THE EFFECT OF TROPONIN I PHOSPHORYLATION ON THE STEEPNESS OF THE FRANK-STARLING RELATIONSHIP

A Thesis Presented to the Faculty of the Graduate School of the University of Missouri

In Partial Fulfillment of the Requirements for the Degree Master of Science

By TIMOTHY D. CORNELL

Kerry S McDonald, Ph.D, Thesis Advisor

MAY 2013
The undersigned, appointed by the dean of the Graduate School have examined the thesis entitled:

THE EFFECT OF TROPONIN I PHOSPHORYLATION ON THE STEEPNESS OF THE FRANK-STARLING RELATIONSHIP

Presented by Timothy Cornell,

a candidate for the degree of master of science in physiology

and hereby certify that, in their opinion, it is worthy of acceptance.

Kerry McDonald PhD

Michael Rovetto PhD

Craig Emter PhD
ACKNOWLEDGEMENTS

Thanks are owed to Kerry McDonald, my thesis advisor and to committee members Dr. Craig Emter and Dr. Michael Rovetto for their support and guidance. Their assistance has been essential to the completion of my degree. I would also like to thank Dr. Laurin Hanft for the knowledge that she has shared. Finally, a note of gratitude is owed to Dr. Steven Segal and Dr. Alex Moore; for their aid as I began my work in the field of physiology.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS........................................................................................................... II

LIST OF FIGURES.................................................................................................................. IV

INTRODUCTION

Preface ........................................................................................................................................ 1

Historical Perspective of the Frank-Starling Relationship ............................................... 3

The Sarcomere ........................................................................................................................ 7

Thick Filament ...................................................................................................................... 9

Thin Filament ....................................................................................................................... 11

Titin .......................................................................................................................................... 14

Cardiac Cycle ...................................................................................................................... 16

Heart Failure ....................................................................................................................... 18

Sympathetic Control ........................................................................................................ 21

STATEMENT OF THESIS ..................................................................................................... 23

MATERIALS AND METHODS

Experimental Animals .................................................................................................... 23

Solutions ........................................................................................................................... 23

Whole Heart Cannulation ............................................................................................ 23

Autoradiography ........................................................................................................... 24

Calculations ..................................................................................................................... 25

Statistical Methods ........................................................................................................ 25

PRELIMINARY STUDIES .................................................................................................. 26

RESULTS ............................................................................................................................ 32

DISCUSSION ...................................................................................................................... 37

REFERENCES ....................................................................................................................... 43
LIST OF FIGURES

Figure 1 ............................................................................................................................... 6
Figure 2 ............................................................................................................................... 8
Figure 3 ............................................................................................................................... 17
Figure 4 ............................................................................................................................... 19
Figure 5 ............................................................................................................................... 27
Figure 6 ............................................................................................................................... 28
Figure 7 ............................................................................................................................... 31
Figure 8 ............................................................................................................................... 33
Figure 9 ............................................................................................................................... 34
Figure 10 ............................................................................................................................. 36
INTRODUCTION

A. Preface

In 1918, Otto Frank’s “Zur Dynamiks des Herzmuskels” described the phenomenon that within physiological limits ventricular pressure is directly related to ventricular volume. This observation in combination with Ernest Starling’s 1914 finding that end-diastolic volume had a direct effect on ventricular output led to the description of the phenomenon known as the Frank-Starling relationship. The Frank-Starling relationship states that an increase in end-diastolic volume will result in a corresponding increase in ventricular stroke volume and/or ventricular pressure. This relationship allows for beat-to-beat regulation of cardiac output, which matches right and left ventricular output and equalizes venous return to cardiac output. Failure to match output between left and right sides of the heart would result in both volume and pressure increases in the pulmonary circulation. For example, a 50% mismatch of a resting ventricular output of 5 liters (L)/minute (average stroke volume ~70 ml) would lead to a doubling of the pulmonary circulatory volume (~1L) in as little as 15 minutes barring any compensation by the vasculature or lymphatic systems, which would be catastrophic for respiratory gas exchange.

The Frank-Starling relationship plays a significant role at rest and during low intensity exercise to match blood supply with peripheral demands, however, during an intense bout of exercise where cardiac output can increase up to seven-fold, Frank-Starling seems to play less of a role in regulating stroke volume in some individuals since end-diastolic volume plateaus or even declines due to such high
heart rates (~200 bpm). On the other hand, several studies have shown that elite athletes often show progressive or bimodal increases in stroke volume up to maximal work levels (Vella and Robergs, 2012). In the cases where stroke volume reaches a plateau or declines at very high work loads, the well defined beta-adrenergic system increases contractility and speeds relaxation to help assure adequate pumping strength and stroke volume. With regards to chronic aerobic exercise, stroke volume increases at a given work load, in part, through elongation of the cardiac myocytes by adding sarcomeres in series and the consequent physiologic dilatation of the left ventricular chamber. The increases in left ventricular chamber size and stroke volume necessitate a lower heart rate to maintain cardiac output and lead to greater end diastolic volume. At near maximal workloads trained hearts of elite athletes also seem to have adapted to have faster filling rates during diastole, in part, due to greater ventricular compliance. While the Frank-Starling relationship was described over 100 years ago the scientific community lacks a full understanding of the cellular and sub-cellular mechanisms that regulate this relationship. This thesis will focus on the hypothesis that the molecular regulation of Frank-Starling relationship, in part, involves post-translational modification of the cardiac thin filament protein, cardiac troponin I (cTnI). The following sections will examine the sarcomere and its strain-dependent regulation of the myocardium and post-translational modifications of myofibrillar proteins.
B. Historical Perspective of the Frank-Starling Relationship

Carl Ludwig appears to have been the first to describe the relationship between end diastolic volume and stroke volume, writing “a strong heart that is filled with blood empties itself more or less completely, (filling of the heart with blood) changes the extent of contractile power”; this conclusion was made when observing isolated perfused frog hearts in the 1850s. Other early contributors to defining the Frank-Starling relationship were Sir Michael Foster, a founder of the British Physiological Society, and Charles Smart Roy, who validated Ludwig’s findings in both frog and mammalian hearts. Ludwig’s original findings and subsequent ones by Foster and Roy were the first to conclusively show that ejection and contraction amplitude were dependent on filling volume; these findings provided the foundation for later work by Otto Frank and Ernest Starling. Otto Frank studied with Carl Ludwig from 1891-1893, and was compelled to study heart mechanics by his previous study of skeletal muscle thermodynamics. Frank made the analogy of volume and pressure with length and tension, respectively and used an improved frog heart preparation to measure isovolumetric and isotonic contractions. In essence, Otto Frank’s data showed that increased ventricular filling increased diastolic pressure and maximal isovolumetric pressure up to a certain point. He plotted his data as heart volume related to peak ventricular tension, which showed an ascending limb and a plateau that slightly descended at greater filling volumes. In the early 1900s Earnest Starling began his work on the mammalian heart, with the goal of explaining the phenomena of why cardiac output stays constant over a range of temperatures, heart rates, and arterial pressures.
Starling’s laboratory used a dog heart-lung preparation, which allowed for control of peripheral resistance and venous inflow. In 1914, Starling and Markwalder stated that “the rise of venous pressure must be regarded as one of the mechanical means that are operative in enabling the heart to maintain an output corresponding to the blood it receives from the venous system.” Subsequent work by Starling’s group further quantified the relationship between venous inflow, venous pressure, and ventricular output and concluded that “the output of the heart is… determined by the amount of blood flowing into the heart” (Katz, 2002). Starling surmised that the laws of the heart were similar to laws of skeletal muscle, which relates that the energy of contraction is a function of the length of the muscle fiber.

From the work of Ludwig, Henry P. Bowditch, Foster, Roy, Frank, and Starling, there was a consensus for the “law of the heart” whereby cardiac output is a function of preload, which has an impact on the length and subsequent energy production of the cardiac myocyte and network of cardiac fibers. The Frank-Starling relationship indeed had many similarities to early descriptions and preceded more contemporary descriptions of the length-tension relationship characteristic of striated muscle. In 1832, Theodor Schwann discovered the relationship between length and tension in skeletal muscle. Ramsey and Street provided a more quantitative description of length and tension in frog skeletal muscle fibers (Ramsey and Street, 1940). The classic study by Gordon, Huxley, and Julian (Gordon et al., 1966) rigorously describes the quantitative analysis in semitendinous single intact skeletal muscle fibers. This study provided systematic
electronic feedback to clamp muscle and sarcomere length in the middle of the fiber and yielded a length-tension relationship similar to that shown in Figure 1. This study was consistent with the sliding filament theory of muscle contraction, which consisted of overlapping thick and thin filaments with lengths of 1.6 um and 1.0 um, respectively. The descending limb of the length-tension relationship showed a decline in force as the overlap between thick and thin filaments progressively decreased. The ascending limb exhibited both a shallow and steep region. The shallow portion was hypothesized to arise from collision between thin filaments from the opposite side of the sarcomere yielding a restoring force, while the steep portion (which commenced at sarcomere length 1.6 um) was proposed to arise from Z disks colliding with thick filaments.
**Figure 1.** Sarcomere length-tension relationships for skeletal muscle and cardiac muscle. The sarcomere length-active tension relationship is steeper in cardiac muscle but this compares a length-twitch tension relationship in cardiac muscle versus a length-tetanic tension relationship in skeletal muscle. (Reproduced from Boron & Boulpaep, Medical Physiology)

A qualitatively similar ascending limb of the sarcomere length-tension relationship was reported in maximally calcium-activated cardiac myofibril preparations (Fabiato and Fabiato, 1975). Length-\textit{twitch} tension relationships exhibit much steeper ascending limbs than length-\textit{tetanic} tension relationships (Allen and Kentish, 1985). This arises because twitch contractions do not allow myofilaments to reach steady-state activation, thus, activations are sub-maximal and during sub-maximal Ca\textsuperscript{2+} activations there is a steep length dependence of Ca\textsuperscript{2+} sensitivity of force in striated muscle (Moss et al., 1983; Harrrison et al., 1988; McDonald and Moss, 1995; Wang and Fuchs, 1995; Konhilas et al., 2002; Hanft and McDonald, 2010). The exact reasons underlying length dependence of Ca\textsuperscript{2+}...
sensitivity of force remain uncertain but there is evidence that as sarcomere length is reduced the spacing between thick and thin filaments increases; this would reduce the probability of myosin interacting with actin to generate force and cooperatively activate the thin filaments to allow neighboring cross-bridges to interact with actin and undergo the force generating transitions (Fuchs and Martyn, 2005). There also is evidence that as sarcomere length is increased myosin cross-bridges and thin filaments are structurally altered to increase the likelihood of cross-bridge interaction (Farman et al., 2003; Farman et al., 2007; Mateja et al., 2012). These factors likely work synergistically to yield a steep length-dependence of twitch tension that occurs physiologically in the beating heart and also is characteristic of twitching skeletal muscle (Close, 1972).

C. The Sarcomere

The function of muscle is to produce force, and generate movement. In order to produce movement muscle is composed of long bundles of myofibrils. A myofibril is a cylindrical bundle of thick and thin filaments (Figure 2). The myofibrillar array is organized into sarcomeres, which is the fundamental functional unit of muscle. The boundaries of the sarcomere are defined by Z-lines, which is an array of non-contracting proteins that run perpendicular to the myofilaments (i.e., thick and thin filaments) and act as an anchoring point for the thin filaments and titin molecules (which runs from the Z line to the middle of the sarcomere). The sarcomere also is composed of thick and thin filaments that work in concert to
generate force. Within the sarcomere there is a region known as the I-band, which consists of thin filaments only. The H-zone contains a thick filament region located around the M-line, which is an array of proteins that denotes the middle of the sarcomere and likely contains mechano-sensing signaling molecules. The A-Band consists of the sarcomere region that comprises the entire length of the thick filament, including the portion where it overlaps with the thin filaments.

Sarcomeres are aligned in parallel and in series throughout the myofibril and this highly ordered array of proteins gives rise to a striated pattern characteristic of skeletal and cardiac muscle observed at the light microscopic level.

Figure 2. High-resolution light micrograph of a sarcomere and an illustration to depict the various regions of the sarcomere. (Reproduced from Anatomy and Physiology, 7E)
i.) **Thick Filament**

**Myosin**

The thick filament spans the entirety of the A-band and is 1.56 µm in length. The major component of the thick filament is the motor protein myosin II, which is composed of two heavy chains (~200 kDa/chain) and four myosin light chains (MLC). The two myosin heavy chains intertwine to form a coiled-coil with a long tail region, a flexible hinge region S2 that allows for movement of the myosin head and two myosin heads (S1) that contain the binding sites for the MLC, ATP and actin. This region is known as the motor region for the myosin molecule (Harrington and Rodgers, 1984). Myosin heads project away from the filament with heads separated by 14.3 nm, and 43 nm of separation between heads with the same axial orientation. Subfragment 1 (S1) contains the ATPase catalytic site and actin binding site, which are essential for cross-bridge cycling as the energy released by ATP hydrolysis and actin-myosin binding provide the basis for contraction. During cross bridge cycling, one cross-bridge power stroke is thought to move the thin filament ~11 nm in relation to the thick filament and to generate between 3 and 4 pico-Newton of force in isometric conditions (for review see Cooke, 1997).

As mentioned earlier each myosin molecule contains four light chains, two of which are associated with each of the globular myosin heads. Myosin light chains (MLC) are classified as either a regulatory light chain or as an essential light chain. Both light chains wrap around the neck region of the myosin head and it has been hypothesized that the placement of the MLCs provides the neck with structural
stability and stiffens the lever arm to aid in force production during the power stroke (Rayment et al., 1993). Regulatory myosin light chain (RLC) also may play a role in modulating Ca\(^{2+}\) sensitivity of force generation and cross-bridge cycling, due in part to its N-terminal region that contains both a helix-loop helix structure that includes a Ca\(^{2+}\) binding EF hand domain and serine phosphorylation sites. Along these lines, both maximal Ca\(^{2+}\) activated force and stiffness fell in skinned skeletal muscle fibers when endogenous RLC was replaced with a mutated RLC that lacked the Ca\(^{2+}\)/Mg\(^{2+}\) site (Diffee et al., 1996). Also, selective phosphorylation of RLC by myosin light chain kinase caused increased Ca\(^{2+}\) sensitivity of force and accelerated rates of force development (Sweeney and Stull, 1986; Metzger et al., 1989; Sweeney and Stull, 1990). This effect has been proposed as a mechanism for muscle post-tetanic force potentiation (e.g., treppe), which is a frequency dependent increase in muscle force production.

**Myosin Binding Protein-C**

Another protein found on the thick filament is Myosin Binding Protein-C (MyBP-C). MyBP-C is localized to 7-10 transverse stripes in two 300 nm zones on either side of the A band. These stripes are spaced 43 nm apart. There are 2-3 MyBP-C molecules located at each stripe. MyBP-C molecules are comprised of ~1173 amino acids having a molecular weight of 130-140 kDa. They are U- or V-shaped molecules having an arm length of ~30 nm and a width of 4 nm. The stoichiometric ratio of MyBP-C to myosin molecules is ~1:8. Functional studies have shown that MyBP-C contributes to the
Ca\textsuperscript{2+} sensitivity of myofibrillar force (Hofmann et al., 1991) and phosphorylation of the cardiac isoform of MyBP-C via protein kinase A (PKA) increases radial positioning of the myosin heads (i.e., proximity of myosin heads to thin filament as well as orientation of myosin heads towards the actin filaments) (Levine et al., 2001; Colson et al., 2008). The primary hypothesis behind these changes is that phosphorylation decreases the affinity of MyBP-C for the thick filament, allowing myosin heads to have greater degrees of radial freedom, which likely increases the probability of actin binding and/or kinetics of force-generating transitions. MyBP-C also is probably essential for proper thick filament assembly and protein turnover in the thick filament, which is highly complex given the constant contraction/relaxation cycle of cardiac myofilaments. Along these line, mutations in cardiac MyBP-C account for nearly 35% of all hypertrophic cardiomyopathy clinical cases, underpinning the role it plays in thick filament structure/assembly and sarcomeric function (Watkins et al., 1995).

ii. Thin Filament

Actin

Proteins that make up the thin filament include actin, tropomyosin and the proteins that make up the troponin complex, TnC, TnI and TnT. The main component of the thin filament is actin, which is comprised of subdomains I-IV. Subdomain I contains the myosin-binding site, which consists of amino acid regions that interact with myosin by formation of electrostatic, van der Walls, and hydrophobic chemical bonds. The thin filament contains actin monomers (G-actin)
that have polymerized to form filamentous (F)-actin. Two actin filaments wind around each other to form a coiled-coil that yields the thin filament backbone. Of the four actin subdomains; subdomains I and II exist on the exterior of the helix while the larger subdomains III and IV make up much of the core or interior of the thin filament (Holmes et al., 1993; Holmes, 1995). The axial distance from the mid-point of one actin monomer to the mid-point of its nearest neighbor is 5.46 nm, which is thought to coincide with the power stroke of the myosin cross-bridge. The “functional unit” is made up of actin, tropomyosin and troponin in a 7:1:1 ratio, where the troponin complex and a new tropomyosin are positioned at every 7th actin monomer to help regulate the myosin binding site.

**Tropomyosin**

In the absence of regulatory proteins, actin and myosin will interact continuously by hydrolyzing ATP in cyclical manner. Tropomyosin is a regulatory protein that “covers” the myosin-binding site on the actin molecule; which provides the tropomyosin with a “gatekeeper” role. Tropomyosin is comprised of two alpha-helical chains that form a coiled-coil. Tropomyosin molecules are continuous along the actin filament due to overlap of the C-domain of trailing tropomyosin molecule with the N-terminal domain of the leading (next) tropomyosin. Three isoforms of tropomyosin have been identified, alpha, beta and gamma, with alpha-tropomyosin being the predominant form in both skeletal and cardiac muscle. In muscle at rest, tropomyosin forms a complex with troponin to cover the cross bridge binding site. Upon calcium binding to TnC a mechanical signal is transmitted that results in the
movement of tropomyosin into the major actin groove exposing regions of the myosin binding site on actin. The formation of strongly-bound myosin cross-bridges yields a further conformational change and movement of tropomyosin deeper into the actin groove, which allows myosin cross-bridges to undergo the transition from strongly bound non-force generating to the strongly bound force generating state (McKillop and Geeves, 1993) (for review see (Gordon et al., 2000)).

Troponin

Cardiac troponin (cTn) is composed of three globular proteins; cardiac troponin C (cTnC), which binds calcium, cardiac troponin I (cTnI), which contains an actin binding region that along with tropomyosin sterically impedes the interaction of myosin with actin, and cardiac troponin T (cTnT), which links the troponin complex to tropomyosin (Tobacman, 1996; Gordon et al., 2000). When cardiac muscle is relaxed the troponin complex acts to block actin's binding site for myosin, inhibiting activation of the thin filament. Once a cardiac muscle cell is electrically stimulated, intracellular calcium rises and calcium binds to the N-terminal calcium specific binding domain cTnC. In response to calcium binding there is a cTnC conformational change that exposes a hydrophobic patch in the N-terminal region that attracts the switch peptide of cTnI that, in turn, moves the cTnI inhibitory peptide away from actin; this helps expose the myosin binding sites on actin. Extensive investigation of the cTnI molecule has revealed 9 phosphorylation sites, which can be modified by a number of cellular kinases (Solaro et al., 2013). Two of the sites phosphorylated by protein kinase A (PKA) are serines 22/23 (in rodents).
When cTnI is phosphorylated, there is a loss in calcium sensitivity and a decrease in actomyosin ATPase activity (Solaro, 2002). To test this hypothesis, a study incorporated mutant cTnI where serines 22/23 were replaced by alanine residues that cannot be phosphorylated (Malhotra et al., 1997). Results showed that PKA dependent phosphorylation was ~90% reduced in the S22A/23A mutant and calcium sensitivity was decreased by PKA with wild type TnI but was unaffected by PKA when the S22A/23A mutant was employed (Malhotra et al., 1997). These data support the regulation of Ca^{2+} activation of force. Other studies of TnI phosphorylation by PKA are known to decrease the myofilament responsiveness to calcium by increasing the rate constant of cTnC-calcium dissociation (Robertson et al., 1982). This process is thought to contribute to faster rates of ventricular relaxation via decreased calcium stimulation of force in response to beta-adrenergic stimulation (Kentish et al. 2001).

### iii). Titin

Titin was discovered in 1971; titin is the third most abundant protein in muscle and is important to muscle cell function in part due to its high degree of elasticity (Wang et al., 1979) (Granzier and Labeit, 2002). Also, titin is the largest protein in the mammalian genome, ranging in size from 2900-3700 kDa. Titin spans each half sarcomere from the Z disc to the M line, which creates a continuous span of a connecting molecule along the entire half-sarcomere (Obermann et al., 1996). Titin creates much of the passive tension in muscle cells over the sarcomere working range (Wang et al, 1993), and the stretching and shortening of titin helps to regulate...
sarcomere function. Titin acts as a spring, providing restorative force to a sarcomere that has been either stretched or shortened. This property would tend to assist the cross-bridges during shortening since a stretched titin would bear some of the resistive load allowing the cross-bridge to cycle faster. After a sarcomere has shortened below titin's equilibrium length titin becomes compressed and resists shortening but also likely assists in sarcomere re-lengthening by recoil forces. Titin recoil likely translates to faster ventricular relaxation and more rapid ventricular filling during diastole.

Titin has a number of different isoforms whose expression levels can change to adjust to the passive stiffness associated with cardiac development, exercise and disease. Isoforms largely differ by the components and length of the N2 sequence, which, in turn, determines passive force. Passive tension of cardiac muscle likely plays an important role in the Frank–Starling mechanism as passive tension creates a radial force that controls the distance between thin and thick filaments and passive force yields resistance that affects ventricular filling (Cazorla et al., 2001). Related to this latter point, lower passive tension as manifested by titin, would yield a more distensible myocardium that can fill more easily (Shiels and White, 2008).

**D. Cardiac Cycle**
There are four phases to the cardiac cycle (Figure 3.) Phase one (i.e., filling) begins when the atrial pressure exceeds ventricular pressure, which opens the atrioventricular (AV) valve and the pressure gradient drives blood into the ventricle. Following the wave of electrical depolarization that travels through the ventricle, the ventricle begins to contract increasing ventricular pressure above atrial pressure, which closes the AV valve. The aortic valve remains closed, thus, this phase (isovolumetric contraction) yields pressure development with no change in ventricular volume. Once the pressure in the left ventricle has exceeded the pressure in the aorta, phase three (i.e., ejection) begins. During phase three, the aortic valve opens and blood is ejected into the systemic circulation driven by the pressure gradient between the left ventricle and aorta. Isovolumic ventricular relaxation is the fourth phase and occurs after pressure in the ventricle falls below pressure in the systemic circulation. The aortic valve then closes and the ventricle relaxes isovolumetrically.
Figure 3. Pressure-volume relationship depicting the four phases of the cardiac cycle and coincident myofilament activation states. During phase 1 or ventricular filling, myoplasmic Ca\(^{2+}\) is low and thin filaments are inactivated due to tropomyosin (Tm) occupying the "blocked" state, which inhibits strong binding of myosin crossbridges (○). Phase 2 of the cardiac cycle (isovolumic contraction) begins following electrical depolarization of the ventricles and an increase in myoplasmic Ca\(^{2+}\). Ca\(^{2+}\) binds to troponin C (TnC) causing conformational changes of troponin I (TnI) and troponin T (TnT). Conformational changes in troponin allow Tm to undergo the transition to the "closed" state whereby myosin cross-bridges can strongly bind actin (●). Strongly bound myosin cross-bridges promote additional movement of Tm to the "open" state. It is only in the "open" state that myosin cross-bridges can undergo force generating transitions (○). The "open" state of Tm is depicted in the myofilament drawings during both phase 2 and phase 3. Phase 3 of the cardiac cycle begins when the semilunar valves open and blood is ejected as myocytes perform work on the blood. Myocyte shortening and power output is driven by conformational changes in myosin cross-bridges that propel thin and thick filaments past each other (→). During the latter part of ejection ventricular pressure falls causing the semilunar valves to close. Pressure falls during phase 4 (isovolumic relaxation) as the ventricles relax following decreased myoplasmic Ca\(^{2+}\) and inactivation of the thin filament as Tm returns to the "blocked" state. (Note: Myosin binding protein-C (MyBP-C) is shown on the thick filament (●)).
E. Heart Failure

Heart failure results from a mismatch between the supply and demand of blood, and ultimately results in a decrease in cardiac output. Heart failure may occur via systolic or diastolic dysfunction or a combination of the two. Classically, heart failure begins with a reduction in the contraction strength of the cardiac myocytes (systolic dysfunction.) With decreased strength of contraction, the cardiac myofibrils compensate via concentric hypertrophy to maintain adequate cardiac output that supplies blood to the systemic and pulmonary circulations. Increases in the size of cardiac myocytes decreases the size and filling capacity of the left and right ventricles. This may lead to diastolic dysfunction and over time provides an as yet undetermined stimulus for cardiac myocytes to decompensate from the increase in pressure via elongation and consequent dilatory remodeling of the ventricles, which increases filling capacity but coincidently increases wall tension. This tends to exacerbate ventricular dysfunction since myocytes with compromised contractile properties must work against progressively greater increased wall tension. The Frank-Starling relationship plays an interesting role in heart failure; it first acts as a compensatory mechanism to elevate ventricular output in the midst of greater end-diastolic volumes/pressure. However, in the case of dilated cardiomyopathy the heart eventually loses its ability to maintain the Frank-Starling relationship (see Figure 4), which further contributes to heart dysfunction and acts as another step in the vicious cycle of ventricular decompensation via more dilatory remodeling. Interestingly, a main aspect of conventional heart failure therapy involves both
angiotensin-converting enzyme inhibitors and beta\textsubscript{1}-adrenergic receptor blockade, both of which slow down ventricular remodeling.

\textbf{Figure 4.} Ventricular function curves showing the Frank-Starling relationship. In normal hearts (orange line) a change in end diastolic volume (EDV) leads to a change in stroke volume (SV) appropriate to meet the metabolic requirements of the tissue. The Frank Starling relationship may be modulated leftward and upward (green line) to yield more stroke volume for a given EDV and a greater change in SV with a change in EDV (e.g. \(\beta\)-adrenergic stimulation). Conversely, the relationship may be shifted downward and rightward (yellow and red lines), which has been defined as heart failure. With heart failure an increase in EDV results in a smaller increase in stroke volume, one that is inadequate to meet the demands of the tissues (Permission granted by Hanft, Korte, McDonald. Cardiovascular Research, 77, 627-636, 2008).
LaPlace’s Law

The changes in the shape and function of the heart during failure are based, in part, on changes in wall tension as defined by LaPlace’s Law, where

\[
\text{Wall Tension (T)} = \frac{\text{Pressure} \times \text{Radius (r)}}{\text{Wall Thickness (w)}}. \quad \text{(Equation 1)}
\]

During heart failure, there is activation of the sympathetic nervous system to preserve cardiac output and compensate for depressed ventricular pump function. The chronic activation of this system, in addition to activation of neurohumoral factors (i.e. renin-angiotensin), initiates a complex remodeling process of myocytes and the ventricles. Typically, the ventricular wall undergoes compensatory concentric hypertrophy, which according to LaPlace’s law would tend to decrease wall tension. Then, for unclear mechanistic reasons, the myocytes/ventricles undergo decompensatory remodeling, whereby the myocytes add sarcomeres in series and elongate. In addition, the loss of myocytes yields infiltration of connective tissue. Both of these processes yield a thinner ventricular wall and enlarged (radius) of the ventricle. Thus, it becomes clear based on Laplace’s law that the increased radius and decreased wall thickness yields large increases in wall tension. This creates an insurmountable problem for ventricular myocytes. Myocytes, already with compromised function, must generate higher forces and power to overcome the elevated wall tension (due to decompensatory ventricular remodeling) in order to eject blood into the systemic circulation during each heartbeat. This vicious cycle of decompensatory remodeling has provided a rationale for current therapy to treat heart failure, which includes beta-adrenergic and angiotensin II blockade.
F. Sympathetic control

Stimulation of the autonomic nervous system, and specifically the sympathetic nervous system and circulating catecholamines, acts to increase the contractile force and rate of the cardiac muscle. This positive inotropic effect is facilitated through the actions of norepinephrine on the G-protein coupled beta-1 receptors. Sympathetic control has three main myocardial function features: (i) increased peak tension, (ii) increased rate of tension development and (iii) faster rates of relaxation. Activation of the beta-1 receptors leads to an increase in the production of adenylyl cyclase (AC). An increase in the activation of AC leads to the production of cAMP, activation of certain protein kinases and phosphorylation of proteins that enact the physiological response of increased contractility. The main purpose of this thesis study was to determine whether the positive inotropic effect of the sympathetic nervous system was partially facilitated by phosphorylation of cTnI.
Statement of Thesis

The main goal of the group of experiments that we conducted was to discern whether biophysical/biochemical results translate to the organ (whole heart) level. The hypothesis that we tested was whether the steepness of the left ventricular power-preload relationship would correlate with the phosphate content of cTnI. This hypothesis was tested by directly comparing cTnI phosphate content with ventricular function curves of the same isolated rat heart.
MATERIAL AND METHODS

Experimental Animals

All procedures involving animal use were performed according to the Animal
Care and Use Committee of the University of Missouri. Male Sprague-Dawley rats (6
wks of age) were obtained from Harlan (Madison, WI), housed in groups of two or
three, and provided with access to food and water ad libitum. Rats were treated
with propranolol for 1, 3 or 7 days by adding 50 mg to 1L of H2O and age matched
with control rats.

Solutions

Perfusion buffer for whole heart experiments contained the following (in
mmol/L): 118 NaCl, 4.7 KCl, 2.25 CaCl2, 1.2 MgSO4, 1.2 H2PO4, 25 NaHCO3, 0.5 Na-
EDTA, 11 glucose, 0.4 octanoic acid, 1 pyruvate; plus 0.1% bovine serum albumin
(dialyzed against 40–50 volumes of the preceding buffer salt solution).

Whole Heart Cannulation

For whole heart experiments, hearts were quickly removed and placed in
beakers containing perfusion buffer over ice. The aorta was cannulated and
perfused with oxygenated perfusion buffer for 10 min in a Langendorff apparatus.
The pulmonary vein was then cannulated, and hearts were switched to a working
heart system. Hearts could not consistently be electrically paced, so their
endogenous pace was used (~150–250 beats/min) at the perfusate temperature
that was set at 34°C for all hearts. Heart rate, blood pressure, aortic flow, and
coronary flow were measured at varied preloads both before and after administration of epinephrine, which was added to perfusion buffer at a final concentration of 0.1 mM. Afterload was kept constant at 80 cmH₂O throughout the experiments. The sequence of the preload protocol was 10, 15, 10, 7.5, 5, 3, 5, 7.5, 10, 15 and 10 (cm H₂O) to characterize ventricular function (i.e., Frank-Starling) relationships with and without Epinephrine.

**Autoradiography**

To examine myofibrillar substrates of PKA, myofibrillar samples from control and propranolol treated rats were incubated with the catalytic subunit of PKA in the presence of radiolabelled ATP, separated by SDS-PAGE, and visualized by autoradiography. Briefly, 10 μg of skinned cardiac myocytes were incubated with the catalytic subunit of PKA (2.5 U μl−1) and 50 μCi [γ-32P]ATP for 30 min. Control experiments where cardiac myocytes also were incubated with PKA in the presence of protein kinase inhibitor PKI (4.4 U μl−1) have been performed in previous studies (Hanft and McDonald, 2010). The reaction was stopped by the addition of electrophoresis sample buffer and heating at 95°C for 3 min. The samples were then separated by SDS-PAGE (12% acrylamide), silver stained, dried and subsequently exposed to X-ray film for 24 hours at −70°C.
Calculations

Left ventricular power (LVP) was calculated using the following equation:

\[
\text{LVP} = \text{mean arterial pressure (cmH}_2\text{O)} - \text{left atrial pressure (cmH}_2\text{O)} \times \text{(cardiac output (ml/min))}.
\]

(Equation 2)

Statistical Methods

The relationships between ventricular function curves and MyBP-C and cTnI phosphate content were examined by linear regression.
PRELIMINARY STUDIES

Two populations of length tension relationships in cardiac myocytes

A recent study examined sarcomere length-tension relationships in rat permeabilized single fast-twitch skeletal muscle fibers, single slow-twitch skeletal muscle fibers, and single cardiac myocytes. Consistent with reports in the literature (Allen and Moss, 1987; McDonald et al., 1997; McDonald, 2000; Konhilas et al., 2002), fast-twitch skeletal fibers exhibited steeper sarcomere length-tension relationships compared to slow-twitch skeletal muscle fibers (Figure 5). Permeabilized single cardiac myocyte preparations exhibited two populations of length-tension relationships, one steep like fast-twitch fibers the other shallow like slow-twitch fibers. Interestingly, cardiac myocytes with shallow length-tension relationships could be shifted to a steep length-tension relationship by PKA-mediated phosphorylation of myofilament proteins (Figure 6). PKA has multiple myofibrillar substrates including titin, myosin binding protein-C (MyBP-C), and
Figure 5. Sarcomere length-tension relationships for rat fast-twitch skeletal muscle fibers, slow-twitch skeletal muscle fibers, and cardiac myocytes. Cardiac myocyte preparations exhibited two populations of length-tension relationships, one steeper even than fast-twitch fibers and the other similar to slow-twitch fibers. Inset shows passive length-tension relationships of rat skinned cardiac myocytes, fast-twitch skeletal muscle fibers, and slow-twitch skeletal muscle fibers. The steepness of active length-tension relationships was independent of passive tension. (Taken from Hanft & McDonald J Physiol 588, 2891-2903, 2010).
Figure 6. (A). An autoradiogram showing radiolabeled phosphate incorporation into rat cardiac myofibrillar proteins upon PKA treatment. Lane 1 and 2 contains permeabilized cardiac myocytes in the presence of $^{32}$P-$\text{[ATP]}$ either without (lane 1) or with PKA (lane 2). (B). Cardiac myocyte force traces during submaximal $\text{Ca}^{2+}$ activations at four different sarcomere lengths before and after PKA treatment. (C). Normalized cardiac myocyte sarcomere length-tension relationships before and after PKA treatment (n=6). PKA-induced phosphorylation markedly steepened the length-tension relationship in these preparations (Permission give by the Journal of Physiology, taken from Hanft & McDonald J Physiol 588, 2891-2903, 2010).
cardiac troponin I (cTnI). Studies have been undertaken to determine if phosphorylation of one of these molecules was sufficient or more important in the control of length-tension relationships in striated muscle. The effect of PKA on the myofibrillar substrates may play an important role in regulating the Frank-Starling relationship of the ventricles.

2D-DIGE

In order to address the distribution of post-translational charged residues in rat cardiac myocytes, 2D- Differential Gel Electrophoresis (2D-DIGE) was performed. Myocytes were isolated from control rats and rats treated with the beta-adrenergic blocker propranolol to prevent myofibrillar phosphorylation. Each of these samples were incubated with a uniquely fluorescing CyDye that ubiquitously binds to proteins. The samples were combined and underwent 2D electrophoresis to separate by size in the vertical axis and by charge horizontally. Control myocytes were incubated with green CyDye and myocytes from propranolol treated rats were incubated with red CyDye; where there is overlap between samples, the protein spots appear yellow; however, where there are differences in charge between the samples the spots remain red or green, respectively. If there are near-neighbor cellular differences in PKA-mediated phosphorylation, 2D-DIGE on samples from control rats combined with those from rats treated with propranolol should show a CyDye color pattern that mathematically yields 25% highly phosphorylated (green) and 75% with low phosphorylation (overlap- yellow). Although there were not large differences in charge of cMyBP-C between control and propranolol treated
samples (as evidenced by all the cMyBP-C protein spots appearing yellow), we did see a distribution pattern whereby ~25% of cells displayed (high charge) highly phosphorylated TnI and ~75% low charge (Figure 7). These results suggest that cTnI may be the critical myofibrillar modulator responsible for two distinct populations of length-tension relationships in cardiac myocytes.

Additional 2D-DIGE experiments combined control myocytes with PKA treated myocytes. The 2D-DIGE pattern for cTnI yielded ~60% highly charged and ~40% lowly charged, again consistent with near-neighbor differences in PKA mediated cTnI phosphorylation. On the other hand, PKA treatment yielded four additional cMyBP-C bands that were not seen in control myocytes (Figure 7C).
Figure 7. 2D-DIGE implicates cTnI as the key signaling molecule responsible for bimodal distribution of length-tension relationships. (A) Myocytes from control hearts (green Cy dyes) were combined with myocytes from rats treated with propranolol (a β-adrenergic blocker) (red Cy dyes). Since propranolol decreases PKA-mediated phosphorylation and yields mostly shallow length tension-relationships (Hanft and McDonald, 2010), and myocytes from control hearts show a nearly 50:50 (steep:shallow) distribution of length-tension relationships, we hypothesized 75% of the signal to be overlapped (yellow, relatively low charged state) and 25% to be non-overlapped (green, higher charged state (i.e., phosphorylated)). This result was observed for cTnI while MyBP-C exhibited nearly all overlap (yellow appearance) (B). Combining control and PKA-treated myocytes nearly yielded the inverse results whereby approximately 40% of the cTnI appeared to have low charge while 60% of cTnI was highly charged. In contrast there was very little overlap in signal between the control and PKA-treated samples for MyBP-C (C).
RESULTS

Whole Heart Experiments

To test the hypothesis that the molecular regulation of Frank-Starling relationship involves PKA-mediated phosphorylation of cardiac troponin I (cTnI), ventricular function curves were correlated with phosphate content of cTnI. Phosphate content of cTnI was manipulated in rat hearts by treatment with the beta-adrenergic blocker, propranolol. Rats were provided propranolol water, at the concentration of 50mg propranolol/L of water for one to seven days. On the day of the experiment age-matched control and experimental animals, hearts were dissected, aortas cannulated, and ventricular function curves were characterized. The following protocol for preload was used 10, 15, 10, 7.5, 5, 3, 5, 7.5, 10, 15 and 10 (cm H₂O) to characterize ventricular function (i.e., Frank-Starling) relationships. Heart rate (HR), systolic and diastolic pressure, aortic output and coronary flow were all assessed; Figure 8 shows the results of the calculated left ventricular power versus preload. There was a reduction in left ventricular power corresponding to the number of days of treatment with propranolol. In order to correct for any variations that may have been attributed to fluctuations with heart rate, preload also was plotted against calculated stroke volume. Again, stroke volume increased with preload but the increase in stroke volume with increased preloads was less following propranolol treatment (Figure 9).
Figure 8. Steepness of the ventricular function curves (Pre-load versus left ventricular power) in rat isolated hearts. Green circles are data points from hearts treated with epinephrine, blue circle are from untreated hearts, grey circles are from hearts of rats treated with propranolol for 3 days, and red circles are from hearts of rats treated with propranolol for 7 days.
**Figure 9.** Steepness of the ventricular function curves (Pre-load versus left ventricular stroke volume) in rat isolated hearts. Green circles are data points from hearts treated with epinephrine, blue circles are data points from untreated hearts, grey circles are from hearts of rats treated with propranolol for 3 days, and red circles are from hearts of rats treated with propranolol for 7 days.
**Autoradiography**

To examine whether the relationship between ventricular function curves and phosphate incorporation into cTnI and cMyBP-C, hearts were frozen with liquid nitrogen immediately following completion of functional assessment. Cardiac myofibrils were prepared and autoradiography was performed to assess baseline phosphate content in cTnI and cMyBP-C by PKA-mediated back-phosphorylation assays using radiolabelled [γ-32P] ATP. Silver stain was used to normalize relative autoradiography signal with protein load in each gel lane. Figure 8 shows the relationship of relative baseline PKA-mediated phosphorylation with steepness of ventricular function curves. The linear relationship between the level of phosphorylation of MyBP-C and the increase in left ventricular power with increased preload yielded an $r^2$ value of 0.614. The relationship between the baseline level of phosphorylation of cTnI and the increase in left ventricular power with increased preload was more tightly correlated, exhibiting an $r^2$ value of 0.991. These results suggest that phosphorylation of cTnI by PKA is more functionally responsible for the steepness of the Frank-Starling relationship than cMyBP-C phosphate content.
Figure 10. The relationship between PKA-mediated myofibrillar phosphorylation and the Frank-Starling relationship in isolated rat hearts. A. The left panel shows a silver-stained SDS gel of cardiac myofibrillar preparations obtained from control and epinephrine treated rat hearts as well as hearts obtained from rats treated with propranolol for 1, 3, and 7 days. The right panel shows the coincident autoradiogram displaying the level of phosphate incorporation into myofibrillar proteins after PKA treatment. B. The relationship between the level of phosphorylation of MyBP-C and the increase in left ventricular power with increased preload. The linear regression ($r^2$ value) = 0.614. C. The relationship between the level of phosphorylation of cTnI and the increase in left ventricular power with increased preload. The linear regression ($r^2$ value) = 0.991.
DISCUSSION

Changes in Cardiac Output with Beta-Adrenergic Stimulation

Beta-adrenergic stimulation has long been known as a cardiac inotropic agent, whereby it increases ventricular contractility yielding greater stroke volume (and more power output) for a given end diastolic volume. The purpose of this study was to investigate a potential molecular mechanism by which cardiac myofilaments augment ventricular contractility. Recent work in our laboratory has shown that PKA-mediated phosphorylation of cardiac myofilaments steepens the sarcomere length tension relationship, and it appears that phosphorylation of cTnI was both necessary and sufficient to mediate this biophysical response (Hanft et al., 2013). The goal of this study was to investigate whether these biophysical/biochemical results translate to the organ (heart) level. The hypothesis was tested that the steepness of the left ventricular power-preload relationship would correlate with the phosphate content of cTnI. This was tested by direct comparison of cTnI phosphate content and ventricular function curves of the same isolated rat heart. The finding that these two parameters were highly correlated (Figure 8) suggests that shifts in length-tension relationships at the myofilament level by covalent modulation of cTnI translate to ventricular function and may be a key molecular mechanism underlying the steepness of the Frank-Starling relationship.

A limitation of this work is that the experimental design and conclusion are based on correlative data and while it is consistent with the myofilament length-
tension relationship (which is modulated by cTnI phosphate content) eliciting a specific Frank-Starling relationship, it fails to prove causation. A more causative experimental design would rely on gain/loss of function studies. For example, a study whereby inducible expression of constitutively phosphorylated or non-phosphorylatable cTnI would more directly assess whether cTnI covalent modulation controls the steepness of Frank-Starling relationship in isolated hearts. A key aspect of these theoretical studies would be to incorporate high levels of exogenous cTnI in a timely manner and to measure ventricular function curves soon after cTnI incorporation in order to prevent compensatory responses such as other post-translational modifications (i.e., phosphorylation, oxidation, glycation, acetylation, N-linked glycosylation). Another advantage of an adenoviral induction of gene expression design versus inducible transgenesis is that the rat model could be used and not confounded by use of mouse myocyte preparations, which are known for high levels of endogenous PKA mediated phosphorylation of cTnI and high ATPase activity of mouse alpha-myosin heavy chain. If indeed cTnI phosphate content is sufficient to control the steepness of Frank-Starling relationships then incorporation of pseudo-phosphorylated cTnI would yield steep ventricular function curves in isolated hearts and conversely incorporation of non-phosphorylated cTnI would yield shallow ventricular function curves, and whose steepness is unresponsive to epinephrine treatment. If epinephrine were able to further alter the steepness of ventricular function curves then a mechanism in addition to cTnI phosphorylation would be implicated to work in combination with cTnI. For instance, the change in intracellular calcium induced by activation of the
beta-adrenergic pathway may increase contractility to a greater extent at high-end diastolic volumes than at low-end diastolic volumes, which would result in a steeper ventricular function curve despite no additional change in cTnI phosphorylation.

Another limitation of this study was the use of beta-adrenergic blocker propranolol, which is an antagonist for both beta-1 and beta-2 adrenergic receptors. The use of a beta-1 specific antagonist such as atenolol could have limited some potential pharmaco-physiological effects. For instance, the activation of beta-2 receptors would affect respiration by dilating small airways and could reduce afterload by vasodilation of arterioles. Such actions could yield physiological compensation (e.g., post-translational modifications) of the heart that could alter the steepness of the Frank-Starling relationship independent of cTnI phosphorylation of serines 22/23.

Potential confounding effects of hypovolemia and variable heart rates

It was noticed, although not quantified, that rats treated with propranolol appeared to drink less during the course of treatment than did control rats. If there was a decrease in drinking volume in the propranolol-treated animals this could alter blood volume between groups with the experimental group becoming hypovolemic. During periods of hypovolemia, stroke volume usually does not change as much in response to increased peripheral demand. Thus, the cardiovascular system becomes more reliant on an increase in heart rate to maintain or increase blood pressure and cardiac output. If indeed the propranolol
treated rat population was hypovolemic during a 1-7 day treatment it might be predicted that propranolol treated rats would have a higher heart rate than the control population to maintain cardiac output. An increase in heart rate set point may have persisted after propranolol treated rat hearts were isolated for working heart measurements. A difference in heart rate can result in a change in filling and power. Because of this possibility and/or difference in heart rates between preparations (for reasons independent of propranolol treatment), we normalized for heart rates by plotting preload against both stroke volume (Figure 10) and left ventricular power (Figure 9). In other words, the calculation for left ventricular power includes heart rate as a multiplier within the equation, which could affect results and interpretation. In calculating stroke volume, heart rate is eliminated as a confounding co-variable as long as both left ventricular power versus preload and stroke volume versus preload relationships are indistinguishable (compare Figures 9 & 10) which was the case in our studies. The results imply that any differences in blood volume and heart rate were corrected for in our experimental design and does not confound the key finding that cTnI phosphate content correlates with the steepness of the Frank-Starling relationship. The interpretation being that this post-translational modification may be an important molecular mechanism underlying the family of Frank-Starling relationship that are commonplace of mammalian heart pump function.
Final thoughts

There are certainly other ways that altering beta-adrenergic responses for one to seven days of propranolol treatment may have altered the steepness of ventricular function curves in isolated rat heart. The downstream signaling molecule of the beta adrenergic system (i.e., PKA) has many substrates in mammalian cardiac myocytes. These include calcium-handling proteins such as the L-type calcium channel, the ryanodine receptor, and phospholamban. It is possible that decreased phosphorylation of the L-type calcium channel and ryanodine could depress ventricular function curves mechanistically by decreasing the amount of activator calcium intake and release of sarcoplasmic reticulum calcium during a given heartbeat. This would depress power for a given end-diastolic volume or pre-load yielding a negative inotropic effect. Interestingly though, the sarcomere length tension relationship is markedly steeper at lower levels of activator calcium (Allen and Kentish, 1985). This would be predicted to steepen the Frank-Starling relationship in spite of a downward shift from control curves. This was not observed as propranolol treatment both depressed ventricular function curves and decreased the slope of the relationship. Another PKA target is phospholamban, which tends to inhibit the sarcoplasmic reticulum calcium ATPase pump and the PKA-mediated phosphorylation of phospholamban is known to attenuate this inhibition yielding faster calcium reuptake rates. Physiologically this would tend to accelerate relaxation and may allow greater diastolic filling for a given pre-load, which would tend to increase contractility (i.e., upward shift of plotted ventricular function curves due to greater end diastolic volume for a given preload). Along
these lines, lowered phospholamban phosphorylation by propranolol treatment may have the opposite effect, i.e., slower relaxation and, thus, less filling for a given pre-load. Whether propranolol actually altered the phosphorylation state of these calcium-handling proteins was not assessed in this study. The concept of altered diastolic filling volumes mediated by PKA-mediated phosphorylation, however, could be a very important mechanism by which cTnI phosphorylation modulates ventricular function to meet peripheral demands. For example, if cTnI phosphorylation steepens the length-tension relationship then as the cells shorten during ejection, there would be more rapid cooperative deactivation of the thin filaments due to exquisite sensitivity to length after cTnI phosphorylation and this would assist relaxation allowing more diastolic filling to keep heart working high on the Frank-Starling relationship. This phenomenon would have added importance in presence of beta-adrenergic stimulation, which increases heart rate yielding much less diastolic time for filling. Finally, endurance trained athletes show progressive rises in stroke volume with increased oxygen consumption (Vella and Robergs, 2012) perhaps due to faster and more efficient filling at a given pre-load, which may be due to higher levels of cTnI phosphorylation, calcium reuptake, and/or greater ventricular compliance.
REFERENCES


