ON THE ROLE OF HEART RATE VARIABILITY AND PYRUVATE ON CARDIAC CONTRACTILITY

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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2014

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Abstract

Despite major improvements in the treatment of heart disease, the prevalence of heart failure (HF) continues to rise, leading to increased disability and cost. Significant effort is placed on the discovery of new strategies that might increase contractility and inotropy.

There is still a scarcity of inotropic therapies available for the long term treatment of congestive heart failure. We examine the effect of beat-to-beat variability on contractility. We then investigate the inotropic mechanism of pyruvate on myocardial contractility.

The specific hypothesis behind the research performed is that heart rate variability (HRV) plays an important role in maintaining normal cardiac contractility and that HRV is not only an indicator of heart disease but also a modulator of contractility.

To investigate the effect of heart rate variability on cardiac contractility we developed a model utilizing heart trabeculae, that allowed us to characterize those effects independent of previous loading conditions, examine the frequency-dependence of HRV and define the load dependence of contractility during heart rate variability by studying HRV at preloads between EDV and ESV in isolated, intact muscles at physiologic temperatures.

First, we detected a slight positive increase in force indicating an effect of heart rate variance in parts of the FFR that are more concave or convex (i.e. where the
relationship between frequency and force is non-linear). We also observed a positive tight correlation between inter-beat duration and strength of the following beat suggesting that HRV does change beat to beat contractile strength, independent of loading conditions. In a subset of experiments, intracellular calcium transients were measured after iontophoretic loading of the calcium indicator bis-Fura-2. Trabeculae were then stimulated following the previous pacing protocols. In this subset we were able to demonstrate that the variation in beat to beat duration was associated with variations in the calcium transients. We then sought to verify if these findings would translate to species that more closely resemble human myocardium. Cardiac trabeculae were isolated from the right ventricle of rabbits and dogs and a similar protocol of variability in beat to beat duration was imposed. We observed no change in force in the dog model and a nonsignificant decrease of force in the rabbit. In a subset of experiments we simulated an increase in preload by applying a precisely timed stretch during the inter-beat period this caused a reversal of this negative affect except for at the more extreme stretch at the high end of the frequency range for rabbit. We propose that these species differences are due to the distinct handling of EC coupling and calcium regulation, and to the different myosin heavy chain isoform composition in the larger mammals.

Pyruvate has cardioprotective and antioxidant properties that are ideal for many settings where traditional inotropes are fraught with undesirable effects. We hypothesized that the majority of the inotropic effect of pyruvate was due to an increase in myofilament calcium sensitivity. By utilizing ultra-thin rabbit heart trabeculae, intracellular iontophoretic fluorescent dye methods with fluorophores sensitive to [pH], changes and [Ca^{++}], we were able to characterize the effects of pyruvate on calcium
transients and developed force and isolate them from those induced by pH changes in normal myocardium. With sarcoplasmic reticulum (SR) block there was little to no change in systolic calcium (or calcium transient development) compared to the relatively large change in force development (up to 200%) seen with infusion of pyruvate. Changes in [pH], did not correlate with developed force and peak systolic calcium changes.

To study the effect of pyruvate on myofilament calcium sensitivity under physiologic conditions we employed a K+ contracture and compared those findings with skinned fiber experiments. Pyruvate induced a leftward shift in the myofilament sensitivity curve that was not reproduced in the skinned fiber experiments. Finally, we set out to determine the effects of pyruvate on force development and myofilament calcium sensitivity in pulmonary artery banded rabbits with right ventricular hypertrophy. We found a similar increase in force development and a shift in myofilament calcium sensitivity observed in control animals. In conclusion, we demonstrate that a leftward shift in myofilament calcium sensitivity is an important mediator of the inotropic effect of pyruvate.
Dedication

This document is dedicated to my parents.
I am in great debt to many who supported me both academically and personally. I would like to thank my advisor, Dr. Paul Janssen for his guidance during my Ph.D training. Dr. Janssen taught me persistence when submitting grants and responding to reviewers. I also want to thank him for his patience tolerating the interruptions in my PhD training for my clinical duties and pursuits. I’d like to thank my committee members: Dr. Peter Reiser for constantly bringing to my attention challenging articles and different approaches towards problem solving. Dr. Jonathan Davis, to whom I am grateful for his enthusiasm in teaching the intricacies of crossbridge cycling and for his support in difficult times. Dr. Mark Angelos for his unwavering support for my research endeavors throughout the years without which I would not have been able to start down this path.

I would also like to thank my colleagues in the labs in particular: “Ko” for teaching me the fine art of dissecting trabeculae, and Kenny Varian for passing on the nuts and bolts on how to perform calcium experiments and the lively discussions. Special thanks to my wife Ligia and my children for their understanding of my commitment to these academic pursuits.
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Publications

Fields of Study

Major Field: Biophysics
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<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BDM</td>
<td>2,3 Butadione Monoxime</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>EC$_{50}$</td>
<td>Effective Concentration to reach 50% of maximum force</td>
</tr>
<tr>
<td>ECC</td>
<td>Excitation Contraction Coupling</td>
</tr>
<tr>
<td>FDAR</td>
<td>Frequency Dependent Acceleration of Relaxation</td>
</tr>
<tr>
<td>F$_{dev}$</td>
<td>Developed Force</td>
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<tr>
<td>FFR</td>
<td>Force Frequency Relationship</td>
</tr>
<tr>
<td>L-Type</td>
<td>Long type</td>
</tr>
<tr>
<td>MCS</td>
<td>Myofilament calcium sensitivity</td>
</tr>
<tr>
<td>MVO$_2$</td>
<td>Myocardial Oxygen Consumption</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium calcium exchanger</td>
</tr>
<tr>
<td>PAB</td>
<td>Pulmonary artery banding</td>
</tr>
<tr>
<td>PDC</td>
<td>Pyruvate Dehydrogenase complex</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic Phosphate (Orthophosphate)</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PLB</td>
<td>Phospholamban</td>
</tr>
<tr>
<td>RT$_{50}$</td>
<td>Relaxation Time to 50% of max force</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
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<td>--------------------------------------------------</td>
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<tr>
<td>RVH</td>
<td>Right Ventricular Hypertrophy</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarco-endoplasmic reticulum calcium ATPase</td>
</tr>
<tr>
<td>SDNN</td>
<td>Standard Deviation N to N intervals</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic Reticulum</td>
</tr>
<tr>
<td>TCA</td>
<td>TriCarboxylic Acid Cycle</td>
</tr>
<tr>
<td>TnC</td>
<td>Troponin C</td>
</tr>
<tr>
<td>TnI</td>
<td>Troponin I</td>
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<tr>
<td>TnT</td>
<td>Troponin T</td>
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Chapter 1: INTRODUCTION

1.1 General Introduction

Approximately 5.1 million Americans have heart failure. More than 670,000 new cases are diagnosed annually, corresponding to over $30 billion of total healthcare costs.(Yancy et al. 2013). Despite major improvements in the treatment of heart disease, the prevalence of heart failure (HF) continues to rise, leading to increased disability and cost.

With over 600,000 deaths from heart failure in 2012 it is only natural that considerable resources are dedicated to the discovery of the mechanisms that underlie the normal and abnormal function of the heart. Significant effort is currently placed on the development of new strategies that might increase contractility and cardiac inotropy.

In 1628 Harvey published his Anatomical Treatise on the Motion of the Heart and Blood in Animals and laid the foundations for the modern understanding of the heart and its integration with the cardiovascular system. Since then there have been many advances that contributed to our current understanding of the structure and function of the heart. At the turn of the 20th century, the fundamental relationships that regulate cardiac performance were captured into what is now known as the length tension relationship and the force interval relationship. The biological mechanisms that govern these physiologic
Determinants of the heart's contractile state have been object of intense study over the past 100 years so that we now have a good grasp on the fundamentals that guide the performance of the heart. The initial chapters of this dissertation continue to explore these same relationships taking advantage of current tools and technologies.

We must not lose sight however, that this progress has not translated into a similar increase in new therapies for those suffering of heart failure. In fact the only inotropic drug recommended for continued use in treatment of heart failure in the 2013 American Heart Association Heart Failure Guidelines is Digoxin (Yancy et al. 2013): a drug that was described in the 18th century. While therapies that increase the contractile state of the diseased myocardium have faltered, treatment of heart failure with drugs that are aimed at decreasing the work and energy expenditure of the heart have found a preeminent role in the management of this syndrome. Such energy sparing treatments include beta blockers, ACE inhibitors and angiotensin II blockers. This has led to resurgence in the hypothesis that energy depletion plays an important role in heart failure. Initially proposed by Herrmann and Decherd in 1939 in the article titled “The Chemical Nature of Heart Failure” it has evolved today into the field of myocardial energetics (Neubauer 2007). This concept will have important implications for the latter part of this work where we discuss the role of pyruvate as an inotropic drug.

Intrinsic performance of the cardiac muscle is defined primarily by

- The inotropic state or contractility
- The length tension relationship
- The force interval relationship
For many years the understanding of the heart and its function has historically been a narrative of the discovery and elucidation of the function structures and mechanisms involved in these fundamental relationships. The β adrenergic status is an important determinant of cardiac contractility but is dependent on external and neurohumoral influences and is not discussed further here. The importance of the relaxation phase during the end of systole has also become the object of intense study recently. While systolic relaxation is integral to the normal function of the heart it is discussed in this work only in the context of the previous relationships.

1.2 The Length Tension Relationship

Observations on the association between ventricular filling and developed force were made by several investigators from the Carl Ludwig Physiological Institute in Leipzig (Elias Cyon, Henry Bowditch, Joseph Coats) (Zimmer 2002) before either Ernest Starling or Otto Frank published their work however it was Frank and Starling who accomplished most of the experimental work unraveling the relationship between cardiac output and ventricular filling pressures.

In 1915 Ernest Starling concluded his now famous Lineacre Lecture by what is known today as the Law of the Heart. “The energy of contraction “of cardiac muscle” “is a function of the length of the muscle fibre.” In fact, the longer the fibres at the beginning of systole, the stronger is the force of the beat.” (Starling 1918).

Nearly 30 years before in 1892 Otto Frank had observed the influence of diastolic filling on the amplitude of contraction. The Frank-Starling mechanism is recognized today as fundamental to hemodynamic equilibrium. It plays a central role in the beat-to-
beat regulation of the heart and allows for changes in preload and afterload to modify cardiac performance in vivo. The basis of the force-length relationship is largely related to the extent of overlap between thick and thin filaments and the number and strength of the crossbridges. Contrary to skeletal muscle, the heart is unable to recruit additional fibers to deal with the changes in demand. It is dependent on the intrinsic mechanisms to perform its function. When compared to skeletal muscle the cardiac length-tension relation is notably steeper between 1.8 and 2.0 \( \mu m \) (Allen et al. 1974). This occurs because the degree of activation of the cardiac myofibrils by calcium increases as muscle length is increased. While an extensive review of the length tension relationship in the heart is not the subject of this work, several processes are thought to contribute to the length-dependence of activation: among them are the calcium sensitivity of the myofibrils that increases with muscle length, the amount of calcium supplied to the myofibrils during systole that also increases with muscle length (Allen and Kentish 1985) and increases in SR calcium uptake (Kentish and Wrzosek 1998). Geometric factors might also lead to a narrower inter-filament gap as the myocyte is being stretched thus increasing the probability for cross-bridge binding (Fuchs and Wang 1996). More recently there is evidence that the actual kinetics of cross-bridge cycling are also influenced by the Frank-Starling mechanism (Milani-Nejad et al. 2013).

1.3 The Interval Force Relation

Already in the third century AD Galen was aware that a premature beat could produce a weak pulse, however it was Bowditch in 1871 that first described the influence
of the interval between a heartbeat and the following contraction (Bowditch 1871). His landmark paper contained many remarkable observations that are still accurate today.

He correctly detected in the frog heart that as the frequency increased so did the developed pressure. Furthermore, he noted that after a long rest the developed pressure increased with repeated regular stimulation (treppe or staircase).

His historical study also described several other phenomena related to the interval strength relation in the heart: one such observation was that the influence of one contraction on the next declined as the interval between them increased.

In 1902 Woodworth, working with a perfused oxygenated dog heart preparation was able to confirm many of these findings in mammals. He was also able to further elaborate on the optimal interval concept in which maximal force can be obtained by finding a precise ideal interval between beats (Puglisi et al. 2013). His work and that of others (Hajdu and Szent-Gyorgyi 1952, Koch-Weser and Blinks 1963) gave birth to many of the concepts that we investigate here further.

Crucial to the understanding of some of the future chapters is the distinction between the force frequency relationship (treppe) and the regulatory influence that occurs in the heart on a beat-to-beat basis.

The frequency dependent effect on the heart (treppe) appears to be, at least in part indirect: With increasing heart rate Ca$^{2+}$ enters the cell at a faster rate than it can be removed. This leads to a progressive accumulation of Ca$^{++}$ in the SR and an increasing Ca$^{2+}$ availability for contraction with a resulting positive inotropic effect. This enhanced contractility helps the heart eject more blood with each contraction and helps compensate for the reduced filling time associated with increased rate. Frequency dependent
acceleration of relaxation is a physiologic mechanism that accompanies the increase in frequency and allows for more rapid diastolic filling at increasing frequency (DeSantiago et al. 2002) and contribute to the effectiveness of treppe.

On a beat-to-beat basis however phenomena such as mechanical restitution assume much more significance. Mechanical restitution can be understood as the time dependent process that describes the recovery of the contractile response after an interval of varying duration. The basis for mechanical restitution can be found in the electromechanical processes that govern the contraction and require both recovery of membrane conductances and calcium cycling (Franz et al. 1983).

Two other related phenomena: post extrasystolic potentiation and post rest potentiation describe the more powerful beat that occurs after a prolonged interval between beats. The belief is that the more powerful beat occurs because of the additional accumulation of calcium that results either secondary to an early and incomplete beat (extra-systole) or after a prolonged pause (post-rest potentiation).

1.4 Contractility Myofilaments and EC Coupling

The capacity to alter the amount of force generated at the individual cell level is one of the hallmarks of cardiac muscle. The definition of myocardial contractility is elusive and perhaps it is easier to think in terms of contractility being equal to the summation of interactions arising during the excitation contraction coupling of the heart at that specific moment.

In the heart calcium-induced calcium release is one of the initial events to occur in order for depolarization to activate contraction (Bers 2002a). Calcium signaling also
controls the final step in excitation contraction coupling at the level of the myofilaments. Here we briefly review some of the more pertinent aspects of this process as it applies to many of the discussions and conclusions set forth in this work.

The initial event in EC coupling is the development of an action potential that depolarizes the plasma membrane. The cardiac action potential displays a characteristic plateau phase which is responsible for the extended refractory period of the muscle fibers. In contrast to skeletal muscle the cardiac action potential lasts for over 300ms and therefore does not permit tetanization of the cardiomyocytes (there is also no use for a sustained contraction in the heart). Each one of the five phases of the action potential is dominated by specific ion currents (Nerbonne and Kass 2005). A simplified scheme is depicted in figure 1.1.

- Phase 0 the upstroke is fast and causes an increase conductance to Na+ through voltage-gated Na+ channels.
- Phase 1 (early repolarization) the rapid, voltage-gated sodium channels are inactivated and opening of voltage-gated K+ channels with K+ efflux occurs.
- Phase 2 (Plateau) a slow inward calcium current (voltage-gated L-type Ca++ channels) balances an outward slow K+ current.
- Phase 3 (Repolarization) increased negative potential causes L type Ca++ channel inactivation and opening of voltage-activated K+ channels (K+ outflux).
- Phase 4 (Resting membrane potential)

The muscle cells are electrically interconnected by gap junctions forming a syncytium. Once electrical excitation occurs in one cell it spreads on to the adjacent cells.
1.5 Calcium Transient

Calcium is the primary ion responsible for the coupling of electrical activity in the heart to myocardial contraction. During systole calcium levels must rise to elicit contraction. Calcium enters the cell through the voltage gated L type Ca$^{2+}$ channels and primarily triggers Ca$^{2+}$ release from the SR. It also contributes calcium directly to the cytosol and eventually to the troponin C subunit of the thin filament Figure 1.2.

During diastole calcium has to be pumped back into the SR as well as extruded from the cytosol back into the extracellular space. This is accomplished respectively by the Sarco-endoplasmic reticulum calcium ATPase (SERCA) pump and by the sodium calcium exchanger (NCX) and (to a lesser extent) by a sarcolemmal Ca$^{2+}$ ATPase pump.

SERCA in turn is regulated primarily by phospholamban (PLB), which lowers SERCA’s activity by decreasing its affinity for calcium. Phosphorylation can remove this inhibition and increase SERCA’s pumping activity.

Different species show significant variations in calcium handling. In general, in smaller mammals like the rat, SERCA activity is responsible for the removal of anywhere from 95 to 98% of activator Ca$^{2+}$ from the cytoplasm while in the rabbit and in humans only 70% is resequestered by SERCA(Bers 2001). This fact explains some of the observations found by researchers using different animal models and some of our own findings regarding the influence of heart rate variability on cardiac contractility. Despite all this complexity, the time to peak [Ca$^{2+}$] is reached much earlier than the peak in force. Ca$^{2+}$ cycling does not therefore, appear to be a rate limiting factor in contraction or relaxation (Janssen et al. 2002).
1.6 The Sarcomere

The primary function of the heart is to produce force and generate pressures to pump blood from its cavities. In order to perform this function the structure of the contractile apparatus is organized into repetitive units approximately 2 \( \mu \text{m} \) long referred to as sarcomeres.

Historically the ultrastructure of the sarcomere has been organized into bands corresponding to the how the structures were first observed through the optical microscope. In Figure 1.3 these parallel units are seen bound by Z-lines. These plate like proteins anchor the thin filaments and the titin molecules. The cardiac contractile unit consists of thick and thin filaments. The thin filament is composed of actin, tropomyosin and troponin distributed in a 7:1:1 ratio. Actin is the filament backbone and consists of polymerized actin monomers in a \( \alpha \) helix formation. The troponin complex is formed by three individual proteins: Troponin C (for Calcium receptor), Troponin T (for tropomyosin binding) and Troponin I (for inhibitor).

About 2000 actin filaments are bound to the Z plate. The region adjacent to the Z line is termed the I band. Dystrophin further anchors the actin filaments to the sarcolemma. The region of the myosin filaments is called the A band. The H zone contains only myosin filaments (1000 per sarcomere) which thicken towards the middle to form the M Line.

The thick filament is composed of myosin (figure 1.4). Myosin is made up of two asymmetric dimers (the heavy chains) each consisting of a tail with 2 \( \alpha \) helical chains wrapped around each other, a hinge area (S2) and 2 globular heads (S1) that contain the actin binding site as well as the site for ATP hydrolysis. In addition each globular portion
is associated with 2 light chains (LC-1 and LC-2). These are thought to be important in force production by adding stability and “stiffness to the lever arm. The “tail” is associated with a multi-domain protein that also binds to actin and is involved in contraction regulation: myosin binding protein C. Titin is a large structural protein that is currently believed to add passive tension and also possibly modulate the distance between the myosin head and the actin binding site (Granzier and Labeit 2002).

1.7 The sliding filament theory

During diastole, myosin is blocked by tropomyosin from interacting with actin. Tm is held in place by TnI. This state of the thin filament blocks cross-bridges from reacting with actin or holds weakly bound cross-bridges in a non-force-generating state (Solaro et al. 2010). During systole, calcium binds to troponin C and causes a conformational change in TnI. This change in conformation shifts tropomyosin and unblocks and exposes the actin to the active site on myosin. This enables the interaction between actin and myosin to occur and the cross-bridge cycle to generate force. The regulatory protein tropomyosin can be located in three positions on the thin filament. In the absence of Ca2+, troponin induces an “off” state where tropomyosin sterically hinders strong cross-bridge binding. Ca2+ binding to TnC causes tropomyosin to move toward the inner domain of actin, and thereby exposes myosin-binding sites. A second tropomyosin shift, further toward the groove, is promoted by myosin binding and required for full thin filament activation (Craig and Lehman 2001). Each cross-bridge power stroke causes approximately 10 nm of reciprocal sliding during an isometric twitch (Caremani et al. 2008) and generates approximately 2.4 pN of energy. Once calcium drops to diastolic
levels, it leaves the binding site on TnC and tropomyosin returns to its original position again blocking the acto-myosin interaction.

In order for the process of actin myosin interactions to occur a series of biochemical and structural interactions must happen. This process has come to be known as the crossbridge cycle (Figure 1.5):

In the relaxed state ATP is attached to the myosin head and the thick and thin filaments are dissociated. The catalytic site of myosin then hydrolyzes the myosin bound ATP to ADP and Pi (inorganic phosphate) and transfers the energy of the terminal phosphate bond to myosin. Myosin is thus energized and cocked into the high energy conformation. This transition from weakly to strongly-bound actomyosin states directly precedes the powerstroke. With calcium binding to troponin c and the subsequent exposure of actin by tropomyosin the myosin head attaches to actin and forms an active complex releasing Pi. The power stroke is initiated as the myosin bound ADP is released and the myosin head pivots and pulls on the actin filament towards the M line. With the release of ADP the myofilaments now form the rigor complex. In this conformation myosin is firmly attached to actin but in a low energy configuration. This release of ADP from actomyosin is rate limiting in the cross bridge cycle (Siemankowski et al. 1985). In order for the cycle to conclude, ATP must now reattach to the myosin head and weaken the bond between actin and myosin. This leads to the detachment of the rigor complex and returns the cross bridge to the initial relaxed state.
1.8 Myofilament Sensitivity

In order to enhance contractility either an increased amount of calcium is required to bind to troponin-C, or modifications at the level of the myofilaments are required to promote an increased interaction with the contractile system at the same level of cytosolic calcium. The latter is commonly referred to as myofilament calcium sensitivity.

There are several sites on the myofilaments themselves that alter their sensitivity to calcium mainly through phosphorylation/dephosphorylation. The regulatory myosin light chain contains a calcium binding site and serine phosphorylation sites. Phosphorylation of these sites by myosin light chain kinase is known to increase calcium sensitivity and increased rate of force development (Sweeney and Stull 1990). Myosin binding protein C has been shown to alter the physical relationship of myosin heads towards the thin filaments when phosphorylated by protein kinase A (PKA). Phosphorylation of one or more of the four available sites in cardiac myosin binding protein C is thought to remove bundling constraints and allow the myosin heads to move closer to the thin filament increasing ATPase activity and force (Colson et al. 2008).

TnI has a unique stretch of N-terminal amino acids, which contains several phosphorylation sites sensitive to different kinases (Solaro et al. 2013). PKA phosphorylation of some of these sites (serine 22/23) has been shown to decrease calcium sensitivity and contribute to frequency dependent acceleration of relaxation in response to beta stimulation.
1.9 *Ventricular Hypertrophy Model*

Ventricular hypertrophy is the process of increase in size of the myocyte secondary to an increase mainly in the number of myofibrils. Ventricular hypertrophy can present itself as a physiological adaptation or as a pathological mal adaptation (decompensation) leading eventually to heart failure. Heart failure is a complex clinical syndrome that has multiple etiologies and stages. The signaling mechanisms involved alongside the molecular, biochemical and functional changes that are characteristic of each of these stages are well beyond the scope of this work. For the purposes of this dissertation we will only briefly comment on changes that occur in a pressure overloaded right ventricular hypertrophy model.

Classically ventricular hypertrophy can be divided into three stages (Katz 2011):

- In the first stage of Developing Hypertrophy, the cardiac output is still normal but the increased pressure load initiates a series of growth signals.

- In the second stage, Compensatory Hypertrophy, may be viewed as a period when the increased workload has already produced visible hypertrophy of the heart and although mechanical function may still be clinically unaltered, more subtle measurements indicating dysfunction such as decreased rate of ventricular shortening velocity (Vmax) and delayed relaxation are present.

- The third phase, Heart Failure, occurs when there is inability of the heart to meet the metabolic demands of the organism due to a decreasing ability to fill normally and to generate force.

The increased demand imposed on the heart leads to remodeling of the myocytes. It is yet unclear what causes the transition from the compensatory phase to that of overt heart failure.
failure with abnormal calcium handling (Morgan 1991), decreased force frequency response (Mulieri et al. 1992) and blunting of the response to β adrenergic stimuli (Bristow et al. 1982).

The pulmonary artery banded model was selected because of its ability to promptly induce ventricular hypertrophy that is easily quantifiable. A detailed explanation of the procedure is available in the material and methods section of chapter 5. Essentially the pulmonary artery was ligated using a polyethylene tube with an outer diameter of 3.2mm as a gauge. This model has been well established and well characterized functionally and histologically in our lab. The hearts had increased TnI levels, significantly blunted force frequency response, elevation in diastolic tension and decreased calcium frequency response at 10 weeks. (Varian et al. 2009a).

1.10 Energy Metabolism in the Heart

The heart is an omnivore. It functions best when it oxidizes different substrates (Depre et al. 1999). From a thermodynamic point of view it converts chemical energy to work with an efficiency rate of approximately 25-30% which is favorable when compared to the likes of a car engine for example (18-20%).

Adenosine Triphosphate (ATP) is the universal energy currency in the heart and the heart uses lots of it: over 6000 g in a day. Despite this fact, at any time the heart has enough ATP stored for only a few beats (Ingwall 2002). Almost all ATP is derived (>95%) from oxidative phosphorylations in the mitochondria (Stanley et al. 2005). ATP hydrolysis is primarily used for contractile shortening (70%) with the remaining 30% utilized for SR calcium uptake and ion homeostasis.
In order to maintain ATP concentrations constant, the heart must match ATP synthesis to the ATP utilization rate. One of the mechanisms the heart uses to maintain ATP constant, despite variations in workload is an energy storage system from which it can quickly transfer energy to meet sudden increases in demand when the demand for ATP exceeds the supply. This system consists primarily of phosphocreatine (PCR). Phosphocreatine is present at approximately twice the concentration of ATP. Creatine Kinase transfers the high energy phosphate bond between PCR and ATP at a rate that is several times faster than the rate of ATP synthesis.

In order to maintain its contractile function, the heart requires a large chemical driving force for the ATPase reactions. In other words, high ratios of ATP to its hydrolysis products are required for the heart to maintain its viability. This is often referred to as the free energy of ATP hydrolysis or \( \Delta G_{\text{ATP}} \). At any moment the \( \Delta G_{\text{ATP}} \) can be calculated from the expression:

\[
\Delta G_{\text{ATP}} = \Delta G^0_{\text{ATP}} - RT \ln \left( \frac{[\text{ATP}]}{[\text{ADP}] [\text{Pi}]} \right)
\]

Where \( \Delta G^0 \) is the standard free energy change of ATP hydrolysis under standard conditions; \( R \) is the gas constant and \( T \) is the temperature (in degrees Kelvin) and \([\text{ATP}],[\text{ADP}],[\text{Pi}]\) are the chemical concentrations in the cytosol. The term \([\text{ATP}]/[\text{ADP}] [\text{Pi}]\) is often referred to as the phosphorylation potential. Since the other terms on the equation are constant, it follows that \( \Delta G_{\text{ATP}} \) is a function of the concentration of the reactants or of the phosphorylation potential.

The well perfused myocardium typically derives 60-90% of its acetyl-CoA from the \( \beta \)-oxidation of fatty acids and 10-40% from the oxidation of pruvate (Stanley et al.)
2005). Pyruvate has three main destinations: lactate formation (note that under non-ischemic conditions the heart is a net consumer of lactate); decarboxylation and oxidation by pyruvate dehydrogenase complex (PDC) to acetyl-CoA or carboxylation to oxaloacetate or malate in an anaplerotic reaction entering the citric acid cycle. There are many factors that inhibit and stimulate PDC directly and indirectly. As an example, adrenergic stimulation leads to increased calcium transients and stimulation of PDC, which leads to increased metabolism of pyruvate that then generates the power for the increase in contractile force.

1.11 Introduction to Methods and Techniques

The study of the contractile properties of the myocardium requires specialized equipment and preparations. And while multiple preparations are suitable for investigating the heart, each has its advantages and shortcomings.

Isolated myocytes do not include connective tissue components or extracellular matrix, do not allow the study of interactions between cells and are not suitable for the study of loaded contractions given that the physiologic range for sarcomere length in the beating heart is 1.85 μm -2.2 μm and in isolated myocytes the same values are typically 1.6 μm (shortened) to 1.8 μm (relaxed) . In contrast, experiments in the whole heart cause the muscle length to change almost on a beat-to-beat basis constantly modifying the force-calcium relationships and inevitably confounding the assessments of shifts in myofilament calcium sensitivity. It is also very difficult to obtain accurate intracellular calcium measurements under these conditions.
The ventricular trabecula model overcomes many of these issues by preserving all of the major cell types and constituents of the in vivo cardiac tissue (Janssen et al. 1998). The studies described herein have been performed by utilizing 2-4 mm long and 100-200 μm wide right ventricular trabeculae that are no thicker than 150-200 μm. These ultra-thin preparations have been shown to prevent core hypoxia at physiologic frequencies and temperatures (Raman et al. 2006). These multicellular preparations have several advantages over isolated myocytes. Crucial to our studies was the ability to measure the response to pharmacologic interventions and to changes in stimulation and loading conditions. Propitiously their linearity allows for the application of different preloads and stimulus algorithms while permitting the simultaneous study of intracellular calcium handling and mechanical twitch parameters.

1.12 Intracellular Calcium Measurements

Before the synthesis of fluorescent Ca^{++} dyes by Tsien (4,5), research in intracellular calcium involved the use of microelectrodes or aequorin. Microelectrodes were difficult to manage and required large invertebrate cells. Aequorin, a Ca^{++} activated photoprotein was typically extracted from jellyfish through a very rudimentary process. An extract of 50,000 jellyfish yielded only 200mg of purified aequorin (Shimomura 1995) and was therefore difficult to acquire. In the early 1980’s fluorescent probes became available and revolutionized the way calcium measurements could be obtained. Several of these new indicators had two very important properties: They could be loaded into cells with minimal disruption and the ratiometric method could be used to convert the fluorescence to Ca^{++} measurements simply by using the fundamental equation of the
ratio method (Gomes et al. 1998) where Ca\(^{++}\) concentration is related to the fluorescence intensity by:

\[
[Ca^{++}] = \beta * K_d * \left[ \frac{(R-R_{\text{min}})}{(R_{\text{max}} - R)} \right]
\]

Where \(\beta\) is the ratio of the fluorescence intensities at the wavelength chosen for the denominator of \(R\) in zero and saturating Ca\(^{++}\). \(R\) is the ratio of the measured fluorescences, \(R_{\text{min}}\) is the ratio of the measured fluorescences in the absence of calcium and \(R_{\text{max}}\) is the ratio Ca\(^{++}\) saturated dye and \(K_d\) is the dissociation constant of the indicator for Ca\(^{++}\) (Gryniewicz et al. 1985) (Takahashi et al. 1999).

Ratio methods reduce the impact on measured fluorescence intensity (and therefore calcium estimates) that might occur from such factors such as nonuniform distribution of the dye within a population of cells as well as effects from photobleaching and leakage of indicator as illustrated in Figure 1.6. (Bright et al. 1989)

For our pH assessments we used BCECF (2',7'-bis(2-carboxyethyl)-5,6-carboxyfluorescein) BCECF is a dual-excitation ratiometric pH indicator Intracellular pH measurements with BCECF are made by determining the pH-dependent ratio of emission intensity (detected at 535 nm) when the dye is excited at ~490 nm versus the emission intensity when excited at its isobestic point of ~440 nm. For our calcium studies we utilized Bis-fura-2. This ratiometric dye displays a wave length shift in the excitation fluorescence spectra from ~340 to ~380, with emission at ~510. When excited at ~340 nm its fluorescence intensity increases when bound to calcium nm and decreases when it is excited at ~380nm. It has a lower affinity for Ca\(^{++}\) (Kd=370nM) and is about twice as fluorescent (brighter) as fura-2. This is important in order to use lower concentrations and thus decrease the Ca\(^{++}\) buffering effects of the indicator. The higher Kd allows for better
resolution at higher intracellular calcium levels especially in the range above 500 nM of Ca\(^{++}\) while still allowing accurate diastolic measurements. Bis-fura-2 is impermeable to the cell membrane and requires microinjection (via a patch clamp technique pioneered by Backx and Ter Keurs 1993) making it technically more challenging to load the myocytes. Once loaded the dye spreads to adjacent cells by means of gap junctions. The membrane impermeable form can also be seen as an advantage since it has better retention in the cytosol and no compartmentalization in the other calcium containing organelles such as the mitochondria and the sarcolemma (SR).

1.13 Myofilament Calcium Sensitivity

In order to assess myofilament calcium response we employ two different techniques: skinned fibers in which the membranes have been removed by detergent as well as intact trabeculae where a pseudo steady state relation between Ca\(^{++}\) and Force can be induced by a potassium contracture. In this approach, detailed by Varian et al (Varian et al. 2006a), a high potassium, low sodium solution causes a slow membrane depolarization and a slow rise in intracellular calcium making it possible for a pseudo-steady state contracture to develop. This technique can be executed at physiologic temperatures and has been shown to be repeatable utilizing the same trabecula with the advantage (over skinned fibers) of preserving the intracellular organelles, sarcolemma and membrane while still allowing for simultaneous intracellular calcium measurements.

In the skinned fiber method, muscle fibers are demembranated with detergents and exposed to varying Ca\(^{++}\) concentrations (typically \(10^{-9}\) to \(10^{-4}\) mol/L). While
keeping the sarcomere length constant, the developed force is measured at each Ca\(^{2+}\) level.

In both methods, the resulting sigmoidal curve can be fit with the Hill equation. These curves are typically defined by several parameters: the half-maximal activating Ca\(^{2+}\) concentration or EC\(_{50}\) which represents the myofilament Ca\(^{2+}\) sensitivity, the maximal developed force (F\(_{\text{max}}\)), and the steepness of the relationship also referred to as the Hill-coefficient (n\(_{\text{Hill}}\)) which measures the degree of cooperativity. These parameters can be altered by both physiologic and pharmacological interventions (Kass and Solaro 2006).

\[ 1.14 \quad \textit{Aims and Objectives of the Experiments} \]

The overarching theme guiding these studies is that by manipulating inter beat duration and myocardial metabolism that potentially can modify contractility and contribute to the arsenal of therapies available.

1. In the first part we investigate under near physiological conditions how modifications in the intermediate beat-to-beat interval can influence contractility in rats.
2. We then research how the hearts of different species behave when subjected to a similar protocol. In these experiments we also introduce load as an additional variable in order to stimulate more accurately preload changes that occur with changes in beat-to-beat duration in a live animal.
3. We demonstrate how pyruvate, a naturally occurring biofuel can be used as a very
potent inotrope and attempt to further explore the mechanism through which it exerts its effects.

4. Finally we examine the effect of pyruvate in both normal hearts as well as in a model of decompensated ventricular hypertrophy.
1.5  Figures

Figure (1.1) Graphic representation of the phases of a cardiac potential.

- Phase 0 the upstroke is fast and causes an increase conductance to Na+ through voltage-gated Na+ channels.
- Phase 1 (early repolarization) the rapid, voltage-gated sodium channels are inactivated and opening of voltage-gated K+ channels with K+ efflux occurs.
- Phase 2 (Plateau) a slow inward calcium current (voltage-gated L-type Ca++ channels) balances an outward slow K+ current.
- Phase 3 (Repolarization) increased negative potential causes L type Ca++ channel inactivation and opening of voltage-activated K+ channels (K+ outflux).
- Phase 4 (Resting membrane potential)
Figure (1.2) Main structures involved in cardiac excitation contraction coupling reproduced from *A genetic framework for improving arrhythmia therapy* Björn C. Knollmann & Dan M. Roden Nature 451, 929-936(21 February 2008) with permission.
Figure (1.3) The Sarcomere: reproduced with permission from *To the heart of myofibril assembly* Carol C. Gregorioemail, Parker B. Antin Volume 10, Issue 9, p355–362, 1 September 2000
Figure (1.4) illustrates protein to protein interactions of the myofilaments. Seven actins in 1:1:1:1 complex with tropomyosin (Tm), TnC, troponin I (TnI), and troponin T (TnT). The thin filament unit on the left is relaxed, and the one on the right has been activated by Ca2+ binding to TnC. On the bottom half: the thick filament with cross-bridges and a portion of titin, emphasizes the rich array of potential protein–protein interactions for control of force and shortening.

Figure (1.5). The crossbridge cycle. Reproduced from Skeletal and Cardiac Muscle Contractile activation: tropomyosin “rocks and rolls” Gordon, AM and Homsher E. Physiology April 1, 2001 vol. 16 no. 2 49-55
Figure (1.6) Ratiometric Dyes: ion binding at wavelength $\lambda_1$ and at $\lambda_3$ cause reciprocal changes in fluorescence. Fluorescence measured at ($\lambda_2$) an isosbestic point is independent of ion concentration. The intracellular dye concentration may leak or photobleach but a ratiometric dye continues to allow measurement of the changes in fluorescence. In the figure intracellular ion concentration change due to a stimulus indicated by the arrow is unambiguously identified by recording the fluorescence intensity ratios $\lambda_1/\lambda_3$ or $\lambda_1/\lambda_2$.

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Chapter 2: VARIABILITY IN INTERBEAT DURATION INFLUENCES MYOCARDIAL CONTRACTILITY IN RAT CARDIAC TRABECULATE

2.1 Introduction

The epidemic proportion that heart failure has reached in western society has prompted an intense search for positive inotropic therapies. Current approaches vary from pharmacologic to mechanical to genetic manipulation. We sought to investigate, in a multicellular preparation, if manipulating the R-R interval within physiologic range may be a potential inotropic strategy. The influence of the duration between successive cardiac beats has long been known to be a strong determinant of contractility. Force-frequency relationships have been investigated at the level of the whole heart (Hasenfuss et al. 1994), as well as isolated tissue (Layland and Kentish 1999), and isolated cell level, in various species. These previous studies clearly indicate that within the physiological range, a decrease in inter-beat duration (i.e. increase in heart rate), results in an increase in contractility. However, this force-frequency relationship only holds true for a steady-state stimulation that develops over many beats, whereas contractile strength on a beat-to-beat basis shows a direct opposite effect, the first beat(s) after switching to a higher stimulation rate are in fact weaker (Narayan et al. 1995). An instantaneous switch from a steady inter-beat-duration to a shorter inter-beat period decreases force of contraction,
while an increase in inter-beat period increases contraction, the latter is called post-rest potentiation (Pieske et al. 1996). In vivo, a small variation in inter-beat duration is normally observed, (beat-to-beat variability). Both force-frequency behavior and post-rest potentiation behavior are non-linear in the time domain. This implies that a given shortening of inter-beat duration does not necessarily cause an equal and opposite change in cardiac contractility compared to an identical lengthening of inter-beat duration. In addition, this non-linear inter-beat-to-force relation depends critically on the baseline inter-beat duration (average heart rate). We postulate that a variable timing component superimposed on an average constant pacing rate does therefore not necessarily result in the same contractility observed at a fixed (non-variable) identical pacing rate. In other words, average contractile force may differ depending on presence and magnitude of beat-to-beat variability; if shorter beats would decrease force development less than an identical lengthening would increase this force development, average force development may exceed the one observed at the identical non-variable average rate (same number of beats over an extended period).

To test our hypothesis that inter-beat variability can actively contribute to cardiac contractility, for proof-of-principle, we isolated multicellular cardiac trabeculae, stimulated them isometrically at fixed intervals, as well as with protocols in which a variable inter-beat duration was imposed (with an average identical to the fixed rate). Our results indicate that under certain conditions that fall well within the physiological range beat-to-beat variability plays a role as a determinant of myocardial contractility.
2.2 Methods

Male Rats (LBN-f1, 175-225 g) were anesthetized by intraperitoneal injection of sodium pentobarbital (60mg/kg), and heparinized via the apex. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Hearts were rapidly excised and placed in Krebs-Henseleit buffer containing: 120 mM NaCl, 5 mM KCl, 2 mM MgSO\(_4\), 1 mM NaH\(_2\)PO\(_4\), 20 mM NaHCO\(_3\), 0.25 mM Ca\(^{2+}\), and 10 mM glucose in continuous equilibrium with 95% O\(_2\)/5% CO\(_2\) resulting in a pH of 7.4. BDM (2,3-butanedionemonoxime) 20mM was added to the dissection buffer to prevent cutting injury. (Mulieri et al. 1989) Thin (average diameter <150 µm, to avoid core-hypoxia(Raman et al. 2006, Stuyvers et al. 2002)), uniform, non-branched, trabeculae (n=19) along the tricuspid valve were carefully dissected, their dimensions measured, and then mounted in the experimental set-up(Janssen et al. 2002). Trabeculae were perfused with the same buffer as above (without BDM) at 37 °C, 1.5 mM Ca\(^{2+}\), and allowed to stabilize for at least 30 min at 4 Hz. During stabilization, optimal muscle length was obtained by stretching muscles to where small increases in length resulted in about equal increases in resting tension and active developed tension. This fixed optimal preload is equivalent to that verified at the end diastolic volume of the in vivo situation (sarcomere length of ~2.2 µm). (Rodriguez et al. 1992)

In the first group (n = 17), after 20 minutes of baseline stimulation at a fixed base frequency of 4 Hz, the trabeculae were stimulated for 2-minute periods alternating between a fixed steady state period (4 Hz) and a variable component period (10-120% of cycle length). Beat-to-beat variability was introduced by random stimuli intervals.
with algorithms such that over an identical time period there was an equal number of
deads compared to the steady state. Each period of variation was both preceded and
followed by a steady state period in order to help estimate general rundown (which is
unavoidable in these preparations). In order to avoid computing the effects of post rest
potentiation that might occur at the exact moment the protocols are switched from
steady state to variable, the 2 initial beats were not taken into account during the
calculations. For this proof-of-principle investigation, beat-to-beat variability was
distributed linearly, and expressed as a percentage of the variation between the longest
and shortest beat (e.g. at 40% variation at 4 Hz (R-R interval of 250 ms), the inter-beat
duration varied between 200 and 300 ms). In the second group of experiments, trabeculae
were stimulated at 4 Hz (n = 17), 6 Hz (n = 6) and 8 Hz (n = 6), thereby encompassing
the physiological range for the rat. The experimental period consisted of an imposed 40%
variability over the fixed (control) period. Once again, over an identical time period there
was an equal number of beats both in the control and experimental protocols. Beat-to-
beat variability protocols were both preceded and followed by fixed rate periods. The
variables from the beat-to-beat variability protocols were measured right after
assessment of the fixed-rate response at the same base frequency, allowing the
assessment and potential correction of rundown of the preparation. If rundown
exceeded 20% per 5 consecutive protocols, the trabeculae were discarded. As a surrogate
for standardized measurements of heart rate variability in the time domain, we calculated
the effective SDNN (standard deviation of normal to normal intervals) spanning each of
the individual 2 minute variable periods at each base frequency. These were then
averaged and labeled as SDNN (mod) index.
In a subsequent set of experiments (n = 3), intracellular calcium transients were measured after iontophoretic loading of the calcium indicator bis-Fura-2 into a preparation. The dye loading technique and protocol that allow for assessment of calcium transients at body temperature have been described previously (Janssen et al. 2002, Stull et al. 2002a). Briefly, at room temperature, a micropipette filled with bis-Fura-2 is introduced into a single myocyte near the center of the preparation, and a small negative current is applied to introduce the dye into the cytoplasm. Via gap-junctions this dye spreads to neighboring cells. Once the dye has uniformly spread (on average 30 minutes), fluorescence intensity at 510 nm is measured during alternate exposure to 340 and 380 nm excitation light. Trabeculae were then stimulated with the previously described variable pacing protocols, Simultaneous force development and intracellular calcium transients were recorded at 37 °C.

Data was collected via custom-written Labview® programs, and stored for off-line analysis. Results are denoted as mean ± SEM unless stated otherwise. Wherever applicable, paired or unpaired t-tests were performed, and a two-tailed value of P<0.05 was taken as level for significance.

2.3 Results

In Figure 2.1 we demonstrate that when pre-beat duration increased, so did contractile strength. In the depicted example, at a frequency of 8 Hz (cycle time of 125 ms), a 40% variation was imposed, making the shortest beat-to-beat interval 100 ms, and the longest interval 150 ms. The highest contractile force was observed after the longest
pre-beat duration (i.e. after the longest diastolic interval), whereas the shortest intervals were followed by the weakest beats.

For each stimulation period, we averaged the mean twitch force. By comparing the developed force at 4 Hz between fixed and variable pacing we noticed small, increases in force due to variable pacing with a trend towards increasing force with increasing variability (with the notable exception of 100% variability). The highest increase was noted at 120% variability (+1.3%, SEM 1.0 n = 17, Figure 2.2) although this did not achieve statistical significance.

The 40% variability index imposed during these protocols is well within the normal physiologic intervals reported in the literature for rats whereas the 120% variability index exceeds the variations reported for ‘in vivo” experiments (Schaan et al. 2004) (Figure 2.3).

The second experimental protocol group had baseline frequencies set at 4, 6 and 8 Hz during the fixed rate intervals with the corresponding 40% variability algorithm imposed during alternate 2 minute periods.

Trabeculae, while exposed to a variable beat-to-beat stimulation protocol, showed a more substantial increase in Fdev from an average of 15.1 to 16.5 mN/mm², an average percentile increase of 10.1% (P<0.05, n = 7, SEM 1.9) at 8 Hz (Figure 2.4) when compared to their fixed-rate stimulation averages. This indicates that despite the fact that the number of beats per minute was the same, beat-to-beat variability increased contractility.

When beat-to-beat variability is imposed on cardiac trabeculae the effect of these interval dependent changes on the calcium transients can be seen in Figure 2.5. In Figure
(2.5A) we demonstrate the positive correlation between a longer cycle length (pre-beat duration) with an increase in the corresponding calcium transient. In Figure (2.5B), a positive correlation between the developed calcium transient amplitude and force of contraction (Fdev) was observed (P<0.05) under all tested conditions. In addition in Figure (2.5C) the variations in interbeat duration and their associated fluctuations in the amplitude of the calcium transients are depicted.

2.4 Discussion

We set out to observe whether a small variation in the inter-beat period (HRV) has a modulating effect on cardiac contractility. We showed that a non-variable pacing algorithm can indeed alter myocardial contractile strength, and thus we demonstrate for the first time that HRV is a direct and positive modulator of cardiac contractility. Results from isolated muscle preparations including those regarding the interval-force relationship have been well substantiated by other investigators utilizing different models (Zaugg et al. 1995); the multicellular preparation reflects most properties of the whole heart(Bers 1997, de Tombe and Little 1994, Sys et al. 1998). Our data therefore clearly suggests that under some of the conditions studied, beat-to-beat variability can actively contribute to cardiac contractile strength. For this proof of principle investigation, our focus was on the effects seen on developed force. Changes in diastolic force were not addressed and should be subjected to further investigation.

Post-rest potentiation (Janssen et al. 1999, Pieske et al. 1996) experiments have shown that when stimulation is interrupted and re-established, the contractile strength of the first beat after the period of non-stimulation greatly depends on the duration of rest.
This is very similar to what we observed, albeit in a much smaller time domain. Where post-rest experiments typically are assessed from 1 to 120 seconds, we observed a similar behavior for the short periods of rest (in case of variable beat duration exceeding the average heart-rate), or lack of rest (in case of beat duration shorter than the average heart-rate), within the sub-second/millisecond domains. Thus, although our results were obtained in a much smaller temporal domain, they are in line with the post-rest potentiation observations.

The molecular regulation of the phenomenon of positive inotropic beat-to-beat variability has to be sought in the regulation of calcium handling. The observed beat-to-beat variation in force correlated very closely with the calcium transient amplitude. This was expected, as regulation of calcium handling is at the basis of post-rest potentiation. Post-rest potentiation occurs by Ca2+ loading of the SR during the rest interval. At the same time, at the level of the Na+/Ca2+ exchanger (NCX), an increase in intracellular calcium will lead to increase in activity which can decrease the intracytosolic calcium. Since both the function of the SR calcium ATPase and the NCX are influenced by many factors, including phosphorylation potential, transmembrane sodium gradient, and the cytosolic calcium level, the competition between the sarcoplasmic reticulum (SR) calcium ATPase and the Na+/Ca2+ exchanger (NCX) all contribute to the development of the subsequent calcium transient. Several of these factors, in turn, change within the time-domain of interest. Thus, the resulting SR calcium load highly depends on many, rapidly changing, factors, and it is thus likely that there is not a simple linear relationship between SR calcium load and inter-beat duration. We speculate that a variable inter-beat duration, therefore, likely causes a small positive
effect on the SR Calcium ATPase, this allows for more calcium to be available for subsequent transient/beat resulting in the elevated average calcium transient. Moreover, at different heart rates, the above factors that modulate SR calcium ATPase and NCX activity are affected differently, impacting on the effect of beat-to-beat variability on contractility, as observed with a gradual effect of variability over the measured frequency range. It is thought that this differential effect on the modulating factors is highly responsible for the differences found between 4Hz, 6Hz and 8HZ.

Calcium is the preeminent signaling ion and it is likely that an introduction of beat-to-beat variability that changes the intracellular calcium homeostasis may have secondary effects. Through various signaling mechanisms and resulting changes in protein expression, introduction of beat-to-beat variability may have secondary effects, but to determine these were well beyond the scope of this proof-of-principle investigation.

In vivo, beat-to-beat variation occurs naturally, and has been termed heart rate variability (HRV). Although the underlying principle of our studies may be similar to those underlying HRV, we cannot unambiguously extrapolate our results to the in vivo situation, and whether introduction of beat-to-beat variation will have a beneficial effect in vivo in humans cannot be answered with this proof-of-principle investigation. Still, HRV is known to correlate with cardiac dysfunction. When humans age, HRV becomes depressed (Corino et al. 2007). A similar reduction in HRV is observed in patients suffering from end-stage cardiac failure (Counihan et al. 1993, Panina et al. 1995, Saul et al. 1988). If this reduction in HRV can be altered by introduction of an increase in the inter-beat variability, this may potentially offer a novel therapeutic corridor. While there
have been reports that irregular pacing may worsen cardiac function in unhealthy patients (Daoud, Weiss et al. 1996, Clark, Plumb et al. 1997, Melenovsky, Hay et al. 2005), no direct measure of contractility was obtained. It remains unclear whether these results were in fact due to a decrease in contractility or to other associated factors such as inter and intraventricular dyssynchrony due to RV pacing, variable ventricular filling and resulting loading conditions or other systemic effects. Thus, although it remains unresolved whether beat-to-beat variation could have similar effects in vivo we show that under near physiological conditions and in a multicellular preparation that preserves most of the fundamental components of the myocardium, beat-to-beat variations can introduce an inotropic effect in vitro with corresponding changes in the measured calcium transients.

In conclusion, our data suggest that inter-beat duration can, under certain conditions, be a determinant and modulator of cardiac contractility.
2.5 Figures

Figure (2.1). Force development is dependent on duration of the pre-beat interval; when previous beat-duration increases, so does peak twitch force development (Fdev). In the depicted example, at a frequency of 8 Hz a 40% variation was imposed. Thus, inter-beat duration varied from 100 to 150 ms, with an average of 125 ms (=8Hz).
Figure (2.2). At a base cycle time of 250 ms (4 Hz), between 10 and 120% variability (reflecting cycle times from 237.5-262.5 ms and 125-375 ms resp.), the small increases in average Fdev were not significant, but on the other hand did not impair contractility either (n = 17).
Figure (2.3). At a variability of 40% and for all frequencies observed (4,6,8) the SDNN(mod) was well within the physiologic range for rats. At 120% variability the SDNN(mod) was elevated.
Figure (2.4). At a variability of 40%, average active developed force again was not significantly increased at an average cycle time of 4 and 6 Hz however at the 8 Hz, the 40% variability significantly increased the average developed force of the trabeculae, * (P<0.05, n = 7)
Figure (2.5). At an average cycle rate of 125 ms (8 Hz), a variation in inter-beat duration of 40% was applied. Panel A demonstrates the positive correlation between the variations in cycle length and the developed calcium transients ($R^2 = 0.72$, $p=0.0006$). The correlation between the developed calcium transient and peak active force development ($F_{\text{dev}}$) is shown in panel B ($R^2 = 0.57$, $p=0.03$). In panel C, the top and bottom panel show the resulting intracellular calcium transients and twitch contractions respectively. The variation in inter-beat duration is associated with variations in the calcium transients.
Chapter 3: CONTRACTILE STRENGTH DURING VARIABLE HEART DURATION
IS SPECIES AND PRELOAD DEPENDENT

3.1 Introduction

Bowditch (Bowditch 1871) described the influence of the interval between beats on force development over 100 years ago. His landmark experiments in frog hearts ascribed prime importance to the effect of the interval between beats on the strength of contraction. Widely recognized as one of the most important relationships in cardiac physiology, the force frequency relationship has been subject of intense investigation in the field of cardiac mechanics. The force-frequency relationship (FFR) describes that an increase in force development is observed as the interval between beats decreases (higher frequency) and has been shown in vivo and in vitro to occur in all healthy mammals (Janssen and Periasamy 2007) and this FFR is impaired in patients with heart failure (Mulieri et al. 1992). Typically, these changes in contractile force develop over several beats and are generally derived from steady-state observations at different frequencies (Janssen 2010b).

Other intrinsic and seemingly opposite phenomena in mammalian myocardium act on a more immediate non steady state beat-to-beat basis. Post rest potentiation, first described by McWilliam in 1888 (McWilliam 1888) describes the stronger force development seen when stimulation is resumed after a longer than usual interval, that is a
period of rest, between beats (Pieske et al. 1996). Mechanical restitution describes the dependency of the recovery of contractile force on the interval between contractions namely that a shorter interval leads to a weaker beat and a longer interval leads to a stronger beat (Cao et al. 2003) (Burkhoff et al. 1984) (Hardman et al. 1998b) Post extrasystolic potentiation is yet another mechanism whereby a shorter interval that precedes a longer interval determines a stronger contraction in the subsequent beat (Meijler 1962).

In previous work in rat we were able to show small gains in average contractile force over the fixed rate period given an equivalent number of beats in each period (Torres et al. 2008b). Here, to translate these findings to species that more closely resemble human cardiac function, we investigated what effect R-R interval modulation (such as what may occur during atrial fibrillation) may have on the inotropic status of the myocardium in rabbits and dogs and seek to verify the impact of this strategy on different species with different calcium handling and EC coupling properties.

3.2 Methods

Male NZW rabbits (2-3 months) were anesthetized with pentobarbital (at 60mg/kg intraperitoneal and 25mg/kg/IV respectively). In dogs (weighing 19.0 ± 0.4 kg, range, 13.6-24.1 kg, 2-3 years old) an intravenous catheter was placed in the cephalic vein and a surgical plane of anesthesia was induced by the bolus injection (over 1-2min) of sodium pentobarbital (50 mg/kg, Nembutal, Abbott, North Chicago, ILL). Under deep anesthesia, the hearts were rapidly removed and perfused retrogradely through the aorta with Krebs Henseleit solution (120mM NaCl, 5mM KCl, 2mM MgSO4, 1mM NaH2
PO4, 20mM NaHCO3, 0.25mM Ca2+, and 10mM glucose) in equilibrium with 95%O2/5%CO2. 2,3-butanedione monoxime (BDM) was added to the dissection solution to stop the heart from beating and to prevent damage during dissection. This investigation conforms with the *Guide for the Care and Use of Laboratory Animals published by the US national Institutes of Health* (*NIH Publication Ns 83-23, revised 1996*). Suitable thin, uniform, non-branched, trabeculae along the free wall of the right ventricle were dissected carefully and without touching the muscle mounted in the bath as previously described for rabbit (Varian and Janssen 2007a) and dog (Billman et al. 2010). Average width was \(\sim 120–140\ \mu m\) in order to prevent hypoxic cores of the muscles (Raman et al. 2006). The dissected specimen contained small cubes of ventricular tissue attached to each end to facilitate mounting the muscle onto the experimental setup. The cube of ventricular tissue at one end of the trabecula was connected to a hook, and the cube at the other end rested in a basket-shaped extension of the force transducer. The muscle was bathed in a continuous flow of oxygenated KH solution (without BDM), and with 1.75mM Ca2+, at 37°C. The dimensions (length, width, and thickness) of the muscle were measured under 30 x magnification. Initially, the muscle was stimulated at 1 Hz at a temperature of 37°C and stretched until small increases in length resulted in about equal increases in resting tension and active developed tension. This fixed optimal preload is equivalent to that near the end diastolic volume in the in vivo situation (sarcomere length of \(\sim 2.2\ \mu m\)) (Rodriguez et al. 1992). Trabeculae were stimulated at physiologic frequencies for each species (1 and 4 Hz in dog, \(n = 9\), and 2 and 4 Hz in rabbit, \(n = 8\)) with or without a 40% variation imposed on inter-beat duration. In order to define the load dependence of contractility during heart rate variability (HRV), a subset of rabbit
trabeculae was subsequently subjected to a variable preload protocol by utilizing a high-speed high-accuracy servomotor as a displacement device (n = 5). One end of the trabeculae was attached to a length displacement device (i.e., a high-speed, high accuracy servomotor). Once peak tension is reached and forces start declining (150 ms after initiation of the twitch), the servomotor reduces the muscle length by 10% (to 90% of optimal) within 2 ms, after which it is immediately stretched at the velocity of one muscle length per second until the next beat is triggered. At that point, the muscle length is kept constant. This range of lengths was chosen to be close to the in vivo sarcomere length range the heart undergoes during a contraction (Rodriguez et al. 1992). The protocol is then repeated (Figure 3.1). By actively stretching the muscle at a rate of 1 muscle length per second, a certain percent stretch of muscle length will result, which is identical to the percentage of muscle that is released. Thus, average preload is identical both during the fixed preload and variable preload protocol.

In all protocols, total number of beats in each fixed versus variable period was the same. A fixed-rate steady-state response at the same base frequency was measured before and after each variable period, and average force was reported.

3.2.1 Data analysis.

Data were obtained and analyzed through custom written software (LABVIEW). Correlation coefficients and slopes were obtained for pre-beat duration and force development. Kaleidagraph statistical package was utilized to perform Students T test
and repeated measures ANOVA where appropriate. All data are reported as means ± SEM.

3.3 Results

First, we compared the average active force development between protocols with base frequency with a random 40% variation in the interval between beats compared. In dog myocardium (Figure 3.2), there was no significant change on average force development at both 1 Hz (cycle time of 1000 ms) and 4Hz (cycle time of 250 ms).

Next, we compared these protocols in the rabbit. Since during in vivo beats, a prolonged beat-interval increases filling time, we mimicked this increase in preload by stretching the muscle during the interbeat period until stimulated. In Figure 3.3, we depict the observed non-significant decrease in strength when imposing a 40% variable protocol until a stretch (increased preload) is applied. Albeit not significant, there is a reversal of the negative tendency at 5% stretch for both baseline frequencies 2 and 4 Hz. Only at the more extreme 13% stretch at 4 Hz (the high end of the frequency range for rabbit) do we again observe a nonsignificant trend towards a negative effect.

In Figure 3.4, we show the correlation between pre-beat duration and average force development in the dog at both 1 and 4Hz during a 40% variable pacing protocol. Note the static relationship at the different frequencies. There is no difference with the increase in frequency.

In Figure 3.5, we show that as the preload stretch increases while baseline frequency increases, there is a much higher correlation between beat duration and
developed force. At 4 Hz, the increase in correlation becomes statistically significant. The longer the prebeat duration, the higher the contractile force observed, and the shorter the interval, the weaker the following beat.

3.4 Discussion

In our previous work, we had suggested and shown that an algorithm that imposed a beat-to-beat variability can lead to an average slight increase in force in rat myocardium (Torres et al. 2008a). In subsequent studies, we showed that in the rabbit, up to three preceding beats contribute to the amplitude of a given beat but that the average amplitude is not significantly affected by a pseudo-variable heart rate compared to the average of that rate applied as steady state (Varian et al. 2009b, Xu et al. 2011). Here, we tested the efficacy of this initial randomized beat duration strategy in the rabbit and dog. The current data suggest that neither canine nor rabbit myocardium seem to benefit (i.e., increase inotropy) from a variable rate with no negative or positive effect of variability on force development or kinetics noticed. The explanation for this species differences is more than likely found in the distinct manner in which EC coupling and calcium regulation are handled in each animal. Of particular interest is the increased contribution of the L-type calcium channels to the overall intracellular calcium pool during each contraction: ~70% in rabbit and dog compared to less only 2–5% in rat (Bers 2002a), (Monasky and Janssen 2009). In addition, it is known that also myofilament isoform composition differs between small and larger mammals, most notably in the myosin heavy chain (MHC) isoform (Lompre et al. 1981). However, since the force of contraction highly depends on time passed since the last contraction, dynamic calcium
handling and distribution of calcium fluxes that are very much time-dependent are thus more likely contributors, versus fixed differences in isoforms of MHC, or other differences in myofilament protein expression, although these cannot be excluded to contribute to the species differences at this point.

When next we investigated the effect of a variable length (load) under a variable pacing protocol on the rabbit heart, we noticed a discrete increase on average force of the variable over the fixed rate base frequency that was constant at both 2 and 4 Hz for the 5% stretch group. At 13% stretch, a similar effect was noticed at the 2 Hz frequency group, whereas, at 4 Hz, there was a nonsignificant weakening effect. This latter finding may have resulted as these higher frequencies from the additional stretch may have caused some damage to the myofilament structure by a too rapid stretch.

Of greater importance was the significantly tighter correlation $R^2$ observed between the pre-beat duration and the developed force once a small variable stretch was applied. This correlation was much tighter as the frequency and stretch increased ($P<0.001$ at 4 Hz). This suggests that while the interval between beats is important, there is considerable effect that is load dependent affecting the subsequent beat.

While several studies have shown an independent and strong effect of the interbeat duration alone and with contribution of the Frank Starling mechanism put into question (Gosselink et al. 1995, Hardman et al. 1998a), there are others with findings similar to ours during in vivo investigations (Muntinga et al. 1999, Petretta et al. 2002). At the heart of the problem lie two seemingly opposite phenomena: on one hand a sustained decrease in interbeat duration (thus an increase in frequency) leads to increased contractility, whereas instantaneous decreases in interbeat duration lead to a weaker (Monasky and
Janssen 2009) immediate first beat (Dumitrescu et al. 2002). We here now show that this interplay appears to be nonlinear in the time domain and that, by manipulating the frequency and variability a host of factors interact dynamically in a non-steady-state of equilibrium that changes every instant. The mechanism of the stretch-dependent “tightening” of the correlation is likely to be found in the interaction between load and calcium handling and could include stretch-dependent calcium channels and ion homeostasis (Luers et al. 2005) on one hand, an interaction of the higher calcium sensitivity at greater sarcomere length (de Tombe et al. 2010), or a greater contribution of predominantly passive elements such as collagen or titin (Granzier et al. 2005).

Potentially, at larger sarcomere length, the increase in the absolute myofilament calcium buffer capacity is nonlinear with stretch and promotes retention of calcium between two subsequent beats. However, more detailed experiments would be required to test such hypothesis.

On a beat-to-beat basis with instantaneous switching intervals away from steady state, the phenomena of mechanical restitution and post-rest potentiation and post-extrasystolic potentiation likely play an important role in determining the immediate contractile state of the heart (Cooper 1993). We speculate that the direction and the magnitude of force modulation possibly relate to the different relative contributions of the sodium calcium exchanger (NCX) versus the sarcoplasmic reticulum calcium ATPase (SERCA) to the contractile process in each species. This paper uniquely highlights the influence of heart rate variability on a beat-to-beat basis and the different effects in diverse species while attempting to account for real life in vivo events such as preload stretching of the fibers.
We conclude that, in larger mammals, no changes in contractile force can be obtained by introducing variation in the beat-to-beat duration, and we confirm the evidence that pre-beat duration has an important effect on developed strength (Varian et al. 2009b). The addition of a pre-beat stretch effectively tightens this association that also becomes more significant as the frequency increases. Short-term manipulation of the beat-to-beat interval thus can have variable effects that are species dependent. Its underlying mechanisms require further in vivo experiments as well as potential investigation of the long-term effects on protein expression and posttranslational modifications that are known to occur as a result of length (Monasky et al. 2010) or frequency changes (Lamberts et al. 2007, Varian and Janssen 2007a).
3.5 Figures

Figure (3.1) Following a stimulus, muscle length (y-axis) is kept constant till well after peak active force development has been reached. At 150 ms after the stimulation, muscle length is quickly reduced to 90% of optimal length, and thereafter re-stretched at 1 muscle length per second. The duration of this stretch is varied resulting in successive beats with different preloads, where the preload is increased linearly with the interbeat duration.
Fig. (3.2): In dog trabeculae, at 40% variability, the average active developed force was not increased at 1 or 4 Hz. Results are expressed compared to baseline.
Fig. (3.3): At a variability of 40% in the rabbit at both 2 and 4 Hz, there was no statistically significant difference between variable and base protocols. The addition of a 5% and/or 13% stretch just prior to contraction did not seem to improve or impair the average force development.
Fig. (3.4): The correlation between pre-beat duration and average force development in the dog is demonstrated for both 1 and 4 Hz. There is no difference with the increase in frequency.
Fig. (3.5): Top half displays the interaction between pre-beat duration and force development at 2 Hz. The correlation coefficients are increasingly tighter as the preload stretch is increasingly applied. On the bottom half, we depict the same phenomena at the higher frequency (4 Hz). The correlation here is more evident and significantly different statistically ($P < 0.01$) from the initial non-stretch correlation value of 0.56.
Chapter 4: THE POSITIVE INOTROPIC EFFECT OF PYRUVATE INVOLVES AN INCREASE IN MYOFILAMENT CALCIUM SENSITIVITY

4.1 Introduction

Pyruvate is naturally present in circulating blood at concentrations that vary between 0.1 to 0.2 mM (Mallet et al. 2005). At these levels, pyruvate does not exert any clinically significant enhancement of myocardial contractile strength. However, in concentrations in the range of 3 to 30 mM (which can be achieved via infusion) pyruvate has been shown to improve the contractile strength of the heart up to 200% (Hermann et al. 1999). The inotropic effect of pyruvate has been demonstrated in both normal and failing hearts. Moreover, it exerts its positive inotropic effects under both hypoxic and post ischemic conditions (Keweloh et al. 2007). Pyruvate’s effects in augmenting the contractile strength of the heart have been shown to be consistent throughout several species including rat, rabbit, swine, and humans (Hermann et al. 2004, Hermann et al. 2000, Liedtke and Nellis 1978, Martín et al. 1998). It potentiates the effect of β-adrenergic drugs (Hermann et al. 2002) and it has been infused intracoronary in humans experimentally to increase inotropic support (Hermann et al. 2004, Schillinger et al. 2011).

Pyruvate has demonstrated additional characteristics that separate it from the current available arsenal of inotropic drugs used for treatment of acute heart failure.
Pyruvate is a known antioxidant (Bassenge et al. 2000, Bunger et al. 1989, DeBoer et al. 1993). It is a readily consumable metabolic fuel that has been postulated to enhance glycolysis even during ischemic events (Slovin et al. 2001) and in stark contrast to the currently widely used β-adrenergic agonists, it has been shown to not be detrimental to the economy of myocardial contraction (Keweloh et al. 2007). This latter characteristic is of crucial importance at a moment when the myocardium oxygen consumption is critically taxed and its energy reserves are at a premium and can potentially translate into very significant therapeutic advantages.

While the inotropic action of pyruvate is well known, the underlying mechanism is incompletely understood. A review by Mallet (Mallet et al. 2005) suggests among the most probable candidates for the underlying mechanism pyruvate’s enhancement of cytosolic ATP phosphorylation potential and Gibbs free energy of ATP hydrolysis (DGATP) and pyruvate’s effects on the sarcoplasmic reticulum (SR) (Mallet et al. 2005). Also discussed elsewhere are changes in the intracellular pH, inhibitory effect on the ryanodine receptor channel activity (Zima et al. 2003), decreased intracellular inorganic phosphate (Mallet and Sun 1999), the enhancement of myofilament calcium responsiveness, and/or changes in cross bridge kinetics (Hasenfuss et al. 2002a). Since some of these potential mechanisms may occur simultaneously, we further investigate the inotropic mechanism of pyruvate and its relationship to the contractile properties of the heart. By examining a time-resolved picture of the positive inotropic effect as well as in parallel intracellular calcium handling under near physiological conditions in combination with assessment of pH and myofilament calcium responsiveness, we found
that indirect enhancement of myofilament sensitivity plays a major role in the underlying mechanism for pyruvate’s inotropic effects.

4.2 Material and Methods

4.2.1 Ethics Statement

All experiments were approved by the 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.

4.2.2 Muscle preparation

Male New Zealand White rabbits (2–3 months old ~2 kg weight) were anesthetized by intravenous injection of sodium pentobarbital (60 mg/kg) following systemic heparinization with 5000 units/kg of Heparin. Hearts were rapidly excised and placed in Krebs-Henseleit buffer (K-H) composed of: 120 mM NaCl, 5 mM KCl, 2 mM MgSO₄, 1 mM NaH₂PO₄, 20 mM NaHCO₃, 0.25 mM Ca²⁺, and 10 mM glucose at a pH of 7.4. 20 mM BDM (2,3-butanedione 2-monoxime) was added to prevent cutting injury during dissection. From the right ventricular free wall, thin, uniform, non-branched, trabeculae were carefully dissected as previously described (Varian et al. 2006a). Muscle dimensions were carefully measured (average width, thickness and length were 0.18±0.02, 0.12±0.02, and 2.07±0.46 mm respectively, n = 22). By using only thin preparations, core hypoxia and ischemia were unlikely to occur and thus impair the
results. Schouten and ter Keurs determined a critical diameter of 0.2 mm for room
temperature experiments on rat (Schouten and ter Keurs 1986), and studies by our lab at
physiological temperature have shown that for the frequencies used in our studies the
thickness of the muscles used would not result in a hypoxic core in the muscles using this
study protocol (Raman et al. 2006, Schouten and ter Keurs 1986, Stull et al. 2002b).
Muscles were placed into the experimental set-up, and superfused with the same K-H
buffer as above at 37°C, without BDM, and with calcium raised to 1.5 mM. The
preparation was allowed to stabilize for at least 30 minutes while electrically stimulated
to contract isometrically at 2 Hz. The muscle was slowly stretched in small increments
until increases in diastolic force equaled similar increases in active force development. It
has been shown that, at this length, the length of the preparation is close to the in vivo end
diastolic volume (representing a sarcomere length of ∼2.2 µm) (Rodriguez et al. 1992).

4.2.2 Intracellular calcium and pH measurements

Background auto-fluorescence was measured at excitation wavelength of 340 and
380 nm. The trabeculae were then iontophoretically loaded with bis-fura-2 (Texas
Fluorescence Labs Inc, Austin, Texas.) as described previously (Backx and Ter Keurs
1993, Janssen et al. 2002). The iontophoretically loaded bis-fura-2 was allowed to spread
via gap junctions throughout the preparation (at 22°C) until the photomultiplier output at
baseline 380 nm excitation reached between 6 and 10 times over background. After
loading of the dye was completed, the system was then switched back to 37°C, and data
collection was started. The muscle was now continuously stimulated at 2 Hz while force
and fluorescent emissions at 510 nm (excitation was alternated between 340 and 380 nm)
were recorded. The emission signals were calibrated and converted to \([\text{Ca}^{2+}]\), by standard methods described by us (Varian et al. 2006a) and others (Backx and Ter Keurs 1993).

In a separate set of experiments, using a similar iontophoresis protocol as described above, the pH indicator BCECF was loaded into 5 muscle preparations. Light was then passed alternatively through 440 and 495 nm band-pass filters and fluorescence was monitored at 540 nm. From the subsequent calculation of the fluorescence ratio of the excitation wavelength of 495 and 440 nm, we obtained a qualitative estimate of intracellular pH. In these experiments the BCECF fluorescence ratio was not calibrated (since qualitative interpretation of the results should not be affected). The relative proton concentration was followed while pyruvate (10 mM) was applied as described above. No SR block was performed in this series of experiments.

4.2.3  **Pharmacological inhibition of sarcoplasmic reticulum function**

To examine the effects of pyruvate independent of the effects on sarcoplasmic reticulum (SR) calcium handling, we performed a series of experiments in which pyruvate's effect was assessed while the SR cycling of calcium was blocked. After loading of the fluorescent dye was accomplished and baseline twitch force and calcium transients were obtained, the inotropic response to pyruvate was assessed. The inotropic effect of pyruvate is dose dependent and has been shown to be present in rabbit ventricular trabeculae in concentrations that vary from 0.3 to 30 mM. At 5 to 10 mM concentration there is a very pronounced effect that is easily reproducible (Hasenfuss et al. 2002a, Keweloh et al. 2007). Pyruvate (10 mM) was initially applied for 20 minutes
and thereafter completely washed out. At this point either 10µM of cyclopiazonic acid (CPA) and 1 µM of ryanodine (n = 6) or 100 nM of thapsigargin (n = 3) were added in order to block the SR (Hasenfuss et al. 2002a, Lytton et al. 1991, Rogers et al. 1995). Both strategies were chosen in order to ensure SR inhibition. After 30 minutes, and once the preparation was stabilized, an assessment of both force and calcium at the new baseline was performed. In the CPA+ryanodine preparations, rapid cooling contractures were performed before and after SR block to confirm that SR calcium handling was blocked. In all muscles, amplitude of the rapid cooling contracture were virtually zero (i.e. within the noise of the transducer), indicating a complete SR block similar to previous studies (Hasenfuss et al. 2002a, Monasky and Janssen 2009). In both groups, pyruvate (10 mM) was again applied, and the response recorded in similar fashion under SR block conditions.

4.2.4 Assessment of myofilament calcium sensitivity

In a subset of experiments, after acquiring baseline values of both force and calcium transients, myofilament calcium sensitivity was measured at baseline and during the maximal pyruvate response. To obtain steady-state force-[Ca]i relationships in intact muscle at body temperature, a potassium contracture protocol was used as described previously (Varian and Janssen 2007a, Varian et al. 2006a). Briefly, K+ contractures were elicited by switching the superfusion solution to a modified K-H solution containing high K+ (142 mM), 0 Na+, and 3 mM Ca2+. The other components of the K-H buffer remained unchanged. This high K+ solution was applied for 30 seconds and then washed out. During the developing steady-state contracture, intracellular calcium and force rise very
slowly and are in near equilibrium. After washing out this high-K\(^+\) solution, the contraction relaxes, and the muscle returns to a normal twitching pattern with unaltered force or kinetics. From this data a force-calcium relationship can be constructed, and analyzed similar to the force-pCa curves typically obtained in skinned myocardium. Once the contractures and measurements were obtained under controlled conditions, pyruvate at 10 mM was added to the superfusate and for the next 20 minutes the experiments were repeated followed by washout.

4.2.5 Skinned Fiber Experiments

To test the direct effects of pyruvate on the Ca\(^{2+}\) sensitivity of force development we employed chemically demembranated trabeculae as previously described (Liu et al. 2012, Norman et al. 2007). Rabbit trabeculae were harvested as described above. The buffers and solutions used for the force-pCa experiments are described in detail by Rall and by Moss (Luo et al. 2002, Metzger and Moss 1992, Norman et al. 2007). Briefly, trabeculae were placed overnight at 4\(^\circ\)C in relaxing solution containing 1% Triton X-100. This removes all membranes from the preparation including those from the SR, nuclei and inner and outer membranes of the mitochondria. The skinned trabeculae were mounted the next day between the arms of a high-speed length controller (model 322C, Aurora Scientific, Aurora, Ontario) and an isometric force transducer (model 403A, Aurora Scientific, Aurora, Ontario) with the sarcomere length adjusted to ~2.2 \(\mu\)m, resulting in an average resting tension of 1.3±0.2 mN/mm\(^2\). The trabeculae were exposed to a range of randomized pCa solutions with the active force calculated as the total force minus the resting force (pCa 9.0). Maximum pCa activations (pCa 4.0) were measured at the beginning, middle and end of each Ca\(^{2+}\) titration and were used to normalize the developed force. Every pCa point was measured twice consecutively, either with or without the addition of 10 mM
pyruvate (reversing the sequence for every other trabeculae). The force-pCa relationship was fit (using an iterative procedure) with a logistic sigmoid mathematically equivalent to the Hill equation, as previously described (Tikunova and Davis 2004, Tikunova et al. 2002). All skinned trabeculae experiments were performed at 15°C.

4.2.6 Data acquisition and analysis

Custom designed programs written in LabView (National Instruments, Austin, Texas) were utilized to collect and perform initial on line and off line data analysis. Data sets generated were then subjected to statistical analysis. Two-way and one-way ANOVA repeated measures with Tukey post-hoc testing or paired T-test were utilized where appropriate. Analysis was performed with the data analysis package provided with Kaleidagraph, (Synergy Software, Reading, PA). A p-value of <0.05 was considered significant. All data are depicted as mean ± SEM. For each protocol, only one preparation from each heart was included for statistical analysis.

4.3 Results

4.3.1 Inotropic effects of pyruvate

Typical twitch force recordings from a single experiment (Figure 4.1) show a transient decrease in active developed force between 2 to 3 minutes after application of 10 mM pyruvate (Time = 0), followed by a pronounced increase in developed force that stabilizes after about 10 minutes of pyruvate infusion. From the parallel assessed bis-fura-2 fluorescence, an increase in intracellular peak systolic calcium is observed as well. In
other experiments (n = 5), using an identical protocol, we tracked pH via relative proton concentration during this same time course. From this data we can see that during the “dip” of force, the proton concentration transiently increases, reflecting a transient intracellular acidification, thereafter the ratio tends to return towards baseline values. ($F_{495/490}$ ratio at baseline = 0.107; vs. “dip” = 0.123 and vs. “peak” = 0.113, n = 5). Figure 4.2, displays the inotropic effects of pyruvate and its correlation with calcium transients from the same experiment. In this series pyruvate consistently increased force development to 146% of baseline values on average (range 112 to 182%, n = 9).

4.3.2 *Inotropic Effect is Preserved under Sarcoplasmic Reticulum Inhibition*

Next, we tested the inotropic effect of pyruvate in the absence of functional SR calcium cycling. After pyruvate washout, the same trabeculae utilized above had the SR pharmacologically inhibited by a 30 minute exposure to the same KH solution with either cyclopyazonic acid and ryanodine (n = 6) or to thapsigargin alone (n = 3). Under SR block (indicated absence of any rapid cooling contracture) the baseline force decreased by an average of 59% from the control period (16.8±3.5 vs. 6.9±2.6 mN/mm²). Subsequently, and in continuous presence of SR block, pyruvate was again applied (10 mM), and the inotropic effect was again tested (Figure 4.3). We found that a similar magnitude of increase in force development is brought on by the infusion of pyruvate whether the SR is unblocked or blocked (16.8±3.5 to 24.5±5.1 vs. 6.9±2.6 to 12.5±4.4 mN/mm², non-blocked vs. blocked SR respectively, p<0.001, n = 9). There was no
appreciable difference in results whether thapsigargin or CPA+ryanodine was used to block the SR.

Importantly, during the non SR blocked (control) period an increase in intracellular calcium (as measured by peak systolic values) occurs during pyruvate infusion (from 599±135 to 731±153 nM, p<0.05) whereas during the SR block period, pyruvate infusion does not significantly change systolic calcium (620±108 to 635±107 nM, p = n.s.). Meanwhile, the increase in force still occurs to a large extent.

Table 1 summarizes the twitch parameters and cytosolic calcium kinetics during both the control and blocked SR periods. Baseline 1 refers to the initial pre pyruvate, pre SR block period. Baseline 2 is after the pyruvate washout and post SR block. Time to peak tension (TTP) and time from peak to 50% relaxation (RT\textsubscript{50}) are both significantly delayed after pyruvate infusion when the SR function is intact. Once the SR block is in place, there is still a trend towards delay in peak force development while the delay in RT\textsubscript{50} remains statistically significant compared to its respective baseline (pre pyruvate) period.

4.3.4 Myofilament calcium sensitivity is increased by pyruvate but not in Skinned Fibers

In figure 4.4A we show force and intracellular calcium data from potassium contracture experiments. The figure shows myofilament calcium sensitivity curves displaying the shift in sensitivity noticed from baseline (solid line), to 20 minutes of pyruvate infusion (peak force development, dashed line). These potassium contracture experiments were conducted after 20 minutes of baseline and at 20 minutes after the
addition of pyruvate. The maximal developed force of these contractions was not significantly different in the presence or absence of pyruvate, and was on average 27.2±4.9 mN/mm² (n = 6). The shift to the left of this curve denotes the increase in the calcium sensitivity that occurs with the change in force during pyruvate infusion.

In figure 4.4B the aggregate myofilament calcium sensitivity, expressed as EC50 at baseline vs. 20 minutes of pyruvate infusion (peak force development) demonstrates the sensitizing effect of pyruvate on the myofilaments with a significant change in their values (701±94 vs. 445±65 nM, p<0.01, n = 6). Expressed as pCa50 values, pyruvate caused an increase from baseline of 6.17±0.06 to 6.37±0.06 (p<0.005, n = 6). The Hill coefficient increased from 3.9±0.4 to 7.2±0.9, (p<0.05, n = 6) from baseline to 20 minutes of pyruvate infusion.

In skinned trabeculae the addition of pyruvate had no effect on the maximal developed tension of the skinned trabeculae (47.1±8.9 mN/mm² in absence of pyruvate, and 46.9±10.3 mN/mm² in presence of pyruvate, n = 8/group, p = n.s.) and as can be seen in figure 4.5, the equivalent parameters obtained from skinned fiber rabbit trabeculae caused a non-significant decrease in the Hill coefficient from 3.4±0.5 to 2.8±0.2 (p = n.s., n = 5, figure 4.5), in contrast to the findings in the intact trabeculae. Similarly, EC50 did not change significantly (2.60±0.81 vs. 2.26±0.57 µM, control vs. pyruvate, p = n.s., n = 5), expressed as pCa values this was 5.60±0.08 vs. 5.66±0.06, p = n.s., n = 5).

4.4 Discussion

Despite the fact that the inotropic actions of pyruvate are well documented, the processes underlying the inotropic effect are incompletely understood. Our findings strongly
suggest that a change in apparent myofilament calcium sensitivity is a prominent physiological mechanism that underlies pyruvate’s enhancement of contractile force at the myocardial level.

It has been shown previously that pyruvate alters the calcium transient, and this finding has been used to explain pyruvate’s inotropic mechanism (Hasenfuss et al. 2002a). However, in most cases in which the inotropic mechanism involves an increase in the calcium transient amplitude, there is a concomitant acceleration of relaxation. Beta-adrenergic stimulation of the myocardium increases the force and developed calcium transient, but also accelerates the decline of the calcium transient and force development (Li et al. 2000, Roof et al. 2011). Likewise, an increase in stimulation frequency increases the developed calcium transient and accelerates both calcium and force decline (DeSantiago et al. 2002, Varian and Janssen 2007a). In accordance with previous studies, we too observed that upon addition of pyruvate the calcium transient increases, but the decline of force does not increase, it actually is significantly slowed (Hermann et al. 2002, Martin et al. 1998, Tejero-Taldo et al. 1999). We demonstrated here that the increase SR calcium handling is not the primary underlying molecular mechanism for pyruvate’s inotropic effect. Moreover, even when the SR was entirely blocked from functioning, pyruvate continued to exert its inotropic effects that were quantitatively similar to those observed in non-blocked SR experiments. This leads us to conclude that there must be mechanisms downstream to SR calcium handling that are primarily responsible for pyruvate’s inotropic effect.

We have previously demonstrated that SR inhibition has a variable effect on the calcium transient that is both species- and frequency-dependent and that the balance
between force and calcium is significantly different in intact trabeculae in larger animals (compared to rats) when studied under near physiological conditions (Monasky and Janssen 2009). In those experiments, inhibition of SR resulted in increased diastolic and peak calcium level as well as decreased developed force in both rats and rabbits. It was suggested that the myofilaments may have become further desensitized to calcium as a result of the increased in diastolic calcium concentration verified when the SR is inhibited. Species differences were further exacerbated at the higher frequencies (3–4 Hz in rabbit) where calcium transient amplitude decreased in rat but increased in rabbit (Monasky and Janssen 2009), and can be explained by a loss of negative feed-back regulation of SR calcium release on the L-type calcium channel, resulting in increased L-type calcium entry.

Our focus thus turned next to evaluate the possibility that a change in myofilament calcium sensitivity might be at the basis of pyruvate’s inotropic effect. Using a recently developed potassium-based force-calcium assessment protocol (Varian et al. 2006a), we observed a sensitization of the force-calcium relationship after 10 minutes of pyruvate exposure. This sensitization (left-ward shift) was very substantial, covering 0.2 pCa units, which is quantitatively similar to the magnitude of myofilament de-sensitization (rightward shift) typically observed with β-stimulation (Hofmann and Lange 1994, Strang et al. 1994). In skinned fibers, we did not observe a significant sensitization, nor a change in apparent cooperativity of the myofilaments, leading us to conclude that pyruvate exerts its effects indirectly on myofilament calcium sensitivity. The differences we observed in maximal force between the skinned and intact fibers are due, at least in part, to different length (optimal versus 2.2 µm) and temperature (37 vs.
15 Celsius) that are known to impact force development (de Tombe 2003, Janssen 2010a, b, Janssen et al. 2002).

Unlike the impact of β-stimulation, which occurs in mere seconds, the inotropic impact of pyruvate takes many minutes to develop. In fact, before the force of contraction increases, it transiently decreases. Pyruvate gains entry to the cardiomyocyte via the monocarboxylate-proton symporter that co-transport pyruvate with a proton (Poole and Halestrap 1993). The initial dip in force characteristically present around 2 to 3 minutes after pyruvate perfusion is thus thought to be caused by a transient acidification of the intracellular pH (Hasenfuss et al. 2002a). This was followed by a gradual rise and the re-establishment of a new baseline pH. The latter finding, a reestablishment of pH, is disputed by Zima (Zima et al. 2003) as well as by Blatter and McGuigan and de Hemptinne et al. (de Hemptinne et al. 1983, Laughlin et al. 1993) where sustained intracellular acidification was observed. Still, even in these previous reports, the reported changes in intracellular pH were deemed either insufficient to explain the magnitude and the differential effects of pyruvate on the contractile apparatus or the latter was not the subject of the investigation. Although not the focus of the current study, we conducted preliminary experiments to verify the relationship between the characteristic initial dip in force caused by pyruvate infusion and changes in intracellular pH. Similar to the previous study by Hasenfuss et al (Hasenfuss et al. 2002a) and utilizing iontophoretically loaded BCECF, we verified the initial drop in twitch force to be coincident with a drop in intracellular pH as represented by the increase in \( F_{495/490} \) ratio. This was followed by a return of the ratio to (close to) baseline levels with no further alterations. From this we conclude the dip in pH is likely responsible for the dip in force development, but that the
sustained inotropic effect of pyruvate is not mediated through altered proton concentration.

In this study we found that pyruvate exposure does augment calcium transients and that this augmentation likely plays some role in the genesis of the inotropic effect when the SR is intact. The increase in Ca\(^{2+}\) transients has been associated with increased SR calcium load and release. This finding is consistent with that of previous investigators (Hasenfuss et al. 2002a, Martin et al. 1998) and was observed in the experiments where the SR was not blocked. This could be explained by the sensitized myofilaments holding on longer to calcium ions, slowing their release, and possibly favoring re-uptake by the SR versus extrusion via NCX. In subsequent beats, the increased SR load would lead to an increase in the calcium transient amplitude. Thus, the increase in the calcium transient may be a secondary effect that is initiated by myofilament sensitization. Our results indicate that even in the absence of SR function, pyruvate will impose the majority of its inotropic effect and this effect is mediated through an increase in myofilament sensitivity. The increase in myofilament sensitivity would also explain the prolonged relaxation times in the presence of pyruvate.

Evidence from the skinned fiber experiments point away from any significant direct effect of pyruvate on the myofilaments themselves. Our data however does not allow us to rule out additional indirect non-myofilament effects to explain in part pyruvate’s inotropic properties, such as its positive effects on phosphorylation potential and its known anti-oxidant effects.

At this point we can only speculate as to the exact molecular mechanism driving this modulation of myofilament sensitivity. Processes that are not immediate in onset
(such as metabolic processes) are the most likely candidates. This would further explain the relatively slow development of the inotropic effect (minutes) seen with pyruvate, rather than much quicker effects such as observed after β-adrenergic stimulation, increased extracellular calcium, or changes in pre-load or frequency.

A decrease in inorganic phosphate has been shown to clearly occur with infusion of pyruvate (DeBoer et al. 1993, Laughlin et al. 1993, Mallet et al. 2005, Zweier and Jacobus 1987) and this decrease leads to an increase in calcium sensitivity of the myofilaments (Ebus et al. 1994, Kentish 1986). In addition, the accompanying change in cytosolic phosphorylation potential with the resulting increase in free energy of ATP hydrolysis could also be contributing factors.

In summary, our results support the hypothesis that the main inotropic activity of pyruvate involves an indirect shift in myofilament calcium sensitivity. This can have important consequences for future development of therapeutic strategies for acute and chronic heart failure by circumventing the decreased effectiveness of current treatments that are believed to result in part from altered SR function in these pathophysiological entities.
Figure (4.1). Single experiment recordings relative to baseline values: From top to bottom: peak bis fura 2 fluorescence ratios (systolic calcium) showing a modest increase over time. The middle tracing displays the typical bimodal distribution of force development after infusion of pyruvate (infused at T=120sec). The bottom tracing is from a separate experiment where changes in pH were monitored with BCECF (an increase in the BCECF ratio corresponds to a decrease in [pH]), showing the drop in [pH], concurrent with the drop in force.
Figure (4.2) Snapshot at $t = 20$ minutes of developed force and intracellular calcium tracings in rabbit trabeculae at 2 Hz during control versus pyruvate infusion. Data collected at $37^\circ$C. Pyruvate at 10 mM.
Figure (4.3) Panel A illustrates the increase in developed force (Fdev) with 10mM of pyruvate. Fdev increases are similar in magnitude under both normal and blocked SR function. Panel B, the same experimental group before and after SR block demonstrating the lack of significant change in systolic calcium levels during pyruvate infusion. (Pyruvate at 10 mM, All data at 37°C).
Figure (4.4) Potassium contracture experiment: A: Myofilament force/calcium curve. The myofilament sensitivity curve shifts noticeably to the left denoting the increase in the calcium sensitivity at 20 minutes of pyruvate infusion (peak force development) Data points collected at baseline and at 20 minutes after the addition of pyruvate 10mM at 37°C. B: EC 50 at baseline and at 20 minutes of pyruvate infusion (peak force development) demonstrates the sensitizing effect of pyruvate on the myofilaments (p<0.01, n=6).
Figure (4.5) EC50 did not change significantly (2.60±0.81 vs. 2.26±0.57 µM, control vs. pyruvate, p = n.s., n = 5), expressed as pCa values this was 5.60±0.08 vs. 5.66±0.06, p = n.s., n = 5).
Table 1

Calcium and Force Parameters

<table>
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<tr>
<th></th>
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<th>$RT_{50}$ (ms)</th>
<th>$RT_{90}$ (ms)</th>
<th>TTP (calcium)</th>
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<td></td>
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<tr>
<td>Peak</td>
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<td>107 ± 7$^*$</td>
<td>187 ± 13</td>
<td>148 ± 29</td>
</tr>
</tbody>
</table>

$TTP$ (tension) time to peak tension, $RT_{50}$ time from peak tension to 50% relaxation, $RT_{90}$ time from peak tension to 90% relaxation, $TTP$ (calcium) time to peak calcium. $n = 9$, $^* p \leq 0.05$ (in comparison to the corresponding baseline). $^{**} p \leq 0.05$ (control baseline versus SR block baseline). All values are average ± SEM.
Chapter 5: THE INOTROPIC EFFECT OF PYRUVATE IN A RABBIT RIGHT VENTRICULAR HYPERTROPHY MODEL

5.1 Introduction

Congestive heart failure (CHF) is a debilitating and ultimately fatal disease with limited therapeutic interventions (Gomberg-Maitland et al. 2001). The few current treatments that ameliorate the inotropic state of the heart (catecholamines, inhibitors of phosphodiesterase) become increasingly less effective as the disease progresses; not only due to their inherent side effects (tachycardia, increase in oxygen consumption, increased energy utilization) but also because of a progressive blunting of the response to the drugs themselves. This reduced inotropic response of the failing myocardium has been associated with, among other mechanisms: a decrease in adrenergic receptors, altered transduction pathways and decreased responsiveness of the sarcoplasmic reticulum (SR) (Maier et al. 2002). They are not suitable for long term treatment of CHF and in some cases (phosphodiesterase inhibitors) even have been removed from the market due to deleterious effects on survival and increased mortality. We have demonstrated in the previous chapter that pyruvate, rather than promoting increased activator calcium, exerts its inotropic effect primarily by sensitizing the myofilaments to the existing intracellular calcium.

In the presence of hypertrophy the effects of pyruvate may be different than those observed in normal hearts. We therefore repeated the studies described in the previous
chapter with trabeculae extracted under the same techniques from a cardiac hypertrophy model. The pulmonary artery banding model was chosen because of its ability to promptly induce hypertrophy that is easily quantifiable.

Our studies in the pulmonary artery banded model confirm that pyruvate can still cause an inotropic effect that it is quantitatively similar to that verified in normal hearts and that myofilament sensitization remains the most likely explanation for the effects observed.

5.2 Material and Methods

This study was performed in accordance with the Institutional Animal Care and Use Committee of The Ohio State University and with guidelines published in the Guide for the Care and Use of Laboratory Animals.

5.2.1 Pulmonary Artery Banding

Prior to surgery, Male New Zealand White rabbits (8-12 weeks old) were treated Chloramphenicol (30mg/Kg) and acepromazine (1.25 mg/kg, subcutaneously). They were then anesthetized with isoflurane (5% in anesthetic chamber). Throughout the procedure the animals were administered 100% oxygen at 400-600 ml/min and isofluorane at a rate of 0.5-1% via an anesthesia mask for laboratory animals. The animals were then placed in dorsal recumbent position and surgical plane of anesthesia was confirmed by the absence of pedal reflexes and animals were strictly monitored throughout surgery with pulse oximetry and standard limb electrocardiography. A
midline sternotomy was made with a #10 blade and the pericardium was carefully incised to expose the pulmonary artery. Using a polyethylene tube with an outer diameter of 3.2 mm as a gauge, the pulmonary was ligated with 4-0 monofilament polypropylene suture. Muscle layers and skin were sutured close while the pericardial sac was left open. In the immediate post-operative period the animals were given buprenorphine intramuscularly (0.01 mg/kg). All rabbits were administered 30 mg/kg of chloramphenicol subcutaneously for the following 3 days. Sham animals were subjected to identical surgical procedure with the exception of the pulmonary artery ligature.

5.2.2 Measurement of twitch contractions, calcium transients, and force-pCa

At 10 weeks post banding rabbits were anesthetized with pentobarbital 50 mg/kg and administered heparin 5,000 units/kg. Hearts were excised and immediately bathed with a modified Krebs Henseleit solution containing (in mM) 137 NaCl, 5 KCl, 1.2 MgSO₄, 1.2 NaH₂PO₄, 20 NaHCO₃, 10 glucose, and 0.25 CaCl₂. and 2 g/L 2,3 butandione monoxime (BDM) to prevent cutting injury (Mulieri et al. 1989). Ultrathin uniform trabeculae were carefully dissected from the free right ventricular wall, and mounted in the set-up as previously described (Janssen et al. 2002, Varian and Janssen 2007b, Varian et al. 2006b). Muscles were perfused with an oxygenated Krebs-Henseleit solution (without BDM) containing 1 mM Ca²⁺ and stimulated at 2 Hz until steady state force was achieved. The muscles were stretched until an increase in passive (diastolic) force was no longer followed by a substantial increase in developed force. Previous studies have shown this corresponds to a sarcomere length of about 2.2 µm, similar to the
end-diastolic sarcomere length in the in vivo beating heart (Rodriguez et al. 1992). Auto-
fluorescence background was measured at 340 and 380 nm. Trabeculae (n = 6 banded, n
= 6 sham) were iontophoretically loaded with the fluorescent calcium indicator bis-fura-2
as previously described (Backx and Ter Keurs 1993, Hiranandani et al. 2006, Layland
and Kentish 1999, Monasky et al. 2008). Briefly, the muscle was allowed to stabilize at
optimal length and body temperature, and stimulation was stopped. Temperature was
then switched to 22.5 °C (minimizing dye leakage) and a glass micropipette was placed
into a single myocyte. A 2-3 nA current was applied over a duration of 15-30 minutes to
help drive the indicator into the cytosol. After the indicator was loaded to a level of 4-8
times background, temperature was rapidly switched back to 37 °C, and stimulation was
resumed, at 1 Hz for 20 minutes to allow time for the bis-fura-2 to spread to neighboring
myocytes via gap junctions. Dissociation constant of this dye under these conditions is
sufficiently fast to measure the calcium transient within 1% accuracy (Monasky et al.
2008). Baseline twitch contractions and fluorescence emissions at excitation
wavelengths of 340 nm and 380 nm were then recorded.

The bis-fura-2 emission signals were recorded, calibrated and converted to
intracellular calcium by determining the R_{min} and R_{max} in each muscle. R_{min} and R_{max}
were obtained by utilizing a calcium chelating agent (EGTA) and high calcium
respectively, and together with the published K_d value for bis-fura-2 (370 nM) used to
construct a curve of Ca^{2+} concentration vs. the ratio of 340/380. By using the equation
[Ca^{2+}]_i=K'[R-R_{min}]/[R_{max}-R] where K'=380(no Ca^{2+})/380(max Ca^{2+})*K_d, we calculated
the amount of corresponding [Ca^{2+}]_i given our fluorescent ratios (Backx and Ter Keurs
1993).
In a separate experiment the force-calcium relationship was determined using potassium induced contractures as described previously (Varian and Janssen 2007b, Varian et al. 2006b). Briefly, the superfusion solution was switched from normal to a modified Krebs Henseleit solution with 110 mM potassium and 40 mM sodium. During the resulting contracture, calcium enters the myocytes 1000 times slower than during a twitch allowing for a pseudo steady state measurement of MCS. After acquiring baseline values of both force and calcium transients as described above, myofilament calcium sensitivity was measured at baseline and during the maximal pyruvate response.

Force- calcium data were plotted and an iterative fitting procedure based on the Hill-equation was used. For each curve pCa\textsubscript{50}, and the Hill-coefficient (n\textsubscript{Hill}) was calculated.

5.2.3  *Pharmacological inhibition of sarcoplasmic reticulum function*

After loading of the fluorescent dye was accomplished and baseline twitch force and calcium transients were obtained, the inotropic response to pyruvate was assessed. Pyruvate (10 mM) was initially applied for 20 minutes and thereafter completely washed out. At this point 10 µM of cyclopiazonic acid (CPA) and 1 µM of ryanodine were added in order to block the SR (Hasenfuss et al. 2002b). In a previous set of experiments rapid cooling contractures were performed to confirm that SR calcium handling was indeed blocked (Hasenfuss et al. 2002a, Monasky and Janssen 2009). After 30 minutes, and once the preparation was stabilized, an assessment of both force and calcium at the new baseline was performed. Pyruvate (10 mM) was again applied, and the response recorded in similar fashion under complete SR block conditions.
5.2.4 *Assessment of myofilament calcium sensitivity*

Myofilament calcium sensitivity curves at 37 °C temperature were obtained by inducing a potassium contracture, as described previously (Varian and Janssen 2007a, Varian et al. 2006a). Briefly, by switching the superfusion solution to a high K⁺ modified K-H solution (K⁺ (142 mM), 0 Na⁺, and 3 mM Ca²⁺ (remaining K-H buffer components unchanged), intracellular calcium and force rise very slowly and are in near equilibrium developing a near steady-state contracture. After washing out this high-K⁺ solution, the contraction relaxes, and the muscle returns to a normal twitching pattern with unaltered force or kinetics. Contractures and measurements were initially obtained under control conditions. Subsequently, pyruvate at 10 mM was added to the superfusate for the next 20 minutes and the K⁺ experiments were repeated followed by washout.

5.2.5 *Data acquisition and analysis*

Custom written programs in LabView (National Instruments, Texas) were utilized to collect data. Kaleidagraph statistical package (Synergy Software, Reading, PA) was utilized to run statistical analysis on the data sets utilizing two-tailed paired T-tests where appropriate. A two-tailed p-value of <0.05 was considered to be significant. All data are depicted as mean ± SEM.
5.3 Results

5.3.1 Inotropic Effect

Pyruvate consistently augments the developed force of the myocardium both in the presence or absence of SR block for Sham (18.6 ± 6.3 to 30.3 ± 6.4, and 12.4 ± 3.7 to 20.2 ± 4.6, n=6, p< 0.05) and PAB subjects (16.3 ± 4.6 to 27.9 ± 7.7 and 11.9 ± 2.4 to 19.4 ± 5.5, n=6 p< 0.05) (Figure 5.1). Analogous to our previous investigations (Torres et al. 2008a), during the control phase of the experiment in both in the sham and PAB groups, the increase in force was associated with an increase in \([\text{Ca}^{++}]_i\) during baseline. During SR block the inotropic response was maintained and just as in our previous work was not accompanied by a significant increase in \([\text{Ca}^{++}]_i\) (see figure 5.1).

5.3.2 Pyruvate infusion was associated with an increase in MCS.

A shift in myofilament calcium sensitivity occurred after exposure to 10 mM pyruvate superfusion as illustrated by the significant change in EC\(_{50}\) in both sham and PAB groups (figure 5.2) (551.2±SEM 69.2 to 366.6±SEM 68.2) and (454.6±SEM 57.6 to 331.4 ±SEM 64.1, n=6, p= 0.01). An increase in the index of cooperativity n\(_{\text{Hill}}\) was seen in the sham rabbits (5.4 to 7.7) and a decrease in n\(_{\text{Hill}}\) from 5.1 to 3.9 was observed in the PAB group. In both groups we verified large changes in force development when compared to their respective baseline demonstrating the powerful inotropic effect of pyruvate.

A representative force calcium curve from the PAB group further demonstrates the shift to the left when plotted against its respective baseline (figure 5.3).
Table 2 displays twitch parameters and cytosolic calcium concentrations for both the control and the blocked SR segment of the experiments in the Sham as well as in the hypertrophy group.

5.4 Discussion

Analogous to our previous work detailed in chapter 4, the behavior of the trabeculae from both sham and PAB animals reaffirm the inotropic effects of pyruvate. The fact that the magnitude of force increase is comparable in both sham and PAB and that there was no depression of the effect in the hypertrophy models suggests that the underlying mechanism(s) that mediate the inotropic effect of pyruvate remain intact and are not measurably down regulated during hypertrophy and early failure.

The delayed relaxation seen after exposure to pyruvate in these isometric cardiac muscle preparations is also characteristic of a sensitizing effect on the myofilaments (Hajjar et al. 1997). It is also in sharp contrast to the speeding up of relaxation typically seen during exposure to beta adrenergic stimulation. This normally leads to increased calcium (promoted via the cAMP-protein kinase A (PKA) pathway) and lends further credence to our hypothesis that this inotropic effect is mediated primarily via changes in myofilament calcium sensitivity.

Additionally, in both Sham and PAB groups we do not see a full inotropic effect until a characteristic delay of about 20 minutes occurs while exposed to pyruvate. This also is consistent with our earlier speculation that metabolic processes such as a decrease in inorganic phosphate and/or changes in the cytosolic phosphorylation potential are
likely candidates for the increase in force and myofilament sensitization seen with pyruvate.

5.5 Conclusion

In both normal and hypertrophied myocardium pyruvate leads to a substantial increase in force that does not appear to be mediated by an increase in $[\text{Ca}^{++}]$ or dependent of the SR but rather relies primarily on an increase of myofilament calcium sensitivity to mediate its effects.
5.6 Figures and Tables

Figure (5.1) The significant increase in developed force (Fdev) with superfusion of 10mM of pyruvate can be seen in both Panel A and Panel B. Fdev increases are similar in magnitude under unblocked and blocked SR function in both sham and in the pulmonary artery banded (PAB) animals. In both groups the larger increases in Calcium occur during the control (non SR Block portion) of the experiment. After SR block no noticeable change in $[\text{Ca}^{++}]_i$ occurs during pyruvate infusion despite the significant increase in force development (Pyruvate at 10 mM, All data at 37°C).
Figure (5.2). EC 50 at baseline and at 20 minutes of pyruvate infusion (peak force development) demonstrates the sensitizing effect of pyruvate on the myofilaments (p<0.01, n=6) in both Sham (Panel A) and PAB (Panel B) groups.
Figure (5.3) Representative Potassium contracture experiments from pulmonary artery banded subjects: A shift to the left of the myofilament sensitivity curve illustrates the increase in the calcium sensitivity at 20 minutes of pyruvate infusion (peak force development) Data points collected at baseline and at 20 minutes after the addition of pyruvate 10 mM at 37°C.
<table>
<thead>
<tr>
<th></th>
<th>TTP (tension)</th>
<th>RT_{50} (ms)</th>
<th>RT_{90} (ms)</th>
<th>TTP (calcium)</th>
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<td>208.2 ± 14.8*</td>
<td>182.2 ± 20*</td>
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</table>

F_{dev}, developed force, TTP (tension), time to peak tension, RT_{50}, time from peak tension to 50% relaxation, RT_{90}, time from peak tension to 90% relaxation, TTP (calcium), time to peak calcium.

n=6 in each group

* p<0.05 when compared to corresponding baseline value
6.1 Main Findings

This section provides a synthesis of the main findings from chapters 2 through 5

1. In the rat cardiac trabeculae, heart rate variability can modulate the contractile state of the heart.

2. In larger mammals such as rabbits and dogs, while the influence of heart rate variability on contractility seems to be less important, the amount of preload heavily influences the contributions of the pre beat interval on the subsequent beat.

3. Pyruvate is an endogenous metabolic fuel that is also a very potent inotropic agent with many unique properties and potential therapeutic applications.

4. The inotropic effect of pyruvate is not mediated through intracellular pH changes and was demonstrated even in the absence of SR function which indicates that while changes in pH and increases in intracellular concentrations of calcium may play a role, there must be another mechanism for its effect.

5. The significant shift to the left of the myofilament force-calcium curve during the pyruvate superfusion in the K+ contracture experiments provides strong evidence for a myofilament sensitization.

6. There was no shift in the Force-pCa curves in skinned fiber experiments suggesting that the there is no direct effect of pyruvate on the myofilaments.
7. In a model of ventricular hypertrophy the magnitude of the inotropic effect as well as the myofilament sensitization seen with pyruvate exposure appear to be preserved.

6.2 The inter beat duration

Heart Rate Variability has been found to be an indicator of health and a prognosticator of disease. Clinical conditions such as congestive heart failure (Bernardi et al. 1992, Saul et al. 1988) and ischemic heart disease (Airaksinen et al. 1987) all have been recognized to lead to a decrease in heart rate variability (HRV). HRV has been acknowledged as an indicator both of the autonomic status of the organism as well as a marker of some prognostic significance in both diseased and non-diseased states (Counihan et al. 1993, Kleiger et al. 1987, Malik and Camm 1993, Shannon et al. 1987).

In chapter 2, we verified that the inter-beat duration has a significant role in subsequent beat force development and is tightly linked to the calcium transient. This suggests that the period between beats is of major importance in determining contractile force.

Our experimental work aimed at investigating if HRV was not only an indicator but possibly a factor influencing cardiac inotropy. The physiological basis for this hypothesis lies in the non-linear relationships that are found in the calcium cycle. The rationale was that if the gain in strength caused by longer beats was more significant in aggregate than the decrease in strength caused by shorter beats then we could potentially utilize this mechanism as a pacing strategy to improve long term contractility.
In the rat, where SERCA is responsible for almost 90% of the intracellular calcium transient, we were able to demonstrate a slight inotropic effect of the variable pacing over the non-variable regular pacing. This suggests that the interplay between the elements that govern the calcium transient can be manipulated to an extent to where inherent contractility can be changed. On the other hand, in this proof of principal protocol, there was a lack of a concurrent change in length, (which would invariably happen in vivo) with the changes in frequency. The interaction between the two factors (length and frequency) is thus the subject of investigation in our subsequent study.

In those studies (chapter 3), we utilized larger animals (more specifically rabbit and dog) with the intent of utilizing models that more closely resemble human physiology.

6.3 Interspecies Differences in Calcium handling

The increased reliance of the rat heart on the SR for its activator calcium (92%SERCA/8%NCX + plasma membrane calcium pump) and the small role that the extracellular exchange plays compared to rabbit (70% SERCA/30% NCX + plasma membrane calcium pump) (Bers 2002b) is likely responsible for most of the differences verified in our heart rate variability experiments. The interaction between cardiac action potential, L-type calcium channels, SERCA, NCX and the contractile apparatus are all unique to each species and great care must be exercised when extrapolating results from these investigations to the clinical setting. The dominant myosin isoform in each species
also likely plays a role. The α myosin heavy chain predominant in rat has twice the ATPase activity and velocity versus its β correlate in rabbit and dog and generates crossbridge force with less energy consumption but at a slower rate (MacGowan and Koretsky 2000).

When we applied our initial random pacing protocol we obtained equivocal results between dog and rabbit and at different frequencies and variabilities. The results of our variable protocol also turned out to be quite different for the larger vs the previous investigation involving the smaller specie. In the canine preparation there was no change detected with the variable protocol whereas with the rabbit at the higher frequencies we found a slight negative inotropic effect over the fixed rate.

Of much more physiological significance was the finding that a much tighter correlation ensued between force and duration of the previous beat once we introduced a length variable in the study algorithm.

In general, changes in length are associated to changes in frequency in a beating heart. This tighter correlation exposes the complex interaction between the length tension and the force frequency relationship that exists in real life and is characteristic of complex systems. The heart is such a system where almost all elements vary temporally, qualitatively and quantitatively. Complex nonlinear systems tend to have unpredictable outcomes from even simple interventions (Goldberger 1996) and varying the beat frequency is one such simple intervention. As an example of this complexity, additional work not included in this dissertation but performed in our lab, also showed that a negative effect on beat duration was seen even from the previous secondary and tertiary cycle lengths (Varian et al. 2009b). With the current challenge faced by patients with
heart failure and the lack of therapeutic options to improve long term contractility we felt that the manipulation of these alternative pacing protocols as possible inotropic maneuvers was warranted.

6.4 Pyruvate and its Unique Properties

“If my heart is dying just pour me a bunch of pyruvate in my veins” (from a heart physiologist at AHA Scientific Sessions, 2008 Oral Sessions)

It is well established that increased cardiac work is accompanied by a concomitant increase in energy use in the form of ATP. Given that approximately 70% of total energy is consumed by cross bridge and calcium cycling (MacGowan and Koretsky 2000), it comes as no surprise that inotropic agents that increase force generation by increasing calcium pumping carry a cost in energy expenditure that correlates with the calcium increase.

Pyruvate is an endogenous metabolic fuel and an anti-oxidant with many complex actions in the cytosol. Among those are changes in the cytosolic ATP phosphorylation potential and Gibbs free energy of ATP hydrolysis (ΔG~ATP) (Mallet et al. 2005). Pyruvate also has influence on the intracellular pH, it can exercise an inhibitory effect on the ryanodine receptor channel activity (Zima et al. 2003), and can lead to decreased intracellular inorganic phosphate (Mallet and Sun 1999).
The magnitude of the pyruvate effect on myofilament sensitization is equivalent to that of β-adrenergic stimulation of the heart. That this can accomplished without a proportional increase in myocardial oxygen consumption (MVO2) is quite remarkable.

6.5 Myofilament Calcium Sensitivity and Myocardial Contractility

One of the reasons for the current lack of effective inotropic therapies is that the existing alternatives all increase activator calcium thus indirectly lead to an increased myocardial oxygen consumption (MVO2), arrhythmias and mal-adaptive signaling cascades (Kass and Solaro 2006). The alternative to this strategy would seem to involve approaches that enhance myofilaments response to a given level of calcium.

Changes in Ca$^{++}$ sensitivity are typically mediated either via length dependent mechanisms or by phosphorylation of myofilament regulatory proteins, however changes that affect crossbridge cycling such as an increase in the number of cross bridges or in the force per crossbridge or the fraction of time the crossbridges remain attached are factors that should be considered when analyzing the mechanisms that can alter the force calcium relationship.

Important clues can be derived by closer analysis of the change in shape of the force calcium curves. If we assume the classic three-state model of muscle activation (McKillop and Geeves 1993) described in the introductory chapters, in which the thin filament regulatory complex controls the rate of cross-bridge transition from weakly attached to strongly attached states via Ca$^{++}$ binding to troponin C (the turnover kinetics
model), we can then theorize that certain changes in the shape of the force-calcium curve will correspond to specific alterations at the level of the myofilaments and crossbridges.

An example would be the change brought forth by increasing the number of crossbridges such as seen in hypertrophy, or an increase in the force of the crossbridges (alkalosis) or increasing the fraction of time the crossbridge remained attached. These would cause an increase in maximum force such as visualized in curve (ii) in figure 6.1. On the other hand those changes that would primarily result in an increase in calcium sensitivity and a shift to the left of the curve (curve (i) in figure 6.1) would be secondary to either an increase in the affinity of troponin C for calcium or an increase in the effectiveness with which the TnC-calcium complex influences the rate of crossbridge attachment. A change in cross bridge kinetics (rate of attachment of strongly bound crossbridges and/or rate of detachment) causes a change in both maximum force and calcium sensitivity. Most pharmacological interventions bring about a mixture of alterations in maximal force, as well as changes in myofilament sensitivity (Lee and Allen 1997).

Pyruvate gains entry to the cytoplasm through a monocarboxylate symporter that has a higher affinity for pyruvate over other monocarboxylates. The expression of this monocarboxylate transporter (MCT1) has been shown to be upregulated in a rat model of congestive heart failure (Johannsson et al. 2001). Pyruvate’s uptake increases exponentially with rising extracellular concentration (Poole and Halestrap 1993). Once in the cytosol there is compartmentalization of the exogenous pyruvate vs glycolytic generated pyruvate with a slow exchange between these two pools. At supraphysiological concentrations pyruvate suppresses glucose oxidation. The exogenous pyruvate is
preferentially oxidized or transaminated to alanine, while the glycolytically derived pyruvate is primarily reduced to lactate (Damico et al. 1996). Oxidation of pyruvate is a mitochondrial process that starts with another monocarboxylate symporter exchanging pyruvate for acetoacetate and β-hydroxybutyrate. In the mitochondria pyruvate undergoes irreversible oxidation and decarboxylation to acetyl CoA in a series of reactions that are catalyzed through the subunits of the pyruvate dehydrogenase complex (PDC). The activity of PDC is highly regulated by the ratio of its products (acetyl CoA and NADH) to its substrates (coenzyme A and NAD⁺). An important kinase, pyruvate dehydrogenase kinase, inactivates PDC by phosphorylating an α subunit. This is reversed by pyruvate dehydrogenase phosphatase which increases activity through the PDC. Rising intracellular calcium concentration may also activate pyruvate dehydrogenase kinase and thus stimulate its activity. Pyruvate can also be carboxylated in the mitochondria thus replenishing the levels of Tricarboxylic Acid cycle (TCA) intermediates citrate, oxaloacetate and malate. In fact, in supraphysiologically perfused hearts pyruvate can be responsible for up to 18% of the total citrate synthase flux (Damico et al. 1996).

In our experiments, by utilizing pyruvate at 10mM we consistently saw an increase in developed force by approximately 150-200%. This effect of pyruvate followed a bimodal pattern with a slight decrease in force at around the first 3 minutes followed by a steady increase in force that plateaued around 15 to 20 minutes. Shortly after exposure to pyruvate was discontinued so were its inotropic effects. We followed the intracellular pH and observed that the dip in force coincided with the transient decrease in pH right after pyruvate superfusion was initiated. We explained this as a
consequence of the increased intracellular H\(^+\) brought about by the sarcolemmal symporter activity just before a new cytosolic equilibrium could be reached. Despite not mentioned in the previous chapters, there is a mirror effect with a peak in force once pyruvate is discontinued.

The increase in contractile force was not abrogated by blocking the SR with thapsigargin or ryanodine despite blunting of the Ca\(^{++}\) transient. By using a high potassium contracture protocol we consistently observed an increase in the calcium sensitivity that was not seen in the skinned preparations.

The increase in force was accompanied by calcium transients that were slower. The force-calcium relationships, when plotted as a force calcium curve was steeper, indicating an increase in the n\(_{\text{Hill}}\) cooperativity index in the normal and sham rabbits. The decrease in cooperativity for the PAB group was not statistically significant and although not completely clear this could have been a function of the hypertrophy itself. Pyruvate in this case would act by enabling the recruitment of the larger number of available crossbridges in the hypertrophied heart before increasing their cooperativity.

According to our previous discussion the changes in the force calcium curves obtained after administration of pyruvate lead to both a leftward shift and a rise in maximal force. These changes have been described with changes in redox state secondary to alterations in the cross-bridge force generation (Kass and Solaro 2006).

Our research does not eliminate the contributions of the SR and intracellular pH to the inotropic effect of pyruvate but rather lead us to infer that myofilament calcium sensitivity changes are likely the major contributing factor to pyruvate’s enhancement of contractile force The prolonged time course for the development of the effect and the
dependency on the integrity of the cytosolic membrane and intracellular machinery suggests that the ultimate molecular mechanisms involve a metabolic process rather than a direct effect of pyruvate on the myofilaments.

Pyruvate has been shown to increase cytosolic energy reserves and to double the ATP phosphorylation potential (Mallet and Bunger 1993) thereby increasing $\Delta G_{\text{ATP}}$. Gibbs free energy of ATP hydrolysis is the main thermodynamic driving force for actomyosin crossbridge cycling.

Pyruvate can also promote its own oxidation. Dichloroacetate is a PDC kinase inhibitor that increases contractile function, and improves oxygen utilization efficiency when administered experimentally following global ischemia (Wahr et al. 1996) but not to the same extent as when pyruvate is administered, suggesting that pyruvate oxidation is not the sole mechanism for the inotropic mechanism of pyruvate. Mallet, utilizing 2.5 mM of pyruvate and 0.6mM of $\alpha$-cyano-3-hydroxycinnamate reported that mitochondrial metabolism of pyruvate is required for its enhancement of cardiac function and energetics in isolated guinea pig hearts ((Mallet and Sun 1999), however Leite-Moreiera et al (Leite-Moreira et al. 2002) (utilizing rabbit papillary muscles and up to 15mM of pyruvate) showed that even after the transport of pyruvate into the mitochondria was selectively blocked by $\alpha$-cyano-4-hydroxycinnamate 0.5 mM the positive inotropic effect was not only preserved but actually enhanced.

Pyruvate prevents oxidative damage by directly neutralizing hydrogen peroxide in a non-enzymatic reaction:

$$\text{Pyruvate} + \text{H}_2\text{O}_2 \rightarrow \text{Acetate} + \text{CO}_2 + \text{H}_2\text{O}$$
Pyruvate increases the rate of GSH formation by indirectly increasing NADPH production (through the hexose monophosphate pathway) and thus providing the cell with the means to indirectly reverse sulfhydryl oxidation (Mallet 2000). These mechanisms can potentially play a role in improving the contractile state in the ischemic and failing myocardium.

The main argument in favor of pyruvate’s enhancement of contractile force by Pi reduction however lies in the fact that Pi has clearly been shown to cause a substantial decline in force development at the myofilament level either by lowering the amount of active force developed per cross bridge (Ebus et al. 1994) or by reducing the stiffness and number of force generating crossbridges (Caremani et al. 2008) and by indirectly inhibiting crossbridge formation by lowering $\Delta G_{\text{ATP}}$.

How does pyruvate lower cytosolic Pi?

1. Pyruvate carboxylation (via TCA anaplerosis) increases citrate. Citrate inhibits phosphofructokinase which in turn slows down glycolysis leading to an accumulation of glucose-6-phosphate and fructose-6-phosphate (Tejero-Taldo et al. 1999).

2. A threefold increase in PCr/Pi ratio in guinea pig hearts perfused by pyruvate vs glucose was demonstrated by Zweier utilizing 31P NMR (Zweier and Jacobus 1987).

Both these mechanisms in effect sequester cytosolic inorganic phosphate away from the myofilaments and bind it to different compounds. By lowering Pi pyruvate shifts the force calcium curve leftwards and decreases the energy cost to produce a contraction.
There are however, quite a few hurdles to overcome before the adoption of pyruvate as a clinically useful therapeutic drug. Pyruvate is a very small molecule with very little barriers to its accumulation in varied tissues. It is also readily metabolized and if given parenterally as an intravenous infusion it would have a large volume of distribution. In other words a large amount of pyruvate would be required to achieve an effective plasma concentration (above 1 mM). In its most common form: Sodium pyruvate, it could potentially lead to sodium overload, a particularly undesirable effect in patients already coping with the consequences of heart failure. The fact that the inotropic effect of pyruvate is present only while it is being administered also limits its use to the acute monitored setting and precludes its availability as a drug that can be prescribed for patient’s use at home. Nonetheless a drug that would help those patients in acute systolic failure, even if used solely as a bridge therapy until another treatment can be implemented would certainly be welcome as an additional drug to the scant therapeutic armamentarium available today to treat these patients.

6.6 Future Directions

6.6.1 Heart Rate Variability and Contractility

We have seen that beat-to-beat variation can have an effect on contractility. We have also established that the beat-to-beat variation in force is closely correlated with changes in calcium transient amplitude (Torres et al. 2008a). Given the heart’s propensity to adapt to changes in its homeostasis, it is reasonable to hypothesize that a more prolonged beat-to-beat variability protocol would lead to secondary changes in protein
expression. Isolated trabeculae can now be successfully cultured in our lab (Janssen et al. 1998). If we introduce a variable stimulation protocol onto the cultured trabeculae for a few days, then changes in protein expression that occur may be studied and contrasted to the associated changes in protein expression that accompany heart failure and its progressive lack of heart rate variability.

Linking beat-to-beat interval duration with underlying cellular mechanisms that modulate force and determine the contractile state of the heart, while simultaneously attempting to assess the contribution from changes in cell length, is still very challenging. The idea that stretching the myofilaments can increase calcium buffering capacity, to the extent of being a meaningful contributor in an already dynamic intracellular milieu, where calcium is in constant flux certainly merits further investigation. To that extent, more precise measurements of sarcomere length would need to be performed alongside improved techniques that still need to be developed to resolve, in real time calcium flows throughout the different cellular compartments and myofilaments.

While these pacing protocol investigations did not provide the inotropic support that initially motivated their investigation, they raised interesting questions about several fundamental properties of the heart. Unraveling the mechanisms and interactions between load and frequency dependency is still as justified today as it was over 100 years ago, when these essential properties of the heart were first described by Frank, Starling and Bowditch.
6.6.2 Pyruvate as an Inotrope

The challenge of translating basic science to bedside treatment can be epitomized by pyruvate. Pyruvate seems to hold enormous promise as an adjunct in the treatment of acute exacerbations of heart failure, a condition very familiar to those practicing emergency medicine. The improvement in myocardial energetics at a time where oxygen and ATP are at a premium cannot be under estimated. Pyruvate has already been used experimentally with success in patients with congestive heart failure during catheterization (Hermann et al. 1999, Schillinger et al. 2011) and has been shown to enhance post-surgical recovery of cardiac function (Olivencia-Yurvati et al. 2003) and suppress myocardial inflammation (Ryou et al. 2010) when added to cardioplegic solutions during cardiopulmonary bypass. Yet the author is unaware of approved clinical use in heart failure.

In our studies, we have found that changes in myofilament sensitivity are at the heart of pyruvate’s inotropic effect. However, we still have not been able to verify the precise molecular mechanism through which the change in sensitivity is accomplished. While a change in intracellular Pi seems to be the most compelling hypothesis, the burden of proof has still to be met. Perhaps initially we need to determine if significant changes in protein phosphorylation are caused by exposure to supranormal levels of pyruvate. In addition, there is conflicting evidence regarding the need for the oxidation of pyruvate in the mitochondria for the inotropic effects to occur. Further investigations could combine the use of pyruvate with and without α-cyano-4-hydroxycinnamate (which blocks the transport of pyruvate into the mitochondria) to better establish the role of cytosolic pyruvate versus those resulting from pyruvate oxidation in the mitochondria.
We could further evaluate the inorganic phosphate content of the cardiomyocyte through magnetic resonance spectroscopy ($^{31}$P-MR) before and during infusion of pyruvate. If a decrease is in fact found, similar changes in Pi could be reproduced in skinned fiber experiments and the resultant change in sensitivity compared to that observed by $^{31}$P-MR. We would then be able to quantify the effects of pyruvate in terms of a similar decrease in Pi.

From our experiments and the evidence already published, it is likely that pyruvate has more than one mechanism contributing to the increase in force. Most of these effects improve the energy and redox state of the heart. In this case, given that the failing heart is also an energy starved heart (Neubauer 2007) what can be better than delivering a metabolic fuel that also increases the myofilament efficiency of ATP utilization by increasing myofilament calcium sensitivity?

These and similar questions will continue to motivate me in generating more inquiries and pursuing more investigations that may have a positive impact on those that struggle with heart failure and cardiac disease.
Figure (6.1) The effects of various inotropic mechanisms on the steady state relation between force and Ca$^{++}$

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