Regulation of Cardiac Contraction in Health and Disease: Studies from Animal Models to Humans

DISSERTATION

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Abstract

Heart disease remains the number one cause of mortality in the United States. Heart failure, a type of heart disease where the heart becomes weak and incapable of pumping blood sufficiently, causes significant morbidity and mortality. We have a better understanding of this disease thanks to the research performed in the past few decades; however, it is not yet complete. Therefore, it is essential to perform further research in order to better understand how the heart regulates its contraction during health and what goes wrong during heart failure.

Cardiac contraction is dependent on the cyclical interactions of the myosin heads with the actin filaments. This interaction drives force production and shortening, which result in pumping of the blood. The rate of this cycle, termed cross-bridge cycling rate, is an important determinant of cardiac output. Therefore, we set out to determine how this rate is regulated and disrupted in heart failure. Most of the previous studies on this parameter have used permeabilized cardiac preparations. This is a very reliable and established technique for assessing the function of the myofilaments. However, the cells in this type of preparation are de-membraned and experiments are conducted in essentially “non-living” cardiomyocytes. Furthermore, experiments are typically performed at sub-physiological temperatures. Therefore, we first developed a technique where cross-bridge cycling kinetics can be assessed in intact cardiac trabeculae under near-physiological conditions and body temperature (37 °C). By combining K⁺
contracture and rate of tension redevelopment ($k_{tr}$) protocols, we were able to reproducibly assess cross-bridge cycling rate in intact cardiac trabeculae.

One of the mechanisms that the heart uses for regulating its contraction is the well-established length-tension relationship where an increase in muscle length results in an increase in cardiac contractile tension. Some studies also show that muscle length affects cross-bridge cycling kinetics while others show lack of this relationship. These conclusions were drawn from experiments that were performed on permeabilized muscle preparations, at sub-physiological temperatures, or both. Therefore, we used our technique in order to assess the effects of muscle length on cross-bridge cycling kinetics under more relevant physiological settings. We demonstrated that cross-bridge cycling kinetics slow down as muscles are stretched in rat myocardium. This length-dependent regulation of cross-bridge cycling in rats does not necessarily mean that it also has relevance to humans. Due to the differences between the hearts of rats and humans, it was necessary to extend our studies to freshly isolated human myocardium.

Our experiments in the right ventricles of non-failing human myocardium showed a similar regulation. An increase in muscle length resulted in a decrease in cross-bridge cycling kinetics. We also performed experiments in the right ventricles of patients with end-stage heart failure, and observed a similar length-dependent regulation of cross-bridge cycling kinetics in these samples. Interestingly, the measured $k_{tr}$ values at each muscle length were similar between non-failing and failing human hearts. In order to complement these $k_{tr}$ studies, we also analyzed the parameters of twitch contraction. These measurements paralleled our $k_{tr}$ data in regards to both the effects of muscle length
on kinetics, and comparison between non-failing and failing myocardium. These results collectively show that muscle length is capable of regulating cross-bridge cycling kinetics in both non-failing and failing human myocardium when assessed in intact muscle preparations under near-physiological conditions.

It has previously been shown that muscle length is a regulator of myofilament protein phosphorylation, most notably Myosin Light Chain-2 and Troponin I. Phosphorylation of both of these proteins have been implicated in determining cross-bridge cycling kinetics. Therefore, it is plausible that muscle length regulates cross-bridge cycling kinetics, in part, via phosphorylation of contractile proteins. Our preliminary results, however, did not identify a role of phosphorylation modifications in the length-dependent regulation of cross-bridge cycling kinetics. Additionally, during the course of our studies, we observed that tension transiently overshoots in the human $k_{tr}$ tracings. These overshoots have been previously documented and investigated in other types of muscles, preparations, and species; however, none exist for intact human cardiac muscles. Therefore, we performed a series of experiments which showed that overshoots in human cardiac muscles are most likely mediated by the cross-bridges.

During the course of our studies, we also documented that twitch tensions declined considerably, in contrast to the contracture tensions, over long periods of experimentation. We determined that a decrease in sarcoplasmic calcium reticulum content is, in part, responsible for the decline in twitch tension in rat myocardium. Additional studies led us to demonstrate that the sarcoplasmic reticulum calcium content during baseline stimulation frequencies is an important determinant of the relative force-
frequency relationship. Decreasing this calcium content, results in an increase in the force-frequency relationship in rat myocardium.

The results of the experiments discussed in this work show that muscle length is an important factor in determining cross-bridge cycling rate of not only small rodents but more importantly, both non-failing and failing human myocardium. This research is, in part, novel in that all experiments used for reaching this conclusion were conducted in intact “living” muscles and at body temperature. This allows us to better understand how the heart regulates its contraction in health and disease.
DEDICATED TO MY PARENTS MARZI AND FEREYDOON
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# Table of Contents

Abstract .............................................................................................................................. ii  
Dedication .......................................................................................................................... vi  
Acknowledgments ........................................................................................................... vii  
Vita .................................................................................................................................... ix  
Publications ...................................................................................................................... ix  
Fields of Study ................................................................................................................ x  
Table of Contents ............................................................................................................ xi  
List of Tables ..................................................................................................................... xviii  
List of Figures .................................................................................................................. xix  
Chapter 1: Introduction .................................................................................................. 1  
1.1 General Introduction ............................................................................................... 1  
1.2 Cardiac Excitation and Contraction ........................................................................ 1  
1.2.1 Activation Sequence of the Heart ....................................................................... 2  
1.2.2 Action Potential .................................................................................................... 2  
1.2.3 Calcium Transient ............................................................................................... 3  

xi
1.2.4 Myofilaments................................................................................................................. 4
1.2.4.1 Myofilament Structure................................................................................................. 4
1.2.4.2 Myofilament Activation ............................................................................................... 6
1.2.4.3 The Cross-Bridge Cycle .............................................................................................. 7
1.3 Regulation of the Heart ...................................................................................................... 9
1.3.1 Frank-Starling Relationship .......................................................................................... 10
1.3.1 Force-Frequency Relationship ....................................................................................... 12
1.3.2 Parasympathetic and Sympathetic Systems ................................................................. 14
1.3.3 β-Adrenergic Receptors .............................................................................................. 14
1.4 Heart Failure .................................................................................................................... 15
1.5 Intact Cardiac Trabeculae ............................................................................................... 19
1.6 Overall Goals and Objectives ........................................................................................ 21
    1.6.1 Develop a Technique for Assessing Cross-Bridge Cycling Kinetics in Intact Cardiac Muscles ................................................................................................................. 21
    1.6.2 Determine the Effects of Muscle Length on Cross-Bridge Cycling Kinetics ........ 22
    1.6.3 Relevance of Length-Dependent Regulation of Cross-Bridge Cycling Kinetics to Non-Failing and Failing Human Myocardium ......................................................... 22
    1.6.4 Determine the Role of Phosphorylation Modifications in Length-Dependent Regulation of Cross-Bridge Cycling Kinetics ................................................................. 23
1.6.5 Investigate Tension Overshoots in Human Myocardium ........................................... 23

1.6.6 Assess the Role of SR Calcium Content at Baseline Frequency in Determining the Force-Frequency Relationship ................................................................................. 24

Chapter 2: Effect of Muscle Length on Cross-Bridge Cycling Kinetics in Intact Cardiac Trabeculae at Body Temperature .......................................................................................... 26

2.1 Introduction ................................................................................................................................. 26

2.2 Materials and Methods ............................................................................................................. 28

2.2.1 Animal Model and Trabeculae Isolation .............................................................................. 28

2.2.2 Experimental Apparatus ....................................................................................................... 29

2.2.3 Experimental Protocol .......................................................................................................... 29

2.2.4 Data Analysis and Statistics .................................................................................................. 31

2.3 Results ....................................................................................................................................... 31

2.3.1 Intact Muscle can be Used to Assess $k_{tr}$ in Rat and Rabbit Myocardium ........ 31

2.3.2 Increase in Muscle Length Increases Maximal Tension and Reduces $k_{tr}$ .......... 32

2.4 Discussion ............................................................................................................................... 33

Chapter 3: Length-Dependent Regulation and Preserved Contractile Kinetics in Right Ventricles of Failing versus Non-failing Human Myocardium ........................................ 41

3.1 Introduction ............................................................................................................................... 41

3.2 Materials and Methods ............................................................................................................ 43

xiii
3.2.1 Human Tissue Collection .............................................................................. 43
3.2.2 Trabeculae Isolation ...................................................................................... 44
3.2.3 Baseline Trabeculae Function ...................................................................... 45
3.2.4 k_tr Experiments in Intact Cardiac Trabeculae ............................................. 46
3.2.5 Statistical and Data Analysis ....................................................................... 47
3.3 Results .............................................................................................................. 48
3.3.1 Length-Tension, Force-Frequency, and β-Adrenergic Response ................. 48
3.3.2 Twitch Kinetics are Unaffected in the Failing Myocardium and Slow Down with Increasing Muscle Length ........................................................................... 48
3.3.3 k_tr is Unchanged in Failing Myocardium; Muscle Length Modulates k_tr........ 49
3.3.4 k_tr Measurements are Reproducible and Temperature Dependent ............. 51
3.4 Discussion ....................................................................................................... 52

Chapter 4: Properties of Tension Overshoot in Human Myocardium; Role of Phosphorylation Modifications in the Length-Dependent Regulation of Cross-Bridge Cycling Kinetics ................................................................................................. 70

4.1 Introduction ...................................................................................................... 70
4.2 Materials and Methods ................................................................................... 71
4.2.1 Isolation of Human Trabeculae .................................................................... 71
4.2.2 Experimental Approach for Investigating Tension Overshoots ................... 72
4.2.3 Experimental Approach for Investigating the Relationship between Phosphorylations and Length-Dependent Regulation of $k_{tr}$ .................................................. 74

4.2.4 Data and Statistical Analysis .............................................................................. 75

4.3 Results .................................................................................................................. 76

4.3.1 Effects of Temperature on Tension Overshoots ................................................. 76

4.3.2 No Tension Overshoots in Resting Muscles...................................................... 76

4.3.3 BDM Decreases Tension Overshoot Amplitudes .............................................. 77

4.3.4 Tension Overshoot Amplitudes Depend on the Relative $K^+$ Contracture Tension Levels .............................................................................................................. 78

4.3.5 Effects of Contra-length Stabilization on $k_{tr}$ ................................................... 78

4.3.6 Effects of PKA/PKCβII inhibition on $k_{tr}$ ........................................................ 80

4.4 Discussion ............................................................................................................. 81

4.4.1 Tension Overshoots in Human Myocardium...................................................... 81

4.4.2 Role of Phosphorylation Modifications in Length-Dependent Regulation of Cross-Bridge Cycling Kinetics .................................................................................. 85

Chapter 5: Cardiac Force-Frequency Relationship is Dependent on Baseline

Sarcoplasmic Reticulum Calcium Content .................................................................... 99

5.1 Introduction ........................................................................................................... 99

5.2 Materials and Methods ...................................................................................... 100

5.2.1 Isolation of Cardiac Trabeculae ....................................................................... 100
5.2.2 Force-Frequency Relationship and Post-Rest Potentiation .......................... 101
5.2.3 Rapid Cooling Contractures ................................................................. 102
5.2.4 Confocal Imaging of Trabeculae ......................................................... 103
5.2.5 Data and Statistical Analysis ................................................................. 103
5.3 Results ..................................................................................................... 104
5.3.1 Relative Force-Frequency Becomes Pronounced as Baseline Twitch Tension Decreases .......................................................... 104
5.3.2 Post-Rest Potentiation Becomes Enhanced as Twitch Tension Decreases .... 105
5.3.3 Myofilament Capability of Generating Tension is Not/Slightly Decreased... 106
5.3.4 Decline in SR Calcium Load Contributes to the Decrease in Twitch Tension ........................................................................... 106
5.3.5 Relative SERCA/NCX Activity is Not Responsible for the Decline in SR Calcium Load ................................................................. 108
5.3.6 No Change in Overall Shape of Cardiomyocytes and T-Tubules ............ 108
5.4 Discussion .............................................................................................. 108
Chapter 6: Conclusion and Future Directions.................................................. 119
6.1 Summary of Principle Findings ............................................................... 119
6.2 Implications of Findings ........................................................................ 120
6.3 Future Directions .................................................................................... 129
References........................................................................................................................................... 137
List of Tables

Table 1. Characteristics of non-failing human hearts I.................................................... 59
Table 2. Characteristics of non-failing human hearts II. ................................................. 60
Table 3. Characteristics of failing human hearts. ............................................................ 61
Table 4. Twitch characteristics of muscle utilized for $k_{ef}$ experiments. ....................... 63
Table 5. Twitch characteristics of muscle from Figure 6A. ........................................... 64
Table 6. Effects of H-89 and PKCβII inhibitor peptide I on twitch parameters.............. 96
List of Figures

Figure 1. Examples of trabeculae in ventricles of animal models and humans. .......... 25

Figure 2. Tracings of K+ contracture and $k_{tr}$ protocols performed in intact rat trabeculae.
 ............................................................................................................................................................................ 37

Figure 3. The $k_{tr}$ protocol can be performed in intact rabbit myocardium ................ 38

Figure 4. Increasing muscle length results in an increase of twitch force and prolongation
of twitch kinetics ........................................................................................................................................................ 39

Figure 5. Increase in muscle length decreases $k_{tr}$ in intact rat trabeculae ................ 40

Figure 6. Length-tension relationship, force-frequency relationship, and β-adrenergic
stimulation in non-failing vs. failing hearts ........................................................................................................... 62

Figure 7. Typical twitch tracing of a human right ventricular trabeculae. ............... 65

Figure 8. K+ contracture and $k_{tr}$ tracings in intact human cardiac trabeculae ........ 66

Figure 9. There is no difference in $k_{tr,max}$ during maximal activation between non-failing
and failing hearts ..................................................................................................................................................... 67

Figure 10. No significant differences in $k_{tr,max}$ among etiologies of heart failure .. 68

Figure 11. Sub-maximal $k_{tr}$ values are not different between non-failing and failing
samples at each respective muscle length ........................................................................................................... 69

Figure 12. Schematic definitions of the terms used for tension overshoot ................ 89

Figure 13. Temperature affects kinetics of tension overshoot .................................. 90
Figure 14. No tension overshoots without contracture or stimulation................................ 91

Figure 15. High resting tension is not sufficient to cause tension overshoot in a resting muscle. .................................................................................................................................................. 92

Figure 16. BDM decreases magnitude of tension overshoot. .............................................. 93

Figure 17. Relationship between tension overshoot amplitude and relative K\(^+\) contracture tension. .................................................................................................................................................. 94

Figure 18. Effects of contra-length stabilization on \(k_{fr} \). .................................................. 95

Figure 19. PKA/PKC\(\beta\)II inhibitors had no effect on \(k_{fr,max} \). .................................... 97

Figure 20. Effect of PKA/PKC\(\beta\)II inhibitors on \(k_{fr} \) during sub-maximal K\(^+\) contracture tensions. .................................................................................................................................................. 98

Figure 21. FFR becomes more positive with decreasing twitch tension. ......................... 114

Figure 22. PRP becomes pronounced with declining twitch tension............................. 115

Figure 23. Decrease in SR calcium load is a contributing factor for the decline of developed twitch tension............................................................................................................... 116

Figure 24. Decline in relative SERCA/NCX activity is not responsible for a decrease in twitch tension................................................................................................................................. 117

Figure 25. No change in cardiomyocyte shape or t-tubules after long period (90 minutes) of isometric contraction. ........................................................................................................ 118

Figure 26. Proposed model of the reduction in cross-bridge cycling kinetics due to increasing muscle length during heart failure in an intact heart. ................................. 136
Chapter 1: Introduction

1.1 General Introduction

Heart failure, a type of heart disease where the heart cannot maintain cardiac output, accounts for approximately 58,000 deaths per year\(^1\). Further research is required in order to better understand how the heart regulates its own contraction, and alterations that occur during heart failure. One aspect of cardiac contraction that can regulate cardiac output is the kinetics of the interaction between myosin and actin. Therefore, it is essential to examine the factors that regulate this kinetic parameter in health and how they are disrupted in heart failure. Another important factor is the relationship between heart rate and contractile force, known as the force-frequency relationship. The mechanisms that contribute to this relationship are mostly delineated, but additional factors that are involved need to be investigated. The following is an introduction to the mechanisms of cardiac contraction, regulation, and abnormalities in heart failure.

1.2 Cardiac Excitation and Contraction

Cardiac contraction begins with an electrical stimulation generated by the heart. Through highly controlled and regulated processes, the heart converts this electrical activity into mechanical energy. The following is a discussion of these events.
1.2.1 Activation Sequence of the Heart

The sequence of cardiac depolarization typically begins at the sino-atrial (SA) node, which is located in the right atrium. The action potential (AP) produced by these cells spreads throughout the atria, which is detected as the P wave on an electrocardiogram (ECG). The AP then spreads to the atrio-ventricular (AV) node. The conduction speed of the AV node is relatively slow, which results in a brief pause between atrial and ventricular depolarizations. This separation, detected as the P-R interval on ECG tracings, is necessary in order to ensure enough time for atrial contraction and proper ventricular filling. The AP then travels at faster speeds through the Bundle of His, left and right bundle branches, and Purkinje fiber system. This ultimately results in spread of the AP throughout the left and right ventricles. The general pattern of ventricular depolarization is from apex to base and from endocardium to epicardium. The ventricular depolarization and repolarization are detected as the QRS complex and T wave, respectively on ECG tracings.

1.2.2 Action Potential

The shape, duration, and underlying mechanisms of the AP can vary depending on cell type and location. For a detailed discussion see. The ventricular AP will be discussed here briefly for thoroughness. During the resting state, or Phase 4, the membrane potential is determined by $I_{K1}$. In Phase 0, the activation of the inward $I_{Na}$ causes a rapid membrane depolarization. This is followed by an initial early repolarization with the activation of outward $I_{to}$ and $I_{Cl}$ as well as inactivation of $I_{Na}$.
(Phase 1). The plateau stage, Phase 2, occurs when the inward $I_{Ca,L}$ is balanced by the outward $I_{Ks}$, $I_{Kr}$, and $I_{Kur}$. Phase 3 is rapid repolarization which involves $I_{Kr}$. After the rapid repolarization, the cell returns to the resting membrane potential.

### 1.2.3 Calcium Transient

At the level of ventricular cardiomyocytes, the AP travels down the transverse tubules (t-tubules), which are invaginations of the cellular membranes near the sarcomeric Z-lines. Depolarization results in opening of the L-Type calcium channels (LTCC) during Phase 2 of the AP and results in an influx of extracellular calcium into the cell. This calcium binds to and opens the ryanodine receptors (RyR) located on the surface of the sarcoplasmic reticulum (SR). The opening of the RyR releases calcium from the SR, which is higher in quantity than the calcium entering the cell through LTCCs. The combination of calcium entering the cell through LTCCs and the SR increases the intracellular calcium concentration. Calcium binds to troponin C, the calcium binding subunit of the troponin complex, and activates the myofilaments. This will be discussed further in the next section.

Calcium is removed from the cytosol by multiple calcium-handling mechanisms. The majority of calcium (~92% in mice and rats; ~70% in large mammals and humans) is pumped backed into the SR by the sarcoplasmic-reticulum-calcium-ATPase (SERCA). The sodium-calcium exchanger (NCX) is the primary mechanism by which the cell extrudes calcium (~7% in mice and rats; ~28% in large mammals and humans of total calcium) back to the extracellular space. The sarcolemmal calcium ATPase and
mitochondrial calcium uniport systems cycle a minor fraction of the calcium (~ 1%) to the extracellular space and mitochondria, respectively. The relative contributions of SERCA and NCX, the two main calcium removal mechanisms, to the decline in intracellular calcium concentration can also vary based on the health status of the myocardium.

1.2.4 Myofilaments

The combination of electrical excitation of the heart and the subsequent calcium transient ultimately results in cardiac contraction by the myofilaments. The following is a discussion of the structure of the myofilaments, their activation, and the cross-bridge cycle.

1.2.4.1 Myofilament Structure

The myofilaments are the contractile apparatus of the cardiomyocytes and are a major component of the cardiomyocytes as they take up 45-60% of the cellular volume. The myofilaments are arranged into sarcomeres which based on appearances of the thick and thin filaments can be divided into different sections. The entire length of the thick filaments is defined as the A-band, and the segment of the sarcomere where there are only thin filaments and no thick filaments is defined as the I-band. The H-zone, located towards the center, is the section of the thick filament where there are no thin filaments. The M-line and Z-lines are located in the center and outer boundaries of the sarcomere,
respectively. During isotonic cardiac contraction the length of the A-band does not change; however, the H-zone and I-band shorten \(^5\).

The thick filament is composed of various proteins most notably: 1) titin which spans from the Z-line to the M-line and is the primary determinant of resting tension within the physiological sarcomere lengths \(^8\); 2) myosin binding protein-C (MyBP-C), which is found exclusively in segments of the thick filament defined as the C-zone and interacts with myosin and titin \(^9\); and 3) myosin hexameric complex, which is composed of two myosin heavy chains (MHC), two essential light chains (ELC/MLC-1), and two regulatory light chains (RLC/MLC-2) \(^10\).

Each MHC molecule has a globular motor domain on its N-terminus which contains the actin binding site and the ATP binding site. A converter domain links this globular motor domain to the lever arm where MLC-1 (closer to the N-terminus of MHC) and MLC-2 (closer to the C-terminus of MHC) bind. The lever arm is followed by the alpha helical tail \(^11,12\). MyBP-C binds to MHC at two sites: one in the tail that forms the thick filament backbone (light meromyosin) and another near the lever arm (S2 subfragment of heavy meromyosin) \(^9\).

The thin filament is composed of several proteins: 1) actin double helix filament which contains the myosin binding sites; 2) a troponyosin coiled-coil dimer which binds to seven actin proteins and overlaps with the neighboring tropomyosins; and 3) the troponin complex which is composed of troponin T (TnT), which binds the troponin complex to tropomyosin, troponin I (TnI), which is the inhibitory subunit, and troponin C
(TnC), which is capable of binding to calcium. A single thin filament regulatory unit contains 7 actins, 1 tropomyosin dimer, and 1 troponin complex 13.

Cardiac myofilament proteins such as MHC 14,15, MLC-1 11, and titin 8 can have multiple isoforms with different properties. Additionally, myofilament activity can be modulated by phosphorylation of some of the myofilament proteins such as titin 8, TnI 16,17, MLC-2 18, TnT 19, and MyBP-C 9. The number of phosphorylation sites varies from protein to protein. For example, human MLC-2 has only one phosphorylation site 18 while others, such as TnI, have several phosphorylation sites 16,17.

1.2.4.2 Myofilament Activation

The thin filament exists in different conformational states during systole and diastole. Based on a three state model, in the absence of calcium, the thin filament is in blocked “B” state where the binding of myosin to actin is sterically blocked by tropomyosin 20. Tropomyosin is kept in this position by the TnI actin binding sites, on one of its sides, and by the TnT N-terminus of the Tn complex of the opposite actin filament on the other side 21. In activated cardiomyocytes, intracellular calcium concentration rises due to opening of LTCC and release of SR calcium content 7, as previously described. Calcium binds to the EF hand site II on the N-terminus of TnC, and this exposes a hydrophobic pocket on TnC that attracts the switch peptide of TnI 21,22. This structural change causes the TnI’s actin binding sites to move away from their interaction with actin. This allows tropomyosin to move away from the periphery towards the groove of the actin filaments, exposing the myosin binding sites on actin. Myosin is
now able to form weak cross-bridges with actin, and the thin filament is now in a closed “C” state. The formation of strong cross-bridges causes tropomyosin to further slide on the actin filament and extend the activation to the neighboring thin filament regulatory units. This is known as the open/strong myosin binding “M” state \(^{20,21,23}\).

1.2.4.3 The Cross-Bridge Cycle

Following thin filament activation, myosin attaches and detaches from the actin filament in a cyclical manner. Myosin is an ATPase molecule capable of converting the chemical energy of adenosine triphosphate (ATP) into mechanical energy. The ATPase activity of myosin is responsible for the majority of ATP consumption in cardiomyocytes \(^{24}\). Myosin, when bound to adenosine diphosphate (ADP) and inorganic phosphate (P\(_i\)), initially binds weakly to actin. This is followed by an isomerization step, resulting in strong cross-bridge formation. Next the P\(_i\) is released, resulting in the power stroke step. The lever arm rotates by about \(\sim 65-75\) degrees around the converter domain. Myosin then undergoes isomerization, followed by the release of ADP. Binding of ATP to myosin induces detachment of myosin from actin. Myosin hydrolyzes the ATP molecule into ADP and P\(_i\), and the myosin is ready to complete another round of this cycle \(^{10,12,23}\).

It is estimated that each cross-bridge cycle causes shortening of \(\sim 5-10\) nm \(^{5,10,12}\). The rate limiting step of this cycle during isometric conditions is considered to be the release of ADP or isomerization step following P\(_i\) release. The release of P\(_i\) has been suggested to be the rate limiting step during shortening, for example, during the ejection phase of the cardiac cycle \(^{10,23,25}\). It should be noted that myofilaments are not maximally
activated during the cardiac cycle, rather the amount of calcium released is sufficient to activate only a portion of the thin filament regulatory units. This allows only a fraction (estimated 20-25%) of the myosin heads to enter the cross-bridge cycle 25.

The rate of the cross-bridge cycle has important cardiovascular implications. The amount of work that is performed by the heart over time (i.e. power output of the heart) is the product of force and velocity. Therefore, both the rate of the cross-bridge cycle and the number of cross-bridges that are generating force determine the amount of blood pumped by the heart 10,26,27. Consequently, a decrease in the cross-bridge cycling rate causes a decline in cardiac output. As discussed later under the “Heart Failure” section, some studies, but not all, indicate that cross-bridge cycling rate is reduced in heart failure.

Multiple factors can affect the rate of the cross-bridge cycle. One factor is the MHC isoform. There are two isoforms of this protein in the heart: β-MHC and α-MHC. Most mammalian species express both MHC isoforms, though relative expression levels vary depending on the species and health status of the myocardium. Small animals (i.e. mouse and rat) and large animals (i.e. rabbit, canine, and human) predominately express α-MHC and β-MHC, respectively 28. These two isoforms are 93% homologous at the amino acid level 29; however β-MHC has a significantly lower ATPase rate and slower contractile kinetics 30-32. Therefore, significant up-regulation or down-regulation of these isoforms can affect cross-bridge cycling kinetics.

Another factor that can regulate the rate of the cross-bridge cycle is phosphorylation of myofilament proteins. Some studies have shown that activation of the β-adrenergic pathway accelerates cross-bridge kinetics 33-36. The β-adrenergic pathway
results in phosphorylation of myofilament proteins, one of which is TnI at Ser23/24. Phosphorylation of TnI has been implicated in acceleration of cross-bridge cycling kinetics in some studies, but not all. It should be noted that some studies showed a complete lack of effect of the β-adrenergic pathway on cross-bridge cycling kinetics. Phosphorylation of MyBP-C has also been shown to be involved in increasing cross-bridge cycling kinetics. A third myofilament protein phosphorylation that can regulate cross-bridge cycling kinetics is MLC-2, which has been shown to accelerate kinetics of the cross-bridge cycle, although one study did not report this effect.

The cross-bridge cycle is highly dependent on temperature, where an increase in temperature results in an increase in the kinetics with a Q of ~2-3. Increasing calcium concentration has also been shown to increase cross-bridge cycle kinetics; however, some studies have shown that this relationship does not exist. Lastly, muscle length can potentially affect cross-bridge cycling kinetics. This will be discussed separately under the discussion of the Frank-Starling Relationship.

1.3 Regulation of the Heart

The function of the heart is to meet the metabolic demands of the body. These demands change regularly throughout the day and the heart needs to adjust its pumping activity accordingly. The following are some of the regulatory mechanisms that the heart and the body use in order to fine tune cardiac output.
1.3.1 Frank-Starling Relationship

The Frank-Starling relationship is one of the regulatory mechanisms of the heart where an increase blood volume in the heart stretches the cardiomyocytes and results in an increase in stroke volume. This relationship can be attributed to an increase in overlap between thick and thin filaments, but more importantly, to an increase in myofilament calcium sensitivity, which will result in a more forceful contraction for the same amount of calcium\textsuperscript{60,61}. Multiple mechanisms can be potentially involved in this increased myofilament calcium sensitivity. For a detailed discussion of these please see these referenced reviews\textsuperscript{60,62}.

The relationship between cardiac muscle length and contractile force is well established; however, the role of muscle length in regulating cross-bridge cycling kinetics is less understood. Several studies have provided mixed results in addressing the question of whether there is a relationship between muscle length and cross-bridge cycling kinetics. Some of these studies show that increasing muscle length decreases cross-bridge cycling rate, while others do not observe any relationship between these two\textsuperscript{56,59,63-68}. The majority of the studies, with only one exception\textsuperscript{59}, were performed in permeabilized preparations. Furthermore, all of these studies were carried out under sub-physiological temperatures. As discussed previously, temperature greatly affects cross-bridge cycling kinetics, and, therefore, it is essential to conduct experiments at body temperature (37 °C). Additionally, all of these studies, with exception of one\textsuperscript{63}, were performed in animal models including mouse, rat, ferret, and, porcine. The only study on humans by Edes et al. was performed on only control human donors (no disease group), low in sample size.
(n = 3), and experimented on permeabilized preparations at sub-physiological temperatures. There is currently a gap in knowledge in regards to the effects of muscle length on cardiac cross-bridge cycling kinetics in intact human preparations performed under near-physiological conditions.

A group of investigators have proposed a model that can explain the mechanisms underlying the relationship between muscle length and cross-bridge cycling kinetics. Titin is the main determinant of the passive force within the physiological sarcomere length, and it becomes stretched as the length of cardiomyocyte is increased. It has been proposed that at longer muscle lengths, titin, through its interaction with MyBP-C, can restrict the movement of myosin and decrease the cross-bridge cycling rate. Consistent with this idea, in MyBP-C deficient mice, myosin is further away from the thick filament backbone and cross-bridge cycling rate is accelerated.

Muscle length is also capable of regulating phosphorylation modifications. Increasing diastolic ventricular pressure in isolated rat heart has been shown to increase and decrease MLC-2 phosphorylation in the epicardium and endocardium, respectively. Increasing muscle length has also been shown to increase MLC-2 phosphorylation in human atrial preparations. Additionally, stretching rabbit ventricular trabeculae has been shown to induce phosphorylation of myofilament proteins MLC-2 and TnI at Ser23/24. Furthermore, Protein Kinase A (PKA) and Protein Kinase C-βII (PKCβII) have been implicated as the kinases responsible for these modifications. Studies suggest that MLC-2 and TnI Ser23/24 can potentially regulate cross-bridge cycling kinetics, as discussed previously. Therefore, it is plausible that muscle length...
can, in fact, regulate cross-bridge cycling kinetics via phosphorylation modifications. As noted earlier, most studies used permeabilized preparations for investigating the effects of muscle length on cross-bridge cycling kinetics. This type of preparation has disrupted pathways and machinery, kinases and phosphatases, responsible for regulating phosphorylation modifications. Performing experiments on intact preparations under physiologically relevant settings can be of great advantage in determining the role of muscle length in regulating cross-bridge cycling kinetics.

1.3.1 Force-Frequency Relationship

The force-frequency relationship (FFR), otherwise known as the Bowditch effect, states that the frequency of stimulation can regulate the force of cardiac contraction. Kinetics of contraction and relaxation are also accelerated with increasing stimulation frequencies \(^{77,78}\). Acceleration of these kinetic parameters are necessary to allow for complete cardiac contraction and relaxation before the next stimulation at high heart rates, and assures proper ejection of the blood during systole and filling of the ventricles with blood during diastole.

The degree and magnitude of the FFR can vary depending on the species. The FFR of small animals is limited; rat cardiac contractile force increases only slightly (i.e. slight positive FFR) and the contractile force of the mouse barely changes (i.e. flat FFR) within their respective physiological frequency ranges. On the other hand, large animals, such as rabbits and canines, and humans have a strong positive FFR where the force of
contraction can dramatically increase within their respective physiological frequency ranges \(^{28,77}\).

Multiple mechanisms contribute to the FFR in the mammalian myocardium. Increasing stimulation frequency causes more calcium to enter the cardiomyocytes per unit of time. SERCA uptakes some of this calcium into the SR, causing an increase in SR calcium content. This increases the amount of calcium available for release during subsequent stimulations. Additionally, the increased calcium activates Calcium-Calmodulin-Dependent Protein Kinase II (CaMKII), which phosphorylates phospholamban (PLB) on its Thr 17 site. This phosphorylation removes phospholamban’s inhibitory effect on SERCA, which further enhances SERCA activity and increases SR calcium content. Furthermore, there is an increase in intracellular sodium concentration at high stimulation frequencies. This limits the forward mode of NCX and calcium efflux while enhancing reverse mode of NCX and calcium influx. These mechanisms collectively result in both an increase in SR calcium content and calcium transient and, therefore, a greater myofilament activation and force of contraction \(^{77,79,80}\). Determination of other factors that can contribute to FFR is essential for better understanding this relationship in health and its alterations in disease. As discussed earlier, relaxation kinetics also become accelerated with increasing frequency which has been attributed to: 1) enhanced SERCA activity \(^{81}\); and 2) myofilament calcium desensitization \(^{82}\). For an in depth discussion of these two mechanisms see this review \(^{77}\).
1.3.2 Parasympathetic and Sympathetic Systems

The autonomic nervous system is responsible for controlling the involuntary activities of the body including the heart. This is in addition to the regulation of the vasculature by the autonomic nervous system which will not be discussed here. The parasympathetic system directly causes a decrease in heart rate (negative chronotropy) and conduction velocity (negative dromotropy) \(^{83,84}\). The activation of the sympathetic nervous system results in increased contractility (positive inotropy), enhanced relaxation (positive lusitropy), increased heart rate (positive chronotropy), and increased conduction velocity (positive dromotropy) \(^{83,84}\). While both α- and β-adrenergic receptors of the heart are activated by the sympathetic nervous system, β-receptors are the main mediators of the positive inotropic response \(^5\). Therefore, the role of these receptors in cardiac function will be discussed in more detail.

1.3.3 β-Adrenergic Receptors

Three subtypes of the β-adrenergic receptor family are expressed in the heart: β1, β2, and β3. The β1-subtype is the most common (≈75-80% of all the β-adrenergic receptors) and is the main modulator of β-adrenergic activity in the heart. β2- and β3-receptors account for ≈15-18% and ≈2-3% of all the β-adrenergic receptors in the heart, respectively \(^{83}\).

Stimulation of the β1-adrenergic receptors results in activation of adenylyl cyclase, which produces cyclic AMP (cAMP) from ATP \(^{83}\) and cAMP, in turn, activates PKA. The phosphorylation of multiple target proteins by PKA alters various cellular
processes. Some of these include phosphorylation of LTCC, which increases $I_{\text{Ca}}$, RyR2 at Ser2809, resulting in increased opening probability, PLB at Ser16, which relieves its inhibition on SERCA, and TnI at Ser23/24, resulting in a decrease in myofilament calcium sensitivity. Collectively, these modifications result in positive inotropic and lusitropic effects in ventricular cardiomyocytes. PKA also phosphorylates titin’s N2B element, resulting in decreased resting tension, and MyBP-C’s M-domain, which accelerates cross-bridge cycling kinetics. While β1-receptors have a diffuse effect throughout the cell, the effects of β2-receptor activation are mainly localized to targets in the vicinity of the receptor, mostly the LTCC. β3-receptors, as opposed to β1- and β2-receptors, cause a decrease in contractility.

β-adrenergic receptors undergo desensitization and/or downregulation in response to sustained and prolonged stimulation, such as in heart failure where there is an increase in concentration of circulating catecholamines. This involves phosphorylation of the receptors by G-protein-coupled-receptor kinases (GRKs) which promote interaction of β-arrestin with the receptors. β-arrestin sterically blocks binding of G-proteins to the receptors resulting in an inability of the receptors to function. Clathrin proteins can also interact with β-arrestin on the receptors and induce receptor internalization.

1.4 Heart Failure

Heart failure can be defined as the inability of the heart to pump adequate amount of blood to meet the metabolic demands of the body. This complex disorder currently affects more than 5 million patients in the United States with estimated projections to
reach 8 million patients by the year 2030. The myocardium undergoes extensive remodeling during heart failure. Discussion of all the changes that occur in heart failure is beyond the scope of this work; however, for more information see the following reviews. Two particular alterations that occur during heart failure in humans will be briefly discussed here as they will be assessed in Chapter 3. First, the positive FFR is converted into a flat or negative FFR due to alterations in calcium handling mechanisms. Additionally, the β-adrenergic response is desensitized due to the mechanisms discussed previously in the “β-Adrenergic Stimulation” section. The research in this thesis, however, focuses mainly on potential alterations in the cross-bridge cycling kinetics, which will be discussed next.

As mentioned previously, the rate of the cross-bridge cycle is an important determinant of cardiac output, and a decline in this rate can contribute to the decreased cardiac output in heart failure. Some of the previous studies show that the cross-bridge cycling rate is reduced in heart failure. It is necessary to focus on studies that were performed on human myocardium due to differences between hearts of humans and animal models, particularly small animal models such as mouse and rat. Two studies have shown that cross-bridge cycling kinetics are reduced in patients with end-stage idiopathic dilated cardiomyopathy by ~43-46% (unclassified NYHA stage) and ~29% (NYHA III-IV). Another group showed that the force-time integral of the cross-bridge was increased by 33% in patients with end-stage (NYHA IV) dilated cardiomyopathy (5 out of 6 samples were idiopathic) and 85% in patients with left ventricular volume-overload (NYHA III-IV). It should be noted that the primary cause of heart failure in
the United States is ischemic cardiomyopathy, however, none of these studies included samples from this type of cardiomyopathy. Despite these conclusions that cross-bridge cycling rate is reduced in human heart failure, there are other studies that disagree as they did not detect any significant differences between cross-bridge cycling rates in non-failing vs. failing samples. One study reported results from a combination of both patients with end-stage (NYHA IV) dilated and ischemic cardiomyopathy. Another study used only end-stage (NYHA IV) dilated cardiomyopathy. There were no significant differences in the study by Wijnker et al. in either dilated cardiomyopathy or ischemic cardiomyopathy (stage classified in only a portion of samples; mostly NYHA III-IV). Another group utilized samples from patients with (NYHA II-III) mitral valve regurgitation. Thus, these inconsistencies warrant further research in order to better characterize changes in the cross-bridge cycling rate in human heart failure.

So if cross-bridge cycling rate is reduced in human heart failure, what is the underlying mechanism? One possible explanation is isoform switching from the fast α-MHC to slow β-MHC. This isoform switching has been observed in animal models as well as humans during heart failure. However, the degree and importance of this isoform switching on alteration of cross-bridge cycling rate is dependent on the species. The cardiac ventricles of small animal models, such as mice and rats, predominately express α-MHC (>94-100%) which can be dramatically down-regulated during heart failure in these animals. In human ventricles, α-MHC accounts for only ~3-7% of the total MHC proteins. Even if all of the α-MHC is replaced by β-
MHC in human heart failure, the effects of this isoform switching on cross-bridge cycling kinetics, if any, are very limited \(^{28,115-117}\).

Another possible mechanism for the reduction of cross-bridge cycling kinetics is that alterations in phosphorylation modifications of contractile proteins can affect cross-bridge cycling kinetics in heart failure \(^{115,118}\). Some of these alterations include a decrease in the phosphorylation of TnI at Ser23/24 \(^{119}\), MyBP-C \(^{120-122}\), and MLC-2 \(^{58,123}\) in patients with heart failure. As discussed previously, these phosphorylations can accelerate cross-bridge cycling kinetics. If cross-bridge cycling kinetics are decreased in heart failure, a decrease in the phosphorylation of these proteins can play an integral role in this dysfunction. It should be cautioned that donor non-failing human tissues used in some of these studies, may possibly have artificially higher than normal levels of phosphorylation of specific sites that are targeted by PKA. This is due to use of inotropic agents during the organ procurement process and a surge of catecholamines in donors with brain damage \(^{28,117,124}\).

Another aspect of myocardial contraction that can be potentially altered in heart failure is the regulation of cross-bridge cycling kinetics by muscle length. All previous studies that have compared cross-bridge cycling kinetics in non-failing and failing human myocardium were performed at constant length \(^{54,58,102-104,106-108}\). As previously discussed, cross-bridge cycling kinetics can be dependent on muscle length (see “Frank-Starling Relationship” section). Two lines of evidence suggest the possibility that the relationship between muscle length and cross-bridge cycling rate can be altered during heart failure. As discussed earlier, a model proposed by the McDonald group implicates titin as the
underlying mechanism of the length-dependent regulation of cross-bridge cycling kinetics. The compliant N2BA titin isoform is up-regulated while the stiff N2B titin isoform is down-regulated in human heart failure. Another consideration is phosphorylation of myofilament proteins in particular TnI at Ser23/24 and MLC-2. Phosphorylation of these two sites 1) are regulated by muscle length; 2) can affect cross-bridge cycling kinetics; and 3) are decreased in heart failure. Therefore, it is conceivable that the regulation of cross-bridge cycling kinetics by muscle length is altered in heart failure. However, there are no studies to date which have compared the role of muscle length in regulating cross-bridge cycling kinetics in non-failing vs. failing human myocardium.

1.5 Intact Cardiac Trabeculae

It is beneficial to study cardiac contraction under conditions that are similar to the physiological conditions of the body. This is particularly important when dissecting the alterations in cross-bridge cycling kinetics in patients with heart failure. As discussed, phosphorylations can regulate cross-bridge cycling kinetics and they are altered in heart failure. However, the majority of studies (not including the force-time integral studies) that have directly measured cross-bridge cycling kinetics in human myocardium, with one exception, were performed in permeabilized muscle preparations. This process uses a detergent to remove the cellular membrane and membrane bound organelles in order to study the myofilaments. This is a reliable and regularly used technique which provides valuable information on myofilament contraction. However, permeabilization
disrupts the phosphorylation modification pathways in these preparations. Additionally, temperature is a powerful modulator of cross-bridge cycling kinetics. Previous studies that directly assessed cross-bridge cycling kinetics in human myocardium, with one exception \(^{106}\), were typically conducted at sub-physiological temperatures. It is essential to have a system where experiments are performed at body temperature (37 °C), under physiological conditions, and in a setting where phosphorylation modifications can be regulated by the cardiomyocytes themselves. The intact cardiac trabeculae system can fulfill these requirements. Please see Figure 1 depicting examples of intact cardiac trabeculae isolated from the right ventricles of commonly used animal models and humans.

The intact cardiac trabeculae model, like any other model, has some advantages and disadvantages. These preparations are composed of cardiomyocytes, fibroblasts, endothelial cells, and extracellular collagen and matrix. The cardiomyocytes are arranged linearly; therefore, all the cardiomyocytes produce force in the same direction. The multicellular nature of cardiac trabeculae allows investigations on a population of cardiomyocytes that are connected to the extracellular matrix and integrated with each other. The intact nature of these preparations preserves the phosphorylation modification machinery of the cardiomyocytes, as opposed to permeabilized preparations, which allows modifications to occur and be fully regulated by the cardiomyocytes themselves. Furthermore, muscles can be perfused with a solution that is similar to the extra-cellular fluid, and experiments can easily be performed at the physiological body temperature of 37 °C. Additionally, stabilization of non-failing human trabeculae can, theoretically,
reverse the effects of catecholamines that occur during procurement of these samples. Cardiac trabeculae also have some disadvantages that must be considered. Based on their location on the luminal side of the ventricles, they are essentially part of the sub-endocardium. It is expected that cardiac trabeculae should be more similar to the sub-endocardium than the sub-epicardium. Several reports have established functional and molecular transmural patterns from the sub-epicardium to the sub-endocardial regions\textsuperscript{127-133}. Additionally, the states of phosphorylation modifications in these samples do not necessarily replicate those of the heart \textit{in vivo}. Caution is necessary when comparing studies in such isolated systems to the whole heart or the entire cardiovascular system \textit{in vivo}. There is also a concern that hypoxia can develop in the center regions of these multicellular preparations. In an intact heart, the extensive coronary vasculature is responsible for supplying nutrients and removing waste products from the myocardium. However, the isolated cardiac trabeculae are dependent on diffusion which can result in hypoxia to occur in the inner core of the preparations. This can be circumvented by obtaining small preparations that allow proper diffusion and cardiac function\textsuperscript{134,135}.

\section*{1.6 Overall Goals and Objectives}
\subsection*{1.6.1 Develop a Technique for Assessing Cross-Bridge Cycling Kinetics in Intact Cardiac Muscles}

The overall goal of this project was to determine how does muscle length regulate cross-bridge cycling rate and if this regulation becomes dysfunctional in heart failure. It was first necessary to develop a technique were cross-bridge cycling rate can be assessed
in intact cardiac preparations under near-physiological conditions. Therefore, we first set out to develop a method for measuring rate of tension redevelopment ($k_{tr}$), an index of cross-bridge cycling rate, in intact rat cardiac trabeculae.

### 1.6.2 Determine the Effects of Muscle Length on Cross-Bridge Cycling Kinetics

Muscle length can potentially regulate cross-bridge cycling rate; however, this relationship is debated. Most of the previous studies were performed in permeabilized muscle preparations and/or under sub-physiological temperatures. Therefore, we utilized our technique in order to determine the relationship between muscle length and cross-bridge cycling rate in intact rat cardiac trabeculae at body temperature.

### 1.6.3 Relevance of Length-Dependent Regulation of Cross-Bridge Cycling Kinetics to Non-Failing and Failing Human Myocardium

There are various differences between the hearts of rats and humans. Hence, studies on rats cannot be directly taken to have relevance to humans. Therefore, it was necessary to assess the effects of muscle length on cross-bridge cycling kinetics in non-failing human hearts. We also extended our studies to patients with end-stage heart failure to assess alterations in cross-bridge cycling rate and its regulation by muscle length. To our knowledge, this is the first study to have ever compared the effects of muscle length on cross-bridge cycling kinetics in non-failing vs. failing human hearts. Moreover, our experiments were conducted in intact cardiac trabeculae and at body temperature (37 °C). This is a novel aspect as compared to previous investigations into
alterations in cross-bridge cycling rate in patients with heart failure which were typically performed either in permeabilized preparations, sub-physiological temperatures, or both.

1.6.4 Determine the Role of Phosphorylation Modifications in Length-Dependent Regulation of Cross-Bridge Cycling Kinetics

Muscle length can regulate the phosphorylation of some contractile proteins involved in determining cross-bridge cycling kinetics. Therefore, one of the mechanisms that muscle length can regulate cross-bridge cycling kinetics is via phosphorylation modifications of myofilament proteins. We set out to perform experiments in order to determine the role of myofilament protein phosphorylation induced by muscle length in regulation of cross-bridge cycling kinetics in both non-failing and failing human myocardium.

1.6.5 Investigate Tension Overshoots in Human Myocardium

We routinely observed tension overshoots following our $k_w$ protocol in human myocardium. While tension overshoots have been previously investigated in permeabilized rat soleus muscles and permeabilized rat cardiac myocytes; there are no reports documenting or investigating these overshoots in intact human myocardium. Therefore, we performed several experiments in order to determine the characteristics of these overshoots and whether they are mediated by the muscles themselves or an experimental artifact of our protocol.
1.6.6 Assess the Role of SR Calcium Content at Baseline Frequency in Determining the Force-Frequency Relationship

We observed that there is a dissociation between the tensions developed during stimulated twitches and \( K^+ \) contractures over time in our studies. The twitch tension decreased over time while the \( K^+ \) contracture tension remained unchanged or only slightly decreased. Investigations into this dissociation led us to show that at least one factor responsible for a reduced twitch tension is a decrease in SR calcium content. We set out to determine the effects of this altered SR calcium content on the relative force-frequency relationship.
Figure 1. Examples of trabeculae in ventricles of animal models and humans.
A-D: Black arrows indicate cardiac trabeculae in the right ventricles of various animal models and humans. RA: right atria, RV: right ventricle, LV: left ventricle. E-H: Right ventricular trabeculae were isolated and mounted on the custom made setup via two hooks on the right to a force transducer and another pair of hooks on the left to a linear motor.
2.1 Introduction

The main function of the heart is to pump blood to meet the demands of the body. This pumping activity depends on cardiac muscle contraction, which, in turn, depends on the interaction of sarcomeric thick and thin filaments, which form cross-bridges that generate force. Consequently, the pumping capability of the heart is determined by the number of cross-bridges capable of generating force and the rate at which they cycle through unbound, weakly-bound, and strongly-bound (force-generating) states. Therefore, alterations in either of these two factors can affect cardiac function.

The Frank-Starling law of the heart—as ventricular volume (corresponding to muscle length) increases, the heart intrinsically strengthens—describes a well-known cardiac regulatory mechanism. Although increased muscle length generally results in improved force development, in parallel with a prolonged time to peak (TTP) force and a slowing of relaxation time, it is unclear whether changes in muscle length per se affect cross-bridge kinetics. Some previous studies, found that cross-bridge cycling kinetics decreased with increased sarcomere length, whereas others found that sarcomere length has no effect on the rate of cross-bridge cycling. These previous studies, however, were performed using permeabilized cardiac preparations, sub-
physiological temperatures, or both. Data obtained under physiological temperature and in intact muscle preparations might help resolve this discrepancy and clarify the effects of muscle length on cross-bridge kinetics.

Various laboratory techniques have been used to study cross-bridge cycling, including Edman’s slack test, actomyosin ATPase activity, rate of tension redevelopment, and sinusoidal perturbation \(54,56,138\). Of these, the rate of tension redevelopment \(k_{tr}\) has been the most widely adopted approach. This technique assesses the rate at which force redevelops after a rapid slack-stretch maneuver has disconnected all crossbridges. The \(k_{tr}\) protocol has been used by many investigators and has provided valuable information with regard to quantifying the kinetic steps in thick and thin filament interactions. However, intact cardiac preparations do not normally produce tetanic (fused) contractions even at very high stimulation rates \(^{139}\). This makes measuring \(k_{tr}\), which requires a stable level of \(\text{Ca}^{2+}\) activation, very difficult. The few studies that measured \(k_{tr}\) in preparations with intact membranes \(^{59,140,141}\) combined high frequency stimulation with irreversible SR poisoning (using cyclopiazonic acid or ryanodine) to maintain stable \(\text{Ca}^{2+}\) concentrations, an approach that is constrained to low (non-physiological) temperatures. Our goal here was to design a protocol that allows repeated assessment of \(k_{u}\) in intact cardiac trabeculae at physiological body temperature. We found that this could be done reliably and reproducibly by using \(\text{K}^+\) contractures, a technique that leads depolarization of the muscle, causing an influx of calcium into the cytoplasm that produces a tetanus-like steady-state contraction. We then used this method to show that an increase in cardiac muscle length leads to a decrease in \(k_{tr}\).
2.2 Materials and Methods

2.2.1 Animal Model and Trabeculae Isolation

For the first part of the study, we assessed three muscles from rabbit hearts. Rabbits were anesthetized using 50 mg/kg pentobarbital sodium, delivered intravenously (into the lateral ear vein). The experiments on rabbit myocardium were performed by Ying Xu, a former GRA of the Janssen laboratory. However, we used rats for most experiments. Male Brown Norway rats (approximately 3 months old and weighing 250 grams; n=11) were anesthetized intraperitoneally with 50 mg/kg sodium pentobarbital. The chest wall was opened by means of bilateral thoracotomy, and the heart was injected with 1000 units of heparin. In all cases, the heart was rapidly removed and perfused via the ascending aorta with Krebs-Henseleit solution containing (in mM) 137 NaCl, 5 KCl, 10 glucose, 20 NaHCO$_3$, 1.2 MgSO$_4$, 1.2 NaH$_2$PO$_4$, 0.25 CaCl$_2$ and 20 2,3-butanedione monoxime (BDM)\textsuperscript{139,142}. The BDM prevents contractions and minimizes cutting injury during dissection\textsuperscript{143}. The Krebs-Henseleit solution was equilibrated with 95% O$_2$/5% CO$_2$ resulting in a pH of 7.4. The right ventricle was opened, and thin non-branched trabeculae (average dimensions 159 µm ± 11 wide, 106 µm ± 7 thick and 1.5 mm ± 0.1 mm long, n = 11; rat) were dissected leaving free ventricular wall at both ends. Muscles with a thickness of greater than 150 µm were excluded from analysis to avoid the effects of core hypoxia\textsuperscript{134}. 
2.2.2 Experimental Apparatus

Muscles were mounted in a custom made bath and connected to a force transducer (KG4, SI Heidelberg) on one end by means of two parallel hooks (to eliminate rotation movement artifacts) and to a linear motor (MOT1, SI Heidelberg) on the other end. Vibrations associated with the movement of the motor and the flow of the superfusate were reduced by placing a small glass slide over the bath, and an electronic signaling anti-oscillation unit with an effective time constant faster than 1.2 ms was used to improve signal resolution (AOSU, SI Heidelberg). The muscles were perfused with Krebs-Henseleit solution as described in the section above (without BDM and containing 2.0 mmol/L CaCl$_2$). The solutions were kept at a constant temperature of 37 °C and equilibrated with 95% O$_2$/5% CO$_2$. Rat and rabbit muscles were stimulated at 4 and 1 Hz, respectively. The optimal lengths of the muscles were determined as previously described. Clear striation patterns cannot always be observed with intact trabeculae preparations, but previous work has shown that optimal length ($L_{\text{opt}}$) corresponds to a sarcomere length of ~2.2 µm, which is close to the sarcomere length at the end of diastole.

2.2.3 Experimental Protocol

The rate of tension redevelopment was measured for each rat muscle at both the optimal length ($L_{\text{opt}}$) and at a shorter length, $L_{90}$ (90% of $L_{\text{opt}}$), close to the in vivo sarcomere length at the end of systole. To determine whether experimental order affected the results, we measured $k_{\text{tr}}$ was measured in the order of: $L_{\text{opt}} \rightarrow L_{90} \rightarrow L_{\text{opt}} \rightarrow L_{90}$ in one subset of rat muscles ($n=6$) and $L_{90} \rightarrow L_{\text{opt}} \rightarrow L_{90} \rightarrow L_{\text{opt}}$ in a second set ($n=5$). The K$^+$
contracture plateau (peak) allows for a steady-state equilibrium between calcium and force. Therefore, we performed all $k_{ir}$ experiments when the muscles were under maximal force-inducing K$^+$ contracture, conditions under which calcium concentration is 1 µM or higher, which is saturating for force in intact preparations$^{75,148,149}$. After the muscles were maintained in Krebs-Henseleit solution for 15-20 minutes at either $L_{opt}$ or $L_{90}$, we induced K$^+$ contracture by switching to a solution containing (in mM) 121.4 KCl, 20.6 NaCl, 10 glucose, 20 NaHCO$_3$, 1.2 MgSO$_4$, 1.2 NaH$_2$PO$_4$, and 6 CaCl$_2$. Once the K$^+$ contracture started, the stimulation was turned off and the contracture was allowed to reach its plateau (maximum tension) phase. At this point the $k_{ir}$ was determined, and the solution was rapidly switched back to Krebs-Henseleit buffer (inducing relaxation of the K$^+$ contracture) and the electrical stimulation restarted. Muscle length was then adjusted to $L_{opt}$ or $L_{90}$ as appropriate, and the preparation was allowed to stabilize at the new length for 15-20 minutes, after which the K$^+$ contracture and determination of $k_{ir}$ protocol were repeated. A typical tracing of an entire K$^+$ contracture containing a $k_{ir}$ is given in Figure 2A for a muscle at $L_{90}$. An example of a $k_{ir}$ tracing is given in Figure 2B. $k_{ir}$ was determined through the following protocol: the muscle was rapidly shortened to 80% of its length during 1 ms (“slack” phase), maintained at this length for 10 ms, and then rapidly restretched to the original length during 1 ms. This maneuver disrupts cross-bridges, resulting in a decrease in force, followed by an exponential increase in force as the cross-bridges reattach$^{150,151}$. Data were collected at a rate of 10 kHz.
2.2.4 Data Analysis and Statistics

All data were collected by means of a custom-made application in LabView (National Instruments). The cross-sectional areas of the muscles were used to normalize absolute force measurements to reduce variations among muscles of different diameter. The rate of tension redevelopment ($k_{tr}$) was obtained by fitting the force redevelopment curve following the slack-restretch maneuver to the equation $F = F_{\text{max}} \cdot (1-e^{-k_{tr}t}) + F_{\text{initial}}$ (in which $F =$ force, $F_{\text{max}} =$ maximal force, and $F_{\text{initial}} =$ initial force) using a nonlinear least-squares fitting method $^{152}$. Fitting a double exponential did not significantly improve the fit. Additionally, the half time of force redevelopment, $t_{1/2}$, was determined by linear transformation of the data and used for calculation of $k_{tr}$ where $k_{tr} = \ln(2) \cdot (t_{1/2})^{-1}$. The differences between multiple groups were analyzed via two-way ANOVA with a significance threshold of $P < 0.05$. The differences between $k_{tr}$ calculated by monoexponential curve fit and linear transformation were determined by paired Student’s $t$-test with significance threshold of $P < 0.05$. The data are presented as mean ± S.E.M.

2.3 Results

2.3.1 Intact Muscle can be Used to Assess $k_{tr}$ in Rat and Rabbit Myocardium

First, we compared $k_{tr}$ in two species: the rat, which expresses the fast $\alpha$-myosin isoform, and the rabbit, which expresses the slow $\beta$-myosin isoform. In Figure 3A and B we show traces of $k_{tr}$ performed in a rabbit muscle. We obtained typical force tracings, similar to those described in permeabilized muscles at sub-physiological temperatures $^{151}$. Analyses of duplicate measurements showed that results were repeatable and
reproducible (Figure 3C). $k_{fr}$ is considerably slower (10.6 ± 1.2 s$^{-1}$, n=3) in rabbit than in rat (27.7 ± 3.3 s$^{-1}$, n=11, $P < 0.01$) under identical conditions (Figure 3D), indicating that the rate of $k_{fr}$ at physiological temperature differs with different myosin isoforms.

Next, we investigated the effect of temperature on $k_{fr}$. We observed an increase in $k_{fr}$ as temperature was increased from 27 °C to 37 °C (n=4 trabeculae, each from different rats), using a stimulation frequency of 1 Hz. We measured a Q10 in the range similar to that observed in permeabilized preparations (average Q10 of 2.2, ranging from 1.9 to 2.8; not depicted). The temperature dependence of our $k_{fr}$ measurements supports the notion that $k_{fr}$ in our experiments reflects cross-bridge cycling kinetics in a similar way as it does in permeabilized preparations at sub-physiological temperature.

2.3.2 Increase in Muscle Length Increases Maximal Tension and Reduces $k_{fr}$

Stretching the muscle from L$_{90}$ to L$_{opt}$ resulted, as expected, in a significant increase in twitch tension (Figure 4A), from 17.0 ± 2.8 to 30.9 ± 3.3 mN/mm$^2$ (L$_{90}$ vs. L$_{opt}$ respectively, $P < 0.05$). In addition, at longer length, as expected$^{78,153}$, the TTP (Figure 4B), which measures the time it takes for maximal twitch tension to develop, was prolonged from 50.2 ± 1.7 ms at L$_{90}$ to 55.7 ± 2.2 ms at L$_{opt}$ ($P < 0.05$). Similar results were observed for RT$_{50}$, which is the time from peak twitch force to 50% relaxation (Figure 4C), which increased from 30.2 ± 1.6 to 37.5 ± 1.5 ms ($P < 0.05$). The increase in muscle length resulted in an increase in the maximal tension obtained during the K$^+$ contracture. The maximum (plateau) K$^+$ contracture tension was 32.7 ± 5.1 mN/mm$^2$ at L$_{90}$ and 67.2 ± 6.6 mN/mm$^2$ for L$_{opt}$ (Figure 5A). Maximum K$^+$ contracture tension was
not affected by time-dependent run-down; repeat measurements showed similar values (35.3 ± 8.0 for L_{90} and 61.2 ± 7.0 mN/mm^2 for L_{opt}, P = 0.80). Maximum K^+ contracture tension between L_{90} and L_{opt} was significantly different (P < 0.05).

K_{tr} decreased as muscles were stretched from L_{90} to L_{opt} (see example in Figure 5B). The average rate of tension redevelopment was 45.1 ± 7.6 s^{-1} at L_{90} and 27.7 ± 3.3 s^{-1} at L_{opt} (Figure 5C). When k_{tr} for each length was measured a second time, the repeat k_{tr} measurements were 47.5 ± 9.2 s^{-1} for L_{90} and 27.8 ± 3.0 s^{-1} for L_{opt}, indicating a high reproducibility (Figure 5C). K_{tr} was significantly different between the two lengths (P < 0.05), but similar between the initial and repeat measurements at each length (P = 0.84). Quantification of the k_{tr} data by linear transformation yielded values in close agreement and not significantly different from the above data (P > 0.4, not shown). Finally, analysis of residual tension (F_{res}) after k_{tr} revealed a ratio of F_{res} to F_{dev} of 0.07 ± 0.05 at L_{90}, and this ratio was not significantly (ANOVA, P = 0.85) different from that at L_{opt} (0.09 ± 0.05).

2.4 Discussion

We have developed a method for studying the effect of muscle length on cross-bridge cycling kinetics in intact cardiac trabeculae at physiological temperatures. We found that: a) it is feasible to assess in k_{tr} repeatedly in intact muscle preparations at physiological temperature using K^+ contractures; and b) under these conditions, an increase in rat muscle length leads to a decrease in k_{tr}.
We found effects of both different myosin isoforms and different temperature on
cross-bridge kinetics similar to those described previously in permeabilized muscle at
sub-physiological temperatures. \( k_{tr} \) for the \( \alpha \)-myosin isoform was significantly faster than
with the \( \beta \)-isoform \(^{55,154,155} \), and increased temperature sped up \( k_{tr} \) \(^{53,55} \). Given a Q10 of
~2-3, the rate of tension redevelopment in our studies (up to 45-50 s\(^{-1}\)) would virtually be
identical to six previously reported values (average of \(~9\) s\(^{-1}\) and range of 7-13 s\(^{-1}\)) for rats
in skinned preparations at colder temperatures \(^{55,57,64,156-158} \) and also be very close to
those obtained in intact SR-poisoned cardiac trabeculae, where \( k_{tr} \) at normal calcium was
~11 s\(^{-1}\) \(^{141} \).

Permeabilized or “skinned” preparations, which have typically been used to
determine cross-bridge kinetics, have produced a wealth of critical knowledge. Although
they are ideally suitable for highly controlled experiments on cross-bridge kinetics, these
preparations are devoid of post-translational modification machinery because
membranous structures have been (partially) removed. This in turn may render inactive
or altogether removes signaling kinases and phosphatases. However, post-translational
modification of contractile proteins is encountered under different conditions of pre-load,
frequency, and \( \beta \)-adrenergic stimulation, and it has been proposed as a mechanism for
altering cross-bridge cycling dynamics \(^{16,75,82,148,159-164} \).

We used a modified K\(^+\) contracture protocol \(^{75,82,149,165} \) to reversibly “tetanize”
intact cardiac trabeculae to assess cross-bridge kinetics at physiological temperature. This
type of contracture induces a reversible steady-state force, without the need for
compounds that interfere with SR calcium cycling \(^{59,140,141,166} \), and can be repeated many
times in the same muscle. The maximum tension developed during the $K^+$ contracture did
not change between duplicate measurements; this suggests that the maximal force
generating capacity of myofilaments was not affected by a prior measurement per se, nor
by the passage of time during the course of our analyses. Furthermore, we found that
maximum tension at a given muscle length was independent of the order of length
changes.

Although the relationship between muscle or sarcomere length and force
development is well known\textsuperscript{136}, the effect of sarcomere length on cross-bridge cycling
rate remains controversial. We found that, when intact muscle length is reduced to 90% of optimal length, the rate of tension redevelopment was significantly accelerated. This is
consistent with previous studies that used permeabilized cardiac preparations at sub-
physiological temperatures\textsuperscript{64,65,68}. However, other studies indicated that sarcomere length
has no effect on rate of cross-bridge cycling\textsuperscript{59,63}. The different results obtained in these
studies could stem from various sources. First, they reflect experiments performed with
different animal species or strains. Additionally, all but one\textsuperscript{59} of the past studies used
permeabilized cardiomyocytes, which do not fully recapitulate intact myocardium. For
instance, they do not have constant volume behavior when stretched; the inter-filament
spacing upon stretch may not reduce as much in skinned preparations compared to a
similar stretch in intact muscle. Furthermore, many of these experiments were performed
at a temperature range of 12-27 °C, at which the behavior of many physiological
processes may differ from that at mammalian physiological temperatures (~37 °C)\textsuperscript{167}.
Moreover, post-translational modification of myofilament targets influences contractile
properties and the in situ status of post-translational modifications may be (partially) lost with preparation of the muscle or myocyte for in vitro experimentation. As a result, assessment of cross-bridge cycling rate could thus be affected by preparation-induced or reduced levels of such modifications \(^{75,124}\). In addition, myofilament compliance may affect cross-bridge cycling rate \(^{168}\), and the presence of compliant structures, such as collagen and titin, in muscle preparations may render \(k_{tr}\) rates at different levels of force apparent rather than absolute. Finally, the residual tension could have an effect on the \(k_{tr}\) \(^{169,170}\); however, residual tension was not significantly different in our studies, and therefore could not have contributed to the lower \(k_{tr}\) observed at longer muscle lengths. At present, we do not have the necessary information and data to propose a molecular mechanism for the acceleration of \(k_{tr}\) we observed at shorter muscle lengths. However, others \(^{65}\) have proposed plausible mechanisms to explain this phenomenon. For instance, at longer muscle length titin induces a strain on myosin-binding protein C which in turn restrains the movement of myosin heavy chains; thus decreasing cross-bridge cycling rate \(^{65}\).

In conclusion, our work reveals that repeated \(k_{tr}\) measurements are feasible in intact myocardium at body temperature and that in intact muscle, \(k_{tr}\) decreases with increasing muscle length.
Figure 2. Tracings of $K^+$ contracture and $k_{tr}$ protocols performed in intact rat trabeculae. 
A: Representative $K^+$ contracture in an intact rat muscle preparation at $L_{90}$. The $k_{tr}$ protocol was executed at the plateau stage of each contracture (indicated by arrowhead). Changes in $K^+$ concentration are indicated by arrows. 
B: Representative $k_{tr}$ tracing in a single intact muscle at $L_{opt}$ (value amounted to 28.2 s$^{-1}$). The motor position is shown at the top of the tracing.
Figure 3. The $k_{tr}$ protocol can be performed in intact rabbit myocardium. A and B: Consecutive $k_{tr}$ tracings in a rabbit trabecula at $L_{opt}$. C: detail of duplicate $k_{tr}$ tracings shows the repeatability and reproducibility of these analyses. D: A comparison with rat trabeculae, under otherwise identical conditions, shows that rabbit $k_{tr}$ (~10/s) is significantly slower than rat (~30/s) (representative of $n = 11$ rat, $n = 3$ rabbit). Rabbit data collected by Ying Xu, Janssen Laboratory.
Figure 4. Increasing muscle length results in an increase of twitch force and prolongation of twitch kinetics.
A: Rat muscle twitch tension increases with length ($P < 0.05$). B: TTP is greater at $L_{opt}$ than at $L_{90}$ ($P < 0.05$). C: RT$_{50}$ (time from TTP to 50% relaxation) increases significantly as muscles are stretched to $L_{opt}$ ($P < 0.05$). *, differences between $L_{opt}$ and $L_{90}$. Data are represented as mean ± SEM, $n = 11$. 
Figure 5. Increase in muscle length decreases $k_{tr}$ in intact rat trabeculae.  
A: Increase in muscle length is associated with a significant increase in maximal tension during K$^+$ contracture (*, $P < 0.05$). B: Superimposed $k_{tr}$ tracings of $L_{opt}$ and $L_{90}$ in a single rat muscle. The tracings show the initial 100 ms of force redevelopment. C: Increasing muscle length results in a decrease in $k_{tr}$ (*, $P < 0.05$). The tensions and $k_{tr}$ were not significantly different between duplicate measurements of each group ($P = 0.80$ [tension] and $P = 0.84$ [$k_{tr}$]). Data is represented as mean ± SEM, $n = 11$. 

A

B

C
Chapter 3: Length-Dependent Regulation and Preserved Contractile Kinetics in Right Ventricles of Failing versus Non-failing Human Myocardium

3.1 Introduction

Heart failure is a complex disorder that currently affects 5.8 million patients and is estimated to reach 8 million by 2030 in the United States \(^{88}\). Our understanding of this complex disease has greatly advanced in the past decades; however, patients currently have a 5 year survival rate of only 50\% \(^{171}\). It is fundamental to have a precise and complete understanding of the alterations that occur in heart failure in order to better guide the identification of therapeutic targets.

Right ventricular ejection fraction is a predictor of reduced exercise capacity and outcome in patients suffering from heart failure \(^{172}\). Recognizing factors that contribute to right ventricular contractility and how they are dysfunctional in heart failure is essential. A 2006 report by the National Heart, Lung, and Blood Institute recognized that current knowledge of the right ventricle in both health and disease is limited \(^{172}\). The left and right ventricles have differences in structure, function, and response to stress and disease; hence, experiments specifically on the right ventricle are required \(^{172,173}\). This study uses right ventricular trabeculae in order to enhance our understanding of the right ventricular contraction in both health and disease.
The cyclical interaction of myosin with actin is the basis of muscle contraction. The power of the heart and hence the cardiac output is dependent on both the developed force and shortening velocity\(^\text{26,27}\). Previous studies showed a reduced cross-bridge cycling rate in patients with idiopathic dilated cardiomyopathy\(^\text{54,102}\). Therefore, a decrease in cross-bridge cycling kinetics could contribute to the decline in cardiac output in heart failure patients. However, these studies were conducted only in idiopathic dilated cardiomyopathy and under non-physiological temperature, where it is known that temperature is a very potent modifier of contractile force and kinetics\(^\text{53,54,174,175}\). Alterations in cross-bridge cycling kinetics in heart failure patients, regardless of etiology, under near-physiological conditions remain unknown and need to be further investigated.

The heart utilizes multiple mechanisms in order to fine tune its pumping activity and adjust to the demands of the body. If and how the human heart regulates cross-bridge cycling kinetics is relatively unknown. The Frank-Starling relationship dictates that force is increased when the pre-load in the ventricles and hence cardiomyocyte length are increased\(^\text{60}\). Some studies show that muscle length also affects cross-bridge cycling kinetics, while others show lack of such effect in various animal models\(^\text{59,64-66,68,174,176}\). In Chapter 2, by using our novel method, we showed that muscle length does regulate cross-bridge cycling kinetics in intact rat myocardium. Cardiac studies in animal models, especially in small rodents, do not always translate to humans\(^\text{28,117}\). It is thus essential to perform and/or verify experiments on both non-failing and failing human cardiac tissues in order to bridge the gap between animal studies and humans. Only one study to date has
investigated the relationship between length and cross-bridge cycling kinetics in humans. This study did not find a relationship between sarcomere length and cross-bridge kinetics. However, it was performed on a small sample size of only healthy human samples and in permeabilized cardiomyocyte preparations, far removed from the physiological conditions under which the heart functions. Questions thus remain as to whether length regulates cross-bridge cycling kinetics in humans as assessed in intact preparations under physiologically relevant conditions, and whether such regulation is altered in heart failure. In this study, we examined the role of muscle length in determining cross-bridge cycling rate under near-physiological conditions in intact trabeculae isolated from the right ventricles of non-failing and failing human hearts.

Our data here indicates that despite the presence of phenotypical depression of the force-frequency relationship (FFR) and β-adrenergic desensitization, contractile kinetics and its regulation by muscle length are actually preserved in the right ventricles of heart failure patients.

3.2 Materials and Methods

3.2.1 Human Tissue Collection

All human tissues were collected, stored, and experimented on with approval from the Institutional Review Board (IRB) of The Ohio State University. Informed consents were acquired from cardiac transplant patients. Non-transplantable donor hearts (n = 9) were acquired from Lifeline of Ohio Organ Procurement, and end-stage failing hearts (n = 10) were acquired from patients undergoing cardiac transplantation at The
Ohio State University Wexner Medical Center. The characteristics of these hearts are provided in Tables 1-3. An additional four non-transplantable donor hearts (not included in the tables) were used for only assessing the effects of temperature on the rate of tension redevelopment (ktr). Explanted hearts were obtained in the operating room and flushed immediately after removal from the chest and thereafter transferred to the laboratory in cold cardioplegic solution containing (in mM): 110 NaCl, 16 KCl, 16 MgCl2, 10 NaHCO3, and 0.5 CaCl2.

3.2.2 Trabeculae Isolation

All experiments were performed on intact muscle preparations. The right ventricle of each heart was transferred from the cardioplegic solution to a cold modified Krebs-Henseleit solution (K-H) previously bubbled with 95% O2-5% CO2 containing (in mM): 137 NaCl, 5 KCl, 0.25 CaCl2, 20 NaHCO3, 1.2 NaH2PO4, 1.2 MgSO4, 10 dextrose, and 20 BDM (2,3-butanedione monoxime) and pH of 7.4. Small and linear right ventricular trabeculae were isolated and kept in this solution at 0-4 °C until the time of the experiment. Muscles were placed in custom-made setups as previously described for animal models174 and the perfusion solution was changed to another modified K-H without BDM and containing 0.25 mM CaCl2. This solution was maintained at 37 °C and continuously bubbled with 95% O2-5% CO2 resulting in pH of 7.4. Stimulation was initiated at baseline frequency of either 0.5 Hz (for muscles used to assess baseline cardiac function) or 1 Hz (muscles used for ktr experiments) and the CaCl2 concentration of the solution was incrementally and slowly raised to 2 mM over the time course of ~15
minutes. Muscles were gradually stretched until an increase in developed force was not matched by an increase in resting tension. This length, designated as $L_{\text{opt}}$ (optimal length), corresponds to sarcomere length of $\sim 2.2 \ \mu m$, which is near or at the \textit{in vivo} sarcomere length at end-diastole\cite{147}.

### 3.2.3 Baseline Trabeculae Function

Right ventricular trabeculae were stabilized at $L_{\text{opt}}$ and 0.5 Hz for ~15 minutes. Afterwards, the length-tension relationship was determined at constant frequency of 0.5 Hz from $L_{90}$ (90% of $L_{\text{opt}}$, corresponding to \textit{in vivo} sarcomere length during end-systolic phase) to $L_{\text{opt}}$. The force-frequency relationship was determined at $L_{\text{opt}}$ from 0.5 Hz to 3 Hz. The $\beta$-adrenergic response was determined by adding incremental amounts of isoproterenol for a final concentration of 1 $\mu$M at $L_{\text{opt}}$ and 0.5 Hz. Data from multiple muscles of the same heart were averaged and used for the final analysis. Non-failing muscles ($n = 10$ from 8 hearts) had dimensions of $288 \pm 32 \ \mu m$ in width, $191 \pm 22 \ \mu m$ in thickness, and $3.7 \pm 0.5 \ mm$ in length. Failing muscles ($n = 13$ from 9 hearts) had dimensions of $538 \pm 44 \ \mu m$ in width, $358 \pm 29 \ \mu m$ in thickness, and $3.2 \pm 0.4 \ mm$ in length. The experiments on twelve out of twenty three muscles were performed by other members of the Janssen laboratory including Benjamin D. Canan, Jennifer A. Conkle, Mohammad T. Elnaksih, Rachel L. Gearinger, Jason Murray, and Eric J. Schultz.
3.2.4 \( k_{fr} \) Experiments in Intact Cardiac Trabeculae

Non-failing right ventricular trabeculae (n = 9 from 9 hearts) used for \( k_{fr} \) experiments had an average width of 406 ± 50 µm, thickness of 267 ± 34 µm, and lengths of 2.7 ± 0.4 mm. The failing right ventricular muscles (n = 10 from 10 hearts) had an average width of 352 ± 26 µm, thickness of 234 ± 17 µm, and length of 3.3 ± 0.3 mm. Two of the muscles were previously subjected to the length-tension (at 0.5 Hz) and FFR (from 0.5 Hz to 3 Hz) experiments as described above. Muscles were stabilized at \( L_{\text{opt}} \) and 1 Hz for 10-15 minutes. In some of the muscles (non-failing: n = 7 out of 9, failing: n = 7 out of 10), a brief FFR (1 Hz, 1.5 Hz, 2 Hz) was initially determined before any \( k_{fr} \) experiments. Thereafter, muscles were re-stabilized at 1 Hz for 10-15 minutes and this frequency was used for the remainder of the experiments. Muscles were exposed to a high K\(^+\)/Ca\(^{2+}\), low Na\(^+\) solution containing (in mM): 20.6 NaCl, 121.4 KCl, 6 CaCl\(_2\), 20 NaHCO\(_3\), 1.2 MgSO\(_4\), 1.2 NaH\(_2\)PO\(_4\), and 10 dextrose in order to induce a contracture. During this contracture, \( k_{fr} \) maneuver was performed at sub-maximal (a subset of muscles) and maximal (all muscles) tension levels as previously described for rats and rabbits. The solution was switched back to K-H solution and muscles were stabilized at new lengths, \( L_{95} \) (95% of \( L_{\text{opt}} \)) and \( L_{90} \) (90% of \( L_{\text{opt}} \)) for 10-15 minutes and \( k_{fr} \) experiments were performed at each of these lengths as described above. Thereafter, muscles were re-stabilized at \( L_{\text{opt}} \), and \( k_{fr} \) experiments were performed at this length for a second time (designated \( L_{\text{opt, repeat}} \)) in order to compare to the first \( L_{\text{opt}} \) measurement and assess reproducibility.
In order to further validate the reproducibility of the results, an additional protocol was performed in a subset of muscles (n = 8) where three consecutive $k_{tr}$ experiments were measured during the maximal level of activation of a single $K^+$ contracture at $L_{opt}$ (n = 8), $L_{95}$ (n = 2), or $L_{90}$ (n = 2). In addition, the temperature dependency of $k_{tr}$ was assessed at maximal $K^+$ contracture tension in another set of non-failing donor hearts, not used for previous experiments, at temperatures of 37 °C, 32 °C, and 27 °C. Muscles were stabilized at $L_{opt}$, 1 Hz, and each of these temperatures for 15 minutes prior to $k_{tr}$ experiments.

3.2.5 Statistical and Data Analysis

Tension and kinetic measurements were collected and analyzed using custom made programs in LabView (National Instruments). Muscle tensions were normalized to the cross-sectional area of the muscles and expressed as mN/mm$^2$. Origin 7 software (OriginLab Corp.) was used to fit $k_{tr}$ tracings to the equation $F = F_{max} \left(1 - e^{-k_{tr}t}\right) + F_{res}$ and calculate EC$_{50}$ of isoproterenol with the equation $F = F_{min} + F_{max} \left(F^a/(F^a + EC_{50})\right)$. Statistical analysis was performed with un-paired t-test, two-way ANOVA, or one-way ANOVA for repeated measures followed by Bonferroni post-Hoc test when appropriate. Statistical significance was set at $P < 0.05$. All data is shown as mean ± S.E.M.
3.3 Results

3.3.1 Length-Tension, Force-Frequency, and β-Adrenergic Response

Increasing muscle length stepwise from $L_{90}$ (90% of optimal length; $L_{opt}$) to $L_{95}$ (95% of $L_{opt}$) to $L_{opt}$ increased developed tensions in both non-failing and failing muscles to the same degree (Figure 6A). The FFR of non-failing myocardium was positive (up to 2 Hz) while failing hearts had a negative response (Figure 6B). Both groups responded positively to increasing isoproterenol concentrations (Figure 6C); however, the EC$_{50}$ of non-failing muscles was significantly lower than that of failing muscles indicating β-adrenergic desensitization and/or down-regulation in the latter group (Figure 6D).

3.3.2 Twitch Kinetics are Unaffected in the Failing Myocardium and Slow Down with Increasing Muscle Length

In parallel, in a different set-up, a separate set of muscles was used for performing $k_{tr}$ experiments. FFR measurements in a subset of these muscles also showed that non-failing hearts had a positive FFR (from 1 Hz to 2 Hz) while failing muscles had a flat FFR within the same frequency range (data not shown). Twitch parameters were measured in these muscles after they were stabilized at each muscle length at 1 Hz as well as right before $K^+$ contracture and $k_{tr}$ experiments (Table 4). Developed ($F_{dev}$) and resting ($F_{rest}$) twitch tensions were not significantly different between non-failing and failing hearts at each respective muscle length (un-paired t-test, $P > 0.05$). Furthermore, no significant differences were detected in respect to contractile ($dF/dt_{max}$, $dF/dt_{max}$/Force, and TTP) and relaxation kinetics ($dF/dt_{min}$, $dF/dt_{min}$/Force, and RT$_{50}$) at each
respective muscle length (un-paired t-test, \( P > 0.05 \)). Definition of these parameters is provided in Figure 7. The \( \text{dF/dt}_{\text{max}} \) and \( \text{dF/dt}_{\text{min}} \) were normalized to the developed twitch tensions in order to obtain a pure kinetic parameter in units of \( \text{s}^{-1} \). Within each group, \( F_{\text{dev}} \) and \( F_{\text{rest}} \) decreased while \( \text{dF/dt}_{\text{max}}/\text{Force} \) and \( \text{dF/dt}_{\text{min}}/\text{Force} \) generally accelerated as muscles were slacked from \( L_{\text{opt}} \) to \( L_{90} \) (ANOVA; Bonferroni post-Hoc, \( P < 0.05 \)). We also analyzed kinetic parameters of the muscles in Figure 6A which were performed at 0.5 Hz (Table 5). Overall, we observed a similar pattern as in Table 4. The two major exceptions were: 1) \( \text{dF/dt}_{\text{min}}/\text{Force} \) did not accelerate as muscles were slacked in both non-failing and failing hearts, and 2) \( \text{dF/dt}_{\text{min}}/\text{Force} \) at \( L_{95} \) was statistically lower (un-paired t-test, \( P < 0.05 \)) in the failing group as compared to the non-failing group.

We did observe some run-down in developed tension over the long time course of the \( k_{tr} \) experiments. This was quantified by repeating the experiments at optimal length for a second time (\( L_{\text{opt,repeat}} \)). Interestingly, this run-down was more pronounced and statistically significant in the non-failing group (ANOVA; Bonferroni post-Hoc, \( P < 0.05 \)) while the failing group did not reach statistical significance (ANOVA; Bonferroni post-Hoc, \( P = 0.1083 \)).

3.3.3 \( k_{tr} \) is Unchanged in Failing Myocardium; Muscle Length Modulates \( k_{tr} \)

After stabilization and measurement of twitch parameters at a particular muscle length (\( L_{\text{opt}}, L_{95}, L_{90}, L_{\text{opt,repeat}} \)), \( k_{tr} \) experiments were performed while the trabeculae were under a brief K\(^+\) contracture (Figure 8A-B). The maximal developed tension during K\(^+\) contracture decreased as muscles were slacked from \( L_{\text{opt}} \) to \( L_{95} \) to \( L_{90} \) (Figure 9A). This
tension was increased when muscles were re-stretched to L_{opt,repeat}. However, the tension of L_{opt} vs. L_{opt,repeat} was reduced in non-failing samples (ANOVA; Bonferroni post-Hoc, \( P < 0.05 \)), but unchanged in failing samples (ANOVA; Bonferroni post-Hoc, \( P = 1 \)). There was no statistical difference (un-paired t-test, \( P > 0.05 \)) in the \( K^+ \) contracture tension between non-failing and failing groups at each muscle length.

In both non-failing and failing samples, \( k_{tr,max} \) (during maximal \( K^+ \) tension) was accelerated as muscles were slacked from L_{opt} to L_{90} (ANOVA; Bonferroni post-Hoc, \( P < 0.05 \)) and decreased as muscles were re-stretched back to L_{opt,repeat} (ANOVA; Bonferroni post-Hoc, \( P < 0.05 \)) (Figure 9C). There were no significant differences (un-paired t-test, \( P > 0.05 \)) in \( k_{tr,max} \) values between non-failing and failing groups at each muscle length (Figure 9B-C). The ratios of \( F_{res} \) (residual force following \( k_{tr} \)):\( F_{K+} \) (\( K^+ \) contracture force before \( k_{tr} \)) of the non-failing group were 0.32 ± 0.03 (L_{opt}), 0.27 ± 0.06 (L_{95}), 0.23 ± 0.07 (L_{90}), 0.30 ± 0.06 (L_{opt,repeat}) (ANOVA, \( P = 0.43 \)). The ratios for the failing group were 0.18 ± 0.04 (L_{opt}), 0.2 ± 0.04 (L_{95}), 0.2 ± 0.05 (L_{90}), and 0.18 ± 0.06 (L_{opt,repeat}) (ANOVA, \( P = 0.97 \)).

Of the 10 failing hearts, 3 were from dilated cardiomyopathy (DCM), 6 were from ischemic cardiomyopathy (ICM) etiologies, and 1 was unclassified. We further subdivided the \( k_{tr,max} \) values from Figure 9C into these two categories. The \( k_{tr} \) was similar across all three groups although the DCM tended to be just slightly lower (no statistical significance) (Figure 10).

We also measured \( k_{tr} \) during sub-maximal \( K^+ \) contracture tension levels in some of the muscles. During sub-maximal activation levels, \( k_{tr} \) was not different between non-
failing and failing samples (Figure 11). Note that due to the nature of the K$^+$ contracture experiments, there was variability in regards to the activation level at which these sub-maximal experiments were performed. Results are binned within relative tension ranges and thus each point does not necessarily represent the same number of muscles. Additionally, the $k_{tr}$ traces were more noisy at very low sub-maximal tension levels which caused a larger degree of variance in the measured $k_{tr}$ values at these low-tension points.

### 3.3.4 $k_{tr}$ Measurements are Reproducible and Temperature Dependent

In all of the muscles used for length studies, $k_{tr}$ was measured twice at $L_{opt}$ during two separate K$^+$ contractures. The $k_{tr}$ values of the first $L_{opt}$ were not statistically different as compared to $L_{opt,repeat}$ in both the non-failing and failing groups (ANOVA; Bonferroni post-Hoc, $P = 1$ for both groups) (Figure 9C). Additionally, three consecutive $k_{tr}s$ were performed in some muscles during the peak of a single K$^+$ contracture ($n = 8$ muscles, $n = 12$ experiments). Overall, there was no difference between these $k_{tr}$ values (ANOVA, $P = 0.44$) and ratios of $F_{res}:F_{K+}$ (ANOVA, $P = 0.09$) (data not shown).

Reducing temperature from 37 °C to 32 °C and then further to 27 °C decreased $k_{tr}$ in our human non-failing muscles from 18.1 ± 2.1 s$^{-1}$, to 14.1 ± 1.4 s$^{-1}$, to 6.6 ± 0.9 s$^{-1}$, respectively ($n = 4$, ANOVA, $P < 0.05$). This reflects a $Q_{10}$ of approximately 2.8 (data not shown).
3.4 Discussion

We report here for the first time that muscle length can regulate contractile kinetics in human myocardium and that kinetics are preserved in the right ventricles of end-stage heart failure patients when assessed in intact muscle preparations and under near-physiological conditions. Right ventricular contractility is an indicator of outcomes in patients with heart failure^{172}, regulation of its contraction in health and alterations during heart failure are under-studied as compared to the left ventricle. Therefore, we performed our current study on the right ventricle in order to better gain insight into regulation of right ventricle contraction in both health and disease.

The right ventricles of failing hearts in this current study had a blunted FFR relationship and β-adrenergic desensitization which are classical hallmarks that have been extensively observed in failing human myocardium^{92-101}. Despite these typical pathological alterations, contractile kinetics assessed during twitch were remarkably similar between non-failing and failing hearts. Various parameters corresponding to contractile and relaxation kinetics can be derived from the twitch contraction of an isolated linear myocardial muscle preparation. Of these parameters, $dF/dt_{\text{max}}/\text{Force}$ and $dF/dt_{\text{min}}/\text{Force}$ provide insight into purely kinetic processes (unit: $s^{-1}$) as they are normalized by the amplitude of the force^{78,153}. Our current data show that there were no differences in these parameters between the non-failing and failing groups when measured at $L_{\text{opt}}$. These measurements were assessed at low stimulation rates of 0.5 Hz and 1 Hz which are near or below the resting heart rates in humans. A retrospective analysis of several previous studies on failing human myocardium confirms our finding:
although they were not explicitly analyzed per muscle, the group averaged values for 
\( \frac{dF}{dt_{\text{max}}}/\text{Force} \) and \( \frac{dF}{dt_{\text{min}}}/\text{Force} \) were either not different or very similar in failing versus non-failing muscles at stimulation frequencies of 0.5 Hz or 1 Hz \(^{94-97,103} \).

The cardiac twitch contraction is dependent upon multiple processes ranging from action potential depolarization, calcium-induced calcium release, and myofilament-based processes \(^7,78 \). Since force development is directly correlated to the number of cross-bridges, and twitch kinetics are coupled to the cycling kinetics, we measured \( k_{\text{tr}} \), an index of cross-bridge cycling kinetics. In order to allow for the closest relevance to \textit{in vivo} contraction, and to preserve pathways that may impact twitch kinetics, we performed experiments on intact trabeculae, under near-physiological conditions, using a technique that we described for animal models in Chapter 2 \(^{174} \). Our \( k_{\text{tr}} \) measurements in intact human muscles are stable and reproducible both during a single K\(^+\) contracture and over prolonged periods of experimentation. The \( k_{\text{tr}} \) was not different at \( L_{\text{opt}} \) between non-failing and failing hearts at maximal or sub-maximal activation levels which is in accordance with our twitch data. This is in agreement with previous studies which did not detect slowing in cross-bridge cycling rate in failing left ventricular preparations as assessed by \( k_{\text{tr}} \) in mixed group of ICM and DCM \(^{58} \), \( k_{\text{tr}} \) in ICM or DCM \(^{107} \), \( k_{\text{tr}} \) in DCM \(^{108} \), and minimal frequency of dynamic stiffness in mitral valve regurgitation heart failure \(^{106} \). Note that all of these studies, as opposed to our current study, were performed on permeabilized preparations, and with one exception \(^{106} \) performed at sub-physiological temperatures. The results of these previous studies and our current study are in contrast to somewhat similar investigations in idiopathic DCM patients. One study showed that
minimal frequency of dynamic stiffness and unloaded shortening velocity are lower in permeabilized right ventricular preparations \textsuperscript{102} and another study showed lower minimal frequency of dynamic stiffness in intact left ventricular preparations in this etiology \textsuperscript{54}. Both of these studies were performed, however, at sub-physiological temperatures. Force-time integral was increased in the left ventricles of DCM in another study that used intact preparations at 37 °C \textsuperscript{103}. The difference between these studies and our current results can possibly be attributed not only to the differences in the experimental methodologies but also to the type of heart failure. The referenced studies were performed on DCM tissues. However, most of the failing samples in our current study were ICM-based and only three were classified as DCM. Interestingly, the \( k_{tr} \) values of DCM samples in our study were very similar and only slightly lower, but not statistically different, as compared to those of the ICM and non-failing groups. Several studies suggest that DCM and ICM human hearts are different in regards to myofilament alterations \textsuperscript{122,177}, mitochondrial dysfunction \textsuperscript{178}, and protein expression \textsuperscript{179-181}. One of these studies comprehensively showed that alterations in various cardiac processes, such as \( \beta \)-adrenergic signaling, myofilament phosphorylation and function, and extracellular collagen content, are more pronounced in DCM than ICM \textsuperscript{122}. It is also possible that kinetics of cross-bridge cycling are, due to a currently unknown reason, differentially affected in DCM and ICM. It should be noted that ICM is the most common type of heart failure \textsuperscript{105}; so even if such cross-bridge cycling kinetics are altered in DCM, those findings may not necessarily translate to the entire heart failure patient population.
In Chapter 2, we showed that muscle length regulates cross-bridge cycling kinetics in rat myocardium. It is possible that these kinetic parameters are different between non-failing and failing human myocardium at lower muscle lengths. We assessed whether this length dependent regulation of kinetics exists in human myocardium and whether it is altered in heart failure. The role of muscle length in modulating $k_{\text{tr}}$ in animal models is controversial with some studies showing either an effect or lack of one $^{59,63-66,68,174}$. Decreasing muscle length increased $k_{\text{tr}}$ to about the same extent in both non-failing and failing human myocardium; hence, $k_{\text{tr}}$ at each muscle length was not statistically different between the two groups. This increase in $k_{\text{tr}}$ at shorter muscle lengths is in accordance with twitch kinetics as they were generally accelerated (with the exception of $\text{dF/dt}_{\text{min}}$/Force in muscles performed at 0.5 Hz) as muscles were slacked from $L_{\text{opt}}$ to $L_{90}$ in both non-failing and failing myocardium. The data overall shows that regardless of muscle length, both the $k_{\text{tr}}$ and twitch kinetics (with the exception of $\text{dF/dt}_{\text{min}}$/Force at $L_{95}$ in muscles performed at 0.5 Hz) were similar between non-failing and failing myocardium. One previous study showed that sarcomere length has no effect on $k_{\text{tr}}$ in healthy human myocardium $^{63}$. It should be noted that this referenced study was performed on permeabilized cardiomyocytes at sub-physiological temperatures of 15 °C and 25 °C, while our current study was performed on intact multicellular preparations and at body temperature (37 °C). Cardiomyocyte permeabilization has the advantage of studying the sarcomeres in isolation from the rest of the cardiomyocytes. However, it does not provide complete information about the behavior of sarcomeres inside the cardiomyocytes where their activity can be regulated
and altered. Furthermore, the permeabilization process has been shown to introduce artifacts such as drastic reduction in myofilament calcium sensitivity\(^{166}\), and the lack of membranes can result in loss of constant-volume behavior, impacting myofilament spacing. The myofilaments in intact trabeculae are fully integrated into the cardiomyocytes and their activity is under constant regulation. Experimental conditions such as temperature and extracellular ionic concentrations were chosen to mimic those of the body while the intracellular conditions are regulated by the cardiomyocytes themselves. Therefore, we believe that intact preparations provide the opportunity to study cardiac dynamics under conditions that are as close to the physiological conditions as can be achieved \textit{in vitro}. To our knowledge, the current study is the only study which has investigated the effects of muscle length on cross-bridge cycling kinetics in sufficiently large numbers of intact non-failing and failing human cardiac preparations under near-physiological temperature and conditions. The implications of length-dependent changes in cross-bridge kinetics may help explain the rapid pressure rise and fall in the ejecting ventricle compared to the typically relatively slower kinetics in isometric muscles. As the ventricle ejects, sarcomere length is reduced, which according to our results could accelerate cross-bridge cycling kinetics. Likewise, at end-systolic length, comparable to \(L_{90}\) in our muscle studies, relaxation would initially occur with fast-cycling cross-bridges, that decelerate only when filling has started (i.e. when the sarcomere is re-lengthened).

Temperature had a prominent effect on our \(k_{nc}\) measurements with a \(Q_{10}\) of \(~2.8\) (performed on non-failing muscles). This is close to the \(Q_{10}\) values (range of ~2-3) which
have been previously reported for \( k_{tr} \) in permeabilized rat cardiac preparations by other investigators as well as intact rat trabeculae in Chapter 2 \(^{53,55,174}\). Furthermore, a previous report indicated that the minimum frequency of dynamic stiffness has a minimum \( Q_{10} \) of 2.7 in human intact preparations \(^{54}\). The effect of temperature on our \( k_{tr} \) recordings in humans suggests that this parameter is measuring a cellular process, most likely cross-bridge cycling kinetics.

There was a transient increase in tension following the slack-restretch \( k_{tr} \) maneuver relative to the pre-\( k_{tr} \) tension. These overshoots were routinely observed in both non-failing and failing human samples. This is not an artifact of the intact myocardial preparation since overshoots were rarely observed, and if so to a much lower magnitude, in our previous study in rats in Chapter 2 \(^{174}\). Tension overshoots following \( k_{tr} \) have been previously reported in permeabilized rat soleus fibers \(^{182}\) and Protein Kinase A treated permeabilized rat cardiomyocytes \(^{66}\). These tension overshoots have been attributed to a temporary increase in the number of bound cross-bridges \(^{182}\). Titin has also been suggested to play a role in another study \(^{66}\). Simulation studies suggest that a compliant filament is necessary for a transient increase in binding of myosin to actin and such tension overshoots \(^{169}\). Rat cardiomyocytes predominately express the stiff N2B titin isoform as opposed to humans which express both compliant N2BA and stiff N2B isoforms \(^{28}\). The need for a compliant N2BA titin for tension overshoot is in accordance with the observation that overshoots did not regularly occur our previous study in rats in Chapter 2 \(^{174}\). Additionally, we will further investigate these overshoots in human myocardium in Chapter 4.
In summary, we report that cross-bridge cycling kinetics are relatively un-altered in right ventricles of patients with end-stage heart failure and that muscle length can regulate these kinetics in both non-failing and failing hearts to the same degree by accelerating the rate when length of the muscle decreases.

Study limitations

The types of physiological experiments used in this study require non-frozen and freshly explanted hearts. It is not feasible to attain completely healthy human hearts devoid of any sort of cardiovascular disease in sufficiently large numbers. Therefore, some of the non-failing samples included in this study did have some type of cardiovascular disease(s) as outlined in Table 2. However, none of the non-failing samples had low left ventricular ejection fraction. Since the object of the study was to compare non-failing and failing human myocardium; we do not believe that such diseases dramatically affected our results. Additionally, all of the non-failing hearts were subjected to some type of inotropic agent before organ procurement. We believe that the stabilization of trabeculae in vitro should reverse most, if not all, of the modifications induced by such agents. Similarly, medication both in the non-failing and failing groups can potentially have an effect which was not taken into consideration in this study.
Table 1. Characteristics of non-failing human hearts I.

<table>
<thead>
<tr>
<th>Heart #</th>
<th>Age</th>
<th>Gender</th>
<th>Race</th>
<th>Heart Weight (g)</th>
<th>LV Wall Thickness (cm)</th>
<th>RV Wall Thickness (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF1</td>
<td>36</td>
<td>Male</td>
<td>Caucasian</td>
<td>415</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NF2</td>
<td>41</td>
<td>Female</td>
<td>African-American</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NF3</td>
<td>58</td>
<td>Female</td>
<td>Caucasian</td>
<td>499</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NF4</td>
<td>65</td>
<td>Female</td>
<td>Caucasian</td>
<td>451</td>
<td>1.8</td>
<td>0.6</td>
</tr>
<tr>
<td>NF5</td>
<td>22</td>
<td>Male</td>
<td>African-American</td>
<td>383</td>
<td>2.1</td>
<td>0.7</td>
</tr>
<tr>
<td>NF6</td>
<td>63</td>
<td>Female</td>
<td>Caucasian</td>
<td>608</td>
<td>1.7</td>
<td>0.9</td>
</tr>
<tr>
<td>NF7</td>
<td>29</td>
<td>Female</td>
<td>Caucasian</td>
<td>271</td>
<td>1.3</td>
<td>0.5</td>
</tr>
<tr>
<td>NF8</td>
<td>51</td>
<td>Female</td>
<td>Caucasian</td>
<td>320</td>
<td>1.1</td>
<td>0.6</td>
</tr>
<tr>
<td>NF9</td>
<td>55</td>
<td>Female</td>
<td>African-American</td>
<td>350</td>
<td>1.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>
Table 2. Characteristics of non-failing human hearts II.

<table>
<thead>
<tr>
<th>Heart #</th>
<th>Cause of Death</th>
<th>Past Medical History*</th>
<th>LVEF (%)</th>
<th>Heart Rate (bpm)</th>
<th>Blood Pressure (mmHg)</th>
<th>Inotropic Intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF1</td>
<td>Fentanyl patch</td>
<td>HTN, 1 cigarette pack per 6 months, chewing tobacco, 12 beers per day on and off, IV heroin and marijuana</td>
<td>60</td>
<td>97</td>
<td>121/70</td>
<td>norepinephrine, dopamine</td>
</tr>
<tr>
<td></td>
<td>intoxication, cardiac arrest, anoxia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NF2</td>
<td>CVA; SAH</td>
<td>HTN, abnormal ECG, borderline LV concentric hypertrophy, mild diffuse mitral valve thickening, trivial mitral valve regurgitation, 2 cigarettes per day, marijuana</td>
<td>55</td>
<td>86</td>
<td>126/78</td>
<td>norepinephrine</td>
</tr>
<tr>
<td>NF3</td>
<td>Head Trauma</td>
<td>HTN, atrial fibrillation, CAD, MI, cardiac stents, pacemaker/defibrillator, evidence of septal infarct on ECG, mini-strokes, 1.5 cigarette packs per day, 6 packs of beer per month</td>
<td>-</td>
<td>111</td>
<td>133/46</td>
<td>dopamine</td>
</tr>
<tr>
<td>NF4</td>
<td>CVA; ICH</td>
<td>HTN, CAD, arrhythmia, abnormal ECG, leaky valve (unspecified), left bundle branch block, poor circulation, 2 cigarette packs per year, 10-20 beers per year</td>
<td>-</td>
<td>89</td>
<td>108/56</td>
<td>dopamine</td>
</tr>
<tr>
<td>NF5</td>
<td>DKA; cardiac arrest</td>
<td>Diabetes Type 1, Diabetic ketoacidosis</td>
<td>-</td>
<td>88</td>
<td>125/65</td>
<td>epinephrine, dopamine</td>
</tr>
<tr>
<td>NF6</td>
<td>MVA; head trauma</td>
<td>HTN, hypothyroidism</td>
<td>-</td>
<td>115</td>
<td>120/80</td>
<td>norepinephrine, dopamine</td>
</tr>
<tr>
<td>NF7</td>
<td>Anoxia, cardiac arrest</td>
<td>Drug abuse, 7.5 cigarette pack years, heavy alcohol use, drug abuse</td>
<td>50-55</td>
<td>143</td>
<td>113/73</td>
<td>norepinephrine, epinephrine</td>
</tr>
<tr>
<td>NF8</td>
<td>Blunt head trauma</td>
<td>HTN, Graves disease, 7 cigarette pack years, heavy alcohol abuse, cocaine</td>
<td>40-50</td>
<td>106</td>
<td>110/67</td>
<td>norepinephrine, dopamine</td>
</tr>
<tr>
<td>NF9</td>
<td>CVA</td>
<td>HTN, high cholesterol, poor fingertip circulation when cold, 1 cigarette pack per week for one year, social alcohol consumption</td>
<td>60</td>
<td>102</td>
<td>130/64</td>
<td>epinephrine, dopamine</td>
</tr>
</tbody>
</table>

Table 3. Characteristics of failing human hearts.

<table>
<thead>
<tr>
<th>Heart</th>
<th>Age</th>
<th>Gender</th>
<th>Race</th>
<th>Etiology</th>
<th>Heart Weight (g)</th>
<th>LV Wall Thickness (cm)</th>
<th>RV Wall Thickness (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>51</td>
<td>Female</td>
<td>Caucasian</td>
<td>DCM</td>
<td>508</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>F2</td>
<td>50</td>
<td>Male</td>
<td>Caucasian</td>
<td>ICM</td>
<td>714</td>
<td>2</td>
<td>0.7</td>
</tr>
<tr>
<td>F3</td>
<td>30</td>
<td>Male</td>
<td>Caucasian</td>
<td>DCM</td>
<td>482</td>
<td>1.4</td>
<td>0.6</td>
</tr>
<tr>
<td>F4</td>
<td>63</td>
<td>Male</td>
<td>Caucasian</td>
<td>ICM</td>
<td>506</td>
<td>1.9</td>
<td>0.7</td>
</tr>
<tr>
<td>F5</td>
<td>50</td>
<td>Male</td>
<td>Caucasian</td>
<td>ICM</td>
<td>636</td>
<td>1.4</td>
<td>0.7</td>
</tr>
<tr>
<td>F6</td>
<td>63</td>
<td>Male</td>
<td>Caucasian</td>
<td>Non-Ischemic</td>
<td>543</td>
<td>1.1</td>
<td>0.8</td>
</tr>
<tr>
<td>F7</td>
<td>62</td>
<td>Male</td>
<td>African-American</td>
<td>ICM</td>
<td>427</td>
<td>2</td>
<td>0.9</td>
</tr>
<tr>
<td>F8</td>
<td>56</td>
<td>Female</td>
<td>Caucasian</td>
<td>DCM</td>
<td>470</td>
<td>1.3</td>
<td>1</td>
</tr>
<tr>
<td>F9</td>
<td>64</td>
<td>Male</td>
<td>Caucasian</td>
<td>ICM</td>
<td>495</td>
<td>1.2</td>
<td>0.9</td>
</tr>
<tr>
<td>F10</td>
<td>67</td>
<td>Male</td>
<td>Caucasian</td>
<td>ICM</td>
<td>630</td>
<td>1.7</td>
<td>0.7</td>
</tr>
</tbody>
</table>

DCM: dilated cardiomyopathy, ICM: ischemic cardiomyopathy
Figure 6. Length-tension relationship, force-frequency relationship, and β-adrenergic stimulation in non-failing vs. failing hearts.
A) Length-tension relationship is not different between non-failing and failing hearts (ANOVA, $P = 0.84269$). B) Force-frequency relationship is negative in failing hearts. C) β-adrenergic response is shifted to the right in failing hearts. * signifies $P < 0.05$ as determined with two-way ANOVA and # signifies $P < 0.05$ as determined with un-paired t-test between non-failing and failing groups. n = 8 non-failing, n = 8-9 failing hearts (1-3 trabeculae per heart). Total of 23 trabeculae: 12 of the experiments were performed by Nima Milani-Nejad. The rest were performed by other members of the Janssen Laboratory: Benjamin D. Canan, Jennifer A. Conkle, Mohammad T. Elnaksih, Rachel L. Gearinger, Jason Murray, and Eric J. Schultz.
Table 4. Twitch characteristics of muscle utilized for $k_{fr}$ experiments.

<table>
<thead>
<tr>
<th></th>
<th>$F_{dev}$ (mN/mm$^2$)</th>
<th>$F_{rest}$ (mN/mm$^2$)</th>
<th>TTP (ms)</th>
<th>$dF/dt_{max}$ (mN/mm$^2$/s)</th>
<th>$dF/dt_{max}/F$ (s$^{-1}$)</th>
<th>$RT_{50}$ (ms)</th>
<th>$dF/dt_{min}$ (mN/mm$^2$/s)</th>
<th>$dF/dt_{min}/F$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-failing</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$L_{opt}$</td>
<td>23.2 ± 1.7</td>
<td>7.5 ± 0.7</td>
<td>212.6 ± 12.1</td>
<td>179.8 ± 16.6</td>
<td>7.8 ± 0.5</td>
<td>137.0 ± 4.0</td>
<td>-127.6 ± 9.1</td>
<td>-127.6 ± 9.1</td>
</tr>
<tr>
<td>$L_{95}$</td>
<td>9.3 ± 1.1*</td>
<td>4.2 ± 0.5* ‡</td>
<td>174.7 ± 8.7*</td>
<td>84.8 ± 11.8*</td>
<td>9.0 ± 0.5*</td>
<td>123.9 ± 5.6*</td>
<td>-51.8 ± 5.4*</td>
<td>-51.8 ± 5.4*</td>
</tr>
<tr>
<td>$L_{90}$</td>
<td>4.9 ± 0.6* † ‡</td>
<td>2.4 ± 0.3* † ‡</td>
<td>155.4 ± 7.1*</td>
<td>48.6 ± 8.1* † ‡</td>
<td>9.5 ± 0.7* † ‡</td>
<td>114.7 ± 5.3* † ‡</td>
<td>-30.8 ± 3.7* † ‡</td>
<td>-30.8 ± 3.7* † ‡</td>
</tr>
<tr>
<td>$L_{opt}$, repeat</td>
<td>8.9 ± 1.1*</td>
<td>6.8 ± 0.8</td>
<td>181.0 ± 8.7*</td>
<td>79.1 ± 12.1*</td>
<td>8.6 ± 0.5*</td>
<td>126.7 ± 3.7</td>
<td>-49.0 ± 5.7*</td>
<td>-49.0 ± 5.7*</td>
</tr>
<tr>
<td><strong>Failing</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$L_{opt}$</td>
<td>18.5 ± 3.0</td>
<td>10.6 ± 1.6</td>
<td>181.0 ± 10.8</td>
<td>154.8 ± 21.0</td>
<td>8.8 ± 0.5</td>
<td>136.4 ± 5.9</td>
<td>-96.6 ± 15.3</td>
<td>-96.6 ± 15.3</td>
</tr>
<tr>
<td>$L_{95}$</td>
<td>10.8 ± 2.3* ‡</td>
<td>5.7 ± 1.4* ‡</td>
<td>159.3 ± 8.8*</td>
<td>96.7 ± 17.2*</td>
<td>9.6 ± 0.5* ‡</td>
<td>120.4 ± 4.4*</td>
<td>-59.8 ± 12.9*</td>
<td>-59.8 ± 12.9*</td>
</tr>
<tr>
<td>$L_{90}$</td>
<td>7.3 ± 1.7* ‡</td>
<td>3.7 ± 1.8* ‡</td>
<td>147.7 ± 8.3*</td>
<td>71.7 ± 14.1* ‡</td>
<td>10.4 ± 0.5* ‡</td>
<td>109.8 ± 2.7* ‡</td>
<td>-43.8 ± 9.3* ‡</td>
<td>-43.8 ± 9.3* ‡</td>
</tr>
<tr>
<td>$L_{opt}$, repeat</td>
<td>14.9 ± 3.4</td>
<td>10.6 ± 2.6</td>
<td>177.3 ± 10.5</td>
<td>127.6 ± 27.3</td>
<td>8.9 ± 0.5</td>
<td>126.0 ± 6.1</td>
<td>-78.9 ± 17.7</td>
<td>-78.9 ± 17.7</td>
</tr>
</tbody>
</table>

All parameters were measured at 1 Hz. n = 9 non-failing, 10 failing (1 trabeculae per heart). P < 0.05 as determined with ANOVA for repeated measures with Bonferroni post-Hoc analysis is indicated with * vs. $L_{opt}$, † vs. $L_{95}$, ‡ vs. $L_{opt}$, repeat of the same group.

$F_{dev}$: developed force, $F_{rest}$: resting force, TTP: time to peak force, $dF/dt_{max}$: maximal velocity of contraction, $dF/dt_{max}/F$: maximal velocity of contraction normalized to developed force, $RT_{50}$: time from peak force to 50% relaxation, $dF/dt_{min}$: maximal velocity of relaxation, $dF/dt_{min}/F$: maximal velocity of relaxation normalized to developed force.
Table 5. Twitch characteristics of muscle from Figure 6A.

<table>
<thead>
<tr>
<th></th>
<th>TTP (ms)</th>
<th>dF/dt&lt;sub&gt;max&lt;/sub&gt; (mN/mm&lt;sup&gt;2&lt;/sup&gt;/s)</th>
<th>dF/dt&lt;sub&gt;max&lt;/sub&gt;/F (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>RT&lt;sub&gt;50&lt;/sub&gt; (ms)</th>
<th>dF/dt&lt;sub&gt;min&lt;/sub&gt; (mN/mm&lt;sup&gt;2&lt;/sup&gt;/s)</th>
<th>dF/dt&lt;sub&gt;min&lt;/sub&gt;/F (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-failing</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L&lt;sub&gt;opt&lt;/sub&gt;</td>
<td>219.3 ± 15.0</td>
<td>109.4 ± 24.7 7.0 ± 0.4</td>
<td>142.4 ± 11.4</td>
<td>-100.4 ± 30.1</td>
<td>-5.5 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>L&lt;sub&gt;95&lt;/sub&gt;</td>
<td>210.5 ± 15.6</td>
<td>81.8 ± 14.4* 7.6 ± 0.6</td>
<td>130.3 ± 10.5</td>
<td>-72.0 ± 19.3</td>
<td>-5.8 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>L&lt;sub&gt;90&lt;/sub&gt;</td>
<td>193.9 ± 13.0* † 64.0 ± 11.8* 8.2 ± 0.6*</td>
<td>126.8 ± 7.8*</td>
<td>51.0 ± 12.9*</td>
<td>-5.9 ± 0.5</td>
<td></td>
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<tr>
<td><strong>Failing</strong></td>
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<tr>
<td>L&lt;sub&gt;opt&lt;/sub&gt;</td>
<td>211.2 ± 13.8</td>
<td>104.1 ± 26.3 7.6 ± 0.6</td>
<td>153.1 ± 9.5</td>
<td>-70.2 ± 17.7</td>
<td>-4.9 ± 0.3</td>
<td></td>
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<tr>
<td>L&lt;sub&gt;95&lt;/sub&gt;</td>
<td>204.8 ± 15.8</td>
<td>71.9 ± 16.3* 7.6 ± 0.6</td>
<td>143.1 ± 8.1</td>
<td>-46.8 ± 11.5*</td>
<td>-4.8 ± 0.3 §</td>
<td></td>
</tr>
<tr>
<td>L&lt;sub&gt;90&lt;/sub&gt;</td>
<td>195.8 ± 14.4</td>
<td>51.9 ± 12.1* 8.2 ± 0.7*</td>
<td>140.4 ± 8.6</td>
<td>-31.5 ± 6.8*</td>
<td>-5.2 ± 0.4</td>
<td></td>
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</tbody>
</table>

All parameters measured at 0.5 Hz. n = 8 non-failing, n = 9 failing (1-3 trabeculae per heart). P < 0.05 as determined with ANOVA for repeated measures with Bonferroni post-Hoc analysis is indicated with * vs. L<sub>opt</sub> and † vs. L<sub>95</sub> of the same group. § signifies P < 0.05 vs. non-failing of the same length as determined with un-paired t-test. TTP: time to peak force, dF/dt<sub>max</sub>: maximal velocity of contraction, dF/dt<sub>max</sub>/F: maximal velocity of contraction normalized to developed force, RT<sub>50</sub>: time from peak force to 50% relaxation, dF/dt<sub>min</sub>: maximal velocity of relaxation, dF/dt<sub>min</sub>/F: maximal velocity of relaxation normalized to developed force.
Figure 7. Typical twitch tracing of a human right ventricular trabeculae. Developed force ($F_{\text{dev}}$) corresponds to the tension that is generated during muscle contraction and resting force ($F_{\text{rest}}$) corresponds to the passive tension of the trabeculae. Time to peak (TTP) is the time it takes for the muscle to reach peak force development from onset of stimulation. RT$_{50}$ is the time it takes for the muscle to relax from peak developed force to 50% of the force. $dF/dt_{\text{max}}$ and $dF/dt_{\text{min}}$ are the maximal velocity of force development and relaxation, respectively (expressed as mN/mm$^2$/s). $dF/dt_{\text{max}}$ and $dF/dt_{\text{min}}$ can be normalized to the developed force resulting in $dF/dt_{\text{max}}/\text{Force}$ and $dF/dt_{\text{min}}/\text{Force}$, respectively (not shown). The units of these measurements are s$^{-1}$. 
Figure 8. K\textsuperscript+ contracture and $k_{tr}$ tracings in intact human cardiac trabeculae.
A) Typical K\textsuperscript+ contracture in a human trabecula. The arrowhead shows when $k_{tr,max}$ was measured. The arrows show the approximate time of assessment of $k_{tr}$ during sub-maximal activation levels; note that these varied highly from muscle to muscle. No $k_{tr}$ maneuver was performed in this particular contracture. B) Representative $k_{tr}$ tracing in a human trabecula. The movement of the motor is shown on the top. Note the transient overshoot during force redevelopment while the position of the motor is constant.
Figure 9. There is no difference in $k_{tr,max}$ during maximal activation between non-failing and failing hearts.
A) Increase in muscle length results in an increase in developed tension during the K$^+$ contracture. B) Representative $k_{tr}$ tracings from non-failing and failing hearts. C) Increasing muscle length decreases $k_{tr}$ in both non-failing and failing hearts. However, there is no difference between non-failing and failing hearts at each length. *, #, and $^\ddagger$ signify $P < 0.05$ vs. L$_{opt}$, L$_{95}$, and L$_{opt,repeat}$ of the same heart group, respectively as determined with one-way ANOVA for repeated measures. n = 9 non-failing, n = 10 failing hearts (1 trabeculae per heart).
Figure 10. No significant differences in $k_{tr,\text{max}}$ among etiologies of heart failure. $n = 9$ non-failing, $n = 3$ DCM, $n = 6$ ICM. Statistical analysis performed with one-way ANOVA.
Figure 11. Sub-maximal $k_{tr}$ values are not different between non-failing and failing samples at each respective muscle length.
n = 6-8 non-failing n = 6 failing hearts (each point represents data from 2-7 hearts, average of 1-3 $k_{tr}$ experiments per heart at each point).
Chapter 4: Properties of Tension Overshoot in Human Myocardium; Role of Phosphorylation Modifications in the Length-Dependent Regulation of Cross-Bridge Cycling Kinetics

4.1 Introduction

The following chapter is a presentation of preliminary data showing: 1) tension overshoots following $k_{tr}$ maneuver are an inherent property of the human trabeculae, and 2) phosphorylation modifications do not have a central role in the length-dependent regulation of cross-bridge cycling kinetics in human right ventricular trabeculae. It is emphasized that the data presented in this chapter are *preliminary* and further experimentation is required.

There was a brief period of increased tension following the slack-restretch maneuver of the rate of tension redevelopment ($k_{tr}$) tracings in human right ventricular trabeculae. Tension overshoots following similar slack-restretch maneuvers of the $k_{tr}$ protocol have been previously documented in permeabilized rat cardiomyocytes upon PKA treatment $^{66}$, permeabilized rat soleus fibers $^{182}$, and unpublished experiments in permeabilized porcine, canine, and rat cardiac preparations $^{182}$. Additionally, slight overshoots are sometimes observed in published $k_{tr}$ tracings of permeabilized mouse cardiac preparations $^{72}$, permeabilized human cardiac preparations $^{183}$, and intact rat cardiac trabeculae $^{141}$; however, these overshoots were not discussed by the investigators.
To our knowledge there are no previous reports documenting and investigating tension overshoots in intact human cardiac trabeculae. It was necessary to perform further experiments in order to determine whether these overshoots are mediated by the muscle itself or an experimental artifact.

The second part of this chapter focuses on dissecting the role of contractile protein phosphorylations in the length dependent regulation of cross-bridge cycling kinetics in human myocardium. Phosphorylation of contractile proteins, such as MLC-2 and TnI Ser23/24, is a potential mechanism for regulation of cross-bridge cycling kinetics. Previous studies show that increasing muscle length also induces phosphorylation of one or both of these proteins. Therefore, it is possible that these length induced phosphorylation modifications can potentially play a role in the length-dependent regulation of cross-bridge cycling kinetics. Two different approaches were used in order to determine whether phosphorylation modifications have a role in the effects of muscle length on cross-bridge cycling kinetics. One of these approaches is based on a previous study by our laboratory which showed that inhibition of PKA and PKCβII can prevent length dependent phosphorylation of MLC-2 and TnI. The other method is a contra-length stabilization protocol, which will be discussed in detail later.

4.2 Materials and Methods

4.2.1 Isolation of Human Trabeculae

The experiments on the relationship between phosphorylation modifications and $k_{ir}$ were an extension from a subset of muscles in Chapter 3. Muscles from additional
human hearts for assessing tension overshoots were acquired and isolated using the techniques outlined in Chapter 3. In brief, trabeculae were isolated from right ventricles of human myocardium while in a cold K-H solution containing 20 mM BDM. Muscles were attached to a force transducer and a linear motor via two pairs of hooks on each side. The perfusion solution was a modified K-H solution (37 °C) without BDM and containing an initial CaCl$_2$ concentration of 0.25 mM. Muscles were stimulated at 1 Hz and the CaCl$_2$ concentration was gradually raised to 2 mM. Optimal length was determined as previously described in Chapters 2 and 3.

### 4.2.2 Experimental Approach for Investigating Tension Overshoots

Both the experimental protocol and data for the effects of temperature on $k_{tr}$ were presented in Chapter 3. The properties of tension overshoots at different temperatures (n = 4 from four non-failing hearts) were analyzed and presented here. The effect of the relative K$^+$ contracture tension levels on the amplitude of tension overshoots was determined as follows. The $k_{tr}$ data from Chapter 3 and the data on the effects of PKA/PKCβII inhibitors on $k_{tr}$ presented in this chapter were combined and amplitudes of tension overshoots were calculated.

The protocols for performing contracture and $k_{tr}$ experiments have been previously described in Chapters 2 and 3. Four muscles (2 non-failing and 2 failing hearts) were used to determine whether tension overshoots occur in resting muscles. These muscles were first used for assessing the impact of PKA/PKCβII inhibitors on $k_{tr}$. Following these measurements, muscle lengths were re-adjusted to $L_{opt}$ and briefly re-
stimulated at 1 Hz. The electrical stimulation was paused and 3-4 successive $k_{tr}$ maneuvers were performed without inducing a $K^+$ contracture. All experiments were performed at 37 °C.

One muscle from a non-failing heart was used to assess whether tension overshoots occur at high resting tensions. The muscle was initially stabilized at $L_{opt}$ and 1 Hz for 15 minutes. The $K^+$ contracture was induced and the $k_{tr}$ was performed at maximal contracture tension. Afterwards, the muscle was re-stabilized at $L_{opt}$ and 1 Hz for 15 minutes. Stimulation was paused and the muscle was stretched until the resting tension was approximately equal to the total $K^+$ contracture tension. A $k_{tr}$ maneuver was performed at this high resting tension level. The muscle length was returned back to $L_{opt}$ and re-stabilized at 1 Hz for 15 minutes. Subsequently, $K^+$ contracture and $k_{tr}$, at maximal contracture tension, were performed. All of these measurements were made at 37 °C.

Two muscles, from two non-failing hearts, were used for assessing the effects of BDM on tension overshoots. All experiments were performed at $L_{opt}$, 1 Hz, and 37 °C. Muscles were initially stabilized at these conditions for 15 minutes. $K^+$ contractures and $k_{tr}$ experiments were performed without any BDM as previously described. Muscles were then re-stabilized in the K-H solution without any BDM for 15 minutes. The high $K^+/high \text{Ca}^{2+}$ solution (i.e. the $K^+$ contracture solution) was changed to one which contained 10 mM of pre-dissolved BDM, and the $K^+$ contracture and $k_{tr}$ experiments were performed. Muscles were re-stabilized in K-H solution without any BDM for 15 minutes while the high $K^+/high \text{Ca}^{2+}$ solution was changed to another solution with 20 mM of BDM. The $K^+$ contracture and $k_{tr}$ experiments were performed in the presence of 20 mM
BDM. Subsequently, muscles and the setup were thoroughly perfused with K-H and high K\(^+\)/high Ca\(^{2+}\) solutions that did not contain any BDM in order to washout any remaining BDM. Muscles were re-stabilized for 15 minutes and the K\(^+\) contracture and \(k_{tr}\) experiments were performed in the absence of BDM. Finally, muscles were re-stabilized in the K-H solution for 15 minutes. The high K\(^+\)/high Ca\(^{2+}\) solution was changed to one that contained 50 mM of BDM, and the K\(^+\) contracture and \(k_{tr}\) experiments were performed. The overall order of the experiments was Control → 10 mM BDM → 20 mM BDM → Washout → 50 mM BDM. All \(k_{tr}\) experiments were performed at the maximal tension of the K\(^+\) contracture.

4.2.3 Experimental Approach for Investigating the Relationship between Phosphorylations and Length-Dependent Regulation of \(k_{tr}\)

The following experiments were conducted on a sub-set of non-failing and failing muscles of Chapter 3. The control experiments without inhibitors at \(L_{opt}\) (i.e. stabilized \(L_{opt}\)) and \(L_{90}\) (i.e. stabilized \(L_{90}\)) have been previously reported in Chapter 3. In one approach, a contra-length stabilization protocol was used as follows. \(k_{tr}\) was initially measured during maximal K\(^+\) contracture tension after the muscle was stabilized at \(L_{90}\) and 1 Hz for ~10-15 minutes (corresponding to \(L_{90}\) of Chapter 3). The muscle was quickly stretched to \(L_{opt}\) while it was still under contracture and \(k_{tr}\) was measured at this length without any stabilization. Similarly, muscles were allowed to stabilize at \(L_{opt}\) and 1 Hz for ~10-15 minutes (corresponding to \(L_{opt,repeat}\) of Chapter 3). After the \(k_{tr}\) at \(L_{opt}\) was
measured during the maximal K⁺ contracture tension, the muscle was quickly slacked to L₉₀ while under contracture and a kᵣ tr experiment was performed without stabilization.

A second approach was used after the kᵣ tr measurements at Lₒpt,repeat were performed. Muscles were allowed to re-stabilize at Lₒpt, 1 Hz, 37 °C without any inhibitors for 15 minutes. Muscles were then stabilized in the presence of 20 µM H-89 and 7.5 nM PKCβII peptide inhibitor I for 15 minutes. These inhibitors were also added to the high K⁺/high Ca²⁺ solution (i.e. the K⁺ contracture solution). The K⁺ contracture was induced and kᵣ tr maneuvers at sub-maximal and maximal tension levels were performed. Muscles were next stabilized at L₉₀, 1 Hz, and 37 °C with the inhibitors for 15 minutes. Afterwards, K⁺ contracture and kᵣ tr experiments were conducted during sub-maximal and maximal tension levels.

4.2.4 Data and Statistical Analysis

All data were collected and analyzed using custom-made programs in LabView (National Instruments). Tensions were normalized to the cross-sectional areas (mm²) of the muscles. All kᵣ tr tracings were fit to the equation \( F = F_{\text{max}} \cdot (1-e^{-\alpha \cdot k_{\text{tr}}(t)}) + F_{\text{initial}} \) using Origin 7 (OriginLab Corp). Differences between tension overshoot kinetics at different temperatures were determined with one-way ANOVA for repeated measures with Bonferroni post-hoc test. Differences for experiments on contra-length stabilization and effects of inhibitors were determined with paired Student’s t-tests. Statistical significance was set as \( P < 0.05 \) for all tests. All data is shown as mean ± S.E.M.
4.3 Results

4.3.1 Effects of Temperature on Tension Overshoots

A schematic presentation of the terms used for characterizing tension overshoots is provided in Figure 12. Note that the motor is stationary at its original length when the overshoot occurs. As previously discussed in Chapter 3, decreasing temperature resulted in a decrease in $k_{ir}$. Additionally, decreasing temperature slowed down the kinetics of the Tension Decline Phase. Decreasing temperature resulted in an increase in $t_{1/2}$ of this phase with a $Q_{10}$ of ~1.9 (range: ~1.7-2.2) (Figure 13A). There was a close relationship between $k_{ir}$ (during the force redevelopment phase) and $t_{1/2}$ of the Tension Decline Phase (inverse of this parameter shown for clarity purposes) (Figure 13B). Temperature did not affect the amplitudes of tension overshoots (Figure 13C).

4.3.2 No Tension Overshoots in Resting Muscles

In some muscles ($n = 4$, 2 non-failing and 2 failing), we determined whether tension overshoots occur when the muscles are resting. In these muscles we initially observed tension overshoots during the $K^+$ contracture (Figure 14A). However, there were no tension overshoots in any of the $k_{ir}$ maneuvers performed while the muscles were resting, without contracture or electrical stimulation (Figure 14B and C).

In one muscle from a non-failing heart, we investigated whether tension overshoots occur if the resting tension of the muscle (non-stimulated, no contracture) is matched to the maximal $K^+$ contracture tension. There was initially a tension overshoot (amplitude: 19.8%) when the muscle was under maximal $K^+$ contracture at $L_{opt}$ (Figure 14B and C).
15A). However, there was no tension overshoot if the muscle was resting (no stimulation, no contracture) even though the muscle was stretched enough in order to match its resting tension to the total tension of the previous K⁺ contracture (Figure 15B). Performing the $k_{ir}$ maneuver during the maximal tension of the K⁺ contracture at $L_{opt}$ for a second time resulted in the reappearance of the tension overshoot (amplitude: 20.0%) (Figure 15C).

4.3.3 BDM Decreases Tension Overshoot Amplitudes

The effects of BDM on tension overshoots were assessed in another group of muscles (n = 2, two non-failing hearts). BDM was present only in the high K⁺/Ca²⁺ solution and absent in the K-H solution. Under baseline conditions, tension overshoots (amplitude of ~22.3 ± 2.3%) were present in both muscles (Figure 16A). In the presence of 10 mM BDM, the tension developed during the K⁺ contracture decreased by ~59.7 ± 5.5%. The tension overshoots were still present though with a lower amplitude (~15.1 ± 6.5%) (Figure 16B). Conversely, there were no tension overshoots in both muscles when the concentration of BDM was increased to 20 mM (Figure 16C). This concentration of BDM decreased the tension generated during the K⁺ contracture by 74.4 ± 5.9%. Washing out BDM resulted in the reappearance of tension overshoots in both muscles (amplitude of ~29.5 ± 2.1%) (Figure 16D). Finally, there were no tension overshoots with the introduction of 50 mM BDM, which decreased the tension developed during K⁺ contracture by ~82.6 ± 0.5% (Figure 16E).
4.3.4 Tension Overshoot Amplitudes Depend on the Relative K\(^+\) Contracture Tension Levels

There was a relationship between relative K\(^+\) contracture tension and the amplitude of the tension overshoots in non-failing and failing myocardium (Figure 17). In both groups, this amplitude increased as the relative K\(^+\) contracture increased up to half maximal contracture tension (~0.6 and ~0.4-0.6 in non-failing and failing myocardium, respectively). Tension overshoot amplitudes then decreased as the relative K\(^+\) contracture tensions increased from half maximal to maximal.

4.3.5 Effects of Contra-length Stabilization on \(k_{tr}\)

We determined whether the length at which muscles are stabilized has an effect on the relationship between muscle length and \(k_{tr,max}\) (during maximal K\(^+\) contracture tension) (Figure 18). The data on \(k_{tr}\) measured at \(L_{opt}\) while stabilized at \(L_{opt}\), and \(k_{tr}\) measured at \(L_{90}\) while stabilized at \(L_{90}\) were first reported in Chapter 3. In both non-failing (\(n = 6\)) and failing (\(n = 7\)) myocardium, \(k_{tr,max}\) at \(L_{opt}\) was very similar regardless of whether it was stabilized at \(L_{opt}\) or \(L_{90}\) (paired t-tests, \(P = 0.7292\) for non-failing, \(P = 0.9716\) for failing). The \(k_{tr,max}\) at \(L_{90}\) was slightly greater if the muscles had been stabilized at \(L_{opt}\) as opposed to \(L_{90}\) in both non-failing (\(n = 6\)) and failing (\(n = 8\)) myocardium. However, these differences did not reach statistical significance in either group (paired t-test, \(P = 0.2266\) for non-failing, \(P = 0.08989\) for failing). The \(F_{res}:F_{K+}\) (ratio of residual force to developed K\(^+\) contracture force) were similar within each respective muscle length regardless of the length at which the muscles were stabilized.
The $F_{\text{res}}:F_{\text{K+}}$ of $k_{tS}$ measured at $L_{\text{opt}}$ were 0.29 ± 0.04 (stabilized at $L_{\text{opt}}$) and 0.32 ± 0.04 (stabilized at $L_{90}$) for the non-failing muscles (paired t-test, $P = 0.7167$), and 0.21 ± 0.08 (stabilized at $L_{\text{opt}}$) and 0.30 ± 0.07 (stabilized at $L_{90}$) for the failing muscles (paired t-test, $P = 0.1932$). The ratios of $k_{tS}$ measured at $L_{90}$ were 0.34 ± 0.03 (stabilized at $L_{90}$) and 0.22 ± 0.06 (stabilized at $L_{\text{opt}}$) for the non-failing muscles (paired t-test, $P = 0.103$), and 0.19 ± 0.06 (stabilized at $L_{90}$) and 0.07 ± 0.15 (stabilized at $L_{\text{opt}}$) for the failing muscles (paired t-test, $P = 0.4273$).

The maximal developed tensions during the $K^+$ contracture at $L_{\text{opt}}$ of both non-failing and failing myocardium were not affected by the contra-length stabilization. The $L_{\text{opt}}$ tensions for the non-failing myocardium were 37.3 ± 5.7 mN/mm$^2$ (stabilized at $L_{\text{opt}}$) and 40.3 ± 6.5 mN/mm$^2$ (stabilized at $L_{90}$) (paired t-test, $P = 0.2241$). The $L_{\text{opt}}$ tensions for the failing myocardium were 41.0 ± 4.5 mN/mm$^2$ (stabilized at $L_{\text{opt}}$) and 40.3 ± 3.9 mN/mm$^2$ (stabilized at $L_{90}$) (paired t-test, $P = 0.8718$). Stabilizing muscles at $L_{\text{opt}}$ and quickly slacking them to $L_{90}$ during the contracture did, however, cause a decrease in the tensions as compared to contracture tensions when stabilized at $L_{90}$. The $L_{90}$ tensions for the non-failing myocardium decreased from 20.4 ± 3.9 mN/mm$^2$ (stabilized at $L_{90}$) to 9.9 ± 2.3 mN/mm$^2$ (stabilized at $L_{\text{opt}}$) (paired t-test, $P < 0.05$). The $L_{90}$ tensions for the failing myocardium decreased from 24.8 ± 3.1 mN/mm$^2$ (stabilized at $L_{90}$) to 16.1 ± 14.2 mN/mm$^2$ (stabilized at $L_{\text{opt}}$) (paired t-test, $P < 0.05$).
4.3.6 Effects of PKA/PKCβII inhibition on $k_{tr}$

A subset of non-failing (n = 5) and failing (n = 5) muscles from Chapter 3 were treated with H-89 and PKCβII peptide inhibitor I. The data in the absence of inhibitors were first reported in Chapter 3. The effects of these inhibitors on twitch kinetics are presented in Table 6. The inhibitors did not cause a significant change in $\text{d}F/\text{d}t_{\text{max}}/\text{Force}$ or $\text{d}F/\text{d}t_{\text{min}}/\text{Force}$ at either $L_{\text{opt}}$ or $L_{90}$.

The effects of these inhibitors on $k_{tr}$ were assessed during the maximal $K^+$ contracture tensions (Figure 19). Treatment with inhibitors did not affect $k_{tr,\text{max}}$ at $L_{\text{opt}}$ in either non-failing or failing muscles (paired t-test, $P = 0.8205$ for non-failing, $P = 0.6487$ for failing). Similarly, inhibitor treatment had no effect on $k_{tr,\text{max}}$ at $L_{90}$ in either non-failing or failing muscles (paired t-test, $P = 0.1659$ for non-failing, $P = 0.9604$ for failing). There was no statistically significant difference between $F_{\text{res}}:F_{K^+}$ within each group. The ratios of non-failing muscles at $L_{\text{opt}}$ were $0.37 \pm 0.07$ (- inhibitors) and $0.23 \pm 0.04$ (+ inhibitors) (paired t-test, $P = 0.103$), and at $L_{90}$ were $0.03 \pm 0.24$ (- inhibitors) and $0.14 \pm 0.16$ (+ inhibitors) (paired t-test, $P = 0.7167$). The ratios for failing muscles at $L_{\text{opt}}$ were $0.29 \pm 0.07$ (- inhibitors) and $0.25 \pm 0.04$ (+ inhibitors) (paired t-test, $P = 0.5196$), and at $L_{90}$ were $0.19 \pm 0.07$ (- inhibitors) and $0.24 \pm 0.08$ (+ inhibitors) (paired t-test, $P = 0.6815$).

The maximal tension developed during the $K^+$ contractures was lower in the presence of the inhibitors. The $L_{\text{opt}}$ tension for the non-failing myocardium decreased from $36.3 \pm 4.8 \text{ mN/mm}^2$ (- inhibitors) to $28.0 \pm 3.5 \text{ mN/mm}^2$ (+ inhibitors); however, this decline did not reach statistical significance (paired t-test, $P = 0.06243$). Conversely,
the decrease in $L_{opt}$ tension of the failing myocardium from $41.5 \pm 5.3 \text{ mN/mm}^2$ (-inhibitors) to $36.8 \pm 5.8 \text{ mN/mm}^2$ (+ inhibitors) did reach statistical significance (paired t-test, $P < 0.05$). The $L_{90}$ tensions decreased from $16.7 \pm 1.5 \text{ mN/mm}^2$ (-inhibitors) to $7.8 \pm 0.9 \text{ mN/mm}^2$ (+ inhibitors) in the case of non-failing myocardium (paired t-test, $P < 0.05$), and from $27.0 \pm 2.8 \text{ mN/mm}^2$ (-inhibitors) to $19.2 \pm 2.5 \text{ mN/mm}^2$ (+ inhibitors) in the case of failing myocardium (paired t-test, $P < 0.05$).

The effects of these inhibitors on $k_{tr}$ were also assessed at sub-maximal $K^+$ contracture tensions (Figures 20A-D). It should be noted that there was a lot of variability in the relative tensions (where the $k_{tr}$ maneuver was performed) from experiment to experiment. This caused the sample size at each activation level to be too low.

4.4 Discussion

4.4.1 Tension Overshoots in Human Myocardium

In the first part of this chapter, we show that 1) kinetics of tension overshoot are temperature dependent, 2) resting muscles, regardless of the amount of tension, do not have tension overshoots, 3) BDM is capable of preventing overshoots, and 4) the amplitude of overshoot is dependent on the relative contracture tension level. These results collectively show that overshoots in human $k_{tr}$ tracings, are mediated by the muscles themselves and not an experimental artifact.

Both the $k_{tr}$ and the Tension Decline Phase $t_{1/2}$ were highly dependent on temperature. If these overshoots were simply an artifact of the experimental protocol, it would be highly unlikely that both parameters would be temperature dependent. Several
studies show that kinetics of cardiac contraction and cross-bridge cycle speed up with increasing temperatures\textsuperscript{53,54,174,175}. The temperature dependency of the kinetics of tension overshoot suggest that these overshoots are mediated by some cardiac process, most likely the cross-bridges.

This was further supported by the lack of tension overshoots in the $k_{tr}$ maneuvers performed when muscles were resting (i.e. neither electrically stimulated nor under contracture). Under these conditions, the myofilaments should be inactive due to low intracellular calcium concentration. Conversely, tensions overshoots were observed under the K\textsuperscript{+} contracture where the myofilaments are activated due to an increase in intracellular calcium concentration\textsuperscript{149}. This suggests that it is necessary to have activated myofilaments in order to induce these overshoots. Additionally, this also disposes of the possibility that overshoots are due to a transient increase in intracellular calcium, such as calcium entry through stretch-activated channels\textsuperscript{184}, following the slack-restretch maneuver. The resting tension of cardiac muscle is very low as compared to the maximal K\textsuperscript{+} contracture tension. It can be argued that overshoots are an artifact of the experimental setup and that a sufficiently high amount of tension, regardless of its source, is required to reveal these artifacts. In order to dispose of this possibility, we performed a $k_{tr}$ maneuver in a resting muscle while its resting tension was matched to that of the K\textsuperscript{+} contracture tension. Despite the matched tensions, there was no tension overshoot present. These experiments further support the notion that tension overshoots are not an experimental artifact. The requirement for activated myofilaments, via a K\textsuperscript{+} contracture, implicates the potential role of cross-bridges in these tension overshoots.
The role of cross-bridges in tension overshoots was further investigated with the use of BDM. Increasing BDM concentration progressively decreased both developed tension during the K⁺ contracture and the amplitude of the overshoots. There were no overshoots observed when the majority of cross-bridges were inhibited in the presence of 20 mM (~75% reduction in tension) and 50 mM BDM (~83% reduction in tension). It should be noted that BDM does have some reported side effects besides inhibiting acto-myosin interaction. In one experiment, we substituted blebbistatin, a more specific myosin II ATPase inhibitor, for BDM. The brief exposure of the muscle to blebbistatin (~30 seconds) during the K⁺ contracture was, however, not adequate to sufficiently reduce tensions during the contractures (data not shown). Longer exposure (in the order of several minutes) is necessary in order to achieve profound force inhibition in intact cardiac preparations with this compound. A final piece of information is that the magnitude of tension overshoots varies with relative contracture tension. If these overshoots were artifacts then it would be unlikely that their amplitudes would be modulated by the relative activation level of the myofilaments. This also implicates that the tension overshoots are mediated by processes that are calcium dependent such as the myofilaments. Our current results collectively show that overshoots in human trabeculae are not an artifact of our experimental protocol, and that the cross-bridges are a likely candidate as the underlying mechanism.

Only a few studies have investigated the properties of overshoots following a \(k_{ir}\) maneuver. Campbell K.S. suggested that cross-bridges are the underlying mechanism, while discounting the role of titin. Additionally, calcium affected the magnitude of
tension overshoots with maximal amplitudes occurring near pCa$_{50}$ in this study. This is very similar to our observation in intact preparations where the magnitude of tension overshoots was greatest during half maximal K$^+$ contracture tension levels. As argued by Campbell, a temporary increase in the number of bound cross-bridges results in these overshoots. The cross-bridge requirement for occurrence of tension overshoots in our study is in accordance with this model. The dependency of the amplitude of the overshoots on the relative tension levels might also implicate cross-bridge feedback on the thin filament as a potential mechanism. Only a portion of the thin filament regulatory units are presumably activated by calcium during the half maximal point of the K$^+$ contracture. Therefore, during the force redevelopment, as more and more myosin heads bind to the actin, they can spread the activation to the neighboring thin filament regulatory units. This will potentially allow the myosin heads in the vicinity to be transiently recruited into the cross-bridge cycle resulting in tension overshoots. However, during higher levels of activation, the majority of the thin filament regulatory units will be already activated due to the higher calcium concentrations. This will limit the importance of cross-bridge feedback mechanism in recruiting the neighboring myosin heads into the cross-bridge cycle. This will explain why the amplitude of tension overshoots decreases as relative K$^+$ contracture tension is increased from half-maximal to maximal levels. However, this putative mechanism does not provide explanation as to why the amplitude increases from the lowest tensions to the half-maximal point. It is possible that substantial amount of myosin heads are required in order for the cross-
bridge feedback mechanism to have its greatest effect. Whether this is a mechanism for the tension overshoot is right now a matter of speculation.

Subsequent modeling studies by Campbell K.S. suggested that compliant filaments are a necessary requirement for this transient increase in attached cross-bridges and tension overshoots. Hanft L.M. and McDonald K.S. have proposed that titin also plays a role in these overshoots. In these studies, PKA treatment of permeabilized rat cardiomyocytes resulted in tension overshoots, and the magnitudes were reduced after trypsin digestion of titin. It should be noted that PKA can cause a decrease in stiffness of titin which agrees with the requirement for a more compliant myofilament apparatus put forth by Campbell. While tension overshoots sometimes occurred in our studies on rat myocardium, they were infrequent and had lower amplitudes. Rat hearts predominately express the stiff N2B titin isoform. Humans, on the other hand, express both the stiff N2B and compliant N2BA isoforms, and it is possible that a compliant titin along with cross-bridges are both necessary requirements for these overshoots to occur in humans. However, we currently do not have the necessary experiments to address the involvement of titin in tension overshoots.

4.4.2 Role of Phosphorylation Modifications in Length-Dependent Regulation of Cross-Bridge Cycling Kinetics

Increase in muscle length has been shown to regulate phosphorylation of TnI at Ser23/24 and/or MLC-2. Previous studies suggest that these phosphorylations can regulate cross-bridge cycling kinetics. We attempted to determine whether
length induced phosphorylation modifications play a central role in the regulation of cross-bridge cycling kinetics by muscle length in both non-failing and failing human myocardium. This was assessed via two separate approaches.

In one approach, we measured $k_{tr}$ at $L_{\text{opt}}$ and $L_{90}$ after contra-length stabilization at $L_{90}$ or $L_{\text{opt}}$, respectively. The rationale behind this approach is that this procedure should allow us to assess $k_{tr}$ at a given muscle length with the post-translational modifications of another length. Stabilization at $L_{\text{opt}}$ will allow post-translational modifications at this length to occur. Quickly slacking the muscle to $L_{90}$ and measuring $k_{tr}$ will allow us to assess cross-bridge cycling kinetics at $L_{90}$ with the post-translational modifications of $L_{\text{opt}}$. This was also performed in reverse when the muscle was stabilized at $L_{90}$ and quickly stretched to $L_{\text{opt}}$. The $k_{tr}$ at each length was not statistically different regardless of whether it was stabilized at $L_{\text{opt}}$ or $L_{90}$. Please note that we currently do not have the necessary experiments to definitely state that phosphorylation modifications of contractile proteins are taking place as human right ventricular trabeculae are stretched from $L_{90}$ to $L_{\text{opt}}$ and that the contra-length stabilization protocol can preserve these modifications.

In a second approach, we utilized a cocktail of PKCβII inhibitor peptide I which reduces phosphorylation of TnI and MLC-2, and H-89 (a PKA inhibitor) which reduces phosphorylation of MLC-2, TnI, as well as MyBP-C in rabbit myocardium. Treatment of trabeculae with these inhibitors did not significantly affect $k_{tr}$ at maximal $K^+$ contracture tension levels. In accordance with these results, the inhibitors did not significantly alter the twitch kinetics parameters, $dF/dt_{\text{max}}/F$ and $dF/dt_{\text{min}}/F$. The effects at
sub-maximal activation levels are less clear due to the variability and low sample size. Please note that the previously mentioned inhibitors were effective at reducing the mentioned phosphorylation modifications in rabbit myocardium. It is currently unknown whether these inhibitors are effective in human right ventricular trabeculae.

Based on previous studies on the role of muscle length in modulation of MLC-2 and/or TnI phosphorylations and the role of these phosphorylation modifications in cross-bridge cycling kinetics, we expected that phosphorylation of contractile proteins as induced by muscle length (if they occur) should play a role in the length-dependent regulation of cross-bridge cycling kinetics. We currently do not have all of the necessary information, such as state of the myofilament phosphorylations, in order to make a definitive conclusion. However, we can make speculations as to why our results deviate from our expected outcomes. Of course, one is that these phosphorylations do not play a central role in the length-dependent regulation of cross-bridge cycling kinetics. However, it is also possible that phosphorylation of contractile proteins did not change or only slightly changed as muscles were stretched from L_{90} to L_{opt}. Note that the previous studies were performed in rat and rabbit ventricles, and human atria which are different than our current study on human ventricles. Questions also arise as to whether the inhibitors were effective at altering the phosphorylation modifications and the contra-length stabilization protocol was capable of preserving the phosphorylation modifications. We currently cannot make a definitive statement in regards to the state of phosphorylation of myofilament proteins at different lengths and during our two
protocols. It should be noted that these experiments are preliminary and the conclusions should be observed with caution. Further experiments are warranted.
Figure 12. Schematic definitions of the terms used for tension overshoot. The movement of the motor is shown on top. Note that the overshoot occurred when the motor was stationary. The overshoot amplitude (\%) was calculated as the difference between overshoot tension and developed tension during K\(^+\) contracture tension (pre-\(k_{0}\)) and normalized to the latter. The Tension Decline Phase \(t_{1/2}\) was calculated as the time it takes for the overshoot amplitude to decrease by 50\% from the peak of the overshoot to the lowest point after the overshoot. Muscle is from right ventricle of non-failing heart and stimulated at 1 Hz, \(L_{opt}\), and 37 °C.
Figure 13. Temperature affects kinetics of tension overshoot.
A) Tracings of the Tension Decline Phase at different temperatures in a non-failing muscle is shown on the left. Decreasing temperature resulted in an increase in $t_{1/2}$ of the Tension Decline Phase. * indicates $P < 0.05$ vs. 27 °C as determined with one-way ANOVA for repeated measures and Bonferroni post-hoc test. B) There was a close relationship between $k_{tr}$ and $t_{1/2}$ of Tension Decline Phase. Inverse of $t_{1/2}$ was used for clarity purposes. C) Temperature had no effect on the amplitude of the tension overshoot. $n = 4$ trabeculae from four non-failing hearts. Data is shown as mean ± S.E.M.
Figure 14. No tension overshoots without contracture or stimulation. 
A) Tension overshoot is present during the contracture. The \( k_t \) shown is at the plateau of the contracture. B) Stimulation was paused and three consecutive \( k_t \)s were performed without a contracture. C) Expansion of the first \( k_t \) maneuver from Panel B shows lack of tension overshoot without a contracture or stimulation. Muscle is from non-failing heart in the presence of 20 µM H-89 and 7.5 nM PKCβII peptide inhibitor I and stimulated at 1 Hz, \( L_{\text{opt}} \), and 37 °C. Data shown is representative of four experiments.
Figure 15. High resting tension is not sufficient to cause tension overshoot in a resting muscle.
A) Performing $k_{tr}$ maneuver during the maximal $K^+$ contracture resulted in a tension overshoot. B) Stretching the resting muscle, no stimulation and no contracture, in order to match its resting tension to that of the $K^+$ contracture tension in Panel A did not cause tension overshoots to occur. C) The muscle length was reset to that of Panel A and $k_{tr}$ protocol was performed during the maximal $K^+$ contracture. Note the reappearance of the tension overshoot. Muscle is from a non-failing heart.
Figure 16. BDM decreases magnitude of tension overshoot.  
A) Tension overshoot observed in control (no BDM).  
B) 10 mM BDM reduces tension overshoot.  
C) Tension overshoot is not observed in presence of 20 mM BDM.  
D) Tension overshoot reappears with washout (no BDM).  
E) Tension overshoot does not occur in presence of 50 mM BDM.  
Muscle is from non-failing donor heart. BDM present in only the high K⁺/high Ca²⁺ solution.  
Order: Control → 10 mM BDM → 20 mM BDM → Washout → 50 mM BDM.  
Data shown is representative of two experiments.  
All experiments were performed at 1 Hz, L_{opt}, and 37 °C.
Figure 17. Relationship between tension overshoot amplitude and relative $K^+$ contracture tension.

Combined data at $L_{opt}$ (- inhibitors), $L_{95}$ (- inhibitors), $L_{90}$ (- inhibitors), $L_{opt}$ (+ inhibitors), and $L_{90}$ (+ inhibitors). Inhibitors are 20 µM H-89 and 7.5 nM PKCβII peptide inhibitor I. Data is from 29 separate experiments on non-failing and 27 separate experiments on failing human myocardium. 1-4 data points per experiment per point shown. All experiments were performed at 1 Hz and 37 °C. Data is shown as mean ± S.E.M.
Figure 18. Effects of contra-length stabilization on $k_{tr}$.
Muscles were stabilized at a particular length and $k_{tr}$ was measured at maximal $K^+$ contracture. This was followed by quickly stretching or slacking the muscles to another length and performing $k_{tr}$ without stabilization. The length at which muscles were stabilized and $k_{tr}$s performed is shown on the bottom of each figure. The $k_{tr,max}$ at $L_{opt}$ did not depend on the length at which muscles were stabilized in either non-failing or failing myocardium. The $k_{tr,max}$ at $L_{90}$ were slightly faster when stabilized at $L_{opt}$; though did not reach statistical significance. All experiments were performed at 1 Hz and 37 °C. n = 6 non-failing for $k_{tr}$s at both $L_{opt}$ and $L_{90}$. n = 7 failing for $k_{tr}$s at $L_{opt}$ and n = 8 failing for $k_{tr}$s at $L_{90}$. All statistical analyses were performed with paired Student’s t-test. Data is shown as mean ± S.E.M.
Table 6. Effects of H-89 and PKCβII inhibitor peptide I on twitch parameters.

<table>
<thead>
<tr>
<th></th>
<th>TTP (ms)</th>
<th>dF/dt&lt;sub&gt;max&lt;/sub&gt;/F (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>RT&lt;sub&gt;50&lt;/sub&gt; (ms)</th>
<th>RT&lt;sub&gt;90&lt;/sub&gt; (ms)</th>
<th>dF/dt&lt;sub&gt;min&lt;/sub&gt;/F (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
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<td><strong>Non-failing L&lt;sub&gt;opt&lt;/sub&gt;</strong></td>
<td></td>
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<td>(-) inhibitors</td>
<td>183.2 ± 11.3</td>
<td>8.4 ± 0.6</td>
<td>119.6 ± 3.0</td>
<td>261.4 ± 10.7</td>
<td>5.6 ± 0.2</td>
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<tr>
<td>(+) inhibitors</td>
<td>176.6 ± 11.6</td>
<td>8.3 ± 0.5</td>
<td>121.4 ± 5.5</td>
<td>314.2 ± 25.0*</td>
<td>5.6 ± 0.5</td>
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<td><strong>Non-failing L&lt;sub&gt;90&lt;/sub&gt;</strong></td>
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<tr>
<td>(-) inhibitors</td>
<td>171.0 ± 13.6</td>
<td>8.9 ± 0.8</td>
<td>117.5 ± 3.7</td>
<td>251.8 ± 17.4</td>
<td>6.0 ± 0.1</td>
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<tr>
<td>(+) inhibitors</td>
<td>154.0 ± 9.6*</td>
<td>11.6 ± 1.3</td>
<td>119.0 ± 6.0</td>
<td>359.8 ± 26.7*</td>
<td>5.6 ± 0.6</td>
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<td><strong>Failing L&lt;sub&gt;opt&lt;/sub&gt;</strong></td>
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<tr>
<td>(-) inhibitors</td>
<td>180.4 ± 18.0</td>
<td>8.8 ± 0.9</td>
<td>123.4 ± 7.0</td>
<td>294.8 ± 52.1</td>
<td>5.7 ± 0.2</td>
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<tr>
<td>(+) inhibitors</td>
<td>173.4 ± 13.6</td>
<td>9.3 ± 1.0</td>
<td>136.8 ± 9.6</td>
<td>355.2 ± 34.1</td>
<td>5.0 ± 0.6</td>
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<td><strong>Failing L&lt;sub&gt;90&lt;/sub&gt;</strong></td>
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<tr>
<td>(-) inhibitors</td>
<td>154.2 ± 12.3</td>
<td>10.4 ± 0.8</td>
<td>107.0 ± 3.0</td>
<td>301.2 ± 63.4</td>
<td>6.2 ± 0.2</td>
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<td>(+) inhibitors</td>
<td>146.8 ± 12.0</td>
<td>10.6 ± 1.0</td>
<td>125.2 ± 9.0*</td>
<td>418.9 ± 48.9*</td>
<td>5.5 ± 0.6</td>
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All groups were stimulated at 1 Hz and 37 °C either in the absence (-) or presence (+) of inhibitors (20 µM H-89 and 7.5 nM PKCβII peptide inhibitor I). * indicates P < 0.05 vs. no inhibitors of the same length and group as determined with paired Student’s t-test. n = 5 non-failing for L<sub>opt</sub>, n = 4 non-failing for L<sub>90</sub>, n = 5 failing for both L<sub>opt</sub> and L<sub>90</sub>. Data is shown as mean ± S.E.M.
Figure 19. PKA/PKCβII inhibitors had no effect on $k_{tr,\text{max}}$. Inhibitors did not have an effect on $k_{tr}$ of L$_{\text{opt}}$ and L$_{90}$ in either non-failing and failing myocardium. Experiments performed during maximal K$^+$ contracture tension. The length at which muscles were stabilized and $k_{tr}$ experiments were performed is shown on the bottom. The presence of inhibitors is shown with – (no inhibitor) and + (20 µM H-89 and 7.5 nM PKCβII peptide inhibitor I). All experiments were performed at 1 Hz and 37 °C. n = 5 non-failing and n = 5 failing. All statistical analyses were performed with paired Student’s t-test. Data is shown as mean ± S.E.M.
Figure 20. Effect of PKA/PKCβII inhibitors on $k_{tr}$ during sub-maximal $K^+$ contracture tensions.
Overall, the inhibitors did not have a significant effect on the $k_{tr}$ during sub-maximal tensions in either the non-failing or failing myocardium. All experiments were performed at 1 Hz and 37 °C. $n = 4$ non-failing and $n = 5$ failing hearts (each point represents average of 1-4 hearts. Average of 1-3 $k_{tr}$s was used for each heart). Data is shown as mean ± S.E.M.
Chapter 5: Cardiac Force-Frequency Relationship is Dependent on Baseline Sarcoplasmic Reticulum Calcium Content

5.1 Introduction

The mammalian heart utilizes multiple mechanisms for fine-tuning its pumping activity in order to adjust to changes in the metabolic demands of the body. One of these mechanisms is the force-frequency relationship (FFR), where the frequency of stimulation regulates the force of contraction. A positive FFR, which is found in most, if not all, mammalian species under physiological conditions, is primarily achieved via increases in calcium transients and sarcoplasmic reticulum (SR) calcium content. This is due to multiple mechanisms, such as increased L-type calcium ion channel flux, pronounced sarcoplasmic-reticulum calcium ATPase (SERCA) activity, and relief of the inhibitory activity of phospholamban on SERCA.

The shape and degree of myocardial FFR is highly dependent on the species itself. Most studies indicate that large species including humans have a strong positive FFR. Conversely, studies in smaller species such as rats have yielded conflicting results with some showing a positive FFR, a flat FFR, and others a negative FFR. Most of these seemingly opposite results can be explained by various experimental factors including temperature, range of stimulation frequency, and
occurrence of core hypoxia in large muscle preparations. Whether other factors can also affect the degree of the FFR in experimental settings remains unexplored.

One potential factor that can alter the FFR is the SR calcium content at baseline stimulation frequency. Changing this parameter without altering experimental conditions is not straight-forward. We decided to take advantage of a phenomenon called muscle “run-down”, which is observed routinely but remains unexplained in isolated cardiac muscle preparations. During muscle “run-down”, twitch force decreases over prolonged periods of isometric contractions. This phenomenon can potentially involve alterations in calcium handling, cellular integrity, myofilament function, and/or a combination of these. Our data shows that while myofilament contraction and myocyte integrity remain unaffected, the SR calcium load is decreased during this phenomenon. Furthermore, we show that the FFR becomes enhanced with declining force during muscle run-down, presumably due to a decline in SR calcium content.

5.2 Materials and Methods

5.2.1 Isolation of Cardiac Trabeculae

All experiments were approved by the Institutional Animal Care and Use Committee of The Ohio State University and are in compliance with the laws of The United States of America and conform to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. Male Brown Norway rats (approximately 2-4 months old) were intraperitoneally injected with euthasol (~400 mg/kg pentobarbital sodium and ~50 mg/kg phenytoin sodium). The chest cavity
was exposed by bilateral thoracotomy and the heart was quickly removed after it was injected with 1000 units of heparin. The heart was perfused via the aorta in a retrograde fashion with a modified Krebs-Henseleit solution containing (in mM) 137 NaCl, 5 KCl, 20 NaHCO₃, 1.2 NaH₂PO₄, 1.2 MgSO₄, 10 glucose, 0.25 CaCl₂ and 20 2,3-butanedione monoxime (BDM) at room temperature. This solution was bubbled with 95% O₂/5% CO₂ where the buffering system results in a pH of 7.4. Trabeculae were isolated from the right ventricle and mounted in a custom made setup as previously described. The perfusion solution during experiments was changed to a modified Krebs-Henseleit solution without BDM and containing 2 mM CaCl₂. This solution was also continuously bubbled with 95% O₂/5% CO₂ resulting in a pH of 7.4. Muscles were gradually stretched until reaching optimal length as previously described and all experiments were performed at this length. Muscles for FFR and post-rest potentiation (PRP) experiments were stabilized at a baseline frequency of 4 Hz and 37 °C. Muscles used for rapid cooling contracture (RCC) experiments were stabilized at 1 Hz and 27 °C. Confocal imaging experiments were stabilized at 1 Hz and room temperature. All force recordings were normalized to cross-sectional area of the muscles.

5.2.2 Force-Frequency Relationship and Post-Rest Potentiation

After muscles were stabilized at 4 Hz, the FFR was determined from 4-8 Hz (n = 9, width: 184 ± 12 µm, thickness: 122 ± 8 µm, length: 1.7 ± 0.2 mm). PRP was assessed as follows: muscles (n = 7, width: 203 ± 24 µm, thickness: 137 ± 17 µm, length: 1.9 ± 0.3 mm) were stabilized at baseline frequency of 4 Hz and stimulation was stopped for 1, 3,
5, 10, 20, 30, and 60 seconds. After these FFR and PRP measurements were made, muscles were re-stabilized at 4 Hz. The K\(^+\) contracture, sometimes with rate of tension redevelopment (\(k_{tr}\)) protocol, was performed as previously described \(^{174}\). Following this initial set of experiments, muscles were re-stabilized at 4 Hz for about 15 minutes and this sequence of experiments was performed two more times.

### 5.2.3 Rapid Cooling Contractures

The SR calcium load of a subset of muscles (\(n = 10\), width: 219 ± 35 µm, thickness: 145 ± 23 µm, length: 2.4 ± 0.3 mm) was determined using RCC. After muscles were stabilized at 1 Hz and 27 °C, the stimulation pulse was terminated and simultaneously the muscle bath temperature was reduced from 27 °C to ~ 0 °C. Afterwards, the temperature was switched back to 27 °C, the electrical stimulation was resumed, and the muscles were allowed to stabilize for 5 minutes. The K\(^+\) contracture and \(k_{tr}\) protocol were performed as previously described \(^{174}\) after which the muscles were allowed to re-stabilize at 1 Hz for 15 minutes. Three more rounds of RCC and K\(^+\) protocols were performed as described above. The reproducibility of the RCC was investigated in some muscles by performing two RCCs separated by 2 minutes of stabilization at 1 Hz. There was a correlation between the developed RCC tensions in these duplicate experiments (\(R^2 \sim 0.97\), \(n = 19\) total experiments on 8 muscles, data not shown).

Paired RCC experiments were performed in a subset of the RCC experiments (\(n = 6\) out of 10) by performing an initial RCC as described above. This was followed by
changing the temperature back to 27 °C for a brief period of time (~2-3 s) and then performing a second RCC while the stimulation is off. The reproducibility of this ratio was determined in some muscles by performing two paired RCC experiments separated by 2 minutes of stabilization. Both of these RCC2/RCC1 ratios were similar with an R² of ~ 0.82 (n = 13 total experiments on 5 muscles, data not shown).

5.2.4 Confocal Imaging of Trabeculae

Muscles were divided into two groups, in one group (n = 3, width: 207 ± 35 µm, thickness: 137 ± 24 µm, length: 2.2 ± 0.6 mm), staining was initiated after obtaining optimal length and a brief stabilization period. In the second group (n = 3, width: 220 ± 44 µm, thickness: 143 ± 29 µm, length: 1.3 ± 0.1 mm), muscles were initially stabilized for 10 minutes followed by 90 minutes of stabilization at optimal length, and then stained. Both groups were stained with RH-237 dye as previously described 203. In some cases muscles were paralyzed with 20 µM of blebbistatin during imaging in order to prevent motion artifact. Multiple z-stacks with an acquisition rate of 200 µs/pixel were taken with and without 3x digital magnification from the surface to the deepest layer which had adequate staining. These experiments were carried out by Lucia Brunello, a post-doctoral researcher from the Sandor Gyorke laboratory.

5.2.5 Data and Statistical Analysis

All force recordings were made using custom-made LabView programs (National Instruments). Confocal images were processed using ImageJ software. kᵣ tracings were
fitted to the equation \( F = F_{\text{max}} \cdot (1-e^{-\lambda t}) + F_{\text{initial}} \) using Origin 7 software (OriginLab Corporation). Statistical analyses were performed with one-way ANOVA for repeated measures with Bonferroni post-hoc test or two-way ANOVA with statistical significance set at \( P < 0.05 \). All data is presented as means ± S.E.M. Muscles were excluded from the final analysis if a complete set of four RCC \((n = 2)\) and three PRP \((n = 1)\) experiments were not obtained.

5.3 Results

5.3.1 Relative Force-Frequency Becomes Pronounced as Baseline Twitch Tension Decreases

Rat cardiac muscles had a positive FFR near their in vivo heart rate range (Figure 21A). The peak occurred at 6 Hz, followed by a slight decrease at 7 Hz, and a more prominent decrease at 8 Hz. The developed tension was lower at all frequencies in the 2\(^{nd}\) and 3\(^{rd}\) experiments (ANOVA, \( P < 0.05 \)); however, the decline was more dramatic at lower stimulation frequencies than at higher ones (Figure 21A). Furthermore, the twitch tension in the 2\(^{nd}\) and 3\(^{rd}\) experiments continued to increase up to 8 Hz. Therefore, the FFRs of the 2\(^{nd}\) and 3\(^{rd}\) experiments were more positive when normalized to their corresponding baseline frequency of 4 Hz (ANOVA, \( P < 0.05 \)). Increasing the stimulation frequency from 4 Hz to 8 Hz resulted in relative increases of 12\%, 81\%, and 107\% for the 1\(^{st}\), 2\(^{nd}\) and 3\(^{rd}\) FFR experiments, respectively (Figure 21B, ANOVA \( P < 0.05 \)).
We analyzed parameters of contraction \((\text{d}F/\text{d}t_{\text{max}}/F)\) and relaxation \((\text{d}F/\text{d}t_{\text{min}}/F)\) kinetics in these experiments. It was necessary to divide these kinetic measurements by the developed forces in order to: 1) obtain a purely kinetic measurement (in units of \(\text{s}^{-1}\)) and 2) normalization purposes accounting for the decline in twitch tension.

Increasing stimulation frequency resulted in an increase in both \(\text{d}F/\text{d}t_{\text{max}}/F\) and \(\text{d}F/\text{d}t_{\text{min}}/F\) (ANOVA, \(P < 0.05\)). The \(\text{d}F/\text{d}t_{\text{min}}/F\) did not change from the 1\(^{\text{st}}\) to the 3\(^{\text{rd}}\) experiments (Figure 21C, ANOVA \(P = 0.67664\)). Interestingly, the \(\text{d}F/\text{d}t_{\text{max}}/F\) of the 2\(^{\text{nd}}\) and 3\(^{\text{rd}}\) experiments became accelerated as compared to the 1\(^{\text{st}}\) experiment (Figure 21D, ANOVA, \(P < 0.05\)). Similar to developed tensions, alterations in \(\text{d}F/\text{d}t_{\text{max}}/F\) were more pronounced at lower frequencies.

5.3.2 Post-Rest Potentiation Becomes Enhanced as Twitch Tension Decreases

FFR is highly dependent on calcium cycling mechanisms\(^{77,79}\), therefore, we probed these processes further by performing PRP experiments which are also dependent on calcium handling. Rat cardiac trabeculae (\(n = 7\)) exhibited PRP (Figure 22A) where rest periods resulted in an increase in post-rest tension relative to pre-rest values. This PRP was not only maintained, but became increasingly pronounced in the 2\(^{\text{nd}}\) and 3\(^{\text{rd}}\) PRP experiments (Figure 22A, ANOVA \(P < 0.05\)). There was still considerable decline in non-normalized post-rest tensions over time in the 2\(^{\text{nd}}\) and 3\(^{\text{rd}}\) PRP experiments as compared to the 1\(^{\text{st}}\) (Figure 22B, ANOVA \(P < 0.05\)). We also kept track of non-stimulated contractions during rest periods which can potentially signify abnormal calcium release. Non-stimulated contraction was recorded in one muscle during the 2\(^{\text{nd}}\)
PRP, which was not observed during the 1\textsuperscript{st} or 3\textsuperscript{rd} PRP in the same muscle (data not shown).

5.3.3 \textit{Myofilament Capability of Generating Tension is Not/Slightly Decreased}

As discussed above, there was a profound decrease in developed twitch tensions over time during the course of both FFR and PRP protocols. We performed K\textsuperscript{+} contractures in order to circumvent the cellular calcium handling processes and assess the force generation capability of the myofilaments. In some muscles, we did not achieve a proper apparent level of myofilament activation during contracture (i.e. contracture tension was much lower than twitch tension). These muscles (4 out of 15) were excluded from this analysis. K\textsuperscript{+} contracture data from FFR and PRP experiments were combined and analyzed together. The tension developed during the K\textsuperscript{+} contracture did not significantly change (ANOVA, \( P = 0.21086 \)) during the course of these experiments. These tensions (in mN/mm\textsuperscript{2}) were 45.9 ± 4.3 (1\textsuperscript{st} experiment), 41.7 ± 3.7 (2\textsuperscript{nd} experiment), and 41.2 ± 3.8 (3\textsuperscript{rd} experiment). The resting tensions at 4 Hz (combined FFR and PRP experiments) (in mN/mm\textsuperscript{2}) were 5.1 ± 0.7 (1\textsuperscript{st} experiment), 4.9 ± 0.8 (2\textsuperscript{nd} experiment), and 3.0 ± 0.8 (3\textsuperscript{rd} experiment) (ANOVA, \( P = 0.05615 \)).

5.3.4 \textit{Decline in SR Calcium Load Contributes to the Decrease in Twitch Tension}

We used RCC in order to determine SR calcium content as a factor in decrease of the twitch tension over time. The developed twitch tensions of the 2\textsuperscript{nd}, 3\textsuperscript{rd}, and 4\textsuperscript{th} experiments were significantly decreased, on average, (\( n = 10 \), ANOVA \( P < 0.05 \)) to
81%, 60%, and 46% of the 1st experiment, respectively (Figure 23A). Interestingly, this decrease in twitch tension was accompanied by a similar decrease in SR calcium content (n = 10, ANOVA P < 0.05). Amplitudes of RCC of the 2nd, 3rd, and 4th experiments were, on average, 67%, 48%, and 35% of the 1st experiment, respectively (Figure 23A). The resting tension of these measurements decreased slightly but did not reach statistical significance (ANOVA P = 0.00051, data not shown). The K⁺ contracture tension in these muscles was also decreased slightly over the course of the experiments (Figure 23A) as the 2nd, 3rd, and 4th experiments were 91%, 88%, and 86% of the 1st experiments, respectively (n = 6, ANOVA P < 0.05). However, this decrease was much less than changes in the twitch tensions and RCC amplitudes. There was a slight decrease in resting tensions before the K⁺ contracture in this subset of muscles, but it did not reach statistical significance (ANOVA P = 0.09306, data not shown). Four muscles were excluded from K⁺ contracture analysis since a proper level of activation was not achieved. We further analyzed the relationship between RCC amplitude and twitch tension immediately before the RCC protocol (40 data points from 10 muscles) and found a linear correlation with an R² ~ 0.76 (Figure 23B). Additionally, there was no difference in the kᵢᵣ values across all four experiments in a subset of these muscles (n = 5, ANOVA P = 0.23052).
5.3.5 Relative SERCA/NCX Activity is Not Responsible for the Decline in SR Calcium Load

In a subset of muscles used for the RCC experiments (n = 6 out of 10 of Figure 23), we also performed paired RCC protocols (Figure 24A). The RCC2/RCC1 ratios were not statistically different over the course of all four experiments (ANOVA, $P = 0.25$), suggesting that a decrease in SERCA pump activity is not responsible for the decrease in SR calcium load (Figure 24B).

5.3.6 No Change in Overall Shape of Cardiomyocytes and T-Tubules

We were able to successfully visualize cardiomyocyte membranes and their t-tubules in intact cardiac trabeculae (Figure 25). We did not detect any significant alterations in the overall structure of cardiomyocytes or t-tubules after 90 minutes of continuous contraction (n = 3) as compared to control (n = 3). It should be noted that with this type of dye and protocol, we were able to properly stain the trabeculae within a maximum distance of ~20-30 µm (variable depending on the trabeculae) from the surface.

5.4 Discussion

In this study we show that: 1) rats have a positive FFR and PRP under baseline conditions, 2) the relative FFR and PRP are enhanced with decreasing twitch tension, 3) there is a dissociation between twitch and $K^+$ contracture tensions, and 4) decline in SR calcium load is one contributing factor for the decrease in twitch tension. Comparison of
all protocols allowed us to determine that the “run-down” of twitch contractile force typically observed in the first few hours in isolated muscle experiments occurs as a result of EC-coupling changes, and is not due to a change in the myofilament capability of generating force. Furthermore, these results suggest that the baseline SR calcium content is a factor in determining the FFR.

Rats had a positive FFR when stimulated near their physiological heart rates and normal body temperature. This is in agreement with previous studies that have shown rats have a positive FFR from 4 to 8 Hz at 37 °C. However, the maximal tension in this study occurred at 6 Hz while tension in previous studies continued to increase from 6 Hz to 8 Hz. We have previously shown that core hypoxia can develop when muscle thickness exceeds 150 µm. Since the average thickness of muscles used for FFR experiments in this study was 122 µm (range of 90-150 µm), the decline in tension from 6-8 Hz is most likely not due to core hypoxia. Our rat samples also exhibited a positive PRP which is in agreement with previously published reports. It must be noted that these referenced PRP studies were performed at different frequencies and/or temperatures than ours.

Since both FFR and PRP are highly dependent on calcium handling processes, changes in both of these parameters suggest alterations in the cardiomyocyte calcium handling mechanisms. Our enhanced FFR and PRP after prolonged incubation periods have been previously reported. In this report, the authors showed that rat left papillary muscles initially exhibit a negative FFR from 0.2-6 Hz at 30 °C. However, after incubation either in presence or absence of fura-2 dye for 3-4 hours, twitch tensions
dropped at all frequencies and the negative FFR was converted into a positive one and the PRP became enhanced.

In our study, twitch tension decreased dramatically while the K\(^+\) contracture tension was not (muscles from Figures 21 and 22) or only slightly affected. There was no difference in \(k_{tr}\) during the course of the RCC experiments. This is in agreement with our study in Chapter 2 where we showed that the K\(^+\) contracture tension and rate of tension redevelopment (\(k_{tr}\)) in rat trabeculae are stable over time at 4 Hz and 37 °C. It is thought that a high extracellular K\(^+\) concentration during this type of contracture induces slow membrane depolarization, activation of calcium channels, and influx of calcium which in turn activates the myofilaments. It effectively bypasses the normal excitation-contraction coupling mechanisms of the myocytes. Therefore, the dissociation between twitch and K\(^+\) contracture tensions suggests abnormalities in calcium handling mechanisms, and indicates that myofilament function is well preserved in prolonged \textit{in vitro} experiments. The contractile parameter \(dF/dt_{max}/F\) accelerated with declining twitch tensions; however, this is most likely not due to cross-bridge kinetics as \(k_{tr}\) in both this study and a previous one remain unchanged over time. Furthermore, this acceleration was more pronounced at lower stimulation frequencies suggesting that FFR dependent processes are responsible.

Arguably, the most important calcium mediated determinant of twitch tension in all mammalian species is SR calcium content, especially in rats where it is estimated that SR calcium contributes to 92% of activating calcium. Therefore, we measured the SR calcium content during muscle run-down using RCC which is an established technique.
for measuring SR calcium content in multi-cellular cardiac preparations where a rapid
temperature decline results in an increase in tension due to release of the SR calcium
content. The decrease in twitch tension in our samples was accompanied with a
similar decline in RCC amplitude over the course of the experiments. The $K^+$ contracture
tension in this series of experiments declined over time. However, this decline of 14%
was much less when compared to the 54% decrease in twitch tension and 65% decrease
in SR calcium load. Our data suggests that one mechanism for the observed decrease in
twitch tension is alteration in SR calcium content and not the tension generation
capability of the myofilaments. It should be pointed out that other factors can contribute,
although they are currently not investigated in this study. Note that the RCC amplitude is
often used as an index of SR calcium content, but it may also depend on the ability of the
myofilaments to bind calcium. If over time a significant decrease in myofilament calcium
sensitivity would occur, it could present an alternative explanation for a decline in RCC
amplitude.

Rat cardiac myocytes are heavily dependent on SERCA for re-sequestration of
calcium back into the SR where SERCA is responsible for removal of 92% of calcium
during relaxation. Therefore, a prime candidate for a decrease in SR calcium content is
an alteration in the relative SERCA and NCX activities. These two systems are in
competition with each other and an alteration in this relationship can affect SR calcium
content. The ratio paired first RCC (RCC2/RCC1) is an established indicator of the
relative SERCA/NCX activities in multi-cellular cardiac preparations. Our paired
RCC data indicates that there is no change in the relative SERCA/NCX activities and the

111
observed decrease in SR calcium load is not mediated by changes in SERCA function. The positive FFR is highly dependent on SERCA function as it is necessary to increase SR calcium content at higher stimulation frequencies. Therefore, the positive FFR that is maintained and even enhanced, during muscle run-down is dependent in part by having a normal SERCA function. This is in contrast with the conclusion that was reached by Taylor and colleagues. They concluded that SERCA activity is initially very high at low frequencies and after incubation of muscles with fura-2 for 3-4 hours, this SERCA activity decreases. Although SERCA activity was not assessed directly, this conclusion was reached by the observation that relaxation velocity of twitches decreased at low frequencies (0.5-2 Hz) but not at higher frequencies (3-6 Hz) after such incubation. The differences between these two studies can be attributed to various factors including: 1) temperature, 2) muscle dimensions, and 3) stimulation frequencies. In fact, the lack of change in relaxation kinetics at higher frequencies of 3-6 Hz found by Taylor and colleagues agrees with our data at 4-8 Hz (Figure 21C).

The cardiac trabeculae model is a multi-cellular system consisting of several hundreds of individual cardiomyocytes. Therefore, it is possible that some of these cells become damaged while others unaffected during muscle run-down which can contribute to the decline in twitch tensions. We assessed the effects of long incubation periods on the overall health and shape of the individual cardiomyocytes by staining trabeculae with a membrane dye. Qualitatively, we did not detect any significant changes in the shape of cardiomyocytes or t-tubules after long periods of contractions as compared to control.
The increase in twitch tension during positive FFR is achieved by multiple mechanisms such as increased calcium influx per unit of time, enhanced SERCA activity, and phospholamban phosphorylation. The net effect is an increase in SR calcium content and calcium transient amplitude which results in greater activation of the myofilaments and, ultimately, force generation. Our data here adds another factor in modulation of the shape and amplitude of FFR in experimental settings, i.e. the SR calcium content at baseline frequencies. While decreasing SR calcium load results in a decline in twitch tension at basal frequencies, it enhances the relative FFR. The SR has a finite calcium capacity and it is plausible that by decreasing the SR calcium content at baseline frequencies, the myocytes have a greater capability to relatively increase their SR calcium at higher frequencies and achieve a more positive FFR. Based on this information, we suggest that experimental factors, capable of affecting baseline SR calcium content, must be taken into consideration when assessing FFR data from multiple studies.
Figure 21. FFR becomes more positive with decreasing twitch tension.
A) Non-normalized twitch data at various frequencies during the 1\textsuperscript{st}, 2\textsuperscript{nd}, and 3\textsuperscript{rd} experiments. B) Normalization of data to 4 Hz reveals an increase in the relative FFR. C) No change in relaxation kinetic parameter $dF/dt_{\text{min}}/F$ across all frequencies. D) Contractile kinetic parameter $dF/dt_{\text{max}}/F$ becomes accelerated with declining twitch tension. * and # indicate $P < 0.05$ with respect to frequency and experiment number, respectively as determined with two-way ANOVA. $n = 9$. All experiments performed at 37 °C.
Figure 22. PRP becomes pronounced with declining twitch tension. A) Post-rest twitches normalized to pre-rest twitches indicate an enhanced PRP with muscle run-down. B) Non-normalized post-rest twitches show the decline in tensions. * and # indicate $P < 0.05$ with respect to rest interval and experiment number, respectively as determined with two-way ANOVA. $n = 7$. All experiments performed at 4 Hz and 37 °C.
Figure 23. Decrease in SR calcium load is a contributing factor for the decline of developed twitch tension.

A) Each measurement was normalized to its corresponding value in the 1st experiment. There was a dramatic decline in developed twitch tension accompanied by RCC amplitude (n = 10). K+ contracture tension decreased to a lesser degree (n = 6). * and # indicate $P < 0.05$ vs. 1st and 2nd measurements respectively, as performed by one-way ANOVA for repeated measures with Bonferroni post-hoc test. B) Correlation between twitch tensions and RCC amplitudes. All experiments performed at 1 Hz and 27 °C.
Figure 24. Decline in relative SERCA/NCX activity is not responsible for a decrease in twitch tension.
A) Simultaneous recording of stimulation pulse, muscle tension, and temperature in a single cardiac trabecula. B) RCC2/RCC1 ratio was not different in successive experiments. n = 6, P = 0.25402 one-way ANOVA for repeated measures. Muscles were stimulated at 1 Hz and 27 °C.
Figure 25. No change in cardiomyocyte shape or t-tubules after long period (90 minutes) of isometric contraction. Multiple z-stacks were taken from each muscle. Images shown are from the most superficial layer (left) and 20 µm deep (right) of each muscle. All scale bars shown are 20 µm. Both muscles were stimulated at 1 Hz and room temperature. Data collected by Lucia Brunello, Gyorke Laboratory.
Chapter 6: Conclusion and Future Directions

6.1 Summary of Principle Findings

Cross-bridge cycling kinetics can be assessed in intact cardiac trabeculae under near-physiological conditions by combining: 1) $K^+$ contracture to tetanize the muscles, and 2) rate of tension redevelopment ($k_{tr}$) as an index of cross-bridge cycling kinetics. The measured $k_{tr}$ is stable over time and highly reproducible in both animal models and humans. Furthermore, using our technique, we show that muscle length can regulate $k_{tr}$ in both a small animal model (i.e. rat) and freshly explanted non-failing human myocardium, as an increase in muscle length resulted in a decrease in $k_{tr}$ in both species. Extension of studies into failing human myocardium revealed that both twitch contractile kinetics and $k_{tr}$ were unaffected in the right ventricular trabeculae from patients with end-stage heart failure. Additionally, the effects of muscle length on twitch contractile kinetics and $k_{tr}$ were preserved in these failing samples. The current preliminary results do not identify a role of phosphorylation modifications in this length-dependent regulation of cross-bridge cycling kinetics in either non-failing or failing human myocardium. We also show that overshoots following $k_{tr}$’s slack-restretch maneuver in human myocardium are not artifacts of the experimental protocol, and cross-bridges are required for their occurrence. Finally, we observed that while twitch tensions decreased over time, contracture tensions, for the most part, remained relatively stable.
Continuation of studies on this dissociation led us to conclude that at least one contributing factor is a decline in SR calcium content. Furthermore, we show that a reduction in SR calcium content at baseline frequencies enhances the relative FFR.

6.2 Implications of Findings

Our technique for measuring $k_{ir}$ is a novel method for assessing cross-bridge cycling kinetics in intact cardiac preparations under near-physiological conditions. The use of intact preparations as compared to their permeabilized counterparts can provide a better picture of the state of the cross-bridge cycling kinetics of the heart. Intact preparations provide the opportunity to study cardiac contraction in a population of “living” cardiomyocytes as opposed to “non-living” permeabilized preparations. Not only are our experiments conducted under physiologically relevant temperature of 37 °C, but the “living” cardiomyocytes have preserved phosphorylation modification machinery. This allows the cardiomyocytes to regulate these phosphorylation modifications, which can potentially be involved in regulating cross-bridge cycling kinetics (see Chapter 1). Future studies can take advantage of this novel method to assess the effects of myofilament phosphorylation on cross-bridge cycling kinetics under near-physiological conditions.

It should be noted that our technique, like any other, has its own set of limitations. Most importantly, we are assessing cardiac function in a small portion of the myocardium (i.e. the cardiac trabeculae) as opposed to a whole organ. While we matched our experimental conditions to reflect those of the body as best as possible, such ex vivo
isometric contractions do not completely reflect and represent the in vivo contraction of the heart. Additionally, precise control of the level of activation that is achievable in permeabilized preparations is not achievable during the K\(^+\) contracture. Exchange of native proteins with mutants, which has been done previously for permeabilized cardiac preparations\(^{39,208}\), is not feasible in these intact muscles, although, a potential method could exist (see Future Directions for a discussion). Therefore, our novel method on intact cardiac trabeculae should not be thought that it can fully replace the permeabilized preparations for all studies. The permeabilized preparations have been and will be an important and essential technique for studying myofilament function. Overall, our technique provides a novel method, with its own set of strengths and limitations, for assessing cross-bridge cycling kinetics in intact preparations under near-physiological conditions.

The cross-bridge cycling rate is an important determinant of cardiac output\(^{10,26,27}\); hence, understanding how it is regulated and if it becomes dysfunctional in disease is essential. Our studies show that muscle length can regulate cross-bridge cycling kinetics in intact cardiac muscles when studied under near-physiological conditions. Increasing muscle length resulted in a decrease in cross-bridge cycling kinetics in both healthy rats and non-failing and failing humans. It was necessary to extend our initial studies in rats to humans, since results in small animal models do not directly translate into humans\(^{28}\). While the heart of a rat and human share many similarities, there are many differences at the level of the myofilaments. The most relevant of these to our studies include
expression of MHC and titin isoforms. A more detailed discussion of the similarities and differences can be found in the referenced manuscript 28.

Currently, we have not identified a specific mechanism responsible for this length-dependent regulation of cross-bridge cycling kinetics. Our preliminary studies in Chapter 4 did not identify a primary role for phosphorylation modifications induced by muscle length. Alternatively, a model put forth by the McDonald K.S. group can explain our observations. In this model, an increase in the muscle length results in titin straining the movement of MHC through its interactions with MyBP-C. This straining effect will decrease the cross-bridge cycling kinetics 26,65,69. In order to assess this model, we reanalyzed data on intact cardiac trabeculae from a previous study on mice expressing truncated MyBP-C 153,209. When muscles from the homozygous mice with this mutation were stretched from L_{85} to L_{opt}; there was no decrease in the dF/dt_{max}/F parameter (P = 0.51238). This is contrast to the wild-type counterparts of the same study where dF/dt_{max}/F decreased as muscles were stretched within the same muscle range (ANOVA, P < 0.05). While this is not assessing cross-bridge cycling kinetics directly, this data suggests that MyBP-C is required for the slowing effects of increasing muscle length on twitch kinetics in mice and further supports the model proposed by McDonald K.S. We currently have not performed the necessary experiments to examine this model in our studies; however, future experiments are warranted (see Future Directions section).

This length-dependent regulation of cross-bridge cycling kinetics can have profound physiological implications in the intact heart. During the ejection phase of systole, the length of cardiomyocytes will continuously decrease as blood is being ejected
from the ventricles. Based on the Frank-Starling Relationship, the force of contraction will concurrently decrease \(^{60,61}\). However, based on our data, the cross-bridge cycling kinetics will accelerate at shorter lengths which will act to enhance pumping activity of the heart and aid in ejection of the blood towards the end of systole. Additionally, the heart needs to relax in order to allow for the ventricles to fill with blood and be ready for the next cycle. The accelerated kinetics at lower muscle lengths will also allow for rapid myocardial relaxation. In essence, this length-dependent regulation of cross-bridge cycling kinetics is an important and integral mechanism used by the heart for regulating its mechanical activity.

One of the goals of this research project was to determine if the length-dependent regulation of cross-bridge cycling kinetics is altered in heart failure. We decided to use end-stage failing human hearts in order to remove any uncertainty in regards to the relevance of animal heart failure models to their human counterparts. Experimental studies on the FFR and \(\beta\)-adrenergic stimulation suggest that the right ventricles of our failing group exhibit the signatures of heart failure \(^{92-101}\). Additional clinical information is necessary in order to definitely determine the extent of right ventricular dysfunction. Our results show that cross-bridge cycling kinetics, as assessed by \(k_{tr}\), is unaltered in the right ventricles of patients with heart failure when measured under near-physiological conditions. The kinetics of twitch contraction mirrored these observations. This is in contrast to previous studies which suggest that cross-bridge cycling kinetics are reduced in the left and right ventricles of patients with heart failure \(^{54,102}\). The differences between these studies can be attributed to: 1) patient population of idiopathic dilated
cardiomyopathy in previous studies as compared to mainly ischemic cardiomyopathy (six out of ten failing samples) in our study, and 2) our methodology of performing experiments under near-physiological conditions in intact muscle preparations. We should also point to previous studies using permeabilized preparations, which showed that cross-bridge cycling rates of the left ventricle are unchanged in multiple etiologies of heart failure in humans. Overall, the combined results of these studies and ours suggest that cross-bridge cycling rate is not affected in patients with heart failure.

Previous studies have typically used the maximal rate of tension rise and decline of the twitch in order to assess the kinetics of twitch contraction and relaxation. However, these parameters (units of mN/mm²/s) are highly influenced by the amplitude of the twitch tension which can be different between non-failing and failing groups. In Chapter 3, we normalized these reported dF/dt_max and dF/dt_min values at baseline stimulation frequencies (0.5 Hz or 1 Hz) to their corresponding developed forces. The resulting dF/dt_max/F and dF/dt_min/F parameters of the non-failing myocardium is very similar between non-failing and failing myocardium in these studies. This collective analysis in combination of our data provide further support that cardiac contractile kinetics are not decreased in heart failure patients.

Arguably, the most influential factor in determining cross-bridge cycling kinetics is the isoform of MHC. This is particularly important in the case of small animal species since α-MHC accounts for >94-100% of the total MHC content. Consequently, there is a large pool of α-MHC that can be switched to β-MHC, which will play a role in decreasing cross-bridge cycling kinetics in small animal models of heart failure.
In the referenced studies, the α-MHC accounted for only 22-57% of the total MHC during heart failure in small animal models. Healthy human ventricles are almost saturated with β-MHC while α-MHC accounts for ~7% or less. Assuming that all of this residual α-MHC is replaced with β-MHC during heart failure then the effects on cross-bridge cycling kinetics, if any, will be limited. Therefore, alterations in cross-bridge cycling kinetics and the underlying mechanisms are different between small animal models and humans. It should be noted that we have currently not measured MHC isoform expression in our samples. But the preservation of cross-bridge cycling kinetics in our studies suggests that either: 1) isoform switching occurs but the change is not sufficient to cause a significant decrease in cross-bridge cycling kinetics in humans, or 2) there is no isoform switching in our failing samples. Another factor that determines cross-bridge cycling kinetics is myofilament protein phosphorylation. We currently do not have data showing the phosphorylation status of myofilaments in non-failing vs. failing myocardium. However, our data suggest that either: 1) these modifications are relatively unchanged in the right ventricle during heart failure, or 2) such phosphorylation modifications do not play a vital role in regulating cross-bridge cycling kinetics. Additional experiments are needed as addressed in the “Future Directions” section.

It is cautioned that our studies were performed in the right ventricle and results cannot be directly translated into the left ventricle. If the degree of dysfunction is greater in the left ventricle, then it is possible that cross-bridge cycling kinetics are differentially affected in the right and left ventricles during heart failure. Future studies on the left ventricle are necessary in order to draw conclusions on possible alterations in cross-
bridge cycling kinetics in this side of the heart. Furthermore, the use of human myocardium does have its own set of challenges and limitations which need to be considered. A detailed discussion of these can be found in the referenced manuscripts 28,117,124.

There are transmural variations in protein expression in the heart. Expression of α-MHC is higher in the epicardium than in the endocardium, a variation which has been observed in both animal models 133,211 and humans 128. It is possible that MHC isoform switching during heart failure 14,15 will be more prominent in the sub-epicardial than sub-endocardial regions. Therefore, cross-bridge cycling kinetics can potentially be differentially affected across the transmural wall during heart failure. We cannot make a statement on possible alterations in the sub-epicardium as our studies on cardiac trabeculae are restricted to the sub-endocardium. If such differential alterations occur then it can have adverse impact on cardiac output in a fully intact heart since proper pumping of the blood requires coordination of different segments of the heart. The ventricular depolarization spreads from the endocardium to the epicardium 3, which results in the sub-endocardial regions to shorten before the sub-epicardial regions 212-214. However, the shortening of these two regions are almost synchronous during the ejection phase of the cardiac cycle 212-214. Consistent with this observation, isolated epicardial myocytes contract faster than their endocardial counterparts 215 which has been attributed to the higher amount of α-MHC in the sub-epicardium 133. Therefore, a biased reduction in cross-bridge cycling kinetics in the epicardium as compared to the endocardium during
heart failure can potentially disrupt the highly coordinated cardiac contraction and result in a decrease in cardiac output in an intact heart. The reduction of cross-bridge cycling kinetics with increasing muscle length in our isolated muscle studies provides a potential mechanism whereby kinetics of contraction are actually reduced in the failing myocardium in vivo. There is an increase in left ventricular end-diastolic volume in systolic failure, and increase in end-diastolic pressure in both systolic failure and diastolic failure which can result in cardiomyocytes to be stretched. This adaptation increases force based on the Frank-Starling Relationship. However, our studies suggest this adaptation will concurrently reduce the cross-bridge cycling rate, which is another important determinant of cardiac output. It is possible that kinetics are reduced in a fully intact heart during heart failure via increasing “functioning length” of the cardiomyocytes. This length-dependent reduction in cross-bridge cycling kinetics can reduce cardiac output during heart failure. Figure 26 provides a schematic presentation of this proposed model. Future studies are necessary for determining the relevance of this model in heart failure.

We showed that tension overshoots following the $k_u$ maneuvers are mediated by the muscles themselves; however, their significance to in vivo cardiac contraction is currently not clear. If these overshoots are mediated by the cross-bridges then it can be speculated that overshoots also occur in isolated muscles during contraction and even in the intact heart during systole. The question then becomes, why are these overshoots not observed during cardiac contraction? One possibility is that there is no reference point for comparison purposes which results in these overshoots being masked. Overshoots allow
the myocardium to recruit more myosin heads to the cross-bridge cycle than would normally be achievable for the same amount of calcium. This will allow the heart to develop more force and pressure during the cardiac cycle. Additionally, the heart will enter the ejection phase of the cardiac cycle sooner and maintain this phase longer which will increase the amount of blood pumped. We currently do not have evidence that overshoots occur during normal cardiac contraction. However, they can be another potential mechanism that the heart uses to regulate cardiac output.

The studies on twitch rundown show that the relative FFR becomes stronger with decreasing SR calcium content at baseline stimulation frequency. This has important consequences on data interpretation in both healthy and diseased myocardium. Any experimental alteration that raises or decreases SR calcium content will affect the amplitude of the FFR. Therefore, in order to allow comparison of FFR data from one study to another, one should be mindful of any factor that can alter the SR calcium content.

Some studies suggest that the SR calcium content is reduced in heart failure. At first look, this might seem to contradict our results as numerous studies show that FFR is reduced in heart failure. One of the reasons that the FFR is dysfunctional in the failing myocardium is the reduced SERCA activity. SERCA activity becomes compromised in heart failure, which results in the inability of the myocardium to increase its SR calcium content at higher frequencies. In our studies, we show that the SERCA activity remains unaltered despite a reduction in SR calcium content. Therefore, it might be that in heart failure, lower SR calcium content acts to enhance the relative
FFR; however, its effects are outweighed by the strong negative effects of a dysfunctional SERCA which will collectively result in a flat-negative FFR.

6.3 Future Directions

There are multiple experiments that can be performed in order to advance our understanding of the regulation of cross-bridge cycling kinetics in health and heart failure. Animal models, either healthy controls or failing, are a more homogenous population than their human counterparts. There is a lot of variability in regards to genetics, social habits, medication, and type, severity, and duration of heart failure than can potentially have an effect. Our current failing sample size of ten is insufficient for determining the influence of all of these parameters. Furthermore, the current study was skewed towards ischemic cardiomyopathy, the most common etiology of heart failure. Therefore, it would be interesting to have a very large sample size of heart failure patients in order to account for influence of these factors.

The length-tension relationship is not the only mechanism used by the heart for regulating cardiac output. Two other important mechanisms are the force-frequency relationship and β-adrenergic stimulation. Both of these have been shown to regulate the twitch contractile and relaxation kinetics \(^{95,96,175,219}\) and some studies implicate β-adrenergic pathway in regulation of cross-bridge cycling kinetics \(^{33-36}\). It would be interesting to utilize our technique to assess cross-bridge cycling kinetics at different stimulation frequencies and isoproterenol concentrations. It would also be interesting to
investigate the collective effect of combination of these regulatory mechanisms on cross-bridge cycling kinetics.

The role of titin in the length-dependent regulation of cross-bridge cycling kinetics needs to be investigated. In one approach, $k_{tr}$ will initially be measured at multiple muscle lengths in intact trabeculae. Afterwards, the trabeculae preparations will be permeabilized with Triton while still in the setup as has been performed by other investigators. $k_{tr}$ measurements will be made in these “skinned” trabeculae at multiple muscle lengths. If titin is the sole determinant of the length-dependent regulation of cross-bridge cycling kinetics, then it is expected that the skinning process should not affect the relationship between muscle length and $k_{tr}$. Next, titin will be briefly digested with trypsin as previously described. Assuming that titin strains MHC at longer muscle lengths, then it is expected that this digestion should accelerate $k_{tr}$ at longer muscle lengths. Additionally, if titin has a role in this process, then we would expect that the effect of length on cross-bridge cycling kinetics should differ depending on whether the myocardium expresses the stiff N2B or compliant N2BA titin isoforms. This can be probed by performing $k_{tr}$ maneuvers using our protocol in a rat model with high levels of N2BA titin isoform. Rats predominately express the N2B titin isoform; however, a line of rats harboring a natural occurring autosomal dominant mutation has been established. The N2BA titin isoform makes up approximately 50% and 100% of the total titin in heterozygous and homozygous rats of this line, respectively. If titin plays a role, then the relationship between muscle length and cross-bridge cycling kinetics should be different in wild-type vs. heterozygous vs. homozygous rats. Alternatively, we could
measure $k_{tr}$ in intact muscle preparations from a mouse model with truncated MyBP-C

If titin is exerting its effects through MyBP-C, then there should be no length-dependent regulation of $k_{tr}$ in these mutant mice as compared to their wild-type counterparts. It is important to note that these studies will be limited to small rodents which have profound differences as compared to human myocardium. It is preferred to perform studies on large animal models harboring the mutations; however, development of such models will take considerable amount of time and financial resources.

It is necessary to assess the relative MHC isoform expressions in our non-failing and failing samples in order to determine the extent of MHC isoform switching. Additional work is necessary for assessing the roles of phosphorylation modifications in regulation of cross-bridge cycling kinetics. We have not yet assessed and compared the baseline phosphorylation modifications of contractile proteins in our non-failing and failing human myocardial tissues. It should be noted that samples of these tissues are currently preserved at -80 °C; however, caution is warranted in regards to analyzing and using such samples. All of the non-failing donor hearts were exposed to at least one inotropic agent (norepinephrine, epinephrine, and dopamine) during the organ procurement process. These agents artificially increase the phosphorylation of targets downstream of the adrenergic receptors which include the myofilament proteins.

Alternatively, trabeculae can be stabilized ex vivo in our experimental setups and flash frozen with liquid nitrogen as previously performed by our laboratory, which will eliminate the confounding effects of the inotropic agents and provide a better picture of the change in phosphorylation modifications that occur in heart failure. Additionally, it
will be necessary to use this technique to determine whether: 1) muscle length modulates phosphorylation modifications of contractile proteins (ex: MLC-2 and TnI) in human myocardium, 2) capability of H-89 and PKCβII peptide inhibitor I to reduce the length-dependent phosphorylation of contractile proteins in humans, and 3) efficiency of the contra-length stabilization protocol in preserving phosphorylation modifications. It is worth noting that this technique does not precisely represent the state of phosphorylation modifications in a fully intact beating heart under normal conditions. Ideally, a biopsy of the myocardium has to be taken from an in vivo heart, not exposed to inotropic agents, and immediately frozen with liquid nitrogen. This can be technically challenging or even impossible for human studies; however, similar experiments can be performed in large animal models, such as canine or porcine, and can complement humans studies.

The effects of phosphorylation modifications on cross-bridge cycling kinetics can be further studied by introducing contractile proteins which contain point mutations that are either phosphomimetic (such as aspartic acid) or non-phosphorylatable (such as alanine) into human myocardium. Of course, making such alterations is unethical and impractical in a human being; however, we can potentially perform these experiments in isolated human cardiac trabeculae. Personal experience suggests these muscles can be preserved in some cases, but not always, for 2-3 days after cardiac explantation if they are stored in K-H supplemented with BDM at 4 °C (data not shown). We can theoretically introduce mutated proteins with the aid of viral vectors and alter the protein expression profile of the trabeculae (Jonathan P. Davis, personal communication). It should be noted that it is necessary to develop a protocol where muscles can be preserved
at 37 °C (as opposed to 4 °C), a temperature where the protein synthesis machinery can function properly. Preliminary studies that have been performed in collaboration with Vikram Shettigar, a GRA from the Jonathan P. Davis laboratory, have not been encouraging. However, if this technique can be mastered, then the coupling of our $k_{ir}$ protocol and introduction of such mutations into intact human trabeculae can help us better understand the role of phosphorylation modifications in regulation of cross-bridge cycling kinetics in human myocardium.

Investigation into sub-epicardium can be performed using either myocardial strips or single cardiomyocytes both isolated from the epicardium. The strips do have limitations. There is a substantial amount of damage during the isolation procedure as multiple cuts have to be made along the length of the preparation. Additionally, as opposed to trabeculae, the cells in this type of preparation are not necessarily arranged linearly and in the same direction. Intact cardiomyocytes can be isolated solely from the epicardial regions and mounted between two attachment points using techniques utilized by others. It is possible to adapt our $K^+$ contracture and $k_{ir}$ protocols to study single cardiomyocytes. Although these studies will be technically challenging and limited to single cells.

The properties of tension overshoot in human cardiac trabeculae can be further determined with some additional experiments. As discussed earlier in Chapter 4, a preliminary experiment with blebbistatin was unsuccessful due to its brief exposure (~30 seconds) to the muscle. This issue can be simply addressed by adding the blebbistatin directly to the K-H solution along with the high $K^+/Ca^{2+}$ solution. Stabilizing the muscle
(in dark) for ~15 minutes in K-H with blebbistatin should be enough time for blebbistatin to inhibit myosin activity\textsuperscript{189,190}. It is expected that blebbistatin should eliminate or reduce the amplitude of the overshoots following the \( k_{tr} \) maneuver during the contracture.

The role of titin in tension overshoots can be probed via two methods. One of these includes performing a \( k_{tr} \) maneuver initially in an intact preparation and then after it has been permeabilized with Triton in the setup\textsuperscript{166}. It is expected that tension overshoots will still occur under these conditions. Next, titin will be digested with trypsin\textsuperscript{220} and \( k_{tr} \) maneuvers will be conducted. If the amplitude of tension overshoot does not change then titin most likely does not have a central role in these overshoots. In a second approach, we can perform our \( k_{tr} \) protocols in intact cardiac trabeculae from heterozygous and homozygous rats with a mutation that up-regulates N2BA expression\textsuperscript{221}. If a compliant titin is required for tension overshoots, then it is expected that overshoots will be mostly absent in wild-type while present at lower amplitude in heterozygous and greater amplitude in homozygous rats.

In Chapter 5, we observed that twitch tensions decline over time, most likely due to a decline in SR calcium content. Other factors besides reduced SR calcium content can contribute to the decline in twitch tension. One potential factor is a decrease in myofilament calcium sensitivity, which can be assessed in intact cardiac trabeculae with the use of \( K^+ \) contracture and in the presence of a calcium indicator\textsuperscript{149}. This will also allow us to assure that the amplitude of the rapid cooling contracture is not affected by alterations in myofilament calcium sensitivity. Furthermore, the current membrane dye staining method restricts proper staining within a finite distance from the surface. It is
currently unknown whether alterations occur deep in the core of the muscles.

Alternatively, the whole heart can be perfused with the dye prior to isolation of the trabeculae. This should allow the dye to have access to the entire trabeculae via the coronary vasculature and allow proper staining of the inner core of the muscles.
Figure 26. Proposed model of the reduction in cross-bridge cycling kinetics due to increasing muscle length during heart failure in an intact heart.
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