki, Arvanitis et al. 2009). We measured SERCA oligomer FRET in the absence and presence of HAX-1, but found no difference in the \( \text{FRET}_{\text{max}} \) and \( K_d \) parameters (figure 15A). We observed, however, that HAX-1 appeared to increase Cer-SERCA and YFP-SERCA expression. To test whether HAX-1 increased expression of SERCA or PLB, we co-transfected HEK cells with HAX-1 and SERCA or PLB. Increasing molar ratios of HAX-1 resulted in dramatically increased protein expression levels of SERCA or PLB (figure 15B). The altered expression, however, did not alter the \( K_d \) or \( \text{FRET}_{\text{max}} \) parameters, suggesting that HAX-1 does not regulate SERCA-SERCA affinity or structure.

To determine whether our measured SERCA-SERCA FRET was due to specific protein-protein interactions, rather than a consequence of non-specific protein crowding in the membrane, we measured Cer-SERCA/YFP-SERCA FRET in the presence of a third species of unlabeled SERCA (figure 16A). Unlabeled SERCA should act as a competitor if specific protein-protein interactions contribute to the detected FRET
Figure 15. Effect of HAX-1 on oligomerization. **A)** Co-expression of HAX-1 with Cer-SERCA and YFP-SERCA did not alter FRET \(_{\text{max}}\) or \(K_d\), however, an apparent increase in protein expression with HAX-1 was noted. **B)** This was further examined over a range of molar ratios of HAX-1 relative to YFP-PLB (mean ± SD).
Figure 16. FRET competition assay. A) FRET from Cer-SERCA and YFP-SERCA was reduced by coexpression of non-fluorescent SERCA. Values reflect the molar ratio of competitor:YFP-SERCA. B) FRET$_{\text{max}}$ was decreased by competition with non-fluorescent SERCA (black) but not by PLB (blue). Hyperbolic fit shown in red.
signal. The transfection ratio of Cer-SERCA to YFP-SERCA was kept at a constant molar ratio of 1:5 while increasing molar ratios of unlabeled SERCA were co-transfected. Increasing the molar ratio of unlabeled SERCA relative to YFP-SERCA caused a progressive decrease in FRET$_{\text{max}}$ (figure 16B, black data points). This reduction in FRET$_{\text{max}}$ was well described by a hyperbola (red fit), consistent with simple competition of unlabeled SERCA for the fluorescently labeled SERCA protomers in the oligomeric complex. The data suggest that the FRET observed here is due to a specific interaction between SERCA molecules. The residual FRET remaining at the highest competitor:YFP ratio tested was 3.5%, and is taken as an estimate of non-specific FRET between non-interacting donors and acceptors. Notably, using PLB as a competitor did not alter the measured SERCA-SERCA FRET at any ratio tested (figure 16B, blue data points), consistent with the experiment shown in figure 13. We conclude that the presence or absence of PLB did not alter the constitutive oligomerization of SERCA under the present experimental conditions.

We further sought to disrupt our FRET signal by titration of anti-SERCA antibodies, as this would provide a useful tool for separating SERCA homo-oligomers in the membrane. AAV-293 cells expressing Cer-SERCA and YFP-SERCA were subjected to permeabilization with saponin and incubation with anti-SERCA2 IID8 antibody (IID8, AbCam). Incubation of this antibody did not alter FRET even after incubation for nearly four hours (figure 17). We cannot draw a conclusion about the region of binding, as the exact sequence identity of the epitope is unreported. We also utilized crude test bleeds
Figure 17. FRET\textsubscript{max} measurements of AAV-293 cells expressing Cer-SERCA and YFP-SERCA. Cells were treated with saponin in the presence of increasing concentrations of anti-SERCA2 (IID8) antibody and incubated at room temperature for the indicated period of time (mean ± SD from N = 3 experiments per treatment).
from rabbit sera targeting various peptides within the P-domain of SERCA. These were raised against the following peptide sequences:

- n-CATEQERTPLQQKLDEF
- n-CLGTRRAKKAIVRSLP
- n-YNEAKGVEKVGAEAT
- n-CPDLDIMNNPRLPPKEPL
- n-DPPRIEVASSVKL
- n-SLLRMPPWENIWLVGS

Incubation with sera from these rabbits did not alter the oligomeric FRET, suggesting that the P-domain is not involved in homo-oligomerization.

**SERCA homo-oligomeric stoichiometry**

To determine the oligomeric stoichiometry of SERCA, we performed progressive acceptor photobleaching with AAV-293 cells transiently expressing Cer-SERCA and YFP-SERCA or Cer-SERCA and untethered YFP. Figure 18A shows the progressive decrease in YFP-SERCA emission (black) as YFP is selectively photobleached. This occurred with a concomitant increase in Cer-SERCA emission (blue) as the donor is close enough for FRET. Figure 18B shows the relationship between the changes in donor and acceptor intensities. The relationship was linear for Cer-SERCA + YFP-SERCA (black). The Cer intensity did not increase for a negative control expressing Cer-SERCA + untethered-YFP, demonstrating that this donor/acceptor pair is not interacting (red). The stoichiometry of the donor-acceptor complex is reflected in a comparison of the donor and fluorescence intensities (Li, Reddy et al. 1999). As an example, figure 19A shows that Cer
Figure 18. Acceptor photobleaching of the SERCA oligomer. A) Progressive acceptor photobleaching of YFP-SERCA (black) resulted in increased Cer-SERCA fluorescence (blue), indicating FRET. Mean ± SEM, N = 28 cells total from 3 independent experiments. B) Photobleaching of the SERCA homo-oligomer resulted in a linear increase in Cer intensity with decreasing YFP intensity (black). No increase in Cer-SERCA intensity was observed for non-fusion control (red).
Figure 19. The relationship of donor/acceptor fluorescence intensities during the process of photobleaching for a construct comprising A) one acceptor and one donor (C32V) or B) a construct comprising two acceptors and one donor (VCV). C) This relationship was also plotted for Cer-PLB and YFP-PLB, a well-known pentameric oligomer. Mean ± SD, N = 12 cells. Lines connect the first and last data points.
fluorescence (plotted on the ordinate) increases linearly with the decrease in Venus acceptor fluorescence (plotted on the abscissa, decreasing from right to left over time) for a control construct (C32V) expressing a tethered donor and acceptor in a stoichiometry of 1:1. In contrast, a construct comprising a donor tethered to two acceptors (VCV) displayed significant curvature (figure 19B), since FRET persists after ablation of a single acceptor, and donor brightness only fully increases late in the photobleaching process when the last acceptors are photobleached. We observed similar curvature with FRET between Cer-PLB and YFP-PLB protomers in a pentameric complex (figure 19C). The linear relationship for the SERCA homo-oligomer observed in figure 18B is consistent with a single acceptor in the oligomeric complex, suggesting that SERCA forms homo-dimers. This relationship was linear over a wide range of protein concentrations, indicating that this is the predominant oligomeric state. Photobleaching did not provide evidence for formation of higher order oligomers or aggregates even at the highest protein concentrations.

**PLB interaction with SERCA**

The above results suggest that PLB does not modulate the physical interaction between SERCA protomers. We set out to determine whether PLB can interact with SERCA homo-dimers or whether PLB interacts with a separate pool of monomeric SERCA. Figure 20A shows co-localization of Cer-SERCA and YFP-PLB in AAV-293 cells. Acceptor-sensitized FRET measurements of the complex showed a protein-dependent binding that saturated at approximately 25% FRET (figure 20B). Acceptor photobleaching revealed a linear donor:acceptor relationship consistent with a single
Figure 20. FRET measurements of the SERCA/PLB regulatory complex. A) Co-expression of Cer-SERCA2a and YFP-PLB in AAV-293 cells. B) Acceptor-sensitized FRET measurements of the regulatory complex. Hyperbolic fit shown in red. C) Acceptor photobleaching of the regulatory complex, indicating the presence of a single acceptor (YFP-PLB). Blue line connects the first and last data points. Data are shown as mean ± SD.
YFP-PLB acceptor in the complex (figure 20C), but this assay cannot rule out the possibility of multiple Cer donors in the complex. In theory, one could swap the fluorophores and perform FRET from Cer-PLB to YFP-SERCA to determine if the inverse FRET experiment also shows a linear relationship (as in a 1:1 hetero-dimer) or a curved relationship consistent with multiple SERCA protomers in the regulatory complex. However, this is impractical for these proteins as PLB also avidly forms homopentamers, so most Cer-PLB donors would not participate in FRET, complicating the analysis. As an alternative, we probed the stoichiometry of the SERCA-PLB complex with fluorescence lifetime analysis, which can reveal subpopulations of donors with different FRET efficiencies. Figure 21A depicts the labeling strategy, with a GFP donor fused to the N-terminus of SERCA and an mCherry acceptor fused to the N-terminus of PLB. The crystal structure of the regulatory complex is taken from Akin et al. and did not reveal the PLB cytoplasmic domain (Akin, Hurley et al. 2013). As such, the actual position of the PLB N-terminus and mCherry fluorescent protein tag are unknown.

Figure 21B shows the fluorescence decay of GFP-SERCA in the absence (black) or presence of mCherry-PLB (red). The decrease in the GFP fluorescence lifetime in the presence of mCherry-PLB (red) is consistent with FRET from GFP to mCherry. Decays were obtained from 99 cells and analyzed by fitting with a single component exponential decay. The fluorescence lifetime of GFP-SERCA alone ($\tau_D$) was $2.56 \pm 0.02$ ns. The fluorescence lifetime ($\tau_{DA}$) of GFP-SERCA decreased with increasing mCherry-PLB protein expression to a minimum value (figure 22A). FRET was quantified according to the relationship $\text{FRET} = 100 \times \left(1 - \frac{\tau_{DA}}{\tau_D}\right)$ where $\tau_D$ is measured from cells expressing
Figure 21. SERCA/PLB regulatory complex fluorescence lifetime measurements. A) Labeling strategy for FRET from GFP-SERCA (grey) to mCherry-PLB (red). B) Representative fluorescence decays of GFP-SERCA in the absence (black) or presence of mCherry-PLB (red). The decay was shortened by coexpression of mCherry-PLB, indicating FRET. Exponential tail fits are shown in gray.
Figure 22. Fluorescence lifetime fitting. A) A single component exponential fit of the fluorescence lifetime shows a decrease in tau (τ) with increasing protein expression. Each point represents a measurement from an individual cell. N = 99 individual cells. Hyperbolic fit shown in red. B) FRET (calculated from data in A) increased with protein expression. Hyperbolic fit shown in red. C) Reduced chi-squared ($\chi^2$) values for 1-component fits worsened as protein expression increased. Reduced $\chi^2$ was improved for a two-component fit. Shown with linear fits. D) Average Reduced $\chi^2$ values obtained from fitting the fluorescence decay to a 1-, 2-, or 3-component exponential decay.
GFP-SERCA only. Figure 22B shows that FRET increased with acceptor expression in a hyperbolic relationship to a maximum of 22.2% FRET, a value that is in harmony with the acceptor-sensitized FRET measurements above (figure 20B) and previous steady-state FRET measurements from our lab (Hou and Robia 2010, Bidwell, Blackwell et al. 2011).

We observed that the single component exponential decay goodness of fit (reduced $\chi^2$) became progressively worse as the level of protein expression increased (figure 22C, black), suggesting that a single species model was a poor description of the complex at higher protein expression levels. Reduced $\chi^2$ was significantly improved by a two component exponential model, which was a good description of the data over a wide range of concentrations (figure 22C, red). Fitting to a third component did not improve $\chi^2$ (figure 22D). Fit residuals for one- and two-component models show that the GFP-SERCA alone was sufficiently described by a one-component model, but GFP-SERCA in the presence of mCherry-PLB was effectively described only by a two-component model (figure 23). Interestingly, the two-component exponential fit yielded a long lifetime ($\tau_L$) of $2.48 \pm 0.09$ ns that was very similar to the GFP-SERCA only lifetime of $2.56 \pm 0.02$ ns and a short lifetime ($\tau_S$) that averaged $1.37 \pm 0.09$ ns. The data are consistent with the existence of a sub-population of high FRET donors ($\tau_S$) and another subpopulation of non-FRET donors ($\tau_L$). Figure 24A is a cell-by-cell measurement of the short lifetime ($\tau_S$) component. We calculated FRET from the high FRET donors ($\tau_S$) over the range in protein expression and found that the values for this population did not
Figure 23. Representative residual plots for 1- or 2- component exponential models of the fluorescence decay for GFP-SERCA alone (black) or in the presence of mCherry-PLB (red).
display protein concentration dependence as observed in the one-species exponential model (figure 24B).

Figure 24C shows the FRET\textsubscript{max} value obtained from the 1-species model and the two populations obtained from the 2-species model. The average FRET efficiency of the high FRET population (Pop. 2) was 46.3 ± 3.9%. This corresponds to an apparent probe separation distance of 54 Å. Interestingly, the present estimate of SERCA-PLB FRET efficiency is nearly twice that determined from acceptor-sensitized FRET measurements. Our previous steady state measurements of the SERCA-PLB regulatory complex (which cannot distinguish subpopulations of donors) yielded distance estimates of 10 Å longer than the present time-resolved quantification. Previous measurements thus overestimated the apparent separation distance between the N-terminus of SERCA and the cytosolic domain of PLB; these two domains are closer than we previously reported.

Two-component fitting also revealed the relative proportion of the high and low FRET populations. As expected, the percentage of donors with short lifetimes (high FRET) increased with protein expression at the expense of the long lifetime (non-FRET) species. However, it is noteworthy that the percentage of high FRET donors approached but did not exceed 50% of the total (figure 24D). Thus, the numbers of high FRET and non-FRET donors converged to approximately equal size populations. This phenomenon may be explained by a model in which a single SERCA protomer undergoes FRET with PLB while a second bound SERCA protomer does not. At low levels of protein expression most SERCA protomers are likely not engaged in dimerization, thus the non-FRET population (\(\tau_L\)) dominates. As protein expression increases, the SERCA protomers are
Figure 24. Bi-exponential decay fitting. A) The short lifetime values from 2-component exponential decays were consistent across a large range of protein expression levels. B) The short lifetime values were used to calculate FRET, which remained constant with increasing protein expression. C) The FRET\textsubscript{max} value calculated from the 1-component FRET curve in figure 22 and the FRET values for the long lifetime (Population 1) and short lifetime (Population 2) species. D) The relative contributions (amplitude) of the short and long lifetimes.
able to “find” each other, thus producing a FRETing SERCA and a non-FRETing SERCA bound to PLB in a 2:1 stoichiometry. At saturated binding this would indicate equal contributions from the two SERCA populations.

To determine whether phosphorylation of PLB alters interaction with SERCA, we measured fluorescence lifetime in AAV-293 cells co-transfected with Cer-SERCA and YFP-PLB containing either the S16A (non-phosphorylatable) or S16E (phosphomimetic) mutations. Fluorescence lifetime imaging microscopy showed a homogenous distribution of the measured lifetime, suggesting that there are not distinct sub-populations of molecules with different lifetimes at the optical resolution of our microscope (figure 25A). The whole-cell average FRET from Cer-SERCA to YFP-PLB-S16A measured using fluorescence lifetime was 27.1%, which was in agreement with previous ensemble measurements. We noted again, however, that both S16A and S16E fluorescence decays were better described by a bi-exponential model, yielding a non-FRET population (Pop. 1) and a high-FRET-population (Pop. 2). This suggests that non-phosphorylated and phosphorylated PLB both remain bound to the SERCA homo-dimer.

The average FRET for population 2 was 44.7 % ±6.15 for non-phosphorylatable PLB and 32.7 % ±3.51 for phosphomimetic PLB (Figure 25B). The average amplitudes of the low FRET and high FRET populations were 66.9% and 33.1% respectively for PLB-S16A and 52.5% and 47.5% respectively for PLB-S16E. We noted that FRET decreased from 44.7% to 32.7% upon phosphorylation. The magnitude of this change was twice what we previously reported from whole cell FRET measurements (Hou, Kelly et al. 2008),
Figure 25. Lifetime measurements with PLB phosphorylation. A) Fluorescence lifetime images in AAV-293 cells expressing donor-labeled Cer-SERCA alone or Cer-SERCA with YFP-PLB-S16A. Note that the color-scale in these images represents the fluorescence lifetime (in nanoseconds) and not the color of the emitted light. B) FRET was calculated from 2-component exponential decays as in figure 23 for Cer-SERCA in the presence of YFP-PLB-S16A (non-phosphorylatable) or YFP-PLB-S16E (phosphomimetic). Mean ± SD, N = 12 cells, unpaired t-test, p < 0.01.
suggesting that phosphorylation of PLB moves the cytosolic domain more than previously thought.

**SERCA function**

To examine the functional effects of SERCA in the presence of PLB, we received purified skeletal SR from rabbit fast twitch muscle and cardiac SR from pig hearts as a gift from Dr. David D. Thomas. We performed ATPase assays on the preparations and observed a characteristic rightward shift in the calcium sensitivity for PLB-containing cardiac SERCA (figure 26A). We noted, however, that the Hill coefficient for cooperativity of activation by calcium was increased for the cardiac tissue ($n_H = 2.64$) relative to the skeletal tissue ($n_H = 1.55$), suggesting that an additional cooperative mechanism may be present with PLB.

To investigate the functional coupling of SERCA we mutated the aspartic acid residue responsible for formation of the phosphoenzyme intermediate to alanine (D351A). This mutant has been previously characterized as a catalytically inactive enzyme that is still capable of binding calcium (Akin, Chen et al. 2010). Co-expression of wild type (wt) SERCA with mutant SERCA would be predicted to alter enzyme activity if there is a functional coupling mechanism present that relies on ATPase activity of both paired protomers. We expressed equal amounts of wt and D351A SERCA in molar ratios of 1:1 with PLB-S16A or PLB-S16E in AAV-293 cells and prepared microsomes. Measurements of the ATPase activity showed calcium sensitivity with a rightward shift for the non-phosphorylatable PLB-S16A (figure 26B). We also noted that at saturating calcium (pCa = 5.0) the sample with S16E appeared to have a larger $V_{\text{max}}$, but this was
Figure 26. Calcium-dependent ATPase measurements. A) Normalized ATPase measurements for microsomes purified from skeletal SR from rabbit fast twitch muscle and cardiac SR from pig hearts. Data points are the mean from N = 3. Hill fits shown as lines. B) ATPase activity from microsomes transfected with equal amounts of wild type and D351A SERCA in a 1:1 molar ratio with PLB-S16A or PLB-S16E. Untransfected cells are shown for comparison. Data points are the mean from N = 3. Hill fits shown as lines. C) \( V_{\text{max}} \) from the data in (B) was calculated by subtracting the untransfected ATPase activity from the transfected activity at pCa = 5.0. Mean ± SD, N = 3, unpaired t-test, \( p = 0.11 \). D) \( V_{\text{max}} \) measurements for microsomes transfected with equal amounts of wild type and D351A SERCA without PLB or in a 1:5 molar ratio with PLB-S16A or PLB-S16E. N = 72 for each treatment.
not significant (figure 2C). However, these samples only expressed PLB at a 1:1 ratio with SERCA. We hypothesized that more PLB might be necessary to observe a full functional effect. We repeated ATPase measurements in microsomes expressing wt and D351A SERCA in a 1:5 ratio with PLB. Measurements at saturating calcium (pCa = 5.0) showed similar $V_{\text{max}}$ values for SERCA in the absence or presence of non-phosphorylatable (S16A) PLB. This suggests that functional coupling is not modulated by PLB binding or unbinding. However, SERCA in the presence of phosphomimetic (S16E) PLB showed a marked increase in $V_{\text{max}}$, indicating that a functional coupling mechanism may be present that is altered by phosphorylation of PLB (figure 26D). Specifically, this functional mechanism that alters $V_{\text{max}}$ is distinct from the physical dimerization observed. That is, dimerization by itself does not alter $V_{\text{max}}$ unless phosphorylation of PLB is engaged.
CHAPTER FIVE

DISCUSSION

Diverse experimental approaches have provided evidence for SERCA homo-oligomerization (Vanderkooi, Ierokomas et al. 1977, Chamberlain, Berenski et al. 1983). Previous studies have provided various estimates of the stoichiometry of the SERCA oligomeric complex, with evidence supporting dimers (Negash, Chen et al. 1996, Yao, Chen et al. 2001, Mahaney, Albers et al. 2005, Chen, Yao et al. 2009), trimers (Mahaney, Thomas et al. 2008), or higher order oligomers (Voss, Birmachu et al. 1991, Mersol, Kutchai et al. 1995, Voss, Mahaney et al. 1995). The present results are consistent with a SERCA homo-dimer, as we observe a linear increase in donor fluorescence with the progressive photobleaching of acceptor (figure 18B). This linear relationship was preserved even at the highest levels of protein expression, suggesting that the dimer does not proceed to higher order oligomerization. The SR membrane in the heart contains abundant expression of SERCA. The junctional SR is estimated to contain more than 50% SERCA and the longitudinal SR is estimated to contain as much as 90% SERCA (Meissner 1975). Based on these estimates, we speculate that most of the SERCA in the heart would be in a dimeric state.

It is interesting to note that the electrophoretic mobility of cross-linked SERCA band 2 (230 kDa) is slightly greater than 2-fold that of the monomeric band 1 (99 kDa) (figure 7B). Previous gel filtration experiments by Yamamoto et al. observed an apparent
dimer fraction with electrophoretic mobility larger than 2-fold that of the monomer (Yamamoto, Yantorno et al. 1984). This may indicate the presence of an accessory protein in mediating homo-dimerization, although we did not identify any such protein. We observed this phenomenon in both HEK cells (devoid of PLB) and rabbit LV cardiomyocytes (containing abundant PLB), indicating that any such protein would have to be present in both systems. Other work in our lab has shown that PLB is also captured in our cross-linked species (data not published); although this alone does not account for the 30 kDa shift.

Multiple types of experiments have indicated the presence of a SERCA homo-dimer in live cells. One problem that has eluded an answer thus far is the interface for SERCA protomer interaction. Incubation of cells with various SERCA antibodies did not produce alterations in dimer structure or affinity. However, the 6 custom antibodies used all targeted the P-domain while the commercial antibody epitope was unknown. This suggests the possibility that the P-domain is not the interface for dimerization; however, this cannot be completely ruled out as antibody binding to SERCA in these cells was not verified through immunohistochemistry. We also generated large fragments of the SERCA peptide in an attempt to compete the SERCA homo-dimer FRET. The short fragments localized to the cytoplasm, even when they contained parts of the TM domain. We did not detect competition by these fragments, although a specific short peptide may be capable of competing for the dimer interaction. If a precise determination of the dimer interface is made, FRET competition by a short peptide fragment could prove useful for verifying the interaction. This would be especially useful
to determine whether these peptides alter the functional effects that we observed in this study.

Mass spectrometry could prove to be a useful tool for examining the site of interaction. Cross-linking with BPM is irreversible, so extraction from the gel should yield peptide fragment sizes that are altered. This technique may prove technically challenging if multiple cross-links are present and include multiple accessory proteins. We predict that another protein is present as the molecular weight of the cross-linked SERCA is shifted by more than two-fold; however, mass spectrometry could prove useful for identifying any such additional proteins. Our previous attempts to mutate four different cysteines on SERCA were not successful in abrogating the cross-link formed by BPM. It is possible that, while our data show a direct physical interaction between SERCA protomers, the observed cross-link is due to reactivity of a cysteine on a partner protein that forms a cross-link to a carbon on SERCA with the benzophenone group. Thus mass spectrometry has great potential to identify any accessory proteins as well as possibly identify the interface for SERCA dimerization.

While some studies have suggested that SERCA dimerization is a regulated process, we did not see any evidence for this. Physical interaction between SERCA protomers does not appear to be modulated, indicating a constitutively present homo-dimer. PLB was not required for homo-dimerization and SERCA conformational poise did not alter binding affinity or homo-dimer structure. HAX-1 was previously shown to regulate SERCA activity via PLB, however we did not detect a structural change in the SERCA homo-oligomer. Although we did note that HAX-1 increases both SERCA and PLB
expression, something that should be examined in more detail. Despite the large structural change to SERCA that we detected with the addition of TG or calcium shown here and previously (Hou, Hu et al. 2012, Pallikkuth, Blackwell et al. 2013), we did not detect a change in the oligomeric state of SERCA, indicating that oligomerization is not modulated by conformational poise. Inhibitors such as TG and melittin do not appear to regulate SERCA function through interactions between SERCA protomers as others have shown. It is interesting to note that the large structural changes to SERCA observed with both calcium and TG did not alter oligomeric FRET, suggesting that the acceptor and donor N-terminally labeled A-domains move in a coordinated fashion with binding of Ca or TG.

In the present study we investigated the regulatory complex in detail using FRET from SERCA to PLB. Steady state acceptor-sensitized FRET measurements of fluorescently-labeled PLB and SERCA showed that FRET increased with protein concentration, similar to previous measurements (Kelly, Hou et al. 2008, Hou and Robia 2010). Photobleaching revealed a linear relationship between the Cer-SERCA donor and YFP-PLB acceptor, indicative of a single PLB in the regulatory complex. However these data do not rule out the possibility of multiple Cer-SERCA donors in the complex.

Fluorescence lifetime measurements enabled us to resolve multiple FRET subpopulations within a single cell. We observed that there are always donors that do not participate in FRET, even at the highest protein concentrations where binding interactions appear to approach saturation. One may consider several possible interpretations of this result. There may simply be a fraction of SERCA that is
inaccessible to PLB. We did not observe any mislocalization of SERCA fluorescence, which was 100% colocalized with PLB, so any such sequestration of SERCA would have to be in membrane microdomains that are below the resolution limit of fluorescence microscopy. However, it seems implausible that the proportion of inaccessible SERCA would decrease with increasing protein expression down to a minimum of almost exactly 50% of total SERCA (figure 24D). A second alternative hypothesis is that only SERCA molecules with the appropriate conformational poise can interact with PLB (Chen, Stokes et al. 2003). Again, it seems surprising that this proportion of pumps in the accessible state would titrate to 50%. Moreover, we have previously shown that calcium only modestly alters the PLB-SERCA binding affinity (Bidwell, Blackwell et al. 2011), suggesting that PLB can bind to SERCA without regard to conformational poise. Therefore, we interpret the present data as indicative of a model in which the SERCA dimer interacts with PLB such that only one of the two donors in the complex may perform FRET with the acceptor on PLB. Several structural schemes could satisfy this; one simple arrangement is diagrammed in figure 27 where a dimer of SERCA protomers interacts with a single PLB. The leftmost donor GFP, which is bound to the left/front SERCA protomer (dotted outline), is >100 Å away from the mCherry (“R”), too far to participate in FRET. The donor bound to the SERCA protomer on the right is 54 Å from the mCherry, supporting robust FRET. Thus, no matter how much increasing protein expression drives the equilibrium toward the bound complex, the population of donors participating in FRET can never exceed 50% of the total. Thus, the maximal FRET measured by steady-state methods (figure 20B)
**Figure 27.** A model of the SERCA-SERCA-PLB regulatory complex in which the GFP on one of the SERCA protomers (dotted outline) is too distant to participate in FRET with PLB.
(Kelly, Hou et al. 2008, Hou and Robia 2010) is half of that quantified from the short lifetime component ($\tau_S$) of a FRET donor fluorescence decay (figure 24C).

Previous estimates of the stoichiometry of the regulatory complex have suggested that PLB binds to SERCA in a 1:1 relationship (Mueller, Karim et al. 2004). However, studies have reported expression of SERCA and PLB to be anywhere from 1:5 to 4:1, indicating that PLB may not actually regulate SERCA in a 1:1 molar ratio in vivo (Louis, Turnquist et al. 1987, Negash, Chen et al. 1996, Ceholski, Trieber et al. 2012). This is supported by previous results using transgenic mice overexpressing PLB in the heart, indicating that the native functional stoichiometry of the regulatory complex is less than one PLB per SERCA (Kadambi, Ponniah et al. 1996). Furthermore, it has been noted that a large quantity of PLB exists as the highly stable pentamer reserve pool, leaving less monomers available for inhibition of the calcium ATPase. Our data may explain how full inhibition of SERCA by PLB is observed even though estimates of PLB indicate that expression might not be high enough for 1:1 binding.

While some studies have suggested that SERCA dimerization is a regulated by PLB phosphorylation (Negash, Chen et al. 1996, Mahaney, Albers et al. 2005), we did not observe a change in either the structure or the binding affinity of the homo-dimer in response to PLB binding or PLB phosphorylation or Ca binding (figure 13). It is possible that SERCA is always physically coupled, but PLB phosphorylation engages functional coupling of the protomers. This hypothesis is compatible with reports that indicate that phosphorylated PLB increases SERCA $V_{\text{max}}$ (Lu and Kirchberger 1994, Antipenko, Spielman et al. 1997, Reddy, Cornea et al. 2003), (though other groups did not observe a
$V_{\text{max}}$ change (Movsesian, Colyer et al. 1990, Morris, Cheng et al. 1991, Movsesian 1992, Reddy, Jones et al. 1995, Odermatt, Kurzydlowski et al. 1996). Importantly, PLB-dependent functional coupling implies that phosphorylated PLB stays bound to SERCA throughout the calcium transport cycle, a concept that is supported by many studies (Li, Bigelow et al. 2004, Mueller, Karim et al. 2004, Bidwell, Blackwell et al. 2011) but not all (Kimura, Kurzydlowski et al. 1997, Jones, Cornea et al. 2002). Our lab has previously reported spectroscopic evidence for a particularly compact and ordered conformation of SERCA when bound to phosphorylated PLB and calcium (Pallikkuth, Blackwell et al. 2013). This conformation may be the structural signature of functionally coupled SERCA dimers.

Future experiments could take advantage of the other two labeled sites (509 and C-term) to more accurately measure FRET, as this current study only examined the N-term label in detail. These other sites may prove useful in triangulating the relative positions of the Cer-SERCA N-term label relative to the YFP-labeled SERCA. Furthermore, our model suggests that one SERCA in the homo-dimer is not capable of FRET with PLB due to distance constraints. Fluorescence lifetime measurements from these other sites may reveal two populations that are both capable of FRET with the YFP-PLB, thus giving a better estimate of the position of the cytosolic domain of PLB relative to the SERCA homo-dimer.

Previous studies suggested that SERCA homo-oligomerization is functionally regulated by phosphorylated PLB. Several key functional studies suggested that SERCA dimer activity is greater than the sum of its parts (Chamberlain, Berenski et al. 1983,
Chen, Yao et al. 2009). However, detergent-solubilized SERCA1a, which is greater than 80% monomer, is still catalytically active (Vilsen and Andersen 1987), suggesting that dimerization is not obligatory for function. However, our own cross-linking studies showed that some dimers persist even under conditions of 1% DPC. Our data show two important functional changes with PLB. First, the cooperativity of calcium-dependent ATPase activity increases in cardiac microsomal preparations compared to skeletal preparations, suggesting that this effect is modulated by interaction with PLB. Gorski et al. also observed an increase in cooperativity with the addition of PLB, although they did not attribute this increased cooperativity to functional coupling of SERCA protomers (Gorski, Glaves et al. 2013). It is not yet clear whether this cooperativity is due to physical association or activation of PLB. Second, we noted that phosphorylated PLB alters $V_{\text{max}}$, in agreement with a secondary role for regulation by phosphorylation (the first being calcium sensitivity). Examination of detergent-solubilized SERCA suggests that the composition of the lipid environment and the detergents used are crucial determinants of SERCA activity (Vilsen and Andersen 1987, Gustavsson, Traaseth et al. 2011). This may help explain the inconsistency in past measurements of $V_{\text{max}}$ and cooperativity in the literature. In fact, Gustavsson et al. showed that certain lipid compositions had far more potent effects on both calcium sensitivity and $V_{\text{max}}$ than did PLB (Gustavsson, Traaseth et al. 2011), indicating that the experimental conditions may play a large role in the results obtained.

Further work in the field should examine the calcium ATPase activity to determine how PLB increases cooperativity. SERCA calcium uptake in the absence of PLB
has a Hill coefficient slightly less than 2 due to enhancement of the calcium binding affinity at site II following calcium binding to site I. However, we detect increased cooperativity in cardiac preparations that appears to be due to PLB. Further work should examine how PLB modulates increased calcium sensitivity as this does not appear to be dependent on phosphorylation, which is distinct from the $V_{\text{max}}$ alterations observed with phosphorylation. Additional experiments should examine ATPase activity over the full range of calcium to determine the effects of S16E on $V_{\text{max}}$. Titration of PLB may provide an interesting tool for determining the range of $V_{\text{max}}$ changes with phosphorylation. Measurements should also be made to compare the present results with the $V_{\text{max}}$ effects in microsomes expressing 100% wt SERCA or 100% D351A SERCA.

As mentioned in the introduction, multiple studies showed that phosphorylation of PLB was unable to fully correct the decrease in SERCA activity observed in samples obtained from HF. Our data may provide an explanation for decreased SERCA activity in HF and the inability of phosphorylation of PLB to overcome reduced activity. Previous studies have shown that SERCA activity is decreased more than expected for the change in expression evidenced during HF (Schwinger, Bohm et al. 1995, Zarain-Herzberg, Afzal et al. 1996) or in vivo (Chamberlain, Berenski et al. 1983). It is tempting to speculate that decreases in SERCA activity observed during HF may be a result of functional coupling. If a percentage of the SERCA protein becomes post-translationally modified such that it cannot functionally couple with other SERCA protomers, then it would not be able to achieve a similar rate of calcium transport. Combined with reduced levels of PLB phosphorylation observed in HF, SERCA activity would be reduced even further as
evidenced in figure 26D. This may also explain why a recent clinical trial to overexpress SERCA was not successful, as the endogenous SERCA in HF acts in a dominant negative fashion to prevent exogenously-expressed SERCA from reaching full rates of calcium transport. In fact, in vivo studies have shown that certain post-translational modifications reduce SERCA activity (Lancel, Qin et al. 2010). I hypothesize that these pumps may act in a dominant negative fashion; that is, they physically couple with other SERCA protomers but do not contribute enzymatically.

**Conclusion**

The functional implication of a single PLB regulating a pair of SERCA is unclear, however one may speculate that this stoichiometry would make PLB relatively more potent in modulating transport activity. In particular, estimates of the in vivo expression ratio of PLB to SERCA range from 1:5 to 4:1, and a substantial portion (~80%) of the PLB is pentameric and therefore inaccessible to SERCA (Cornea, Jones et al. 1997). Regulation of two SERCA pumps by a single PLB would explain this apparent regulatory protein expression mismatch. The stoichiometry may also account for the greater cooperativity of Ca-ATPase activity in cardiac muscle compared to skeletal muscle lacking PLB. If PLB engages functional coupling of the two SERCA molecules, the number of Ca-binding sites per transport unit is effectively increased. Indeed, some superinhibitory PLB mutants can deliver Hill coefficients that exceed 2 (Trieber, Afara et al. 2009). Finally, the present results have implications for human disease, particularly heart failure, in which SERCA activity is decreased as a result of changes in SERCA:PLB ratios, SERCA expression levels (Hasenfuss, Reinecke et al. 1994) or changes in SERCA
specific activity (Schwinger, Bohm et al. 1995, Schmidt, Hajjar et al. 1999). The present results suggest that deviation from optimal protein expression level or damage to a fraction of the SERCA population may yield a larger than expected functional deficit as a consequence of altered conformational coupling.

Our data support a quaternary regulatory complex containing a single PLB bound to a pair of SERCA protomers. We observe SERCA2a dimers in the absence/presence of PLB and the dimers do not require PLB phosphorylation in order to assemble. Moreover, cross-linking experiments suggest that SERCA2a readily forms dimers in the native environment of the sarcoplasmic reticulum membrane of the cardiac muscle cell. SERCA dimers are functionally coupled by PLB, which increases cooperativity of calcium binding and alters $V_{\text{max}}$. 


associated with super-inhibition of calcium cycling and ventricular arrhythmia."  


VITA

Daniel Blackwell was born on November 23, 1986 to Kenneth and Janet Blackwell in Auckland, New Zealand. He is the oldest of four children: Stephen, Andrew, and Peter. Daniel married Cindy Lynn DeRuiter on August 8, 2015. Daniel received his B.A. in biology and chemistry with an emphasis on biomolecular studies from St Olaf College in 2009. After graduating, he joined the laboratory of Dr. Seth L. Robia at Loyola University Chicago in Maywood, IL and began working as a research specialist before deciding to pursue his graduate studies in 2011.

Daniel joined the Department of Cell and Molecular Physiology where he began studying SERCA oligomerization under the mentorship of Dr. Robia. In 2013 he received a Predoctoral Fellowship from the American Heart Association and, in 2015, an Arthur J. Schmitt Dissertation Fellowship from Loyola University Chicago. He has presented his work at multiple national and international meetings.