

Thermodynamic Effects of Phospholamban on Ca-ATPase Kinetics

Patrick L. Apopa

Thesis Submitted to the School of Medicine at West Virginia University in partial fulfillment of the requirements for the degree of

Master of Science
In
Biochemistry

James Mahaney, Ph.D., Chair
Lisa Salati, Ph.D.
Grazyna Szklarz, Ph.D.
William Wonderlin, Ph.D.

Department of Biochemistry and Molecular Pharmacology

Morgantown, WV
2002

Keywords: Ca-ATPase, phospholamban, activation energy, phosphoenzyme, baculovirus-insect cell expression system

Copyright 2002 Patrick L. Apopa

ABSTRACT

Thermodynamic Effects of Phospholamban on Ca-ATPase Kinetics

Patrick L. Apopa

The Ca-ATPase of sarcoplasmic reticulum removes cytosolic calcium to promote muscle relaxation. In the heart, the Ca-ATPase is regulated by phospholamban, which inhibits the Ca-ATPase by decreasing Ca-ATPase calcium sensitivity. However, the kinetic and thermodynamic mechanisms of inhibition are not understood. The purpose of this research was to test the hypothesis that phospholamban regulates Ca-ATPase kinetics by increasing Ca-ATPase activation energy. The baculovirus-insect cell expression system was used to produce samples containing Ca-ATPase alone or Ca-ATPase with phospholamban. The temperature-dependence of Ca-ATPase activity and catalytic site density was measured in the absence and presence of phospholamban at sub-saturating calcium and used to calculate the temperature-dependence of Ca-ATPase turnover. Arrhenius analyses showed that phospholamban increased Ca-ATPase activation energy from 31 ± 3 J/mol (Ca-ATPase only) to 52 ± 5 J/mol (Ca-ATPase + phospholamban). The results supported the hypothesis and provided new insight into the mechanism of phospholamban inhibition of Ca-ATPase.

Dedication

To my wife Jackie and Daughter Joy

Acknowledgements

I wish to express my appreciation and sincere thanks to Dr. Jim Mahaney, whom I have had as my advisor since I joined this program. I have benefited tremendously from his advice, discussions and encouragement during the entire course of this study. He made learning such a wonderful experience for me. I was most spirited by his patience and encouragement during difficult times.

I want to thank Drs. Wonderlin, Szklarz and Salati for agreeing to serve on my committee at a very short notice.

My sincere appreciation and thanks also go to my Lab colleagues for their time, support and friendship that made working with them just fun. I particularly want to thank Jammie Huffman for tirelessly doing the cell work and making sure I had enough good samples to do my experiments. The assistance given to me by Jason Waggoner and Jason Southall is greatly acknowledged; I owe them many thanks. A lot of thanks also goes to Konni Turner for all the help she gave to me during my entire stay in the Lab and for everything she did ranging from pCa assays to washing dishes to organizing Lab events like picnics, lunches and candy. Your contribution added to my happy stay in the Lab.

I would like to thank all the graduate students (past and present) in the department of Biochemistry and Molecular pharmacology with whom I have had and continue to have helpful interactions, both intellectual and social through the course of this work.

Also, I would like to thank the entire faculty and staff of the Department of Biochemistry and Molecular Pharmacology for all their help during this work.

Finally, special thanks are due to my family, my wife Jackie and daughter Joy for their patience and Love all the time.

Table of Contents

Abstract	ii
Dedication	iii
Acknowledgements	vii
List of Figures	vii
List of Abbreviations	viii
Chapter 1: Introduction	1
Ca ²⁺ -ATPase	8
Mechanism of Ca ²⁺ -ATPase	10
Phosphorylation of the ATPase	14
Phospholamban (PLB)	15
Overview of the Thesis research	18
Aim 1	19
Aim 2	20
Aim 3	20
Chapter 2: Methods	21
Reagents and solutions	21
Protein expression and isolation	21
Electrophoresis and immunoblotting	22
Ca-ATPase activity assays	23
Ca-ATPase phosphoenzyme levels	25
Chapter 3: Results	27
Protein Expression, Characterization and Assay	27
Effect of Phospholamban on the Temperature-dependence of Ca-ATPase Activity	31
Effect of Phospholamban on the Temperature-dependence of Ca-ATPase EP formation	34
Effect of Phospholamban on Ca-ATPase Activation Energy	37

Chapter 4: Discusion	41
Overview	41
The baculovirus-insect cell Ca-ATPase / phospholamban	
Expression system	41
Ca-ATPase turnover number	42
Effect of phospholamban on Ca-ATPase Activation Energy	43
Anti-phospholamban Antibody had no effect on Ca ²⁺ -ATPase activity	47
Chapter 5: References	51
Appendix	56
Permission to use copyright material from <i>Nature</i>	56
Permission to us copyright material from authors	57
<i>Curriculum vitae</i>	58

List of Figures

Chapter 1

Figure 1:	A schematic of myocyte action potential	3
Figure 2:	Secondary structure of the Ca^{2+} -ATPase	9
Figure 3:	2.6 Angstrom Structure of the Ca-ATPase	11
Scheme1:	E1/E2 Scheme for the Ca^{2+} -ATPase	13

Chapter 3

Figure 4:	SDS-PAGE and immunoblot of SERCA2a and phospholamban in cardiac SR vesicles and High Five insect cell microsomes	29
Figure 5:	Effect of Phospholamban on Ca^{2+} -ATPase activity at 37 ⁰ C	32
Figure 6:	Effect of phospholamban on temperature dependence of Ca^{2+} -ATPase activity	35
Figure 7:	Effect of phospholamban on temperature dependence of Ca^{2+} -ATPase turnover number (K_{cat}), Arrhenius Analysis	39

Chapter 4

Figure 8:	Phospholamban as an allosteric modulator of Ca-ATPase	45
-----------	---	----

List of Abbreviations

2A7-A1	monoclonal antibody against Ca-ATPase
2D12	monoclonal antibody against phospholamban
CSR	cardiac sarcoplasmic reticulum
EGTA	ethylene glycol bis((-aminoethyl ether)- <i>N,N,N',N'</i> -tetraacetic acid
EP	phosphoenzyme
k_{cat}	turnover number, Ca-ATPase activity divided by steady-state EP level
K_{ca} value	ionized $[Ca^{2+}]$ giving half-maximal activation of the Ca-ATPase
MOPS	3-(<i>N</i> -morpholino)propanesulfonic acid
PLB	phospholamban
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERCA	sarco(endoplasmic reticulum Ca-ATPase
SR, CSR	sarcoplasmic reticulum, cardiac sarcoplasmic reticulum

Chapter 1: Introduction

There is a high prevalence of heart disease worldwide and mortality due to heart related ailments is on the rise. There is a need for increased understanding of the etiologic mechanisms associated with these varieties of cardiac dysfunction, and this should translate to a more targeted therapy.

The heart tissue is composed of different cell types, namely the smooth muscle cells, fibroblasts and cardiac myocytes. The cardiac myocytes are the contractile cells of the heart. It is prudent therefore to understand the structural component of the myocyte in relation to its function. The myocytes are surrounded by a basement membrane, which is composed primarily of type I collagen, the glycoproteins laminin and fibronectin and proteoglycans. The basement membrane provides a barrier that influences the exchange of macromolecules between the extracellular space and the cell. It also provides an interface for cell adhesion and continuity with intracellular matrix. There is also the sarcolemma, which consists of the plasma membrane and the basement membrane. The sarcolemma is composed of the lipid bilayer, which contains hydrophilic heads and hydrophobic tails. This composition allows it to interact with intracellular and extracellular environment. The hydrophobic tails however, make the sarcolemma impermeable to charged molecules. The sarcolemma forms two specialized regions of the myocyte, the intercalated disc and the transverse tubular system. The intercalated disc has a specialized cell-cell junction, which functions as a strong mechanical linkage between myocytes yet has low resistance to allow for rapid conduction of action potential between myocytes.

The transverse tubules, (or T-tubules) are invaginations of the sarcolemma into the myocytes, which form a barrier between the intracellular and the extracellular spaces. These extensions bring in close apposition the L-type Ca^{2+} channels and the sarcoplasmic reticulum Ca discharge system and this makes the T-tubular system an important structural component in excitation-contraction coupling.

The major component of the sarcolemma is the lipid bilayer. Therefore, as with other lipid bilayers, it provides a barrier for diffusion. The sarcolemma contains various membrane proteins, which include receptors, pumps and channels. This specialized feature of the sarcolemma is essential to the contractile process of the myocyte (Walker and Spinale, 1999). The pumps and channels of the myocyte sarcolemma can be best reviewed when placed in the context of the phases of action potential represented in Figure 1.

The resting membrane potential or phase 4 of the action potential is maintained primarily by the inward K^+ rectifier and secondarily influenced by the Na^+/K^+ adenosine triphosphatase (ATPase). During the resting membrane potential, the sarcolemma is only permeable to K^+ , thus it is the K^+ equilibrium potential that determines the resting membrane potential of the myocyte. The inward K^+ rectifier allows for the K^+ diffusion into the cardiac myocytes. The Na^+/K^+ ATPase then generates an outward current through the extrusion of three Na^+ ions for two K^+ ions to maintain the resting potential. There is also the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and the sarcolemmal Ca^{2+} -ATPase, which provide the basis for Ca^{2+} extrusion from the myocyte. The $\text{Na}^+/\text{Ca}^{2+}$ exchange is a bidirectional channel; with the relative amounts of either ion carried across the membrane determined by the concentration of either side of the membrane (Philipson, 1990).

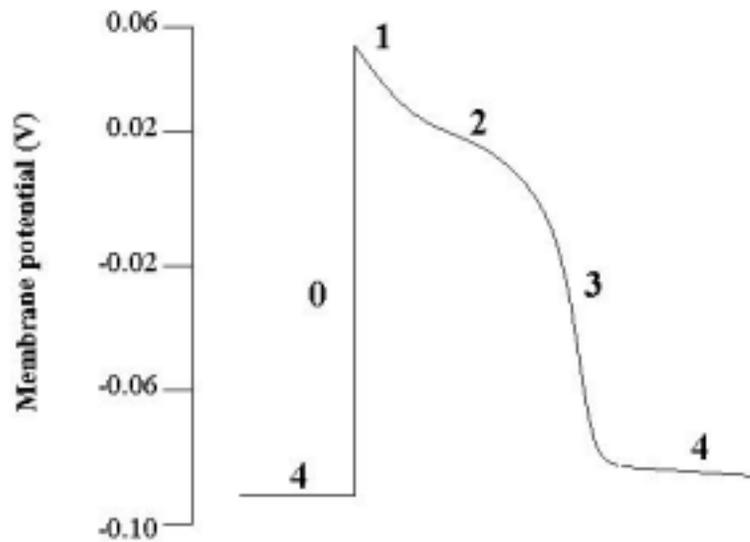


Figure 1: A schematic of myocyte action potential. The cardiac action potential consists of 5 phases. Phase 0, the upstroke, correspond to rapid depolarization. The upstroke is followed by phase 1, a brief early repolarization, phase 2 or plateau, phase 3 or rapid repolarization and phase 4, which correspond to the resting membrane potential. This action potential is the result of sarcolemmal protein interactions that have been summarized in the text.

During the action potential, the upstroke of phase 0 (Figure 1) is created by the fast Na^+ channel. In an all or non-mechanism, this upstroke occurs when the membrane potential achieves a preset threshold voltage and the Na^+ channels rapidly activate ($<1\text{ms}$). The channel remains activated for duration of only 2-10ms, thus the name 'fast' Na^+ channel. This activation allows Na^+ to flow into the cell along both electrical and chemical concentration gradients. This influx of Na^+ through the fast Na^+ channel triggers the ionic processes responsible for the other phases of the action potential (Walker and Spinale, 1999). Rapid inactivation of Na^+ channels and slower activation of two outward currents are the basis for early repolarization (phase 1). The positive membrane potential, the Cl^- concentration gradient, and increased membrane permeability to Cl^- allows for the entry of Cl^- into the cell. A transient efflux of K^+ through specific channels occurs along the K^+ electrochemical gradient. All these three events are responsible for the brief and small repolarization of the membrane potential during phase 1 of action potential.

Phase 2 of the action potential is mainly determined by the influx of Ca^{2+} through the L-type Ca^{2+} channels (Balke and Shorofsky, 1997). Additionally, there is a counterbalancing outward K^+ current that flows through the 'anomalous' K^+ rectifier (Katz, 1992). These channels are both activated during the upstroke of the action potential and reach peak current concurrently during phase 2 of the action potential.

Phase 3 of the action potential, which is the repolarization phase, is the result of increased K^+ conductance through the delayed rectifier K^+ channel. These channels are activated towards the end of phase 2 and they allow K^+ ion to flow out of the cell along the concentration gradient. At this stage of the action potential, the other inward currents,

Na^+ and Ca^{2+} are inactivated, making the delayed rectifying K^+ current responsible for the restoration of the membrane potential to the resting state.

The excitation-contraction coupling is the mechanism by which the action potential leads to contraction of the myocyte. It is basically achieved through an increase in cytosolic Ca^{2+} levels from nanomolar to micromolar concentrations (Berne and Levy, 1997).

Calcium is pivotal to regulation of the rhythmic contraction/relaxation cycle in the heart. Ultrastructural evidence indicates that the L-type calcium channels embedded in the transverse tubules are functionally and physically associated with the ryanodine receptors/ Ca^{2+} release channels on the sarcoplasmic reticulum (SR) in contractile muscle. In cardiac myocytes, communication between the L-type Ca^{2+} channel and ryanodine receptor stimulate release of Ca^{2+} from the SR. This is known as the Ca^{2+} -induced Ca^{2+} release (CICR) mechanism. This is where the action potential reaches the myocyte and a wave of depolarization at the T-tubular system result in the activation of the sarcolemmal voltage sensitive Ca^{2+} channel, also referred to as the dihydropyridine receptor (DHPR) and leads to Ca conductance (Mukherjee and Spinale, 1998). This rapid but small influx of Ca^{2+} through the L-type Ca^{2+} channels causes activation of Ca^{2+} release channels which then release large amounts of Ca^{2+} into the cytosol (Berridge, 1997; Bers, 1991).

For CICR to produce optimal response, it is postulated that either larger calcium releases must inactivate the Ca^{2+} release channels (Fabiato, 1985), or the released calcium must dissipate rapidly from the vicinity of the ryanodine receptor during random and calcium-independent close intervals of the release channels (Morad and Cleeman, 1987).

The increase in the cytosolic Ca^{2+} level results in the Ca^{2+} binding to the Troponin complex and following the release of Ca^{2+} from the sarcoplasmic reticulum, a series of interactions occur within the contractile proteins of the sarcomere that serves as the fundamental basis of muscle contraction (Berne *et al.*, 1997). The fundamental proteins of the contractile apparatus are myosin, actin, tropomyosin and the troponin complex. Myosin, the thick filament, is composed of a filamentous tail and a globular head region. The myosin head contains the actin binding site and the catalytic site for ATP hydrolysis, which drives the muscle contraction. Actin is the major contractile protein found in the thin filament. It has two forms, namely the monomeric (G) form and the polymeric (F) form, composed of individual G actin monomers. F-actin forms the backbone of the thin filament. Each monomer of G-actin within the F-actin has two myosin binding sites. The interaction between the myosin globular head and the actin unit in the presence of ATP results in crossbridge formation and sarcomere shortening. Tropomyosin is another protein found in the thin filament. It lies on either side of actin, thus adding rigidity to the thin filament. Tropomyosin influences actin-myosin cross bridge formation by physically interdigitating between the actin-myosin cleft (Katz, 1992). The troponin complex, also present in the thin filament is composed of three proteins namely troponin T, I, and C. Troponin regulates the extent of crossbridge formation and contributes to the structural integrity of the sarcomere. Troponin T binds the troponin complex to tropomyosin and anchors the complex to the thin filament. Normally, phosphorylated troponin I weakens the affinity of troponin C for Ca^{2+} . The binding of Ca^{2+} to troponin C results in a conformational change of the complex, which exposes the actin /myosin binding cleft,

promoting subsequent actin-myosin interaction, thus initiating cross bridge formation and muscle contraction.

Muscle relaxation depends upon the reduction of cytosolic calcium levels. This is realized mainly by the SR Ca^{2+} pump which transports Ca^{2+} back to the sarcoplasmic reticulum in an ATP dependent manner. The sarcolemmal Ca^{2+} -ATPase also removes a significant portion of Ca^{2+} . Additional Ca^{2+} binding proteins like calmodulin and calsequestrin are also important. The complex formed when calmodulin binds intracellular Ca activates the sarcolemmal Ca^{2+} -ATPase to extrude cytosolic Ca^{2+} (Feher *et al.*, 1990; Sheu *et al.*, 1986). Calsequestrin binds Ca within the cardiac SR lumen where it is located (Katz *et al.*, 1986; Feher *et al.*, 1990), and is the major Ca^{2+} reservoir between events.

In the failing hearts, patients exhibit both systolic and diastolic dysfunction. This is brought about by, among other things, cell death (microinfarction and scar) (Knowlton *et al.*, 1992), dysfunctional cardiac metabolism (inadequate energy supply) (Alpert *et al.*, 1962), abnormalities in cytoskeleton and contractile proteins (Unverferth and Lee, 1988), alteration in myocyte excitation-contraction coupling (Limas *et al.*, 1987), dysfunctional myocyte signal transduction (Mitchell *et al.*, 1995) and altered intracellular calcium transport and regulation. Of the various mechanisms stated above, intracellular calcium transport and regulation is the most dynamic and potentially reversible, thus the most treatable. Therefore, understanding the mechanism of calcium transport and its regulation is an important step towards finding a solution to heart disease.

Ca²⁺ATPase

The sarco(endo)plasmic reticulum Ca²⁺-ATPases (SERCA) are 110 kDa, P-type ATPases that are responsible for the movement of Ca²⁺ from the cytosol to the lumen of the sarcoplasmic reticulum (SR). They are called P-type ATPases because they have an aspartate residue, within a DKTG consensus sequence that is phosphorylated by ATP during catalysis. The Ca²⁺ATPase of muscle sarcoplasmic or endoplasmic reticulum consist of a single polypeptide chain, unlike other ATPases, which exist as α , β -heterodimers Na⁺/K⁺ATPase. The SERCA pumps are coded for by 3 genes, named SERCA 1, 2 and 3. SERCA 1 is expressed mainly in fast twitch skeletal muscle. SERCA 2 consist of two isoforms, SERCA2a which is expressed in slow-twitch, cardiac and smooth muscle while SERCA2b has non-muscle distribution, including the brain. SERCA3 has a wide tissue distribution.

The amino acid sequences of most P-type ATPases have been known for some time and extensive mutational studies have been carried out to identify amino acids that are critical for ion transport. As shown in Figure 2 below, the Ca²⁺-ATPase consists of three globular cytoplasmic domains connected by helical stalk segments to ten transmembrane α -helices (M1-M10). The first domain is a 125-residue loop connected to stalk regions S2 and S3, known as the activator domain. Next there is the phosphorylation domain, which is connected to stalk regions S4 and S5 and contains Asp-351, which becomes phosphorylated by ATP. Finally there is the nucleotide-binding domain (N domain) that is connected directly to the P domain.

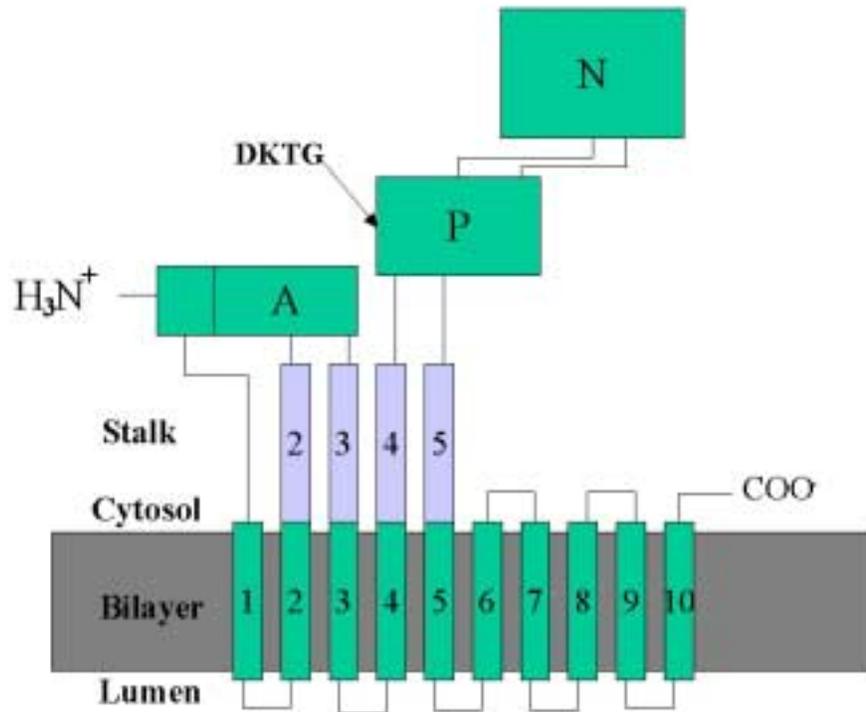


Figure 2: Secondary structure of the Ca^{2+} -ATPase based on the crystal structure by Toyoshima *et al* (1998). The three cytoplasmic domains A (activation), P (phosphorylation) and N (nucleotide binding) are connected to the transmembrane α -helical stalks (numbered 2-5 in accordance with the transmembrane α -helix to which they are attached). The N domain forms an insert into the P domain. The location of the conserved phosphorylation motif is shown.

The crystal structure of the SERCA1 Ca^{2+} -ATPase was reported in 2000. The ten transmembrane α -helices, three cytoplasmic globular domains and the small loops on luminal side were well resolved in structure (Figure 3). All the 994 amino acids in the crystal structure shown above were identified in the electron-density maps.

The structure of the Ca^{2+} -ATPase crystallized in the presence of Ca^{2+} and the ATP analogue 2'3'-O-(2,4,6-trinitrophenyl)-adenosine monophosphate (TNP-AMP) shows TNP-AMP binding to the surface of the N domain, more than 25 Å away from Asp 351 (Figure 3) (20) TNP-AMP is presumed to bind to the nucleotide binding site, since binding of analogues of TNP-AMP is competitive with binding of ATP. Because the adenosine moiety is known to bind around Lys-515, Lys-492 and Phe-487 in the N domain, and because the phosphorylation site (Asp-351) on the P domain is distant by about 25 Å in the crystal structure of the Ca^{2+} bound (E1) state (Toyoshima *et al*, 2000), very large motion of domain closure is necessary for the γ -phosphate of bound ATP to reach the phosphorylation site. This was recently confirmed by Toyoshima and coworker, who also showed the close interaction of the N, P and A domains in the crystal structure of the Ca^{2+} free Ca^{2+} -ATPase.

Mechanism of the Ca^{2+} -ATPase

The mechanism of the Ca^{2+} -ATPase is usually discussed in terms of E1-E2 model developed from the Post-Albers scheme for the (Na^+ , K^+)-ATPase, as shown in Scheme 1. The model proposes that the Ca^{2+} -ATPase can exist in two distinct forms, E1 and E2. The E1 conformation contains two high affinity binding sites for Ca^{2+} that are exposed to the cytoplasm, whereas in the E2 conformation these two sites are transformed to low

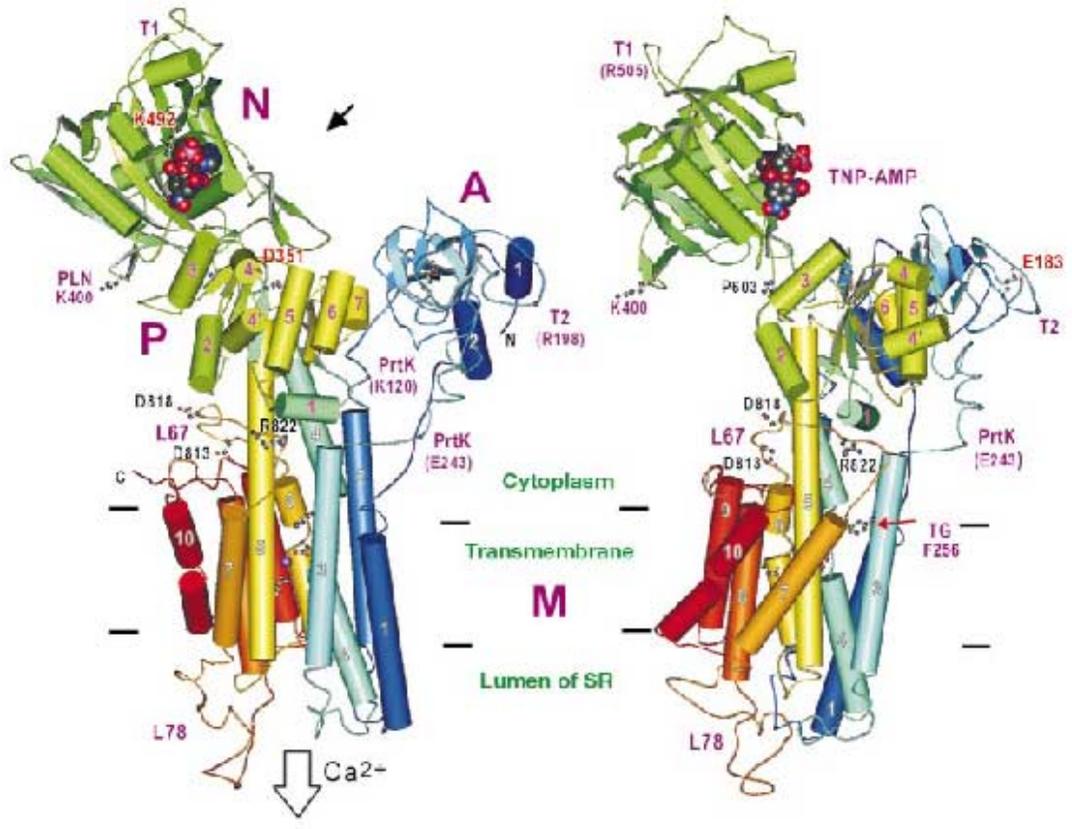


Figure 3: Crystal structure of Ca²⁺-ATPase

In the figure, the cylinders represent α -helices and arrows β -strand. The cylinders are not used for one-turn helices. The color changes gradually from N-terminal (blue) to C-terminal (red). The three cytoplasmic domains are labeled A, N and P. The transmembrane helices (M1-M10) and those in domains A and P are numbered. The orientation of the model shown above is such that Helix M5 is parallel to the plane of the paper. The model in the right panel is rotated by 50° around M5, which is 60Å long and serves as a scale. Several key residues are shown in ball-and-stick, and 2',3'-O-(2,4,6-trinitrophenyl)-Adenosinemonophosphate (TNP-AMP), which is a non-hydrolysable analog of ATP is shown. D351 is the residue of phosphorylation. Two purple spheres represent Ca²⁺ in the transmembrane binding site. The binding sites for phospholamban (PLN) and thapsigargin (TG) are marked. Also marked are the major digestion sites for Trypsin (T1 and T2) and proteinase K (PrtK). These digestion sites are important because they have been used to show changes in the cytoplasmic domain, which results in changes of accessibility of the enzyme to the cleavage sites. Reprinted with permission from *Nature* (Toyoshima, C., Nakasako, M., Nomura, H., Ogawa, H. 2000. Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution. *Nature* **405**: 647-654), www.nature.com/nature.

affinity sites exposed to the lumen of the SR. This is referred to as the alternating site model. Following the binding of MgATP to the Ca^{2+} -bound form of the ATPase (Step 1), the ATPase is phosphorylated at D351 forming E1P (Step 2), which undergoes a change in conformation to E2PCa₂ (Step 3), a state in which the two Ca^{2+} binding sites are of low affinity and inwardly facing. Following the loss of Ca^{2+} to the lumen of the SR (Step 4), the ATPase is dephosphorylated (Steps 5 and 6) and recycled to E1, which binds Ca^{2+}

The phosphorylation and dephosphorylation events of the enzyme are reversible. The phosphorylated enzyme can react with ADP in the presence of Ca^{2+} to regenerate ATP. Also, incubation of the Ca^{2+} ATPase with phosphate (Pi) in the absence of cytoplasmic calcium, lead to phosphorylation of the ATPase to form E2P (Lacapere *et al.*, 1981).

Phosphorylation of the ATPase

It has been shown using chemical labeling that the adenine and γ -phosphate moieties of ATP bind to separate domains on the ATPase, the N and P domains respectively. Analogues of ATP with reactive groups on the nucleotide ring invariably label residues in the N domain. ATP analogues block phosphorylation by ATP, but they do not affect smaller pseudo substrates like acetyl phosphate or Pi (Stokes and Green, 2000). However, if the reactive group is close to the γ -phosphate of ATP, then the enzyme residues labeled are in the P domain and neither ATP nor Pi can phosphorylate the modified ATPase.

The catalytically active E1Ca₂•ATP conformation (Figure 3) can be produced by a pathway in which either Ca^{2+} or ATP binds first to the ATPase (Reinstein and Jencks,

1993). However, the activated state can only be reached following the binding of both ATP and Ca^{2+} . Binding of MgATP and activation of the enzyme is relatively slow in either pathway. This is because ATP binding is not simply an encounter process but, rather, involves a conformational change on the ATPase, triggered by ATP binding to the ATPase (Reinstein and Jencks, 1993).

Phospholamban (PLB)

Phospholamban is a small (52 residue) membrane-bound protein that modulates the activity of the Ca^{2+} -ATPase in cardiac muscle SR.

Following the discovery of phospholamban, early work focused on its apparent role in regulation of calcium transport across the sarcoplasmic reticulum and sketching its complex protein structure. Other recent work has focused on the role of phospholamban in the regulation of myocardial function by catecholamines (Davis *et al.*, 1990) and the role of phospholamban in skeletal and smooth muscle (Lompre *et al.*, 1994).

Initially, phospholamban was implicated in the regulation of calcium transport across the cardiac SR by the correlation of cAMP-induced stimulation of calcium transport by the CaATPase, with the predominant phosphorylation of PLB by cAMP-dependent protein kinase (Kirchberger *et al.*, 1974). Because of its known role then as the principle substrate of cAMP-dependent protein kinase in cardiac SR vesicles, Tada *et al.* (1975) named the protein phospholamban, meaning 'phosphate receptor'. The level of calcium transport stimulated by the cAMP-dependent mechanism was commensurate with the degree of phosphorylation of PLB (Tada and Katz, 1982). Phospholamban was also found to be the principle substrate of an endogenous myocardial

calcium/calmodulin-dependent protein kinase. The phosphorylation of PLB by the cAMP-dependent protein kinase occur independently and additively (Imagawa *et al*, 1986), as does concomitant stimulation of calcium transport and ATPase activity (Kranias, 1985)

Structurally, PLB consists of a hydrophilic N-terminal domain (Met1 to Asn 30) linked to the hydrophobic C-terminal domain (Leu31 to Leu 52), which is likely to be a transmembrane α -helix . PLB when unphosphorylated binds to the ATPase and inhibits it. Phosphorylation of PLB by Ca^{2+} / Calmodulin-dependent or cAMP-dependent protein kinase results in full ATPase activity, presumably as a result of uncoupling of PLB from the ATPase. The binding of PLB to the Ca^{2+} -ATPase in cardiac SR reduces the apparent affinity of the ATPase for Ca^{2+} . This has been determined from plots of Ca^{2+} uptake rate as a function of Ca^{2+} concentration. Other studies suggest that PLB controls Ca^{2+} ATPase activity by decreasing by two fold the rate of phosphoenzyme decomposition. Froelich and Taylor (1975,1976) have shown in kinetic studies that Ca^{2+} ATPase phosphoenzyme decomposition is a two-step process in which E2P is first hydrolyzed to E2.Pi followed by the release of Pi from the enzyme forming an E2 intermediate. PLB however, does not cause a decrease in ATPase activity at saturating concentrations of calcium.

PLB forms homopentamers that are stable in SDS-PAGE (Wegener and Jones, 1984). Spectroscopic analysis has verified that PLB is primarily oligomeric in lipid bilayer (Cornea *et al.*, 1997). Simmerman *et al* (1996) showed by the use of site directed mutagenesis, the regions in PLB that are essential for pentameric stability. They also proposed that PLB pentamer is a left-handed coiled-coil, which is stabilized specifically by interactions between leucine residues 37, 44 and 51 and isoleucines 40 and 47. Autry

and Jones (1997) have shown that it is monomeric phospholamban that binds to and regulate the Ca^{2+} -ATPase

Phospholamban has been purified (Inui *et al.*, 1985; Jones *et al.*, 1985), and its primary structure has been determined by cDNA cloning and sequencing (Fujii *et al.*, 1987). It consists of five identical monomers (Wegener and Jones, 1984; Fujii *et al.*, 1986), each of which contains two distinct domains, the hydrophilic N-terminal domain (domain I) and the hydrophobic C-terminal domain (domain II) (Fujii *et al.*, 1986; Simmerman *et al.*, 1986; Tada *et al.*, 1988). The first 20 residues from the N-terminal (domain IA) are predicted to form an α -helical structure containing serine 16 and threonine 17 phosphorylated by cAMP- and calmodulin-dependent protein kinases, respectively. The next 10 residues are less structured (domain IB). The last 22 residues form a hydrophobic transmembrane domain (domain II). Recently, a direct protein-protein interaction has been demonstrated between phospholamban and Ca^{2+} -ATPase, which is diminished by phosphorylation of phospholamban (James *et al.*, 1989).

The physiological role of phospholamban in cardiac health and disease has been the source of much speculation. Phospholamban clearly is a mediator in the regulation of myocardial function by catecholamines through the cyclic AMP cascade. This is consistent with the distribution of phospholamban in slow-twitch skeletal muscle as well as cardiac muscle which both exhibit cAMP-dependent stimulation of cardiac transport. Phospholamban however, has not been identified in fast-twitch skeletal muscle, which lack this response pathway (Jorgensen and Jones, 1986; Kimura *et al.*, 1996). The physiological role of phospholamban has been demonstrated by recent work with cardiomyocytes and phospholamban knockout mice, and it was found that ablation of

phospholamban greatly augments the intracellular Ca^{2+} transient and myocardial contractility and at the same time attenuates the cardiac response to β -adrenergic agents such as isoproterenol. Likewise, the overexpression of phospholamban or the expression of non-phosphorylatable phospholamban in transgenic mice lead to a variety of cardiac disease states, including cardiac hypertrophy and heart failure (Brittsan and Kranias, 2000).

Overview of Thesis Research

Despite considerable progress toward understanding the functional interaction of phospholamban with the Ca-ATPase, fundamental questions remain concerning the kinetic basis by which phospholamban inhibits the Ca-ATPase. The primary functional effect of phospholamban on the Ca-ATPase is to decrease the apparent Ca^{2+} affinity of the enzyme (see Figure 1, below). There have been many kinetics studies on the effects of phospholamban on the partial reactions of the Ca-ATPase cycle (Tada et al., 1975, 1979, 1980; Jones et al., 1978; Kranias et al., 1980; Sumida et al., 1980; Cantilina et al., 1993; Antipenko et al., 1997, 1999; Mahaney et al., 2000) designed to elucidate this effect. However, these studies have failed to provide a definitive mechanism by which phospholamban decreases the apparent Ca^{2+} affinity of the Ca-ATPase.

The most widely accepted view of the mechanistic basis for Ca-ATPase inhibition by phospholamban was reported by Cantilina et al. (1993), who proposed that phospholamban decreases by ten fold the rate of a Ca^{2+} -dependent conformational change that activates the Ca-ATPase for ATP-dependent phosphoenzyme formation. In so doing, phospholamban makes the Ca-ATPase less sensitive to Ca^{2+} , giving the

appearance of a change in Ca^{2+} affinity. However, the effect of phospholamban on the Ca^{2+} -dependent activation of the Ca-ATPase has not been tested quantitatively. To do this, one must first determine whether phospholamban affects the net activation energy of the Ca-ATPase during steady-state enzyme cycling. In light of a positive result, one can focus specifically on the activation energy of the Ca^{2+} -dependent conformational transition that precedes Ca-ATPase phosphorylation by ATP.

The goal of this thesis research was to test the hypothesis that phospholamban increases the activation energy for steady-state enzyme cycling. To test this hypothesis, we have studied the effect of phospholamban on the temperature dependence of Ca-ATPase steady-state activity and phosphoenzyme levels. These parameters were used to calculate the enzyme turnover number as a function of temperature, in order to determine the activation energy for steady-state enzyme cycling. For these studies, we have employed the baculovirus-insect cell expression system to produce samples containing the cardiac Ca-ATPase expressed in the absence (control) and presence of co-expressed phospholamban. An advantage of using this system was that it produced sufficient quantities of sample for detailed kinetics studies, while at the same time allowing us to examine the Ca-ATPase co-expressed alone versus Ca-ATPase co-expressed with phospholamban. The effect of phospholamban on Ca-ATPase turnover was explored further by using anti-phospholamban monoclonal antibody to uncouple phospholamban from the Ca-ATPase. In particular, we have pursued the following specific aims:

Aim 1: Determine the influence of phospholamban on the temperature-dependence of Ca-ATPase steady-state activity. For these studies, we utilized an NADH-based

enzyme-coupled assay in a temperature-controlled UV-VIS spectrophotometer to determine if Ca-ATPase steady-state activity is affected by phospholamban.

Aim 2: Determine the effect of phospholamban on Ca-ATPase pre-steady state phosphorylation kinetics. For these studies, we utilized ^{32}P -ATP to measure steady-state Ca-ATPase phosphorylation levels, as affected by phospholamban.

Aim 3: Combine the results of Aims 1 and 2 to determine the effect of phospholamban on Ca-ATPase activation energy. Ca-ATPase velocity (nmol ATP split / mg protein / sec) divided by the phosphoenzyme level (nmol EP / mg protein) provided the Ca-ATPase turnover number, k_{cat} , as a function of temperature. An Arrhenius plot of k_{cat} versus $1 / T$ provided the activation energy for the Ca-ATPase in the absence and presence of phospholamban.

The results of these studies indicated that phospholamban increased the activation energy for steady-state Ca-ATPase activity. Based on this positive result, a more detailed analysis of the activation energy of individual steps in the Ca-ATPase cycle is warranted. The results of this study, and the studies that will follow this project, should provide new insights into the mechanisms of catalysis and regulation of calcium transport in cardiac muscle SR, which is a critical step toward understanding cardiac performance and malfunction.

Chapter 2: Methods

Reagents and Solutions.

¹²⁵I-Protein A and [γ -³²P]ATP were obtained from ICN. Other reagents were obtained either from Sigma or Fisher Scientific and were of the highest purity available. The antibodies used in this study were a gift from Dr. Larry Jones, Indiana University School of Medicine.

Protein Expression and Isolation.

Recombinant baculoviruses containing cDNA inserts for either canine cardiac Ca-ATPase (SERCA2a) or canine phospholamban were prepared by Jamie Huffman in our laboratory using the Baculogold Baculovirus Expression Kit from PharMingen. Wild type canine SERCA2a and wild type canine phospholamban were expressed in High-Five insect cells grown in suspension ($1.5\text{-}2.0 \times 10^6$ cells/ml) at 27°C in serum free medium (Invitrogen). Microsomes were isolated from insect cells harvested 48 hours after infection with baculoviruses. For expression of Ca-ATPase alone, a multiplicity of infection (MOI) of 10 (viruses/cell) was used. For co-expression of Ca-ATPase with phospholamban, a MOI of 15 was used for SERCA2a and 5 for phospholamban (WT). Virus-infected High-Five cells in 600 ml suspension (1×10^9 cells) were sedimented and washed twice with ice cold 1x PBS, by centrifuging for 10 minutes at 1500 rpm and 25°C in an IEC GP8R refrigerated centrifuge. The washed cells were resuspended in 60 ml of ice cold 10 mM NaHCO₃ and 0.2 mM CaCl₂, followed by the addition of 60 ml of ice

cold 500 mM Sucrose, 300 mM KCl, 6 mM MgCl₂, and 60 mM histidine. Both buffers contained 10 µg/ml aprotinin, 2 µg/ml leupeptin, 1 µg/ml pepstatin A, and 0.1mM pefabloc. The cells were transferred to a cold room (4°C) and homogenized for 90 seconds with a Brinkman polytron (full speed) and placed back on ice. The homogenate was then centrifuged for 20 minutes at 3000 rpm and 4°C in a Sorvall SS-34 rotor. The supernatant was collected, and 30 ml of 3 M KCl was added to it and it was centrifuged for 20 minutes at 9000 rpm and 4°C in a Sorvall SS-34 rotor. The supernatant was collected and the final High-five insect cell microsomes were pelleted by centrifugation for 38 minutes at 26000 rpm and 4°C in a Beckman Ti-45 rotor. Pellets were resuspended in 5 ml of buffer containing 250 mM sucrose, 30 mM histidine (pH 7.4) and stored in small aliquots at -50°C. Protein concentrations were determined by the method of Lowry et al. (1951), using BSA as a standard.

Electrophoresis and Immunoblotting

Prior to electrophoresis, samples were solubilized at 37°C for 5 minutes in a dissociation medium that consisted of 62.5 mM Tris (pH 6.8), 5% glycerol, 5% sodium dodecyl sulfate (SDS), 40 mM dithiothreitol, and 0.0025% bromophenol blue. SDS-PAGE gel electrophoresis was conducted by the method of Porzio and Pearson (1977), using 8% polyacrylamide (BioRad Mini-Protean II system). Kaleidoscope prestained molecular weight markers (BioRad) were used as standards. Gels were stained using gelcode blue stain reagent (Pierce), or proteins were transferred (BioRad Mini-Trans blot system) to nitrocellulose (SERCA2a blots) or PVDF (PLB blots) membranes (BioRad) for immunoblotting. The transfer protocol was carried out according to instructions

provided by the manufacturer, with the exception that methanol was omitted from the transfer buffer. Mahaney et al. (2000) reported that methanol in the transfer medium decreased SERCA2a and PLB protein transfer. The nitrocellulose membranes were probed with an anti-SERCA2a monoclonal antibody 2A7-A1 for detection of SERCA2a. The PVDF membranes were probed with anti-phospholamban monoclonal antibody 2D12 for detection of phospholamban (Movsesian et al., 1994). Antibody binding was visualized by using [¹²⁵I] – protein A and quantitated using Molecular Dynamics Phosphoimager SI. Cardiac SR was used as a standard since it is known to contain 36% SERCA2a by weight and 2% PLB by weight, based on quantitative immunoblots using purified Ca-ATPase and phospholamban as standards (Waggoner and Mahaney, unpublished results). Typically, our insect cell microsomes contained about 12-15% SERCA2a and 1 – 1.5% phospholamban per total protein by weight. Converting to moles of SERCA2a ($M_r = 110,000$ g/mol) and phospholamban ($M_r = 6,080$ g/mol) per mg of microsomal protein, these results indicated a molar ratio of 1:2 SERCA2a to PLB, similar to that found in native cardiac SR (Mahaney et al., 2000).

Ca-ATPase Activity Assays.

[Ca²⁺]-dependent ATPase activity of SERCA2a in the High-Five insect cell microsomes was measured by two different methods. The [Ca²⁺]-dependence of Ca-ATPase activity was measured colorimetrically at 37°C, using a malachite green-ammonium molybdate assay (Lanzetta et al., 1979; Mahaney et al., 1995). SERCA2a incubation tubes contained 0.05 mg microsomal protein per ml protein in 50 mM 3-[N-morpholino]propanesulfonic acid (MOPS) (pH 7.0), 3 mM MgCl₂, 100 mM KCl, 1 mM EGTA, and 0-1.0 mM CaCl₂ to give the desired ionized [Ca²⁺], as previously determined

(Autry and Jones, 1997). To start the ATPase reaction, 5 mM MgATP was added to the incubation tube. After 10 minutes of reaction at 37°C, a 50 µl aliquot of the incubation mix was transferred into an assay tube containing 1.6 ml malachite green-ammonium molybdate reagent at room temperature. After 30 seconds the reaction was quenched by the addition of 200 µl of 34% sodium citrate into the assay tube. For the determination of phosphate, a standard curve was constructed using aliquots from a 0.4 mM phosphate standard solution, which was assayed in a similar fashion as above. After 30 minutes of color development, the absorbance was measured at 660 nm. [Ca²⁺]-dependent ATPase activity data were fit by the Hill equation using the program KFIT written by N. C. Millar:

$$v = \frac{V_{\max}}{1 + 10^{n(K-[Ca])}}$$

where v is the enzyme velocity at any given [Ca²⁺], V_{\max} is the maximum velocity at saturating [Ca²⁺], n is the Hill coefficient, K is the [Ca²⁺] required for half-maximal velocity, and [Ca²⁺] is the ionized Ca²⁺ level for each particular assay. The best fits of the data were chosen on the basis of optimization of the determination coefficient, R^2 , and/or minimization of the sum-of-squares error, Π^2 .

For the Arrhenius analyses, Ca-ATPase activity of the microsomal samples was measured spectrophotometrically with a continuous ATPase assay using an ATP-regenerating system (Madden et al., 1979). Assays were performed in 1 ml of buffer containing 50 mM MOPS (pH 7.0), 3 mM MgCl₂, 100 mM KCl, 1 mM EGTA, and 0.5 mM CaCl₂, which provided a [Ca²⁺]_{free} of 0.270 nM, as previously determined (Autry and

Jones, 1997). In addition, the assay cuvettes contained 0.2 mM phosphoenolpyruvate, 0.2 mM NADH, 8 units pyruvate kinase, 20 units lactate dehydrogenase, which served as an ATP regenerating system and provided a means to monitor the progress of the assay. For each assay, 50 μ g SR protein was added to the assay cuvette pre-equilibrated at the desired experimental temperature and incubated for 5 minutes. To start the assay, 5 mM MgATP was added to the cuvette, and the rate of change of absorbance of NADH at 340 nm was recorded to estimate the rate of ATP hydrolysis.

For each activity assay, the microsomal samples were permeabilized with the Ca^{2+} ionophore (20 μ g per mg total protein) prior to the start of the assay to allow Ca-ATPase activity to be measured in the absence of a Ca^{2+} gradient. To test the effect of phospholamban on Ca-ATPase activity, SERCA2a + PLB samples were incubated for 20 minutes without or with affinity purified anti-phospholamban monoclonal antibody 2D12 (courtesy L. Jones), at an antibody-to-protein weight ratio of 1:1 (Autry and Jones, 1997).

Ca-ATPase Phosphoenzyme Levels.

Ca-ATPase phosphoenzyme formation experiments were carried out at the indicated temperatures using a circulating water bath to control the reaction temperature to within $\pm 1^\circ\text{C}$. Prior to phosphoenzyme formation, the microsomal samples were permeabilized by the addition of calcium ionophore A23187 (20 μ g / mg protein). Next, 0.25 ml of a solution containing 0.2 mg microsomes/ml in 50 mM MOPS (pH 7.0), 3 mM MgCl_2 , 100 mM KCl, 1 mM EGTA and 0.5 mM CaCl_2 , which provided a $[\text{Ca}^{2+}]_{\text{free}}$ of 0.270 nM, as described above, was placed in a 7 ml glass scintillation vial (VWR) and set in the water bath. To initiate phosphoenzyme formation, 0.25 ml of a solution containing

50 mM MOPS (pH 7.0), 3 mM MgCl₂, 100 mM KCl, 1 mM EGTA, 0.5 mM CaCl₂, and 20 μM [γ -³²P]ATP (20,000 cpm/nmol) was rapidly added (less than 1 sec) to the microsome-containing vial with vigorous vortexing. The final conditions following mixing were 0.1 mg/ml microsomes, 10 μM [γ -³²P]ATP, 50 mM MOPS (pH 7.0), 3 mM MgCl₂, 100 mM KCl, 1 mM EGTA, and 0.5 mM CaCl₂ ($[\text{Ca}^{2+}]_{\text{free}} = 270 \text{ nM}$). The reaction was allowed to proceed for 15 sec before quenching by the rapid addition of 0.5 ml of ice-cold 9% perchloric acid + 6 mM H₃PO₄, followed by vigorous vortexing. After quenching, the reaction tubes were placed on ice. Blank tubes were prepared by first adding 0.5 ml of quench solution to the 0.25 ml microsome-containing solution, vortexing vigorously, then adding 0.25 ml of the ATP-containing solution. A 25 μl aliquot of 10 mg/ml bovine serum albumin was added to each quenched sample to act as carrier protein during the processing of the sample vials. The quenched samples were pelleted by centrifugation for 10 min at 3000 x g, 4 degrees C in an IEC GP8R refrigerated centrifuge, and then washed three times by similar centrifugation using an ice-cold solution of 5% trichloroacetic acid, 6% polyphosphoric acid, 4 mM H₃PO₄, and 5 mM non-radioactive ATP. Pellet recovery following washing was greater than 95%, determined by protein assay. The final pellets were dissolved in 5 ml 1 N NaOH and the ³²P-phosphoenzyme was assayed by counting the Cerenkov radiation.

Chapter 3: Results

Protein Expression, Characterization and Assay.

For this study, canine cardiac Ca-ATPase (the SERCA2a isoform) and canine phospholamban were expressed in High Five insect cells using recombinant baculoviruses (Autry and Jones, 1997) and isolated as High Five insect cell microsomes. The Ca²⁺-ATPase was expressed either alone (Ca²⁺-ATPase only) or co-expressed with wild-type phospholamban (WT-PLB). The amount of Ca²⁺-ATPase and phospholamban in the insect cell microsome samples was determined by immunoblotting (Figure 4), using the Ca²⁺-ATPase and phospholamban in native cardiac SR vesicles as standards. Waggoner and Mahaney (unpublished results) have used quantitative immunoblotting techniques to determine the Ca-ATPase and phospholamban content of the cardiac SR vesicles, which are 36% and 2% by weight total protein, respectively. Based on phosphorimage analysis of the immunoblot shown in Figure 4, our insect cell microsomes contained about 15% Ca-ATPase and 1.5% phospholamban per total protein by weight. Converting to moles of SERCA2a ($M_r = 110,000$ g/mol) and phospholamban ($M_r = 6,080$ g/mol) per mg of microsomal protein, these results indicated a molar ratio of 1:2 Ca-ATPase to PLB, similar to that found in native cardiac SR (Waggoner and Mahaney, unpublished results).

The ATPase activity of the expressed Ca-ATPase and the functional coupling between Ca-ATPase and phospholamban in the Sf21 cell microsome samples was assayed at 37°C at a series of $[Ca^{2+}]_{free}$ in the presence and absence of anti-

phospholamban monoclonal antibody 2D12, which reverses the inhibitory interaction between phospholamban and Ca-ATPase (Briggs et al., 1992; Sham et al., 1991). Each

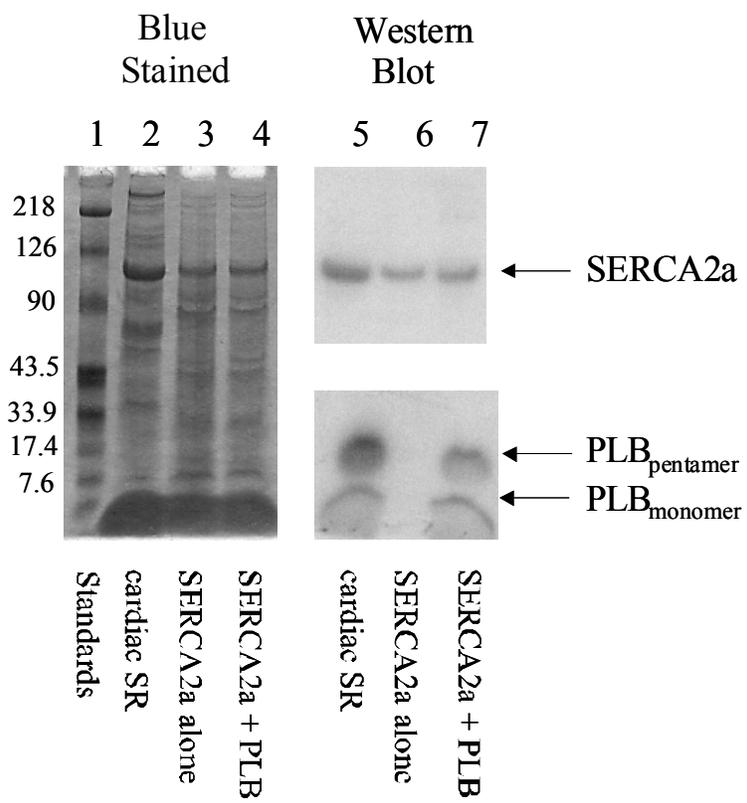


Figure 4: SDS-PAGE and immunoblot of Ca-ATPase and phospholamban in cardiac SR vesicles and High Five insect cell microsomes. Left: Coomassie blue-stained SDS gel (10 μ g protein per lane) showing cardiac SR vesicles (lane 2), insect cell microsomes containing SERCA2a alone (lane 3) or SERCA2a + phospholamban (lane 4) Right: corresponding immunoblot of the SDS gel (left) showing cardiac SR vesicles (lane 5), insect cell microsomes containing SERCA2a alone (lane 6) or SERCA2a + phospholamban (lane 7). PLB_{pentamer} denotes pentameric phospholamban and PLB_{monomer} denotes monomeric phospholamban. The blots utilized SERCA2a antibody 2A7-A1 (top) or phospholamban antibody 2D12 (bottom), and were developed with ¹²⁵I-protein A. The immunoblots were quantified by PhosphorImage analysis. The cardiac SR sample was included as a means to estimate the amount of SERCA2a in the expressed samples. Given that cardiac SR contains 30% SERCA2a and 1.5% phospholamban by weight, the two expressed samples contained approximately 14% SERCA2a and 0.7% phospholamban by weight by comparison.

expressed Ca-ATPase sample displayed the usual sigmoidal $[Ca^{2+}]$ -dependence, and the maximum steady-state activity (V_{max}) of the various samples was similar (approximately 0.4 – 0.5 $\mu\text{mol}/\text{mg}$ total protein/min) when measured at saturating ionized $[Ca^{2+}]$ (Figure 5). Treatment of the expressed samples with monoclonal antibody produced no effect on the maximal activity of the sample. Lack of effect of the monoclonal antibody on the Ca-ATPase V_{max} in cardiac SR vesicles and Ca-ATPase expressed in insect cells is well known (Autry and Jones, 1997; Mahaney et al., 2000).

Ca-ATPase expressed without phospholamban had a high apparent Ca^{2+} affinity ($K_{ca} = 200$ nM), which was unaffected by treatment of the sample with phospholamban monoclonal antibody. When coexpressed with wild-type phospholamban, the Ca-ATPase activity curve was shifted to the right relative to that of Ca-ATPase expressed alone, resulting in an increase in K_{Ca} to 420 nM. Treatment with anti-phospholamban antibody 2D12 shifted the Ca^{2+} activation curve to the left, resulting in a decreased K_{Ca} value similar to that obtained for Ca-ATPase in the absence of phospholamban. The results show that phospholamban was functionally coupled to Ca-ATPase in the expressed samples.

Effect of Phospholamban on the Temperature-dependence of Ca-ATPase Activity.

To determine the effect of phospholamban on Ca-ATPase activation energy, we measured Ca-ATPase activity and phosphoenzyme (EP) levels at a series of temperatures between 5°C and 40°C using insect cell microsomes containing Ca-ATPase expressed alone (Ca-ATPase only) and in the presence of phospholamban (Ca-ATPase + PLB). The effect of phospholamban on Ca-ATPase activation energy was explored further using

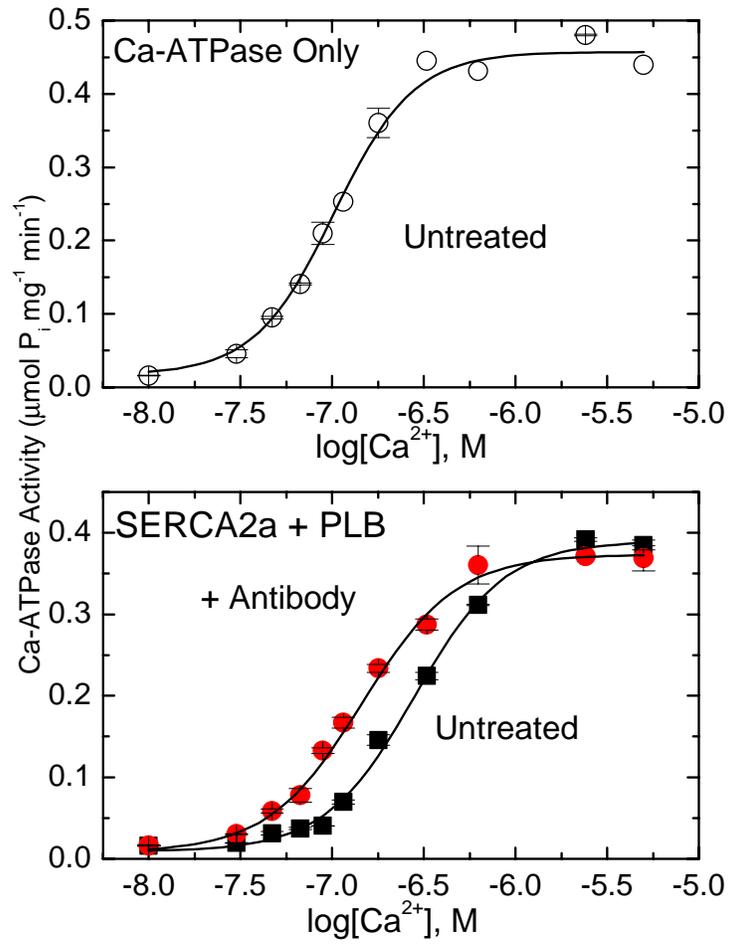


Figure 5: Effect of phospholamban on Ca-ATPase activity at 37°C. Ca-ATPase activity was measured colorimetrically as described in Chapter 2 (Methods), using Hive Five insect cell microsomes containing Ca-ATPase expressed alone (Ca-ATPase only, open squares, top panel) or Ca-ATPase coexpressed with phospholamban (Ca-ATPase + PLB, filled symbols, bottom panel). The Ca-ATPase samples exhibited a sigmoidal dependence on $[Ca^{2+}]$, which was characterized in terms of maximum activity (V_{max}) and the $[Ca^{2+}]$ required for half maximal activity (denoted K_{Ca}). The $K_{0.5}$ for Ca-ATPase in the presence of phospholamban (filled squares, bottom panel) was approximately 420 nM, compared to 200 nM for Ca-ATPase alone (empty squares, top panel) or 220 nM for Ca-ATPase + phospholamban treated with a monoclonal antibody to uncouple phospholamban from the Ca-ATPase (filled circles, bottom panel). Phospholamban had no effect on V_{max} for the Ca-ATPase + phospholamban sample. The results confirm that phospholamban inhibits Ca-ATPase by decreasing the Ca^{2+} sensitivity of the enzyme.

an anti-phospholamban monoclonal antibody to uncouple phospholamban from the Ca-ATPase. These measurements were conducted at an ionized Ca^{2+} level of 270 nM, which is near the $K_{0.5}$ value for the Ca-ATPase in the absence and presence of phospholamban (Figure 5).

As shown in Figure 6, the Ca-ATPase activity of the microsomal samples increased with increasing temperature. The temperature dependence was similar for samples containing Ca-ATPase expressed alone (Figure 6, top) or Ca-ATPase coexpressed with phospholamban (Figure 6, bottom). At higher temperatures (25-40°C), phospholamban decreased Ca-ATPase activity by about 20% relative to samples treated with 2D12 to uncouple phospholamban from the Ca-ATPase. At lower temperatures (5-20°C), however, there was no significant difference between untreated and antibody-treated samples. The activity of the antibody-treated Ca-ATPase + phospholamban sample was similar to that of the Ca-ATPase only sample at each temperature, ensuring that the antibody successfully uncoupled phospholamban from the Ca-ATPase at all temperatures studied.

Effect of Phospholamban on the Temperature-dependence of Ca-ATPase EP formation.

During the Ca-ATPase reaction cycle, the enzyme is phosphorylated by ATP, which drives Ca^{2+} translocation. Using radio labeled ATP, one can determine the amount of phosphorylated Ca-ATPase during steady state enzyme cycling (Methods). In effect, the EP level provides information about the catalytic site density of the enzyme, since the fraction of the enzymes phosphorylated during steady-state cycling gives rise to product

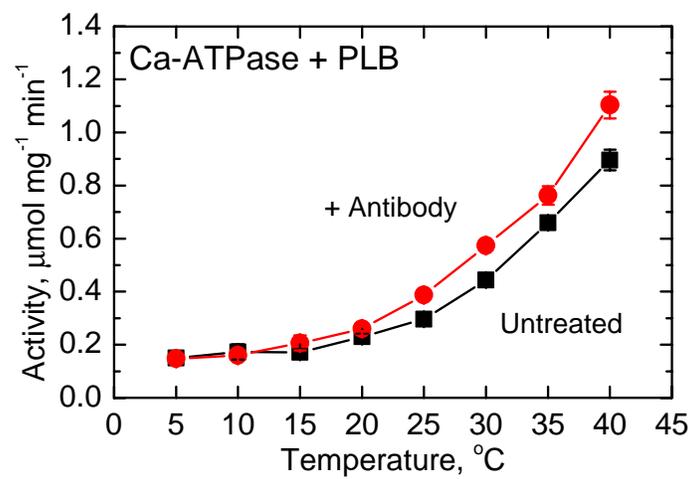
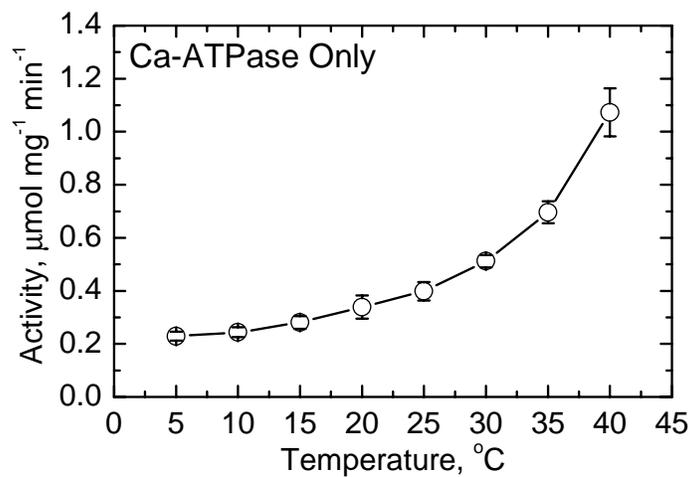


Figure 6: Effect of phospholamban on the temperature dependence of Ca-ATPase activity. Ca-ATPase activity was measured at $[\text{Ca}^{2+}]_{\text{free}} = 270 \text{ nM}$ at the indicated temperatures with a coupled enzyme assay as described in Chapter 2 (Methods), using Hive Five insect cell microsomes containing Ca-ATPase only or Ca-ATPase + phospholamban (Ca-ATPase + PLB). The $[\text{Ca}^{2+}]_{\text{free}}$ was selected to be near the $K_{0.5}$ value for both sample types, where phospholamban has significant effects on Ca-ATPase activity (Figure 2). This experiment was carried out using different (and more active) sample preparations than those shown in Figure 2, which is reflected in differences in the 37°C activities between the two figures. The symbols and sample conditions correspond to those defined in Figure 5.

release and enzyme turnover. Therefore, the steady-state phosphoenzyme (EP) level of each microsomal Ca-ATPase sample was measured as a function of temperature at 270 nM ionized Ca^{2+} using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Because temperature was not expected to affect the catalytic site density of the Ca-ATPase samples (Mahaney et al., 1995), we focused our measurements on three temperatures, 5°C, 20°C and 40°C. As expected, a similar value of EP was obtained at each temperature studied for each individual sample type. For the Ca-ATPase + phospholamban sample, the steady EP level was decreased approximately 25% (0.06 ± 0.01 nmol EP / mg total protein) compared to the antibody-treated Ca-ATPase + phospholamban sample (0.08 ± 0.02 nmol EP / mg total protein). This phospholamban-dependent inhibition of Ca-ATPase EP level was similar to the effect of phospholamban on Ca-ATPase activity (Figure 5), and is consistent with previous studies of the effects of phospholamban on Ca-ATPase phosphorylation by ATP using insect cell microsome samples (Mahaney et al., 2000). Thus, the individual values of steady-state EP levels measured at the three temperatures were averaged to provide one EP level for each sample type, which were used in subsequent calculations of Ca-ATPase turnover. When expressed alone, the Ca-ATPase steady-state EP level was 0.09 ± 0.02 nmol EP / mg total protein, which was similar to the steady-state EP level of the antibody-treated Ca-ATPase + phospholamban sample.

Effect of Phospholamban on Ca-ATPase Activation Energy.

For each sample type studied, the ratio of Ca-ATPase activity (nmol ATP hydrolyzed per mg protein per second) to EP level (nmol ^{32}P bound per mg total protein) was calculated to determine the temperature dependence of the Ca-ATPase turnover

number, k_{cat} (s^{-1}). The turnover number data was analyzed by an Arrhenius plot (Figure 7), and the slope (E_a/R , where R is the gas constant, 8.314 J per K per mole) of the plot provided the activation energy. The Arrhenius plot derived from the Ca-ATPase only and Ca-ATPase + phospholamban samples each consisted of two linear phases, with the break occurring at 15°C, as has been documented previously (Bigelow et al., 1986). Use of two linear phases as opposed to one single line was justified by least squares analysis, which showed that the correlation coefficients of the two phases was significantly better than the correlation coefficient of a single line for each sample type (see legend of Figure 7). From the slopes of the individual plots, it was apparent that the Ca-ATPase in the absence of phospholamban (Figure 7, top) had a smaller activation energy above 15°C (31 ± 3 J / mol) relative to that of the Ca-ATPase in the presence of phospholamban (Figure 4, bottom), which was 51 ± 5 J / mol. Treatment of the Ca-ATPase + phospholamban sample with 2D12 (Figure 4, bottom) had no significant effect on Ca-ATPase activation energy (52 ± 5 J / mol). The finding that the activation energy of the antibody-treated Ca-ATPase + phospholamban sample was significantly different than the Ca-ATPase only sample was surprising in light of the activity and EP level experiments, which showed that the 2D12 antibody completely relieved Ca-ATPase inhibition by phospholamban.

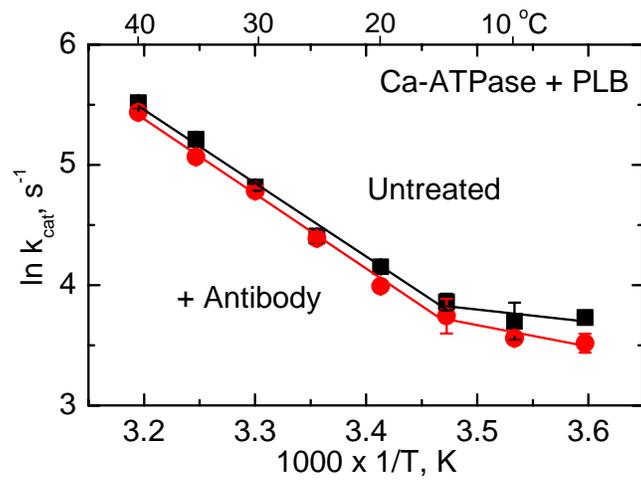
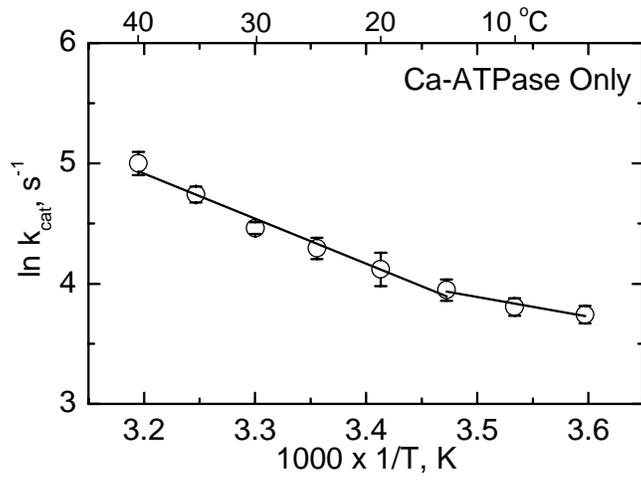


Figure 7: Effect of phospholamban on the temperature dependence of Ca-ATPase turnover number (k_{cat}). The temperature-dependent Ca-ATPase turnover number at $[Ca^{2+}]_{free} = 270$ nM in the absence of phospholamban (Ca-ATPase Only, top panel) and in the presence of phospholamban (Ca-ATPase + PLB, bottom panel), was calculated from the activity data shown in Figure 6 and the catalytic site density of each sample type (described in the text). The symbols and sample conditions correspond to those defined in Figure 5. When plotted as shown in the figure (an Arrhenius plot), the slope corresponds to $-E_a / R$, where E_a is the activation energy and R is the gas constant. For each sample type, the plot consisted of two distinct and well-resolved phases with a break at 15 °C. This was confirmed by least-squares analysis, which showed a significantly better correlation coefficient (r) for individual fits between 5-15 °C and 15-40 °C as opposed to one single fit from 5-40 °C. The activation energies derived from the plots were: Ca-ATPase only, 13 ± 2 J/mol between 5-15 °C ($r = 0.98$) and 31 ± 3 J/mol between 15-40 °C ($r = 0.991$); untreated Ca-ATPase + phospholamban, 9 ± 1 J/mol between 5-15 °C ($r = 0.75$) and 51 ± 5 between 15-40°C ($r = 0.99$); and antibody-treated Ca-ATPase + phospholamban, 15 ± 2 J/mol between 5-15 °C ($r = 0.93$) and 52 ± 5 J/mol between 15-40 °C ($r = 0.99$).

Chapter 4: Discussion

Overview.

The purpose of this study was to test the hypothesis that phospholamban inhibits Ca-ATPase by increasing the activation energy required for enzyme turnover. To test this hypothesis, we conducted an Arrhenius analysis of the Ca-ATPase turnover number to determine Ca-ATPase activation energy in the absence and presence of phospholamban. The effect of phospholamban on the Ca-ATPase was further explored using a monoclonal antibody, 2D12, against phospholamban, which is known to relieve phospholamban inhibition of the Ca-ATPase similar to phospholamban phosphorylation. The results indicated that Ca-ATPase activation energy was significantly higher when phospholamban was present compared to that when phospholamban was absent. Surprisingly, there was no significant difference in Ca-ATPase activation energy for Ca-ATPase + phospholamban samples treated with anti-phospholamban antibody versus those that were not treated, despite a significant increase in steady state Ca-ATPase activity following treatment with the antibody.

The Baculovirus-insect Cell Ca-ATPase / Phospholamban Expression System.

The samples used in this study were prepared using the baculovirus-High Five insect cell (fall army worm fibroblast) expression system for the Ca-ATPase and phospholamban. The advantage of using an expression system rather than native cardiac sarcoplasmic reticulum (SR) vesicles was the ability to study Ca-ATPase activation energy in the absence and presence of phospholamban. Because phospholamban is an

endogenous integral membrane protein in cardiac SR, it is not possible to remove it from the membrane without the use of strong detergents or reagents that would change the fundamental physical properties of the membrane. The High Five cell expression system was based on a similar system using Sf21 insect cells (fall army worm ovarian cells), which was developed and characterized by Autry and Jones (1997) and in more detail by Mahaney et al. (2000). The High Five cells produced a greater amount of expressed Ca-ATPase (and phospholamban) per mg of total protein, with better specific Ca-ATPase activity compared to protein expressed by the Sf21 cells. Thus, a single preparation of expressed protein provided sufficient material for a complete set of experiments, and the kinetics data obtained in each experimental set was of sufficient quality to allow for reproducibility even under the most stringent experimental conditions. The Mahaney laboratory and collaborators (manuscript in preparation) have characterized the physical and kinetic properties of the expressed Ca-ATPase, which were found to be nearly identical to the kinetic properties of the Ca-ATPase in native cardiac SR vesicles. Thus the High Five cell Ca-ATPase and phospholamban expression system was a valid model for studying the physical and kinetic mechanism by which phospholamban regulates the Ca-ATPase.

Ca-ATPase Turnover Number.

For this study, Ca-ATPase turnover was defined as the steady-state ATPase activity of the enzyme (nmoles ATP split per mg total protein per second) normalized to the catalytic site density of the enzyme in the samples (nmol EP per mg total protein). The resulting turnover number, k_{cat} , had units of sec^{-1} . A simpler analysis using only the

temperature-dependent enzyme velocity would have sufficed for this study. As such, one could normalize the Ca-ATPase activity of a given sample to the amount of expressed enzyme within that sample to facilitate the direct comparison of two different expressed samples. However, Mahaney et al. (2000) showed that despite nearly equal amounts of Ca-ATPase (per wt basis) in the insect cell microsome preps, Ca-ATPase in the presence of phospholamban has markedly lower steady-state activity than microsomes containing Ca-ATPase alone, even under experimental conditions that uncouple phospholamban from the Ca-ATPase (e.g., high $[Ca^{2+}]$, anti-phospholamban antibody, phospholamban phosphorylation). Thus, the catalytic site density (steady-state phosphoenzyme formed) was an important factor to consider to understand the effect of phospholamban on the amount of kinetically competent Ca-ATPase in each sample type.

Effect of Phospholamban on Ca-ATPase Activation Energy.

The results of these experiments indicated that the presence of phospholamban increased Ca-ATPase activation energy by 167%, from 31 ± 3 J/mol (Ca-ATPase only) to 52 ± 5 J/mol (Ca-ATPase + phospholamban). However the mechanistic basis of this effect is not yet clear. The Ca-ATPase enzyme cycle consists of a number of partial reactions, some of which are quite sensitive to the environmental conditions. Most notably, Froehlich and Taylor (1976) and Froehlich and Heller (1985) have shown that the three slowest steps in the enzyme cycle are calcium release from E2P, E2P·Ca₂, and phosphoenzyme hydrolysis, E2P to E2 + P_i, and the E2 to E1 transition. Because the rates of these transitions are all similar and highly interdependent, it is generally accepted that the rate of enzyme turnover is determined by the combination of the rates of these

steps. Furthermore, the rate each of these steps is dependent on pH, $[Ca^{2+}]$, [ATP] and temperature. Because $[Ca^{2+}]$ and pH were buffered, temperature was the only variable that changed significantly in these experiments. Nevertheless, temperature has significant effects on the relative rates of these three steps, and thereby changes which of the three steps is dominant for rate limitation for overall enzyme turnover. Thus, while we found that the presence of phospholamban changed the activation energy of the Ca-ATPase, we cannot be certain whether one single step changed or a combination of steps within a series of reactions changed in response to phospholamban. The solution for this problem is to study the effect of phospholamban on the temperature dependence of each of the partial reactions in the Ca-ATPase cycle independently. This would show how phospholamban affects the activation energy of each individual step in the enzyme cycle, which in turn would reveal the mechanistic basis by which phospholamban inhibits Ca-ATPase steady-state turnover.

Based on the effect of phospholamban on the $[Ca^{2+}]$ -dependence of Ca-ATPase activity (Figure 5), it is clear that phospholamban is a negative allosteric modulator of Ca-ATPase. Using this model, the Ca-ATPase would have two fundamental forms in equilibrium: a tense (or T) state, characterized as being less active with a decreased affinity for substrate (i.e., Ca^{2+}) and a relaxed (or R) state, having greater activity and a higher substrate affinity (Figure 8). In the presence of phospholamban, the enzyme has decreased activity and a lower apparent Ca^{2+} affinity, indicating that phospholamban shifts the T to R equilibrium toward the T state, typical of a negative allosteric modulator. Because negative allosteric modulators are reversible inhibitors of enzymes, the presence of substrate at high enough levels can shift the T to R equilibrium back toward the R

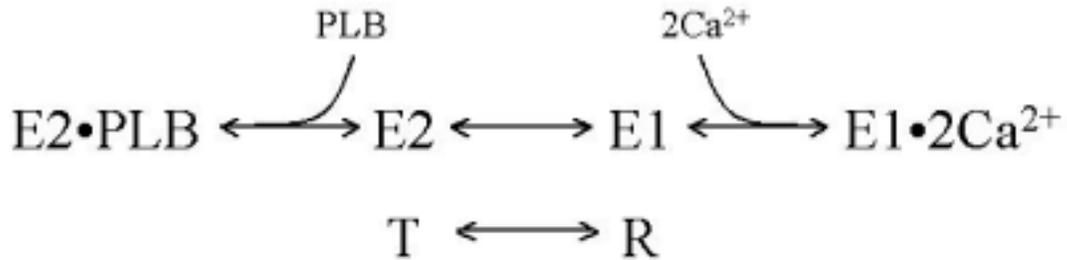


Figure 8: Phospholamban as a negative allosteric modulator of Ca-ATPase. The Ca-ATPase exists in two fundamental forms. The E1 state has high affinity for Ca^{2+} whereas the E2 state has low affinity for Ca^{2+} . As such, the E1 form of the enzyme is considered the relaxed form (R), which readily binds 2 Ca^{2+} ions cooperatively. Conversely, the E2 form is considered the tense form (T), which must first undergo a structural transition to R before Ca^{2+} can bind to the enzyme. Work in the Mahaney laboratory (unpublished results) has shown that phospholamban increases the E2 character of the enzyme, thus increasing the fraction of Ca-ATPase in the T form. However, increasing $[\text{Ca}^{2+}]_{\text{free}}$ reverses this effect and saturating $[\text{Ca}^{2+}]_{\text{free}}$ completely overcomes the effect of phospholamban on the Ca-ATPase. This behavior is consistent with phospholamban acting as a negative allosteric modulator of the Ca-ATPase.

state, effectively reversing the effects of the modulator. This is true for the effect of phospholamban on Ca-ATPase activity. At high $[Ca^{2+}]$, there is essentially no difference in the Ca-ATPase V_{max} in the presence or absence of phospholamban.

Using a combination of rapid mixing kinetics techniques and time-resolved electron paramagnetic resonance spectroscopy, Mahaney et al. (1995) showed that Ca-ATPase oligomeric interactions are important for high affinity Ca^{2+} binding and activation of ATP-dependent phosphoenzyme formation. Subsequently, Mahaney et al. (unpublished results) showed that during steady-state enzyme cycling, the Ca-ATPase operates as a tetrameric complex. At any given time, the four subunits within the tetramer correspond to the principal intermediates in the Ca-ATPase cycle: two E1 units ($Ca_2 \cdot E1 \cdot ATP$ and E1P) and two E2 units (E2P and E2). By modulating the reaction conditions, the authors showed that the distribution of E1 and E2 states could be shifted more towards E2, with a net decrease in apparent Ca^{2+} affinity, or toward E1, with a net increase in apparent Ca^{2+} affinity. Therefore, the Ca-ATPase T state is defined as an oligomeric complex with more E2 units, whereas the Ca-ATPase R state is an oligomeric complex with more E1 units. Both studies showed that the activation of T to R was rate limiting for enzymatic cycling.

Recent studies in the Mahaney laboratory have focused on the effect of phospholamban on Ca-ATPase oligomeric interactions using a variety of kinetic and spectroscopic approaches. Southall et al. (2001) used saturation transfer EPR to show that the presence of phospholamban results in a spatial reorientation of Ca-ATPase subunits within a Ca-ATPase oligomer relative to Ca-ATPase in the absence of phospholamban. Mahaney and coworkers (2002) using rapid mixing kinetics, and

Waggoner et al. (2001) using fluorescence spectroscopy, showed that in the presence of phospholamban, the Ca-ATPase had an increased E2 character relative to Ca-ATPase in the absence of phospholamban. The results of these studies suggest that phospholamban shifts the Ca-ATPase T to R equilibrium toward the T state.

Mahaney et al. (1995 and unpublished results) showed that the interconversion of Ca-ATPase oligomeric units from the less active (T state) to more active (R state) configuration was rate-limiting for the initiation of the enzyme cycle. Therefore, we propose that the activation energy measured in the present study corresponds to the energy required for the physical conversion of Ca-ATPase from the T to the R state preceding high affinity Ca^{2+} binding and ATP-dependent enzyme phosphorylation during the steady state. Because phospholamban shifts the T to R equilibrium more strongly toward the T state, greater energy is required for the activation of T to R in the presence of phospholamban than in the absence of phospholamban. A key test of this proposal would be to conduct the activation energy studies at a saturating $[\text{Ca}^{2+}]$, where phospholamban is effectively uncoupled from the Ca-ATPase (e.g., $[\text{Ca}^{2+}] > 1 \mu\text{M}$; Figure 2). Under these conditions, one would expect no phospholamban-dependent change in Ca-ATPase activation energy.

Anti-phospholamban Antibody Had No Effect on Ca-ATPase Activation Energy.

One aspect of the study that is difficult to account for is the lack of an effect on Ca-ATPase activation energy when the Ca-ATPase + phospholamban samples were treated with anti-phospholamban antibody (Figure 7). We found that treatment of the Ca-ATPase + phospholamban samples with anti-phospholamban antibody did not result in a

decrease in Ca-ATPase activation energy to a value similar to that observed for the Ca-ATPase alone. This lack of change was not due to a failure of the antibody to uncouple phospholamban from the Ca-ATPase. A sufficient amount of antibody was used to maximize the stimulatory effects of uncoupling phospholamban, based on previous antibody titration experiments (Autry and Jones, 1997; Mahaney et al., 2000). As shown in Figure 3, treatment of the Ca-ATPase + phospholamban with the anti-phospholamban antibody resulted in a 20% stimulation of Ca-ATPase activity at 270 nM $[Ca^{2+}]_{free}$, confirming the stimulatory effects of uncoupling phospholamban from the Ca-ATPase. Likewise, we found that antibody treatment stimulated Ca-ATPase EP formation by about 25%, which was a similar increase as that observed for Ca-ATPase activity following antibody treatment. These trends were similar over most of the temperature range studied. Because turnover is defined as the ratio of Ca-ATPase velocity to EP level, and phospholamban decreased both kinetic parameters by a similar amount, the resultant Ca-ATPase activation energy did not change significantly following antibody treatment.

One possible interpretation of this result is that phospholamban may decrease Ca-ATPase activity by completely inhibiting a fraction of enzyme, leaving the remaining enzyme fully active, rather than by inhibiting partially all the enzyme units. That is to say, because $Ca\text{-ATPase activity} = k_{cat} \times [E]_T$, a phospholamban-dependent decrease in the effective enzyme concentration would reduce enzyme activity without changing the turnover number (or presumably the temperature dependence of the turnover). It is well documented (see Chapter 1, Introduction) that phospholamban exists in equilibrium between active monomers and inactive pentameric forms. Thus, even at a molar

stoichiometry of one to two phospholamban per one Ca-ATPase, one would expect only a fraction of Ca-ATPase to have bound phospholamban. This is consistent with phospholamban acting as a negative allosteric modulator of Ca-ATPase. While the R to T equilibrium is shifted toward the inactive T state, the remaining R state is completely active. As outlined above, a way to test this proposal would be to repeat the activation energy experiments at saturating $[Ca^{2+}]_{free}$, where phospholamban has essentially no effect on Ca-ATPase activity (Figure 2) or Ca-ATPase EP level (Mahaney et al., 2000).

An outstanding question which remains is why the activation energy measured for Ca-ATPase expressed alone was so different from that measured for the antibody-treated Ca-ATPase + phospholamban sample. It is possible that there was a fundamental difference in the physical environment of the Ca-ATPase in the absence and presence of phospholamban, that may have affected the thermodynamic or kinetic properties of the enzyme. However, this is unlikely because kinetics studies (Froehlich et al., 2002) and spectroscopic studies (Waggoner et al., 2001; Southall et al., 2001) using nearly identical samples have shown that the two sample types are highly similar in terms of physical and kinetics properties. Alternatively, it is possible that the thermodynamic and kinetics properties of the Ca-ATPase were fundamentally different in the absence of phospholamban than in the presence of phospholamban, even when phospholamban was physically uncoupled from the Ca-ATPase following antibody treatment. This seems unlikely, however, because the $[Ca^{2+}]$ -dependence of Ca-ATPase activity for the Ca-ATPase in the presence of phospholamban treated with antibody was nearly identical to that of the Ca-ATPase alone (Figure 5). Clearly additional studies comparing the Ca-

ATPase only and the antibody-treated Ca-ATPase + phospholamban sample are needed to understand this lack of effect.

Future directions: The studies of this project have provided good evidence that phospholamban increases the activation energy of the Ca-ATPase. More detailed studies directed at understanding the effect of phospholamban on individual reactions in the Ca-ATPase cycle will help elucidate the mechanistic and thermodynamic bases by which phospholamban inhibits Ca-ATPase activity.

Chapter 5: References

- Alpert N. and Gordon M. (1962). Myofibrillar adenosine triphosphate activity in congestive heart failure. *Am J Physiol* **202**, H940-6
- Arkin, I. T., Rothman, M., Ludlam, C. F. C., Aimoto, S., Engelman, D. M., Rothschild, Autry, JM., and Jones, LR. 1997. Functional coexpression of the canine cardiac Ca-pump and phospholamban in *Spodoptera frugiperda* (SF21) cells reveals new insights on ATPase regulation. *J. Biol. Chem.* **272**: 15872-15880.
- Balke, CW., Shorofsky, SR. 1998. Alterations in calcium handling in cardiac hypertrophy and heart failure. *Cardiovasc. Res*; **37**: 290-299.
- Berne, A.M., Levy, M.N. 1997. Cardiovascular Physiology. *Mosby-YearBook*. St. Louis.
- Berridge, M.J. 1997. Elementary and global aspects of calcium signaling. *J. Physiol* (London). **499**: 291-306.
- Bers, D.M.1991. Excitation-Contraction coupling and cardiac contractile force Norwel: *Kluwer academic publishers*.
- Davis, B. A., I. Edes, R. C. Gupta, E. F. Young, H. W. Kim, N. A. E. Steenaart, G. Szymanska, and Kranias E. G. (1990). The role of phospholamban in the regulation of calcium transport by cardiac sarcoplasmic reticulum. *Mol. Cell. Biochem.* **99**: 83–88,
- Dupont, Y., Pougeois, R., Ronjat, M. and Verjovsky-Almeida, S. (1985) ATP. *J. Biol. Chem.* **260**, 7241-7249
- Fabiato, A. (1985). Time and calcium dependence of activation and inactivation of calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. *J. Gen. Physiol.* **85**, 247-289.

- Feher, J.J., Fabiato, A. 1990. Cardiac sarcoplasmic reticulum: calcium uptake and release. In: Langer, G.A., editor. Calcium and the heart. New York: Raven Press; 199-268
- Froehlich, J. P., and E. W. Taylor. 1976. Transient state kinetic effects of calcium ion on sarcoplasmic reticulum adenosine triphosphatase. *J. Biol. Chem.* **251**: 2307-2315.
- Froehlich, J., Mahaney, J., Kutchai, H., Jones, L., Southall, J., and Albers, J. 2002. Cardiac Ca-ATPase (SERCA2a) Expressed without Phospholamban (PLB) Behaves Like SERCA1 with Respect to Interactions with ATP and ADP in Transient Kinetic Experiments. *Biophys. J.*, **80(2)**: 264a.
- Froehlich, J.P., and Heller, P.F. 1985. Transient State Kinetics of the ADP-Insensitive Phosphoenzyme in Sarcoplasmic Reticulum: Implications for Transient-State Calcium Location. *Biochemistry.* **24**: 126-136.
- Fujii, J., Kadoma, M. Tada, M., Toda, H and Sakiyama, F (1986) *Biochem. Biophys. Res. Commun.* **138**, 1044-1050
- Huggins, J. P., Cook, E. A. Piggott, J. R Mattnsley, T. J. and Engalnd, P. J. Phospholamban is a good substrate for cyclic GMP-dependent protein kinase in vitro, but not in intact cardiac or smooth muscle. *Biochem. J.* 260: 829–835, 1989. *J. Biol. Chem.* **263**, 18364–18368
- Jorgensen, A.O and Jones, L. R.(1987). Immunoelectron microscopical localization of phospholamban in adult canine ventricular muscle. *J. Cell Biol.* **104**: 1343–1352.
- K. J., and Smith, S. O. (1995) *J. Mol. Biol.* **248**, 824–834
- Katz A.M. Physiology of the Heart. *New York: Raven Press; 1992.*

- Katz, AM., Takenaka, H., Watras, J. 1986. The sarcoplasmic reticulum. In: Fozzard, HA., Haber, E., Jennings, RB., Katz, AM, editors. The heart and cardiovascular system: Scientific Foundations. New York: *Raven Press*; 61-100.
- Kimura, Y., Kurzydowski, K Tada, M. and Maclenan, D. H. (1996) Phospholamban regulates the Ca^{2+} -ATPase through intra-membrane interactions. *J. Biol. Chem.* **271**: 21726–21731,
- Kirchberger, M. A., Tada, M. and Katz, A. M. Adenosine 3': 5'-monophosphate-dependent protein kinase catalyzed phosphorylation reaction and its relationship to calcium transport in cardiac sarcoplasmic reticulum. *J. Biol. Chem.* **249**: 6166–6173, 1974.
- Knowlton A, Connelly C, Romo G, (1992). Rapid expression of fibronectin in rabbit heart following myocardial infarction with and without reperfusion. *J Clinical invest.* **89**, 1060-1068.
- Kovacs, R. J., Nelson, M. T., Simmerman, H. K. B., and Jones, L. R. (1988)
- Kranias, E. G. Regulation of Ca^{2+} transport by cyclic 3', 5'-AMP-dependent and calcium-calmodulin-dependent phosphorylation of cardiac sarcoplasmic reticulum. *Biochim. Biophys. Acta* 844: 193–199, 1985.
- Lacapere, J.J., Gingold, M.P., Champeil, P. and Guillain, F. (1981) *J. Biol. Chem.* **256**, 2302-2306
- Limas C, Olivari M-T, Goldenberg, T. Benditt, D. Simon, A. 1987. Calcium uptake by cardiac sarcoplasmic reticulum in human dilated cardiomyopathy. *Cardiovasc Res* **21**, 601-605.

- Lompre, A.-M., Anger, M. and Levitsy, D. 1994. Sarco(endo)plasmic reticulum calcium pumps in the cardiovascular system: function and gene expression. *J. Mol. Cell. Cardiol.* **26**: 1109–1121.
- MacLennan, D. H., Brandl, C. J., Korczak, B. and Green, N. M. (1985) *Nature* **405**, 647-655.
- Mahaney, J.E., Froehlich, J.P., and Thomas, D.D. 1995. Conformational Transitions of the Sarcoplasmic Reticulum Ca-ATPase Studied by Time-resolved EPR and Quench Flow Kinetics. *Biochemistry.* **34**: 4864-4879.
- Mitchell, M. B., Meng, X., Ao, L., Brown, M. J., Harken, A. H. 1995. Preconditioning of isolated rat heart is mediated by protein kinase C. *Circ Res.* **76**, 73-81.
- Morad, M. and Cleeman, L. (1987) *J. Mol. Cell Cardiol.* **19**, 527-553.
- Mukherjee R., Spinale, FG. 1998. L-type calcium channel abundance and function with cardiac hypertrophy and failure. *J. Mol. Cell. Cardiol.* **30**: 1899-1916.
- Philipson, K.D. The cardiac Na⁺/Ca²⁺ exchanger. In: Langer, GA, editor; Calcium and the heart. New York: Raven Press; 1990. p. 85-108.
- Reinstein, J. and Jencks, W. P. (1993) The binding of ATP and Mg²⁺ to the calcium adenosine triphosphatase of sarcoplasmic reticulum follows a random mechanism. *Biochemistry* **32**, 6632±6642
- Schaper J, Froede R, Hein S. 1991. Impairment of myocardial ultrastructural changes of the cytoskeleton in dilated cardiomyopathy. *Circulation.* **83**, 504-514
- Sham, J. S. K., Jones, L. R., and Morad, M. (1991) *Am. J. Physiol.* **261**, H1344–H1349
- Sheu, S., Blaustein, MP. 1986. Sodium/calcium exchange and regulation of cell calcium and contractility in cardiac muscle with a note about vascular smooth muscle. In:

- Fozzard, HA., Haber, E., Jennings, RB., Katz, AM., editors. The heart and cardiovascular system: Scientific Foundations. New York: Raven Press; 509-536.
- Southall, J., Huffman, J., Jones, L., and Mahaney, J. 2001. EPR Studies of the Cardiac Ca-ATPase Expressed in Insect Cells: Effect of Phospholamban on Ca-ATPase Oligomeric Interactions. *Biophys. J.*, **80(1)**: 432a.
- Stokes, D.L and Green, N.M. (2000). Modeling a dehalogenase fold into the 8Å density map of CaATPase defines a new domain structure. *Biophys J.* **78**, 1765-1776
- Tada, M., and A. Katz, A. M. Phosphorylation of the sarcoplasmic reticulum and sarcolemma. *Annu. Rev. Physiol.* **44**: 401–423, 1982.
- Tada, M., Kirchberger, M. A, and. 1975. Phosphorylation of a 22,000-dalton component of the cardiac sarcoplasmic reticulum by adenosine 3', 5'-monophosphate-dependent protein kinase. *J. Biol. Chem.* **250**: 2640–2647.
- Toyoshima, C., Nakasako, M. and Ogawa, H. (2000) Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6Å resolution. *Nature* **405**, 647-655.
- Toyoshima, C., Nakasako, M. and Ogawa, H. (2000). *Nature* (London) **405**, 647-655
- Unverferth, D., Lee, S., and Et, W. (1988) Human myocardial adenosine triphosphate activities in health and heart failure. *Am. Heart J.* **115**, 139-146.
- Waggoner, J. Huffman, J., Kupec, J., Jones, L. and Mahaney, J. 2001. Fluorescence Studies of the Cardiac Ca-ATPase Expressed in Insect Cells: Effect of Phospholamban on Ca-ATPase Conformational States. *Biophys. J.*, **80(1)**: 432a.
- Walker, CA., Spinale, FG. 1999. The structure and function of the cardiac myocyte: A review of fundamental concepts. *J. thorac. Cardiovasc Surg.* **118**: 375-382.
- Wegener, A. D., and Jones, L. R. (1984) *J.Biol. Chem.* **259**, 1834-1841.

Appendix

28th October 2002

Dear Patrick Apopa,

Nature, hereby grants permission for the reproduction of figure 2 from, Nature 405:647-655(2000), providing that the authors agree, (please see original article for contact details) and the following credit line is used:

Reprinted by permission from Nature (reference citation) copyright (year)
Macmillan Publishers Ltd.

For electronic purposes, the word Nature must be hyperlinked to the Nature homepage at www.nature.com/nature

Yours sincerely,

Puja Bharadwa
Nature Permissions

Puja Bharadwa
Nature Permissions
The Macmillan Building
4-6 Crinan Street
London
N1 9XW

Tel: 44 (0)207 8334561
Fax: 44 (0)207 843 4596
permissions@nature.com

<http://www.nature.com>

Dear Patrick

>Dear Authors,

>

>My name is Patrick Apopa and I am a graduate student at West Virginia

>University. Currently i am working on the writing of my Thesis

>entitled, Thermodynamic effects of Phospholamban on CaATPase kinetics.

>I have been granted permission by Nature to reproduce part

>of Figure 2 from your paper published in 8 JUNE 2000 (Vol. 405, pp

>647-655). I therefore kindly ask you to grant me permission on the same.

>Appropriate attribution to Nature and the said authers will be provided.

>Thankyou in advance.

>Patrick Apopa

Of course you may use Fig 2 of my Nature paper (405:647-655,2000) in your thesis.

Yours sincerely

Chikashi Toyoshima

Professor of Supramolecular structure

Institute of Molecular and Cellular Biosciences

The University of Tokyo

Curriculum Vitae
Patrick L. Apopa
4 November 2002

Department of Biochemistry and Molecular Pharmacology
PO Box 9142
West Virginia University, Morgantown, WV 26506
Phone: 304-293-0225 (w) 304-293-5847 (h)
Fax: 304-293-6846
e-mail: papopa@mix.wvu.edu

Personal Data:

Birthdate and Place: February 23, 1968; Siaya, Kenya
Address: 367 Newton Avenue,
Morgantown, WV, 26505
Family: Married, one child.

Education:

1992 BSc. Zoology and Biochemistry, University of Nairobi, Kenya
1998 MSc. Biochemistry, University of Nairobi, Kenya
2002 MS. Biochemistry, West Virginia University, Morgantown, WV
Advisor: James E. Mahaney, Ph.D
Thesis: Thermodynamic effects of phospholamban on
Ca-ATPase Kinetics (focus is on cardiac Ca-ATPase
isoform)
Areas of specialization: Enzyme Kinetics, Protein
Mobility,
Lipid Bilayer Dynamics,

Teaching Experience, WVU:

Department of Biochemistry

2001 - Biochemistry 339 undergraduate Lab, Lab assistant

Service Activities, WVU:

Department of Biochemistry

2001-2002 Biochemistry Graduate Student Representative

Seminars Presented:

- 2001 - present: Biochemistry Research Forums (one per year)
- 2002 - present: Biochemistry Research Forums (one per year)

- 2001 - Biochemistry 496 Graduate Student Seminar: 30 Sept. 1999
“Malaria: The export of a trafficking pathway in *Plasmodium* infected erythrocytes”

Manuscripts in Preparation:**Work History:**

- 1993-1996: Medical sales Representative, Philips Pharmaceuticals
- 1997-2000: Medical Representative, Hoechst Marion Roussel Pharmaceuticals
- 2000-Present: Graduate student, WVU, Department of Biochemistry and Molecular Pharmacology

Personal References:

Jim Mahaney (Ph.D)
1414 Brockton Dr.
Morgantown, WV 26508
(304)-292-5767 (Home)
(304)-293-7756 (Work/Daytime #)

Work Related References:

Jim Mahaney (Ph.D)
1414 Brockton Dr.
Morgantown, WV 26508
(304)-292-5767 (Home)
(304)-293-7756 (Work/Daytime #)

John Durham (Ph.D)
20 Chasewood Lane
Morgantown, WV 26508
(304)-291-8350 (Home)
(304)-293-7757 (Work/Daytime #)

Jason R. Waggoner
Apt. F 1293 VanVoorhis Rd.
Morgantown, WV 26505
(304)-598-2760 (Home)
(304)-293-0225 (Work/Daytime #)