EFFECTS OF ISCHEMIC METABOLITES AND CHRONIC EXERCISE ON CARDIAC MYOCYTE FUNCTION

A Dissertation Presented to
The Faculty of the Graduate School
University of Missouri-Columbia

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

by
AARON C. HINKEN

Kerry S. McDonald, Ph.D., Dissertation Supervisor
MAY, 2005
The undersigned, appointed by the Dean of the Graduate School, have examined the
dissertation entitled

EFFECT OF ISCHEMIC METABOLITES AND CHRONIC EXERCISE ON CARDIAC
MYOCYTE FUNCTION

presented by AARON C. HINKEN

a candidate for the degree of Doctor of Philosophy

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

Kelly S. McDonald, Ph.D.

Michael J. Rovetta, Ph.D.

Douglas K. Bowler, Ph.D.

Leona J. Rubin, Ph.D.

Ronald H. Freeman, Ph.D.
ACKNOWLEDGMENTS

I would like to thank my advisor, Kerry McDonald, for the support and guidance provided throughout my graduate career, making me a scientist and better scholar. Thanks also for creating an environment of honesty and friendship which extends from the workplace. I would also like to thank my doctoral committee members Michael J. Rovetto, Douglass K. Bowles, Leona J. Rubin, Ronald H. Freeman, and Michael S. Sturek for their helpful comments and guidance. I would additionally like to thank Dr. Rovetto for an open door and lengthy conversation.

Thanks to F. Steven Korte, F. Spencer Gaskin, and Todd J. Herron for friendship and camaraderie that makes the good times better and the difficult times easier. Also, thanks to the rest of the graduates students, departmental staff, and faculty for all their help and kindness over the years.

Thanks to Michelle Rapisardo, Joseph Schuster, and M. Casey Childers for keeping the McDonald laboratory atmosphere enjoyable. Also, your assistance was essential for my productivity.

Finally, I would like to thank my parents whose eternal encouragement, support and faith has been fundamental to all of my successes.
EFFECTS OF ISCHEMIC METABOLITES AND CHRONIC EXERCISE ON CARDIAC MYOCYTE FUNCTION

ABSTRACT

The pumping action of the heart provides water, O$_2$ and nutrients to all tissues and helps remove metabolic breakdown products and other wastes. During times of low blood flow (ischemia), concentrations of wastes increase in tissues, which can lead to decreased striated muscle contractile function. Acute contractile dysfunction during ischemia is likely mediated by build-up of inorganic phosphate (P$_i$) and protons (H$^+$) (i.e., decreased pH). The focus of this dissertation is examination of the myofibrillar mechanisms by which ischemic metabolites alter the work capacity of cardiac myocytes, which ultimately comprise ventricular pump function. In addition, contractile properties and changes thereto with metabolite concentration were investigated in myocytes expressing either of the two isoforms of myosin heavy chain (α-MyHC and β-MyHC), that show altered expression in response to chronic ischemia. Studies reported here demonstrate differential response to metabolites with P$_i$ and H$^+$ alone and together decreasing power generating capacity of α-MyHC while only in combination did they diminished β-MyHC myocyte power. The greater tolerance toward ischemic conditions in β-MyHC myocytes was attributed to a P$_i$ and H$^+$ induced increase in the velocity of loaded shortening.

P$_i$-induced changes in contractile properties of α-MyHC myocytes were more thoroughly assessed to probe the cross-bridge states that tend to limit power output at intermediate loads where muscles perform work. Power near isometric loads is most likely determined by the balance between attachment and detachment of force-generating cross-
bridges that limit isometric force, while power near maximum velocity is most likely limited by detachment of compressively strained cross-bridges that oppose shortening. Addition of P_i had no effect on maximal shortening velocity but progressively increased velocity at all loads greater than ~10% isometric. This increase in loaded shortening velocity resulted in increased normalized power at the corresponding loads (i.e., 10-100% isometric). These results indicate cross-bridge steps associated with force-generation are most important in determining power output at intermediate loads where muscles operate in vivo.

In contrast to ischemia, changes following exercise training are thought to improve cardiac function. A pig model of exercise training was examined to determine if changes intrinsic to the myofilaments were partially responsible for changes in global cardiac function. Increased peak power generating capacity was observed in myocytes from exercise trained animals as compared to sedentary controls. The increased power output of myocytes from exercise trained pigs coincided with an increase in PKA-induced phosphorylation of myofibrillar proteins, which may partially explain increased power. Overall, these results provide evidence for myofibrillar mechanisms that, in part, underlie changes in myocardial performance associated with acute and chronic ventricular stress.
LIST OF TABLES

Table 2.1 Summary of skinned cardiac myocyte dimensions ………………………29

Table 2.2 Effects of P_i addition on mechanical properties of skinned cardiac ………35
myocyte preparations

Table 3.1 Effects of P_i addition and lowered pH on force-velocity, …….……….53
power-load and k_tr properties of α-MyHC cardiac myocytes.

Table 3.2 Effects of P_i addition and lowered pH on force-velocity, …….……….54
power-load and k_tr properties of β-MyHC cardiac myocytes.

Table 4.1 Animal morphologies with indices of training and myocyte……………..77
measurements.

Table 4.2 Myocyte mechanical properties from sedentary and exercise…………..79
trained miniature Yucatan swine.

Table 5.1 Summary of calculated Huxley constants from myocytes with……………..96
addition of P_i

Table 5.2 Summary of calculated Huxley constants with ischemic ……………….97
metabolites in α- and β-MyHC myocytes.

Table 5.3 Summary of calculated Huxley constants from sedentary and ……………..99
exercise trained myocytes.
LIST OF FIGURES

Figure 1.1  Cardiac structure and ventricular pressure-volume relationship ……………..2
Figure 1.2  Structure of the sarcomere…………………………………………………….5
Figure 1.3  Components of the thick and thin filaments……………………………..7
Figure 1.4  Scheme of actomyosin cross-bridge cycle ……………………………..12
Figure 2.1  Effect of 5 mM added P_i on unloaded shortening velocity ……………………34
in a skinned myocyte preparation.
Figure 2.2  Effect of added P_i on myocyte force-velocity and…………………………36
power-load relationships
Figure 2.3  Effect of added P_i on rate of force redevelopment in skinned………………38
cardiac myocytes.
Figure 2.4  Schematic addressing which steps in the cross-bridge cycle limit………..41
power output.
Figure 3.1  Effect of experimental conditions on absolute force-velocity ………………55
and power-load relationships in an α-MyHC myocyte.
Figure 3.2  Normalized force-velocity and power-load relationships …………………..58
from α-MyHC myocytes.
Figure 3.3  Normalized force-velocity and power-load relationships from ………..60
β-MyHC myocytes.
Figure 3.4  Comparative plots of force and power production between ………………62
α- and β-MyHC myocytes.
Figure 4.1  Representative absolute force-velocity and power-load…………………..80
in myocyte preparations from sedentary and exercise trained pigs.
Figure 4.2  Autoradiogram of PKA treated skinned myocytes from sedentary ………..84
and exercise trained pigs with back phosphorylation quantification.
Figure 5.1  Interaction of constants from Huxley equation on force-………..95
velocity and power-load relationships.
Figure 5.2  Effect of a P_i mopping system on force-velocity and power-load ……….106
relationships in permeabilized myocytes.
Figure 5.3  Relationship between myocyte size and $P_i$ effects; determination.108 of diffusion limitations.

Figure 5.4  Examination of potential synergy of $P_i$ and $H^+$ in combination on.110 skinned cardiac myocyte force production.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS .................................................................................. ii

ABSTRACT ........................................................................................................... iii

LIST OF TABLES ................................................................................................. v

LIST OF FIGURES ............................................................................................... vi

TABLE OF CONTENTS ..................................................................................... viii

CHAPTER 1: Introduction .................................................................................. 1
  1.1 Cardiac structure and function ................................................................. 1
  1.2 Myocardial structure and function ............................................................ 4
  1.3 Actomyosin interaction: cross-bridge cycling ............................................. 7
  1.4 Cardiac function during ischemia ............................................................... 16
  1.5 Cardiac function with chronic exercise ..................................................... 18

CHAPTER 2: Inorganic phosphate speeds loaded shortening in rat skinned cardiac myocytes .................................................................................................................. 23
  Introduction ....................................................................................................... 24
  Methods ............................................................................................................. 26
  Results .............................................................................................................. 32
  Discussion ......................................................................................................... 39

CHAPTER 3: β-myosin heavy chain myocytes are more resistant than α-MyHC myocytes to changes in power output induced by ischemic conditions ............. 45
  Introduction ....................................................................................................... 46
  Methods ............................................................................................................. 48
  Results .............................................................................................................. 51
  Discussion ......................................................................................................... 59

CHAPTER 4: Porcine cardiac myocyte power output is increased following chronic exercise training ............................................................................................................. 69
  Introduction ....................................................................................................... 70
  Methods ............................................................................................................. 71
  Results .............................................................................................................. 76
  Discussion ......................................................................................................... 83

CHAPTER 5: Discussion ..................................................................................... 89

LITERATURE CITED .......................................................................................... 111

VITA .................................................................................................................... 122
Chapter 1. INTRODUCTION

1.1 Cardiac Structure and Function

The heart is an organ whose primary function is to move blood; it does so by forming a hollow opening surrounded by striated muscle. Gross morphology of the mammalian heart (Figure 1.1) displays two atria and two ventricles primarily composed of myocardial tissue and vascularized by the coronary vessels. The chambers pump blood by utilizing a sequence of relaxation and contraction known as the cardiac cycle. The cardiac cycle consists of four phases: ventricular filling, isovolumic contraction, ejection and isovolumic relaxation. The cardiac cycle is well illustrated by a left ventricular volume-pressure relationship shown in Figure 1.1. Left ventricular filling occurs when the pressure in the left atrium exceeds the pressure in the ventricle, which opens the atrioventricular (A-V) valve allowing blood flow from the atria into the ventricle. During this phase the volume of blood in the ventricle rises with little change in ventricular pressure (Figure 1.1). Upon electrical stimulation the ventricular myocardium contracts causing ventricular pressure to increase above that in the atria, thereby closing the A-V valve. During this phase (i.e., isovolumic contraction) left ventricular pressure rises rapidly with no change in volume as both the A-V and semilunar valves are closed. When ventricular pressure rises above aortic impedance the aortic valve opens and blood is expelled from the left ventricle into the aorta. During this ejection phase the ventricle shortens against the load of blood in the aorta, which is determined in large part by the arterial resistance. The ventricle then begin to relax and pressure declines. When
Figure 1.1

1. Filling
2. Isovolumic Contraction
3. Ejection
4. Isovolumic Relaxation

Left ventricle
Left atria
Right ventricle
Right atria
Semilunar valves
Atrioventricular valves
aorta

Pressure
Volume
Stroke Volume

1. Filling
2. Isovolumic Contraction
3. Ejection
4. Isovolumic Relaxation
pressure falls below that in the aorta the semilunar valve closes and left ventricular pressure falls with no change in volume as both the semilunar and A-V valves are closed. The differences in ventricular volume between the end of diastole and the end of systole is termed stroke volume (SV). The total amount of blood pumped by the heart over a period of time is known as cardiac output (CO), which is the product of SV and heart rate (HR). Because cardiac output appears to be exquisitely matched to the systemic demand for blood, concordant changes in CO and systemic demand are accomplished by either altering HR or SV. Both HR and SV are tightly regulated by several mechanisms. HR is primarily controlled by the autonomic nervous system (ANS) via dual innervation. Parasympathetic input from the vagus nerve slows heart rate via the terminal neurotransmitter acetylcholine (ACh), while sympathetic input increases heart rate with the use of norepinephrine (NE). In addition, HR also may be increased by epinephrine (EPI) which is released by the adrenal medulla and transported to the heart via the blood. Alternatively, as all myocytes are activated during a heartbeat, SV is regulated by factors intrinsic to individual myocytes. For example, changes in myocyte length, intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_i\)), phosphorylation status of myofibrillar proteins, and ionic milieu all appear to modulate the force-generating capacity of individual myocytes and thus the workload and SV of the ventricle. Regulation of contractile function at the level of the cardiac myocyte is the primary focus of this dissertation. The studies of this dissertation examine how acute changes in the ionic environment associated with ischemia as well as chronic stress levels induced by exercise training modulate myocyte function, which ultimately modulate SV. The overall hypothesis put forth is that force-generating transitions are most important in determining power output generated by cardiac myofibrils.
1.2 Myocardial Structure and Function

The myocardium is made up of single cardiac myocytes interconnected mechanically by intercalated discs and electrically by gap junctions (Figure 1.2). Cardiac myocytes like all striated muscle cells consist of myofibrils, mitochondria, sarcoplasmic reticulum, nuclei and other cytosolic constituents (Figure 1.2) with myofibrils and mitochondria comprising greater than 80% of all cellular components (15). Myofibrils are arranged in a longitudinal fashion with alternating light and dark regions creating a striated appearance. These light and dark bands are the result of highly structured arrays of thick and thin filaments (63) (Figure 1.2). The light regions, or I bands, contain thin filament proteins from neighboring sarcomeres connected at the Z-line. The dark regions, or A bands, contain thick filaments that are overlapped with thin filaments except in the H zone, which consists of thick filaments only. The amount of overlap between thick and thin filaments is determined primarily by the length of the sarcomere, which is defined as the region between two Z-lines. Sarcomere length (SL), and its consequent changes in filament overlap, is one important modulator of myocyte function. Thus, extreme care must be taken in controlling this variable (i.e., SL) during experiments examining myocyte function. The studies in this dissertation used a permeabilized or skinned myocyte preparation in which SL was closely monitored during all mechanical measurements.
Figure 1.2

![Diagram showing muscle fiber structure with labels for Z-line, Thin filaments, Thick filaments, I band, A band, H Zone, and Sarcomere.]

- Z-line
- Thin filaments
- Thick filaments
- I band
- A band
- H Zone
- Sarcomere
Regarding the filaments, the thin filament consists of two actin filaments in a double helical array, which are connected at the Z-lines of sarcomeres (Figure 1.3). Along with actin, thin filaments contain tropomyosin (Tm) and troponin (Tn), the latter consists of three subunits, TnC, TnI, and TnT. The Tm strand is a dimer in a coiled coil arrangement that binds seven actins and its position along the actin filament plays an important role in regulating contraction. For instance, in the absence of Ca\(^{2+}\) myofilaments are in a relaxed state as Tm blocks the cross-bridge binding sites on actin (81, 166). Binding of Ca\(^{2+}\) changes myofilaments to an activated state by initiating Tm movement away from the cross-bridge binding site of the actin strand, thereby releasing the steric blocking (81, 166). The Tn complex resides in close proximity to Tm with one Tn binding for one Tm strand, that covers seven actin monomers; this makes up a thin filament functional unit. Tn is comprised of three subunits termed TnC, TnT and TnI. The cardiac TnC subunit contains three Ca\(^{2+}\) binding sites, two bind Ca\(^{2+}\) or Mg\(^{2+}\) with high affinity and one binds Ca\(^{2+}\) with low affinity (61). Binding of Ca\(^{2+}\) to the low affinity site produces conformational changes in TnI and TnT that initiate the movement of Tm permitting actin and myosin interaction (61). The TnI subunit forms a complex with both actin and TnC and inhibits actin-activated ATPase when Ca\(^{2+}\) is not present. Ca\(^{2+}\) binding to TnC appears to increase TnC-TnI interaction strength and remove inhibitory effects of TnI on ATPase activity and cross-bridge cycling (114). In addition, cardiac TnI contains phosphorylation sites (serines 22 and 23) for cAMP-dependent protein kinase (PKA) and Ca\(^{2+}\) -activated protein kinase (PKC) (serines 43 and 45, and threonine 144). \(\beta\)-adrenergic stimulation yields PKA-induced phosphorylation of TnI (134) that modulates cardiac function by reducing the Ca\(^{2+}\) affinity for TnC as well as reducing the
Figure 1.3
cooperative binding of TnI to actin-Tm causing a reduction in relaxation time (71, 135). In addition, several studies have suggested that PKA-induced phosphorylation also speeds cross-bridge cycling kinetics. For instance, the β-agonist isoproterenol was shown to increase Ca\(^{2+}\)-activated actomyosin ATPase activity \textit{in vitro} (162). In addition, β-adrenergic stimulation was found to increase the frequency for minimum stiffness (\(f_{\text{min}}\)) in intact papillary muscles during barium contractures (8, 60) and PKA was found to increase loaded shortening velocity in rat skinned cardiac myocytes during both maximal and submaximal Ca\(^{2+}\) activations (52), implying an increase in cross-bridge cycling rates during loaded contractions. In agreement with loaded shortening and power being altered, a recent study reported that left ventricular power output was enhanced in mouse hearts expressing a mutated TnI that mimics constitutive bis-phosphorylation of TnI by PKA (145). Moreover, β-adrenergic stimulation augmented contractility more in control than in transgenic mouse hearts expressing slow skeletal TnI, (which is not phosphorylated by PKA), but only during ejecting contractions and not during isovolumic contractions (80). These results imply that PKA induced phosphorylation plays its most important role in modulating cross-bridge interaction during loaded contractions.

The thick filaments are composed primarily of myosin. Myosin is a large molecule with a long tail and two globular heads (Figure 1.3). It can be separated into two sets of heavy chains (MyHC) and their two respective light chains, an essential (MLC1) and a regulatory (MLC2) light chain. Application of a proteolytic enzyme, such as trypsin, divides the myosin molecule into two sections termed light meromyosin (LMM) and heavy meromyosin (HMM). LMM is the smaller fragment of the two and comprises the tail region
of the molecule whose main purpose is thought to be maintenance of thick filament rigidity. Alternatively, HMM contains the S2 and S1 portions of the myosin molecule obtained with papain digestion, with the S1 fragment containing the neck and globular head region. The light chains are located toward the neck region of S1 (Figure 1.3) and help to stabilize the neck and may confer some regulation of functional activity (149), such as increasing Ca\(^{2+}\)-sensitivity of force (138) and the rate of force development (143). However, it is the globular head region of the HMM fragment that contains the exclusive ability to hydrolyze ATP and bind actin. Myosin hydrolysis of the terminal phosphate on ATP is thought to provide the energy for binding to actin and drive muscle contraction through conformational cycles of myosin cross-bridges. In mammalian ventricles there are three myosin isoforms expressed, composed of either a homo or heterodimer of the two MyHC isoforms, V\(_1\) (which has two α-MyHCs), V\(_2\) (a heterodimer consisting of an α-MyHC and β-MyHC), and V\(_3\) (containing two β-MyHCs). The two MyHC isoforms are individual gene products from the same chromosome (14, in humans) exhibiting 93% amino acid sequence homology (88). Even though highly homologous, examination of the amino acid sequence reveals several regions of difference. These include limited regions of the myosin head near the ATP-binding site (known as loop 1) and near the actin-binding site (loop 2), the α-helix in the neck region, the hinge between S2 and LMM, and the rod potion of LMM (88). It is unknown how these variations in MyHC structure produce robust functional differences including 2-3 fold differences in myofibrillar ATPase activities (117, 129), rates of force development (40, 93, 118, 123), unloaded shortening velocities (105, 130) and power outputs (51, 72).
The thick filament also contains myosin binding protein C (MyBP-C). MyBP-C is localized to the middle two-thirds of each half sarcomere. MyBP-C is a U-shaped molecule that can form trimers that are thought to wrap around the thick filament. The trimers are spaced at 14.2 nm repeats, which coincides with spacing patterns of myosin heads (160). Also, MyBP-C contains three phosphorylation sites available for either PKA or PKC that are thought to modulate molecular arrangement. Although the exact role of MyBP-C in muscle is not fully understood, it has been postulated that MyBP-C plays both functional and structural roles. Functionally, it has been shown that the presence of MyBP-C can alter Ca\(^{2+}\)-sensitivity of force in a PKA dependent manner, with increased PKA-induced phosphorylation decreasing Ca\(^{2+}\)-sensitivity of force (75). Additionally, gene targeted deletion of cardiac MyBP-C yielded myocytes with faster rates of force development and greater power output (73). These functional effects are thought to involve the interactions between MyBP-C and the S2 portion of MyHC. MyBP-C has been shown to bind to the S2 segment of myosin near the lever arm domain of the myosin head and this interaction is thought to be altered by PKA-induced phosphorylation (44, 47). Theoretically, MyBP-C binding to S2 may keep the myosin heads closer to the thick filament backbone limiting the range of movement thereby decreasing the probability of myosin binding actin. It follows then that MyBP-C extraction, truncation, or phosphorylation may create cross-bridges less constrained with a greater probability of binding to actin. In addition to functional duties, the binding of MyBP-C to S2 and possibly titin is thought to play a structural role in sarcomeric assembly as well. Mutations in the MyBP-C gene generating truncated proteins with lesser binding to MyHC result in disarrangement of structure at the myofilaments (168).
Additionally, dephosphorylation of MyBP-C that can occur with ischemia can produce similar sarcomere disarrangement (24).

1.3 Actomyosin interaction: Cross-bridge cycling

During muscle contraction myosin cross-bridges cyclically interact with actin in a process that is driven enzymatically by ATP hydrolysis. The chemomechanical states in the cross-bridge cycle have been studied extensively in skinned muscle fiber preparations and the transition rates between these states are qualitatively similar to rates derived from muscle protein studies in solution (46). A current model of the chemomechanical steps in cross-bridge cycle is provided in figure 1.4 with rate constants for some defined steps. Importantly, these rate constants reflect values obtained in psoas muscle fibers at 20° C under isometric conditions. As the cross-bridge cycle represents a series of enzymatic steps that are influenced by experimental conditions, changes in conditions certainly may alter rates. In fact, the kinetics of the cross-bridge transitions have been shown to depend on such things as velocity of muscle shortening (i.e., strain dependence) and temperature and furthermore these strain and temperature dependences appear to vary among transition steps (33, 172). For example, rate of ATP utilization is largely strain dependent, with utilization increasing with velocity of shortening (33), while ATP hydrolysis is primarily strain independent (34). In addition, force generating states are greatly affected by changes in temperature, while ATP binding is only minimally altered (172).
Figure 1.4

Weak Binding

1. AM $\leftrightarrow$ AM ATP $\leftrightarrow$ AM ADP P$_i$

3. AM ADP P$_i$ $\leftrightarrow$ AM*ADP + P$_i$

Strong Binding

2. M ATP $\leftrightarrow$ M ADP P$_i$

4. H$^+$

5. P$_i$

Non-Force Generating

6. AM ADP $\leftrightarrow$ AM

Force Generating

10$^6$ M$^{-1}$·s$^{-1}$ 100 s$^{-1}$ 40 s$^{-1}$ 5 s$^{-1}$
According to the cross-bridge model, actin and myosin are very tightly bound (i.e., in the rigor state) in the absence of nucleotide (i.e., the AM state). The binding of ATP to the nucleotide binding cleft of myosin (with has a second order rate constant of $10^6 \text{M}^{-1} \cdot \text{s}^{-1}$) decreases the affinity of myosin for actin, and causes rapid detachment of the myosin head (step 1). The unattached or weakly attached myosin head then hydrolyzes ATP into ADP and $\text{P}_i$ at a rate of 100 s$^{-1}$ (84). ATP hydrolysis is associated with a change in myosin formation to a more compact structure (55). Hypothetically this change in structure corresponds to a “repriming” of the cross-bridge from a post-powerstroke formation to one ready and able to move through force-generating steps. Myosin heads with either ATP or ATP hydrolysis products bound exist in equilibrium between weakly bound or unbound states (13), as indicated in Figure 1.4. The weakly bound state with a full compliment of nucleotide products is thought to occur only transiently in intact fibers while moving into a strongly bound state having the same products (step 3) (13). Though a strongly bound, non-force generating state is indicated in Figure 1.4, evidence for this state is limited as it again is likely a very transient state. However, a weak to strong binding step has been postulated to be the step regulated by $\text{Ca}^{2+}$ (84). Following this, an isomerization of the strongly bound state (having ADP and $\text{P}_i$ bound) is thought to occur producing force (step 4) (4, 42, 156). This isomerization utilizes a structural change characterized by attached heads becoming more ordered in the catalytic domain (7) and rotation of the neck region of the myosin molecule (65). In addition, evidence suggests a proton (H$^+$) is lost in this transition from a weakly to strongly bound force-generating state (1). This step containing the isomerization is considered the fundamental force-generating step or powerstroke of the cross-bridge cycle. The release of $\text{P}_i$ is closely related to the force-generating isomerization, with $\text{P}_i$ release
potentially stabilizes the structure (step 5) (20). The rate of movements through steps 4 and 5 (isomerization and \( P_i \) release) are difficult to separate experimentally; though step 5 is at least 40 s\(^{-1}\) (50). Finally, an irreversible isomerization occurs with ADP release following closely behind (54). Further movement of the cross-bridge is thought to occur during this step leaving cross-bridges in rigor-like formations (67). This later isomerization and product release have been proposed to be the rate-limiting step in isometric contractions having a rate constant of 5-25 s\(^{-1}\) (20, 56). Force generation beginning at step 4 is maintained following \( P_i \) release (step 5) and the isomerization coupled to ADP release (step 6), with the AM*ADP+\( P_i \) (which is reversible) and AM*ADP (which is irreversible) the largest populations of cross-bridges during isometric contraction (42). Interestingly, addition of \( P_i \) to active muscle inhibits force by 50-70\% (69, 109) indicating that of force-generating cross-bridges only a small proportion are in force-generating states beyond the irreversible isomerization associated with ADP release. Furthermore, the observation that addition of physiological concentrations of \( P_i \) can inhibit force indicate that this transition (step 5) is not associated with a large drop in free energy, making it highly reversible (16, 20). Binding of ATP and subsequent cross-bridge detachment follow and thus the cycle repeats itself.

As just described, force is generated by cross-bridge rearrangement performing a so-called powerstroke. The amount of force generated depends upon the number of cross-bridges in these states (steps 4-6). In addition to generating force the powerstroke can move the thin filament in the opposite direction of the thick filament (i.e. thin filaments are propelled toward the middle of the sarcomere). Shortening occurs if the load that cross-bridges are cycling against is less than the maximum force that can be generated by the given
number of cross-bridges bound. The load dependence of shortening manifests as an inverse relationship between force and velocity of sarcomere shortening (Figure 2.4). The point at which the load is so high that no shortening occurs indicates maximal isometric force (the interception of the x (force)-axis). Isometric force is ultimately determined by the number of force-generating cross-bridges, and is limited by the balance between the rates of force-generating transitions and detachment of positively strained cross-bridges (63). Conversely, the y(velocity)-intercept of the force-velocity relationship represents cross-bridges cycling against essentially no load. As velocity increases toward this point, shortening is likely limited by cross-bridge detachment rates because cross-bridges that remain bound become an internal load that opposes shortening as filaments slide past each other. Thus, the speed of unloaded shortening reaches its limit when negative resistance forces equal positive forces (63). However, it is unknown which chemomechanical transitions are most important in determining force and shortening speeds at intermediate loads where myocytes produce optimal power output. As elevations in [P_i] (from 0 to ~15 mM) increase the rate of force development (4, 53, 147, 151) but have no effect on unloaded shortening velocity in skinned fast-twitch skeletal muscle fibers (17, 158), experiments in the presence of increased [P_i] should indicate at which loads shortening velocities are most influenced by P_i transitional states, i.e., those which are associated with strong-binding to force producing states (steps 3 to 5). Thus, investigation of the effects of elevated [P_i] on loaded shortening and power output in cardiac myocytes provides mechanistic insights into the transitions that are most important in determining power output in cardiac myocytes, with particular interest in loads where power output is optimal since these are the loads that in vivo myocytes are thought to encounter during the ejection phase of the cardiac cycle.
1.4 Cardiac function during Ischemia

Low coronary blood flow can produce cardiac stress and lead to large changes in the pump performance of the ventricle. For example, patients with coronary artery disease demonstrate reduced left ventricular function as indexed by decreased ejection fraction and cardiac output (98). Echocardiography (144) and three-dimensional MRI (100) determined that global decreases in function are the likely result of regional dysfunction created by abnormally contracting segments of the myocardial wall. Therefore chronic ischemia has the ability to produce segmental abnormalities creating global dysfunction and acute coronary ligation can produce similar results. For instance, acute reduction of regional blood flow by coronary ligation decreased segmental shortening in open-chest pigs within the first 15 heart beats, resulting in a significant fall of left ventricular systolic pressure and rates of pressure development (3). In addition, Langendorff-perfused working heart preparations displayed rapid declines in developed pressure and slowed rates of pressure decline (157). The mechanisms for functional changes such as described above are proposed to include (but not limited to): decreased intracellular [ATP], decreased Ca$^{2+}$ availability, decreased pH, lowered free energy of ATP hydrolysis, and increased [P$_i$]. The actual mechanisms producing dysfunction are most likely dependent upon many factors such as duration or severity of ischemia or the experimental model being studied. Further evaluation of the mechanisms responsible for changes in myocardial function during early acute ischemia have been provided by studies utilizing nuclear magnetic resonance (NMR) spectroscopy in combination with functional measurements. One such study measured decreased segmental shortening in an open-chest pig model of graded ischemia that correlates with decreased pH
and increased $P_i$ (127). A subsequent study using working heart preparations showed little change in [ATP], free energy of ATP hydrolysis, and $Ca^{2+}$ transients with decreased pressure generation during moderate ischemia (35). In this study $[P_i]$ and pH were again found to decrease in relation to diminishing function (35). Of interest is the fact that in this study, pressure changes during ischemia occurred in the presence of similar $[Ca^{2+}]$ indicating a potential desensitization of the contractile apparatus to $Ca^{2+}$. Once again increased $[P_i]$ and $[H^+]$ provide potential mechanisms for this functional observation as it has been reported that both $P_i$ and $H^+$ decrease $Ca^{2+}$ sensitivity of force in skinned cardiac muscle preparations (43, 165). These studies and others have utilized skinned preparations to examine the effects of $P_i$ and $H^+$ on contractile properties of muscle. For example, both $P_i$ and $H^+$ decrease ATPase activity in skinned rat trabeculae however, they do so to a lesser extent than their effect on maximal $Ca^{2+}$-activated force (27). Also, $H^+$ has been reported to slow $V_o$ in cardiac muscle strips (120), whereas $P_i$ has no effect on skeletal fibers at low concentrations (<15mM) (17, 89, 158) and a small slowing effect at very high concentrations (>50mM) in skeletal muscle fibers (17). Because measurements of maximum shortening velocity, $Ca^{2+}$-sensitivity of force and maximal force production provide some important information concerning the effects of $P_i$ and $H^+$ on myocardial contractile properties during ischemia, they do not address directly how these factors modulate rates of force development and rates of loaded shortening, which more closely assess how myocytes perform during isovolumic contraction and ejection phases of the cardiac cycle. The following work directly examined the effects of $P_i$ and $H^+$ on rates of force redevelopment, loaded shortening, and power output in rat skinned cardiac myocytes.
When examining the effects of ischemia on the myocardium it is important to recognize that many factors including contractile protein isoform expression may contribute to the functional response. Studies using skinned skeletal fibers have reported lesser depressant effect of $P_i$ (103, 147) or $H^+$ (91) on force in slow-twitch fibers than in fast-twitch fibers. This may be especially significant in mammalian hearts as MyHC isoform changes toward $\beta$-MyHC isoform (which is the same isoform as in slow-twitch fibers) following prolonged ischemia, infarction, and during heart failure (119, 167, 169). The potential differential effects of ischemic metabolites on power output of cardiac myocytes have not been previously examined even though a switch towards a more resistant isoform would appear to be advantageous teleologically in situations of ischemic heart disease. Thus, we examined the effects of increased metabolite concentrations on force, loaded shortening velocity and power output of single skinned cardiac myocytes primarily expressing either $\alpha$- or $\beta$-MyHC. For these studies permeabilized myocyte preparations were used. These preparations offer several advantages including 1) control of intracellular ionic composition and substrates, 2) control of sarcomere length, and 3) assessment of MyHC composition on the same preparation that functional measurements were performed.

1.5 Cardiac function with chronic exercise

During exercise the heart matches increased tissue demand for blood by increasing cardiac output. This is accomplished acutely by increasing HR and SV (124). Upon the start of exercise there is increased sympathetic outflow, which activates $\beta$-adrenergic receptors leading to an increase in heart rate, shortening of the refractory period, and enhancement of
myocardial contractility. Following chronic exercise training, a myriad of changes occur that increase cardiac contractility such that smaller increases in HR are needed to accommodate elevations in systemic demand at a given workload (128). Many mechanisms could be responsible for such results, though, they most likely vary dependent upon exercise type, duration and intensity.

Increased stroke volume is thought to be due, in part, to training-induced alterations of the intrinsic contractile function of the myocardium (36, 66, 68, 95). Alterations of contractile function following exercise have primarily been limited to measurements of either force production or unloaded shortening velocity. While only a few studies have focused on the effects of exercise training on the entire force-velocity relationship as it is velocity of shortening under a load that is most applicable to in vivo function (121). Two studies utilizing a rat model of chronic exercise training reported increased velocity of shortening for a given load in myocardial preparations from trained animals (25, 95). However, alterations in loaded shortening have not been investigated in other animal models, specifically larger animal models such as swine that may more closely mimic human physiology. The purpose of the study presented here was to evaluate a pig model of exercise training for mechanical changes intrinsic to the myofilaments. The use of a porcine model of exercise training to investigate physiological changes that occur in large mammals may be applicable to humans as pigs and humans have similar heart morphology and similar adaptive responses to chronic exercise with respect to maximal oxygen consumption (VO$_2$max), SV, HR and skeletal muscle oxidation capacity (for review see (87)). This model has previously been shown to produce increased exercise tolerance, moderate cardiac hypertrophy, increased coronary
blood flow capacity, increased oxidative capacity of skeletal muscle and lower heart rates during exercise at submaximal intensities (77). These changes occurred without alterations in myofibrillar ATPase, Ca$^{2+}$ regulatory systems, or the metabolic system (77). However, SV was increased at submaximal workloads (77).

Greater SV following exercise training could be the result of a number of things including greater ventricular volume, less aortic resistance or greater myocardial contractility. Multiple structural and functional adaptations within the heart could be responsible for such changes, such as: myocardial hypertrophy, changes in Ca$^{2+}$ handling, altered myofibrillar protein expression and covalent modification of myofibrillar proteins (as reviewed(96)). Alterations in myocyte morphology may contribute to enhanced contractility. For example, myocyte hypertrophy occurring with endurance training can increase the tension-generating capacity of cardiac muscle (101, 148). As opposed to changes in width, changes in length have also been reported following exercise training (68, 97). Greater cell length contributes to enlargement of the ventricular chamber allowing greater ventricular filling. Additionally, changes to Ca$^{2+}$ handling with exercise training have been reported. Data from experiments measuring Ca$^{2+}$ transients and sensitivity have been mixed, with a few studies reporting an increase in myofilament Ca$^{2+}$ sensitivity (26, 68, 163) while others have seen no difference in sensitivities (101) or Ca$^{2+}$ transients (79, 163) in ventricular myocytes from exercise trained as compared to sedentary control rats. As opposed to myocyte size or Ca$^{2+}$ handling, changes in myofibrillar protein content also occur following exercise training. Shifts toward greater $\alpha$-MyHC content have been reported in multiple endurance exercise training models (9, 49, 66). Greater $\alpha$-MyHC content increases the rate
of force redevelopment following a mechanical perturbation ($k_{tr}$) in cardiac muscle preparations (40). And, while maximal Ca$^{2+}$-activated force is not different between α- and β-MyHC myocytes, power generating capacity is about three times higher in α-MyHC myocytes and working heart preparations (72). Increased $k_{tr}$ or loaded shortening would enhance contractility thus providing a potential molecular mechanism for the exercise induced enhancement of SV. In addition to MyHC, cardiac TnT (cTnT) protein isoform expression was recently found to be altered in a pig model of diabetes and exercise (74). Moreover, cTnT isoform expression correlated with fractional shortening, whereby higher levels of the larger isoforms were found in heart that had greater function (74). Furthermore changes in cTnT isoform content occur in humans during the progression to heart failure (6) further indicating a potential causative role in functional changes. Finally, sympathetic innervation increases during exercise, increasing β-adrenergic activity of the heart. In cardiac myocytes the response pathway includes β-adrenergic receptor activation of adenylate cyclase (AC) and increased intracellular concentrations of cAMP. Basal and peak AC activities increase in young and aged rats following treadmill exercise training (11, 126). Higher levels of cAMP allow increased PKA activity. As discussed previously, PKA-induced phosphorylation of cTnI and MyBP-C increase myofibrillar functional capacity by increasing the rate of force development (110) and loaded shortening velocity causing the production of greater power output (52). The main objective of this study was to determine if myocardial contractility differs from sedentary (SED) and exercise trained (EX) pig myocytes as indexed by isometric force, loaded shortening velocity, power output and rate of force development during maximal Ca$^{2+}$ activation. Another objective of the study presented here was to determine if alterations in cardiac myofibrillar proteins may account for any
functional differences observed. Specifically, we examined relative MyHC content, relative cardiac cTnT isoform content, and PKA-induced baseline phosphorylation of the myofibrillar proteins cardiac cTnI and MyBP-C.
Chapter 2. INORGANIC PHOSPHATE SPEEDS LOADED SHORTENING IN RAT SKINNED CARDIAC MYOCYTES.

ABSTRACT

Force generation in striated muscle is coupled with inorganic phosphate (P_i) release from myosin, because force falls with increasing [P_i]. However, it is unclear which cross-bridge transitional states limit loaded shortening and power output. We examined the role that P_i plays in determining force, unloaded shortening, loaded shortening, power output, and rate of force development in rat skinned cardiac myocytes. Myocytes (n =6) were attached between a force transducer and position motor and contractile properties were measured over a range of loads during maximal Ca^{2+} activation. Addition of 5mM P_i had no effect on maximal unloaded shortening velocity V_o (Control V_o = 1.83 ± 0.75; 5mM added P_i V_o = 1.75 ± 0.58 ML/sec, n =6). Conversely, addition of 2.5, 5, and 10 mM P_i progressively decreased force but resulted in faster loaded shortening and greater power output (when normalized for the decrease in force) at all loads greater than ~15% isometric force. Peak normalized power output increased 16% with 2.5 mM added P_i and further increased to a plateau of ~35% with 5 and 10 mM added P_i. Interestingly, the rate constant (k_tr) of force redevelopment progressively increased from 0 to 10 mM added [P_i] with k_tr being ~360% greater at 10mM than 0 mM added [P_i]. Overall, these results suggest that the P_i release step in the cross-bridge cycle is rate-limiting step for determining shortening velocity and power output at intermediate and high relative loads in cardiac myocytes.
INTRODUCTION

During muscle contraction myosin cross-bridges cyclically interact with actin in a process that is driven energetically by hydrolysis of ATP. The chemomechanical states in the cross-bridge cycle have been investigated extensively in skinned muscle fiber preparations and the transition rates between these states are qualitatively similar to rates derived from muscle protein studies in solution (46). A working model for the chemomechanical steps in the cross-bridge cycle is shown in Figure 1.4 (153), that is based on extensive work in the field (for reviews see (16) and (46)).

According to this model, the transition from weak-binding, non-force generating cross-bridges to strong-binding, force generating states is associated with the release of P$_i$ (steps 3-5) (53). Force generation is maintained through the release of ADP (step 6) (21, 83), which is followed by binding ATP and subsequent cross-bridge detachment. The binding of ATP and its hydrolysis are thought to be relatively fast processes in skinned fibers (34, 45, 84). Thus, the rate limiting step in the cross-bridge cycle is thought to occur following ATP hydrolysis, either during an isomerization of the AM ADP P$_i$ state (step 3), which is associated with the weak to strong binding transition, or an isomerization step of the AM ADP state just prior to ADP release (i.e., step 6) and cross-bridge detachment (for reviews see (16) and (46)). Another factor in this cross-bridge schematic is the fact that transition rates vary as a function of load on the muscle, becoming faster as muscle load is reduced. For instance, the rate constant of force decline ($k_{\text{Pi}}$) following rapid increase in solution P$_i$ varied linearly as a function of load (62). Thus, the rate limiting steps may be different as the load on the myofilaments changes. For instance, unloaded muscle shortening is likely
limited by cross-bridge detachment rates, because cross-bridges that remain bound ultimately become an internal load as filaments slide past each other. Thus, the speed of shortening of an unloaded muscle is thought to reach its limit when compressive resistance forces equal positive forces (63). At the other intercept of the force-velocity curve, isometric force is ultimately determined by the number of force-generating cross-bridges, which is limited by the balance between the rates of force-generating transitions and detachment of positively strained cross-bridges. It is unknown which chemomechanical transitions are most important in determining force and shortening speeds and, thus, power output at intermediate loads where muscles perform work. As elevations in [Pₐ] (from 0 to ~15 mM) increase the rate of force development (4, 53, 147, 151); but have no effect on unloaded shortening velocity in skinned fast-twitch skeletal muscle fibers (17, 89, 158), experiments in the presence of increased [Pₐ] should indicate at which loads shortening velocities are most influenced by Pₐ transitional states, i.e., those which are associated with weak to strong-binding to force producing states (steps 3 to 5). Thus, we investigated the effects of elevated [Pₐ] on loaded shortening and power output in permeabilized single cardiac myocytes in order to gain mechanistic insights into the cross-bridge steps that are most important in determining power output in cardiac myocytes, with particular interest in loads where power output is optimal as these are the loads that in vivo myocytes are thought to encounter during the ejection phase of the cardiac cycle.
METHODS

Cardiac myocyte preparation

Normal Sprague-Dawley rats were obtained from Harlan (Madison, WI), maintained, and humanely killed according to guidelines set by the Animal Care and Use Committee of the University of Missouri. Single skinned cardiac myocytes were obtained by mechanical disruption of hearts as described previously (85). Rats were anaesthetized by inhalation of isoflurane (0.05 mg) for 2-4 minutes in an airtight one-liter container and their hearts were excised and rapidly placed in ice cold relaxing solution. The ventricles were dissected away from the atria, cut into 2-3 mm pieces and further disrupted for 5-10 seconds in a Waring blender. The resulting suspension of cells was centrifuged for 65 sec at 165 x g, after which the supernatant fluid was discarded. The myocytes were skinned by suspending the pellet of cells for 3 min in 0.5 % ultrapure Triton X-100 (Pierce Chemical Co.) in relaxing solution. The skinned cells were washed twice with cold relaxing solution, resuspended in 10-15 ml of relaxing solution and kept on ice during the day of the experiment. Myocytes were used within 12 hours of isolation.

Solutions

Relaxing solution in which the ventricles were disrupted, skinned, and suspended contained (in mmol/L): EGTA 2, MgCl$_2$ 5, ATP 4, imidazole 10, and KCl 100 at pH 7.0.

Compositions of relaxing and activating solutions used in mechanical measurements were as follows (mmol/L): EGTA 7, free Mg$^{2+}$ 1, imidazole 20, MgATP 4, creatine phosphate 14.5, pH 7.0, Ca$^{2+}$ concentrations of 10$^{-9}$ M (relaxing solution) and 10$^{-4.5}$ M (maximal activating solution), and sufficient KCl to adjust ionic strength to 180 mM. Activating solutions used in
phosphate experiments were identical to those described above except for inclusion of 2.5, 5 and 10 mM concentrations of potassium phosphate (KH$_2$PO$_4$) before adjustment to ionic strength of 180 mM. The final concentrations of each metal, ligand and metal-ligand complex at 13°C were determined with a computer program (31). Immediately preceding activations, muscle preparations were immersed for 60 s in a solution of reduced Ca$^{2+}$-EGTA buffering capacity, identical to normal relaxing solution except that EGTA is reduced to 0.5 mM. This protocol resulted in more rapid steady state force development and helped preserve the striation pattern during activation.

**Experimental Apparatus**

The experimental apparatus for physiological measurements of myocyte preparations was similar to one previously described in detail (99) and modified specifically for cardiac myocyte preparations (85). Briefly, myocyte preparations were attached between a force transducer and torque motor by gently placing the ends of the myocyte into stainless steel troughs (25 gauge). The ends of the myocyte were secured by overlaying a 0.5 mm long piece of 3-0 monofilament nylon suture (Ethicon, Inc.) onto each end of the myocyte, and then tying the suture into the troughs with two loops of 10-0 monofilament suture (Ethicon, Inc.). The attachment procedure was performed under a stereomicroscope (~100x magnification) using finely shaped forceps.

Prior to mechanical measurements the experimental apparatus was mounted on the stage of an inverted microscope (model IX-70, Olympus Instrument Co., Japan), which rested on a pneumatic anti-vibration table with a cut-off frequency of ~1 Hz. Force
measurements were made using a capacitance-gauge transducer (Model 403-sensitivity of 20 mV/mg plus a x10 amplifier and resonant frequency of 600 Hz; Aurora Scientific, Inc., Aurora, ON, Canada). Length changes during mechanical measurements were introduced at one end of the preparation using a DC torque motor (model 308, Aurora Scientific, Inc.) driven by voltage commands from a personal computer via a 12-bit D/A converter (AT-MIO-16E-1, National Instruments Corp., Austin, TX, USA). Force and length signals were digitized at 1 kHz using a 12-bit A/D converter and each was displayed and stored on a personal computer using custom software based on LabView for Windows (National Instruments Corp.).

Images of the myocyte preparations were recorded digitally on a personal computer while relaxed and during activation using a Hamamatsu CCD camera (model 2400) and video snapshot software (Figure 1). Videomicroscopy was completed using a 40x objective (Olympus UWD 40) and 25x intermediate lenses. During and after each experiment, the images were reviewed to obtain sarcomere length measurements from the myocyte while relaxed and activated; myocyte length and width for cross-sectional area calculations also were obtained from these images. Sarcomere lengths of these preparations was set to yield passive forces near zero. The dimensions of the skinned cardiac myocyte preparations are reported in Table 2.1.

**Mechanical measurements**

All mechanical measurements were made at 13 ± 1°C. Power output of single skinned myocyte preparations was determined at varied loads as previously described (85).
Briefly, myocytes were placed in activating solution and once steady-state force developed, a series of force clamps (less than steady-state force) were performed to determine isotonic shortening velocities. Using a servo-system, force was maintained constant for a designated period of time (150-250 msec) while the length change was continuously monitored. Following the force clamp, the myocyte preparation was slackened to reduce force to near zero to allow estimation of the relative load sustained during isotonic shortening; the myocyte was subsequently re-extended to its initial length.

<table>
<thead>
<tr>
<th>Table 2.1. Summary of Skinned Cardiac Myocyte Dimensions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcomere Length</td>
</tr>
<tr>
<td>Length (µm)</td>
</tr>
<tr>
<td>131 ± 28</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 6.

Isotonic shortening velocities were measured in activating solutions containing varied amounts of additional P\(_i\) (0, 2.5, 5 and 10 mM). Each cell underwent a series of loaded contractions in each of the P\(_i\) solutions, whose order was chosen at random. In this way each cell served as its own control and pairwise statistical analysis could be performed. Isometric force was measured in activating solution with no additional P\(_i\) prior to and following measurements of isotonic shortening velocities to detect rundown of the preparation. Myocytes were discarded if a 20% or greater decrease in isometric tension occurred.
The kinetics of force redevelopment were obtained using a procedure previously described for skinned cardiac myocyte preparations (73). While in Ca$^{2+}$ activating solution, the myocyte preparation was rapidly shortened by $\sim 15\%$ of the myocyte’s initial length ($L_o$) to produce zero force. The myocyte preparation was then allowed to shorten for $\sim 20$ ms; after 20 ms the preparation was rapidly re-stretched to $\sim 105\%$ of its initial length ($L_o$) for 2 ms and then returned to $L_o$. The slack-re-stretch maneuver caused dissociation of cross-bridges and subsequent force redevelopment was due to reattachment of cross-bridges and transition to force-generating states. Force redevelopment measurements were carried out prior to the series of loaded contractions at each $P_i$ concentration.

Unloaded shortening velocity ($V_o$) was measured during maximal Ca$^{2+}$ activations using the slack test method (28, 140). Once steady-state force was reached the myocyte preparation was rapidly ($< 2$ msec) slackened to a predetermined value between 5% and 20% of its initial length. The time between the imposition of the slack step and the onset of force redevelopment was measured from the intersection of two lines fitted by eye through the zero force baseline and the initial phase of force redevelopment (Figure 2.3). The length of release was plotted against the duration of unloaded shortening and $V_o$ was determined from the slope of a line fitted to the data by linear regression analysis.

**Data Analysis**

Myocyte preparation length traces were fit to a single decaying exponential equation:

$$L = Ae^{-kt} + C$$
where L is cell length at time t, A and C are constants with dimensions of length, and k is the rate constant of shortening ($k_{\text{shortening}}$). Velocity of shortening at any given time, t, was determined as the slope of the tangent to the fitted curve at that time point. In this study, velocities of shortening were calculated at the onset of the force clamp (i.e., $t = 0$).

Hyperbolic force-velocity curves were fit to the relative force-velocity data using the Hill equation (57):

\[(P + a)(V + b) = (P_o + a)b\]

where P is force during shortening at velocity V; $P_o$ is the peak isometric force; and $a$ and $b$ are constants with dimensions of force and velocity, respectively. Power-load curves were obtained by multiplying force x velocity at each load on the force-velocity curve. The optimum force for mechanical power output ($F_{\text{opt}}$) was calculated using the equation (164):

\[F_{\text{opt}} = (a^2 + a \cdot P_o)^{1/2} - a\]

Curve fitting was performed using a customized program written in Qbasic, as well as commercial software (Sigmaplot).

Force redevelopment traces were fit by a single exponential function:

\[F = F_{\text{max}} (1 - \exp(-k_{\text{tr}}t)) + F_{\text{res}},\]

where F is tension at time t, $F_{\text{max}}$ is maximal tension, and $k_{\text{tr}}$ is the rate constant of tension redevelopment. $F_{\text{res}}$ represents any residual tension present immediately after the slack-restretch maneuver.
Statistics

One–way repeated measures ANOVA were used to determine significant effects on force, absolute and normalized power output, and $k_a$ from varied Pi concentrations. The Student-Newman-Keuls test was used post hoc to assess the differences among means. A paired t-test was used to compare $V_o$ values before and after addition of 5 mM Pi. $p < 0.05$ was chosen as indicating significance. Values are expressed as means ± SD unless otherwise indicated.

RESULTS

The addition of Pi resulted in a progressive decrease in maximal Ca$^{2+}$ activated force in skinned cardiac myocyte preparations. Force declined from a control value of 21 ± 7 kN·m$^{-2}$ to 14 ± 5 kN·m$^{-2}$, 12 ± 5 kN·m$^{-2}$, and 7 ± 3 kN·m$^{-2}$ in the presence of 2.5, 5, and 10 mM added [Pi], respectively. These effects of added Pi on skinned myocyte preparations were quantitatively similar to those from permeabilized multicellular cardiac muscle preparations at pH 7.0 (103).

Previous studies have reported that maximal velocity of shortening (i.e., unloaded) is minimally affected by addition of up to 15 mM added Pi in fast-twitch skeletal muscle fibers (17, 89, 158). We addressed whether Pi alters unloaded shortening velocity ($V_o$) in cardiac myocyte preparations using slack tests before and after the addition of 5 mM Pi. Figure 2.1 shows force traces following rapid slack steps in a cardiac myocyte preparation during maximal Ca$^{2+}$ activation in the presence and absence of 5 mM added Pi. Consistent with skeletal muscle reports, $V_o$ was unchanged in response to added Pi in cardiac myocyte
preparations (Control $V_o = 1.83 \pm 0.75$; 5 mM added $P_i$, $V_o = 1.75 \pm 0.58$ ML/sec). This finding suggests that cross-bridge interaction transitions that limit maximal shortening velocity (i.e., detachment of compressively strained cross-bridges) are minimally affected by addition of $P_i$.

A main focus of this study was to examine the effects of added $P_i$ on rates of loaded shortening and power output of cardiac myofibrils. Since previous studies using skinned fiber preparations imply that added $P_i$ speeds force-generating transitions (4, 94) but has no effect on detachment rates of compressively strained cross-bridges (see above), the addition of $P_i$ would be expected to increase normalized power only at loads where loaded shortening is limited by force-generating transition rates. We measured skinned myocyte shortening velocities over a range of loads and plotted averaged force-velocity and power-load curves in response to added $P_i$ (Figure 2.2). For these curves, force was normalized for the $P_i$-induced decrease in isometric force, which allowed direct observation of how $P_i$ alters shortening velocity and power at given relative loads. Addition of $P_i$ sped loaded shortening and increased normalized power output over load ranges above ~10% isometric force. Peak normalized power output increased 16% with 2.5 mM added $P_i$ and was further elevated to a plateau of ~35% greater than 0 mM $P_i$ with the addition of 5 and 10 mM (Figure 2.2). These results taken together with the $V_o$ results imply that force-generating transition rates (which are $P_i$ dependent) as opposed to detachment rates (of compressively strained cross-bridges) determine power output over most load ranges in cardiac myocytes. Interestingly, though, the increase in loaded shortening with added $P_i$ was not great enough to offset the $P_i$-induced
Figure 2.1. Effect of 5mM added P\(_i\) on unloaded shortening velocity (V\(_o\)) in a skinned myocyte preparation.

A. Length traces and force traces are shown during a slack test before and after addition of 5 mM P\(_i\). Unloaded shortening times following length changes were similar before and after the addition of P\(_i\). B. Slack plot before and after addition of P\(_i\). V\(_o\) for this skinned myocyte preparations was 1.29 ML/s before and 1.18 ML/s after the addition of 5 mM P\(_i\). Additionally, in this same myocyte preparation, loaded shortening was measured and peak normalized power output was ~35% greater after addition of 5 mM P\(_i\) (data not shown), which is consistent with the results from the six myocyte preparations reported in Table 2.2.
Table 2.2. Effects of Pi addition on mechanical properties of skinned cardiac myocyte preparations.

<table>
<thead>
<tr>
<th>Added [Pi]</th>
<th>Maximum Force (µN)</th>
<th>Maximum Force (kN·m²)</th>
<th>F&lt;sub&gt;opt&lt;/sub&gt; (P/P&lt;sub&gt;o&lt;/sub&gt;)</th>
<th>a/P&lt;sub&gt;o&lt;/sub&gt;</th>
<th>V&lt;sub&gt;opt&lt;/sub&gt; (ML·s⁻¹)</th>
<th>Peak absolute power output (pW)</th>
<th>Peak absolute power output (mW·mg⁻¹)</th>
<th>Peak normalized power output (P/P&lt;sub&gt;o&lt;/sub&gt;·ML·s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>7.0 ± 1.4</td>
<td>21 ± 7</td>
<td>0.28 ± 0.03</td>
<td>0.18 ± 0.05</td>
<td>0.35 ± 0.07</td>
<td>85 ± 12</td>
<td>1.95 ± 0.82</td>
<td>0.102 ± 0.009</td>
</tr>
<tr>
<td>2.5 mM</td>
<td>4.8 ± 1.0&lt;sup&gt;†&lt;/sup&gt;</td>
<td>14 ± 5&lt;sup&gt;†&lt;/sup&gt;</td>
<td>0.32 ± 0.03</td>
<td>0.32 ± 0.11</td>
<td>0.36 ± 0.06</td>
<td>71 ± 14&lt;sup&gt;†&lt;/sup&gt;</td>
<td>1.61 ± 0.61&lt;sup&gt;†&lt;/sup&gt;</td>
<td>0.118 ± 0.019&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>5 mM</td>
<td>4.1 ± 1.3&lt;sup&gt;††&lt;/sup&gt;</td>
<td>12 ± 5&lt;sup&gt;††&lt;/sup&gt;</td>
<td>0.33 ± 0.04</td>
<td>0.36 ± 0.19</td>
<td>0.43 ± 0.07&lt;sup&gt;††&lt;/sup&gt;</td>
<td>71 ± 12&lt;sup&gt;†&lt;/sup&gt;</td>
<td>1.65 ± 0.77&lt;sup&gt;†&lt;/sup&gt;</td>
<td>0.140 ± 0.025&lt;sup&gt;††&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 mM</td>
<td>2.4 ± 0.9&lt;sup&gt;†††&lt;/sup&gt;</td>
<td>7 ± 3&lt;sup&gt;†††&lt;/sup&gt;</td>
<td>0.33 ± 0.03</td>
<td>0.35 ± 0.11</td>
<td>0.41 ± 0.04&lt;sup&gt;†††&lt;/sup&gt;</td>
<td>41 ± 13&lt;sup&gt;†††&lt;/sup&gt;</td>
<td>0.99 ± 0.58&lt;sup&gt;†††&lt;/sup&gt;</td>
<td>0.136 ± 0.022&lt;sup&gt;†††&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are means ± SD. *p < 0.05 vs 0mM Pi, †p<0.05 vs 2.5 mM Pi, ‡p < 0.05 vs 5 mM Pi.
Figure 2.2. Effect of added Pi on myocyte force-velocity and power-load relationships.

A. Force-velocity and normalized power-load curves shifted upward in response to increased concentrations of added [Pi]. B. Absolute force-velocity and power-load curves in presence of 0 and 2.5 mM added Pi. Data points are means ± SEM.
fall in force to maintain *absolute* power output (Figure 2.2). Peak absolute power output (85 ± 12 pW, with no additional P$_i$) decreased 16% (71 ± 14 pW) with 2.5mM P$_i$ added, 16% (71 ± 12 pW) with 5mM P$_i$ and 52% (41 ± 13 pW) with 10 mM P$_i$. Thus, added P$_i$ increases power output at a given relative load but not at a specific absolute load due to its marked effect of depressing force generation capacity.

We also addressed the effects of added P$_i$ on the rate of myocyte force development following a mechanical perturbation to assess the potential role of this process in determining loaded shortening and power output. The rate constant of force redevelopment ($k_{tr}$) increased in the presence of rising P$_i$ concentrations (Figure 2.3). $k_{tr}$ increased from 6.1 ± 1.4 s$^{-1}$ in controls to 8.5 ± 1.3 s$^{-1}$ in the presence of 2.5 mM Pi, representing a 40% increase from control. Addition of 5 mM and 10 mM P$_i$ further increased $k_{tr}$ 94% (11.8 ± 3.1 s$^{-1}$) and 379% (28.1 ± 7.8 s$^{-1}$) above control, respectively. Interestingly, the residual force (i.e., the force just prior to force redevelopment) was consistently higher as added P$_i$ increased. Residual forces were 20 ± 3%, 30 ± 3%, 37 ± 4% and 47 ± 11% of isometric force at 0, 2.5, 5 and 10 mM added P$_i$, respectively. The exact reason for this is unclear but may involve faster transition(s) to force generating cross-bridges as a function of [P$_i$]. Because of this potentially confounding influence of residual force, we also examined the rate of force redevelopment following a ~10% slack step. The rates of force development were 4.77 ± 1.81 with no added P$_i$ and 8.57 ± 2.30 with 5 mM P$_i$ ($p = 0.003$, n = 6); this result is quantitatively similar to that observed with the slack re-stretch maneuver. Overall, these results are quantitatively different from the P$_i$-induced increase in normalized power output,
Figure 2.3

**A.** Length trace and force development traces during maximal Ca\(^{2+}\) activation in the presence of 0, 5, and 10 mM added Pi in the same cardiac myocyte preparation. **B.** The rate constant of force redevelopment (k\(_{tr}\)) progressively increased as a function of added [Pi]. Data points are means ± SD.
suggesting that cross-bridge steps that determine power output differ from the transitions that limit force redevelopment in cardiac muscle.

DISCUSSION

This study examined the effects of added $P_i$ on force, unloaded shortening, loaded shortening, power output, and rates of force development in rat skinned cardiac myocyte preparations. Addition of $P_i$ lowered isometric force, had no effect on unloaded shortening velocity but increased loaded shortening, power output, and rates of force development. The decrease in force exceeded the decline in peak absolute power output because of the $P_i$-induced increase in loaded shortening velocity. The increase in loaded shortening velocity yielded greater peak myocyte power output after normalizing for changes in isometric force. These results, taken together, suggest that peak power output is more determined by force-generating steps in the cross-bridge cycle than cross-bridge detachment rates since $P_i$ is known to speed force-generating transitions but appeared to have no effect on detachment of compressively strained cross-bridges.

The effects of $P_i$ on contractile properties have been extensively examined in both skinned skeletal and cardiac muscle preparations. Interestingly, contractile properties of cardiac muscle preparations appear to be more sensitive to added $P_i$ than skeletal muscle preparations. For instance, Nosek et al. (103) reported that 10 mM added $P_i$ decreased cardiac muscle force ~60% compared to a 40% force decline in skeletal muscle preparations in the same study. We also observed that force declined ~65% in cardiac myocyte preparation in response to 10 mM added $P_i$. While data from skeletal muscle fiber
preparations report a wide range of force decline in response to added P$_i$ (~30-55%), more recent data using myofibrils from fast-twitch skeletal muscle have reported greater effects of P$_i$ (eg., 70% decline in force) with 10 mM P$_i$ (146). The exact reasons are unclear for the differences in P$_i$ responsiveness between fibers and myofibrils preparations but may involve procedures used to reduce contaminating P$_i$ as described by Pate et al. (109) and used in the myofibril experiments (147). Also there is likely an inverse relationship between preparation diameter and force decline in response to added [P$_i$] because of greater accumulation of P$_i$ from myofibrillar ATPases (and, thus, higher baseline [P$_i$]) in thicker preparations (70, 139).

Regarding other contractile properties, added P$_i$ has been reported to increase $k_{pi}$ (the rate constant of force decline after rapid increases in solution P$_i$) to a greater extent in cardiac myocyte preparations than in fast-twitch skeletal muscle fibers (4, 154). Additionally, the increase in $k_{pi}$ with added [P$_i$] (from 0 mM to 10 mM) was upwards of three-fold in our skinned cardiac myocyte preparations versus a 50% increase in fast-twitch skinned skeletal muscle fibers (151). Taken together, these results suggest contractile properties appear to be more sensitive to elevations in [P$_i$] in cardiac muscle than skeletal muscle at least over a range of 0-10 mM added P$_i$. This may have implications in the response of these two striated types of myofibrils to metabolite (i.e., P$_i$) build up associated with ischemia. For cardiac muscle, [P$_i$] is thought to rise to as high as 30 mM during ischemia (2), such a rise in [P$_i$] would likely result in a marked fall in force-generating cross-bridges but the faster rates of force development and loaded shortening would tend to compensate to possibly help sustain adequate stroke volume and cardiac output.
A primary purpose of this study was to assess which step(s) in the cross-bridge cycle may be most important in determining myocyte shortening and power output at various loads. In looking at a force-velocity curve (Figure 2.4), shortening velocity at low loads is thought to be limited by detachment of compressively strained cross-bridges, which appears to be limited by ADP release from actomyosin (131, 170) and/or by mechanical detachment of highly compressed cross-bridges (18). Conversely, loaded shortening and power at high loads are thought to be determined by force-generating steps in the cross-bridge cycle, which are thought to be coupled to $P_i$ release (53) and/or an isomerization that is in rapid equilibrium with $P_i$ release from the actomyosin complex (94, 154). The question then arises as to what load does the cross-bridge step(s) that limit power shift from force-generating transitions to detachment rates?

**Figure 2.4**

![Graph showing force-velocity relationship with detachment and force-generating transitions](image)
Our experiments were designed to alter one of these processes (i.e., force-generating transitions) but not the other (i.e., detachment rates). In this regard, elevations in \( P_i \) (from 0 to \(~15\) mM) have been reported to speed the rates of force generation in skeletal (147, 151) and cardiac muscle preparations (4) but had minimal effects on unloaded shortening velocity in fast-twitch skeletal muscle fibers (17, 89, 158). We also found no effect of 5 mM \( P_i \) on unloaded shortening velocity in skinned cardiac myocyte preparations (Figure 2.2). Thus, we examined how added \( P_i \) affects power-load curves; if force-generating steps were the primary determinants over a large range of loads, then added \( P_i \) would increase power over much of the curve. If, on the other hand, force-generating steps only determine power at high loads, accelerating these steps with added \( P_i \) should only increase power at high loads. Interestingly, loaded shortening and power output increased with 2.5 mM added \( P_i \) over all relative loads greater than \(~10\%\) isometric force (Figure 2.1). In fact, both loaded shortening and power output continued to increase and reached a plateau at all loads greater than 10\% isometric force when added \([P_i]\) was increased to 5 mM. These results suggest that force-generating steps associated with \( P_i \) release are most important in limiting power output over most loads in cardiac myofibrils.

Our results are also consistent with similar studies on skeletal muscle fiber preparations that sought insight into which chemomechanical transitions might determine power output at various loads. For example, a study by Ford et al. (41) examined power output over a range of loads in rabbit skinned fast-twitch skeletal muscle fibers and discovered that osmotic compression of fibers resulted in reduced velocity and power at low loads but had little effect at intermediate and high loads. Since osmotic compression slowed
unloaded shortening velocity this implies that cross-bridge detachment limits power output at low loads. Consistent with this idea, lowering ATP concentrations or substituting ATP with CTP also slowed unloaded shortening velocity and decreased power output only at low loads in rat skinned slow-twitch skeletal muscle fibers (152). A recent study also found that increased [P_i] sped loaded shortening in fast-twitch skeletal muscle fibers at loads greater than ~15% isometric force (38). Taken together, these studies suggest that power output in striated myofibrils is rate-limited at low loads by the same chemomechanical transitions that limit unloaded shortening speeds whereas at intermediate and high loads it is limited by chemomechanical transitions that are associated with force-generation.

Interestingly, the addition of P_i also increased the rates of force development but to a much greater extent than it did in peak normalized power. The exact reasons for the different P_i dependencies of force development and power are unclear. One possibility is that force development is limited by the cooperative activation of near-neighbor regulatory units on the thin filament by strongly binding cross-bridges (37, 39, 141) and addition of P_i increases the number of strongly binding non-force generating cross-bridges (A M ADP Pi). This is consistent with the finding that P_i addition, like addition of strongly binding NEM modified cross-bridges (142), eliminated the slow phase of unloaded shortening following slack steps in skinned fast-twitch skeletal muscle fibers during submaximal Ca^{2+} activations (89).

Loaded shortening, on the other hand, is not likely limited by the number of strongly bound non-force generating cross-bridges (A M ADP Pi) at least during maximal Ca^{2+} activations (86). Rather, shortening velocity and power output (over most loads) appear to be
determined by $P_i$ release steps in the cross-bridge cycle, which are modulated to a different extent than thin filament activation rates by addition of $P_i$.

In summary, this study implies that myocyte power output is determined by $P_i$-sensitive force-generating transitions in the cross-bridge cycle as opposed to detachment of compressively strained cross-bridges. However, even though $P_i$ speeds loaded shortening at given relative loads, the increase in velocity does not appear to be great enough to compensate for the $P_i$-induced decrease in force; this yields an overall decrease in absolute power generating capacity, providing a myofibrillar mechanism for depressed myocardial work capacity and function during ischemia.
Chapter 3. **B-MYOSIN HEAVY CHAIN MYOCYTES ARE MORE RESISTANT THAN A-MYHC MYOCYTES TO CHANGES IN POWER OUTPUT INDUCED BY ISCHEMIC CONDITIONS.**

**ABSTRACT**

During ischemia intracellular concentrations of inorganic phosphate (P$_i$) and hydrogen ions (H$^+$) increase. Also, changes in myosin heavy chain isoform (MyHC) from towards the β-MyHC isoform have been reported following ischemia and infarction associated with coronary artery disease. The purpose of this study was to investigate the effects of myoplasmic changes of P$_i$ and H$^+$ on the loaded shortening velocity and power output of cardiac myocytes expressing either α- or β-MyHC. Skinned myocytes were obtained from adult, male Sprague-Dawley rats (control or PTU treated to induce β-MyHC) and mounted between a force transducer and servo-motor system. Myocytes were subjected to a series of isotonic force clamps to determine shortening velocity and power output during Ca$^{2+}$ activations in each of the solutions: (i) pCa 4.5, pH 7.0, (ii) pCa 4.5, pH 7.0, 5 mM P$_i$, (iii) pCa 4.5, pH 6.6, and (iv) pCa 4.5, pH 6.6, 5 mM P$_i$. Added P$_i$ and lowered pH each caused isometric force to decline to the same degree in α-MyHC and β-MyHC myocytes, however, β-MyHC myocytes were more resistant to changes in absolute power output. For example, peak absolute power output fell 53% in α-MyHC myocytes while power fell only 38% in β-MyHC myocytes in response to elevated P$_i$ and lowered pH (i.e., solution (iv)). The reduced effect on power output was the result of a greater speeding of loaded shortening.
velocity induced by P$_i$ in β-MyHC myocytes and an increase in loaded shortening velocity at pH 6.6 that occurred only in β-MyHC myocytes. We conclude that the functional response to elevated P$_i$ and lowered pH during ischemia is MyHC isoform dependent with β-MyHC myocytes being more resistant to declines in power output.

**INTRODUCTION**

The capability of the heart to pump blood is dependent upon its ability to contract against a load and thus generate power. During times of reduced blood flow to the myocardium this pumping capability is compromised. Multiple factors are involved in reducing contractile function of the myocardium during ischemia many of which are dependent upon the duration and severity of ischemia. However, a principle cause for the fall in force production during early ischemia is thought to be the accumulation of metabolic byproducts specifically inorganic phosphate (P$_i$) and hydrogen ions (H$^+$) (27, 30, 76, 104, 132). Some of the effects of alterations in myoplasmic electrolyte composition occurring during myocardial ischemia on contractile function have been examined using skinned cardiac muscle. Experiments utilizing skinned strips of cardiac trabeculae in the presence of 0-30mM P$_i$ demonstrated a progressive decrease in force production with increasing [P$_i$] (27, 69, 70, 103). However not all effects appeared to be detrimental as [P$_i$] up to 10mM increased loading shortening at loads greater than 10% maximal isometric tension in single skinned cardiac myocytes (58). The increase in loaded shortening velocity at elevated P$_i$ attenuated the fall in peak absolute power (the product of force and velocity), however the compensation was not enough to completely prevent a decline in absolute power production. Increases in H$^+$ (i.e. acidosis) that occur during ischemia also decrease cardiac myocyte function. In contrast to P$_i$, H$^+$ has
been reported to decrease unloaded and loaded shortening velocity in cardiac trabeculae preparations (120) in addition to decreasing isometric force production (32, 69, 90, 104). Studies examining increases of both P_i and H^+ working in concert have primarily been limited to assessments of alterations in force production (69, 103, 150). In cardiac muscle these two additively decrease isometric force (69, 103) while the effects upon loaded shortening and power output are unknown. Thus, we tested the hypothesis that added P_i and H^+ will act additively to decrease peak absolute power output in single skinned cardiac myocytes.

When examining the effects of ischemia on the myocardium it is important to recognize that many factors including contractile protein isoform expression may contribute to the functional response. Studies using skinned skeletal fibers have reported lesser depressant effect of P_i (103) or H^+ (91) on force in slow-twitch fibers than fast-twitch fibers. This may be especially significant in mammalian hearts as myosin heavy chain (MyHC) isoform changes toward β-MyHC isoform (which is the same isoform as in slow-twitch fibers) following prolonged ischemia, infarction, and during heart failure (119, 167, 169). The potential differential effects of ischemic metabolites on power output of cardiac myocytes have not been previously examined even though a switch towards a more resistant isoform would appear to be advantageous teleologically in situations of ischemic heart disease. Thus, we examined the effects of increased metabolite concentration on force, loaded shortening velocity and power output of single skinned cardiac myocytes primarily expressing either α- or β-MyHC. Utilizing Triton-skinned cardiac myocytes from control and PTU-treated rats (to induce β-MyHC expression) force-velocity relationships were
determined in the presence of P$_i$ (5mM) or H$^+$ (pH 6.6) alone and together to determine the effect of simulated ischemic conditions on myocyte function. The results show that P$_i$ and H$^+$ act additively to depress force in both α- and β-MyHC myocytes. Additionally, 5mM P$_i$ increased loaded shortening velocity in both α- and β-MyHC myocytes. Lowered pH had no effect on loaded shortening in α-MyHC myocytes but increased loaded shortening in β-MyHC myocytes. From these results it was concluded that α-MyHC and β-MyHC myocytes are equally sensitive to depression of force by P$_i$ and H$^+$ but β-MyHC myocytes are more tolerant to changes in metabolite concentrations as indexed by power generating capacity.

**METHODS**

**Cardiac myocyte preparation**

Sprague-Dawley rats were obtained from Harlan (Madison, WI) and maintained according to guidelines set by the Animal Care and Use Committee of the University of Missouri. Single skinned cardiac myocytes were obtained from rats treated for ~3 weeks with 5-n-propyl-2-thiouracil (PTU; 0.6g/L in drinking water) or age-matched controls by mechanical disruption of hearts as described previously (85). PTU treatment has been successfully employed to shift MyHC expression from primarily α-MyHC to the β-MyHC isoform (51). Rats were anaesthetized by inhalation of isoflurane (0.05 mg) for 2-4 minutes in an airtight one-liter container and their hearts were excised and rapidly placed in ice cold relaxing solution. The ventricles were dissected away from the atria, cut into 2-3 mm pieces and further disrupted for 5-10 seconds in a Waring blender all in the presence of ice-cold relaxing solution. The resulting suspension of cells was centrifuged for 65 sec at 165 x g,
after which the supernatant was discarded. The myocytes were skinned by suspending the pellet of cells for 3 min in 0.5 % ultrapure Triton X-100 (Pierce Chemical Co.) in relaxing solution. The skinned cells were washed twice with cold relaxing solution, suspended in 10-15 ml of relaxing solution and kept on ice during the day of the experiment. Myocytes were used within 12 hours of isolation.

**Solutions**

Relaxing solution in which the ventricles were disrupted, skinned, and resuspended contained (in mmol/L): EGTA 2, MgCl2 5, ATP 4, imidazole 10, and KCl 100 at pH 7.0. Compositions of relaxing and activating solutions used in mechanical measurements were as follows (mmol/L): EGTA 7, free Mg2+ 1, imidazole 20, MgATP 4, creatine phosphate 14.5, Ca2+ concentrations of 10^-9 M (relaxing solution) and 10^-4.5 M (maximal activating solution), and sufficient KCl to adjust ionic strength to 180 mM. Starting from a very acidic pH (~4) final solution pH was given by KOH addition to either 6.6 or 7.0. Activating solutions containing P_i were identical to those described above except for inclusion of 5 mM of potassium phosphate (KH2PO4) before adjustment to ionic strength of 180 mM. The final concentrations of each metal, ligand and metal-ligand complex at 13°C were determined with a computer program (31). Immediately preceding activations, muscle preparations were immersed for 60 s in a solution of reduced Ca2+-EGTA buffering capacity, identical to normal relaxing solution except that EGTA is reduced to 0.5 mM. This protocol resulted in more rapid steady state force development and helped preserve the striation pattern during activation.
**Experimental Apparatus, mechanical measurements and data analysis**

See experimental apparatus, mechanical measurements, and data analysis section in Chapter 2.

**SDS-PAGE and MyHC quantification.**

After mechanical measurements, MHC isoform expression was determined for each myocyte preparation as previously described (51). Briefly, myocytes were removed from the experimental apparatus, suspended in 8 µl of SDS sample buffer, and stored at -80°C for subsequent SDS-PAGE analysis. The gels for SDS-PAGE were prepared with 3.5% acrylamide in the stacking gel and 12% acrylamide in the resolving gel. Samples were separated by SDS-PAGE at constant voltage (250 V) for 8.0 h. Gels were initially fixed in an acid-alcohol solution, followed by glutaraldehyde fixing. MyHC isoforms were visualized by ultrasensitive silver staining, and gels were subsequently dried between mylar sheets. The relative expression of each MyHC isoform was determined using QuantiScan (Biosoft) software and an Epson scanner to measure the relative intensity and area of each MyHC band.

**Statistics**

One–way repeated measures ANOVA were used to determine significant effects on force, absolute and normalized power output, and $k_0$ from varied $P_i$ and $H^+$ solutions. The Student-Newman-Keuls test was used post hoc to assess the differences among means. Student t-tests were used to assess differences in metabolite effect between myocytes.
expressing α-MyHC or β-MyHC. $P < 0.05$ was chosen as indicating significance. Values are expressed as means ± SD unless otherwise indicated.

**RESULTS**

**Myocyte characteristics and PTU induced changes in MyHC expression**

PTU treatment of rats has been employed frequently to induce MyHC isoform switching with minimal changes in other myofilament protein expression or overall myocyte morphology (51, 59, 123). Addition of PTU to the drinking water decreased the expression of α-MyHC protein while increasing β-MyHC isoform expression. MyHC content of myocytes used in mechanical experiments was determined by SDS-PAGE separation; an example of MyHC separation gels is shown in figure 1B. Myocytes from non-PTU treated animals expressed 91 ± 4% α-MyHC protein, while those from age matched animals following ~3 weeks of PTU treatment yielded myocytes with 11 ± 5% α-MyHC. Similar to a previous study (51), non-treated and PTU-treated myocytes were the same in length (α-MyHC 148 ± 39 μm; β-MyHC 161 ± 43 μm) and width (α-MyHC 24 ± 9 μm; β-MyHC 22 ± 4 μm). Resting sarcomere length (SL) of the preparations when set to yield passive forces near zero was 2.33 ± 0.07 μm and 2.25 ± 0.04 μm in α-MyHC and β-MyHC myocytes, respectively, and was not altered by maximal Ca$^{2+}$ activation (i.e., pCa 4.5) (α-MyHC 2.32 ± 0.08 μm; β-MyHC 2.26 ± 0.05 μm), which is indicative of low compliance at the points of myocyte attachment.
Effects of ischemic conditions on mechanical properties of cardiac myocytes

Previous experiments on skinned cardiac trabeculae (69, 70, 103) and myocytes (58, 150) have shown increased [P$_i$] and/or [H$^+$] decreased maximal Ca$^{2+}$ activated force. Comparable force and declines in force were observed in this study during addition of P$_i$ and H$^+$ alone and together, with α-MyHC and β-MyHC myocytes having equal decreases. Force and force per cross-sectional area in rat skinned cardiac myocytes are summarized in Tables 3.1 (α-MyHC myocytes) and 3.2 (β-MyHC myocytes). In α-MyHC myocytes maximal Ca$^{2+}$ activated force per cross-sectional area was 62%, 62% and 40% of control force in solutions containing 5mM P$_i$ (solution $ii$), at pH 6.6 (solution $iii$), and 5mM P$_i$ at pH 6.6 (solution $iv$), respectively. β-MyHC myocytes produced 58%, 59% and 35% of maximal Ca$^{2+}$ activated tension at pH 7.0, 0mM P$_i$ with (solution $ii$) 5mM P$_i$, (solution $iii$) pH 6.6, and (solution $iv$) 5mM P$_i$ at pH 6.6, respectively. These results indicate that P$_i$- and H$^+$-induced decreases in force are independent of mammalian MyHC isoform.

Force-velocity and power-load relationship characteristics are given in Tables 3.1 and 3.2, for α-MyHC and β-MyHC myocytes, respectively. Figure 3.1 displays a representative absolute force-velocity and power-load relationship from a non-PTU treated myocyte (i.e., α-MyHC myocyte) under all four of the experimental conditions. Addition of P$_i$ or lowering the pH of the activator solution resulted in a leftward shift of the absolute force-velocity relationship (Figure 3.1) because of lower isometric force. Also, even though addition of 5mM P$_i$ and the lowering of the pH to 6.6 reduced force to the same extent, there was significantly greater peak absolute power output with P$_i$ than with lowered pH (bottom of
Table 3.1. Effects of Pi addition and lowered pH on force-velocity and power-load properties of α-MyHC cardiac myocytes.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Maximum Force (kN•m⁻²)</th>
<th>Peak absolute power output (μW•mg⁻¹)</th>
<th>a/Po</th>
<th>F,opt (P/Po)</th>
<th>V,opt (ML•s⁻¹)</th>
<th>Peak normalized power output (P/P₀,ML•s⁻¹)</th>
<th>k,ir (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.0, 0mM</td>
<td>25.3 ± 12.5</td>
<td>2.68 ± 1.3</td>
<td>0.31 ± 0.12</td>
<td>0.32 ± 0.03</td>
<td>0.35 ± 0.06</td>
<td>0.114 ± 0.02</td>
<td>6.1 ± 1.5</td>
</tr>
<tr>
<td>pH 7.0, 5mM</td>
<td>15.6 ± 6.6*</td>
<td>2.14 ± 1.0*</td>
<td>0.46 ± 0.16*</td>
<td>0.35 ± 0.02</td>
<td>0.41 ± 0.08*</td>
<td>0.145 ± 0.02*</td>
<td>10.5 ± 4.8*</td>
</tr>
<tr>
<td>pH 6.6, 0mM</td>
<td>15.7 ± 8.1*</td>
<td>1.65 ± 0.7*</td>
<td>0.36 ± 0.13</td>
<td>0.33 ± 0.03</td>
<td>0.35 ± 0.06†</td>
<td>0.115 ± 0.02†</td>
<td>7.2 ± 1.9†</td>
</tr>
<tr>
<td>pH 6.6, 5mM</td>
<td>10.0 ± 4.6†‡</td>
<td>1.26 ± 0.6*</td>
<td>0.28 ± 0.09†</td>
<td>0.32 ± 0.03†</td>
<td>0.42 ± 0.07*‡</td>
<td>0.132 ± 0.02*‡</td>
<td>13.4 ± 7.3*‡</td>
</tr>
</tbody>
</table>

Values are mean ± SD (n = 9). Differences were considered significant at P < 0.05. P₀, isometric force. F,opt, force at which peak power occurs. V,opt, velocity at peak power. ML, muscle length. * significantly different than pH 7.0, 0mM Pi. † significantly different than pH 7.0, 5mM Pi. ‡ significantly different than pH 6.6, 0mM Pi.
### Table 3.2. Effects of P_i addition and lowered pH on force-velocity and power-load properties of β-MyHC cardiac myocytes.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Maximum Force (kN•m⁻²)</th>
<th>Peak absolute power output (µW•mg⁻¹)</th>
<th>a/P_0</th>
<th>F_{opt} (P/P_o)</th>
<th>V_{opt} (ML•s⁻¹)</th>
<th>Peak normalized power output (P/P_o•ML•s⁻¹)</th>
<th>k_r (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.0, 0mM</td>
<td>30.1 ± 9.2</td>
<td>1.48 ± 0.7</td>
<td>0.10 ± 0.06</td>
<td>0.22 ± 0.05</td>
<td>0.21 ± 0.04</td>
<td>0.049 ± 0.01</td>
<td>2.5 ± 1.3</td>
</tr>
<tr>
<td>pH 7.0, 5mM</td>
<td>17.3 ± 5.0*</td>
<td>1.46 ± 0.5</td>
<td>0.18 ± 0.07*</td>
<td>0.28 ± 0.03*</td>
<td>0.30 ± 0.04*</td>
<td>0.085 ± 0.02*</td>
<td>3.8 ± 1.4</td>
</tr>
<tr>
<td>pH 6.6, 0mM</td>
<td>17.8 ± 5.1*</td>
<td>1.28 ± 0.4</td>
<td>0.11 ± 0.04†</td>
<td>0.24 ± 0.02</td>
<td>0.30 ± 0.05*</td>
<td>0.070 ± 0.01†</td>
<td>3.1 ± 1.0</td>
</tr>
<tr>
<td>pH 6.6, 5mM</td>
<td>10.6 ± 3.8*†‡</td>
<td>0.92 ± 0.3*†‡</td>
<td>0.13 ± 0.03†</td>
<td>0.25 ± 0.02</td>
<td>0.35 ± 0.06*†‡</td>
<td>0.092 ± 0.02*‡</td>
<td>3.4 ± 1.3</td>
</tr>
</tbody>
</table>

Values are mean ± SD (n = 8). Differences were considered significant at P < 0.05. P_o, isometric force. F_{opt}, force at which peak power occurs. V_{opt}, velocity at peak power. ML, muscle length. * significantly different than pH 7.0, 0mM P_i. † significantly different than pH 7.0, 5mM P_i. ‡ significantly different than pH 7.0, 0mM P_i.
Figure 3.1

A

B

C

D

α-MyHC
β-MyHC

pH 7.0, 0 mM Pi
pH 7.0, 5 mM Pi
pH 6.6, 0 mM Pi
pH 6.6, 5 mM Pi

Shortening Velocity (µm s⁻¹)

Force (µN)

Power Output (pW)

Force (µN)
Figure 3.1 Effect of experimental conditions on absolute force-velocity and power-load relationships in an α-MyHC myocyte.

A. Photographs of a single cardiac myocyte during relaxation (pCa 9.0) and at maximal Ca\(^{2+}\) activation (pCa 4.5). B. Representative silver stained MyHC gel following SDS-PAGE separation. Lanes contain MyHC bands from single myocytes from rats following PTU treatment for 10 days (lane 1), 0 days (lane 2) and 21 days (lane 3). The MyHC pattern in lane 3 is from the myocyte shown in the photographs and whose experimental data is shown in (C) and (D). C. Representative length traces during force clamps of a rat skinned cardiac myocyte preparation during maximal Ca\(^{2+}\) activations in the presence of 0 and 5 mM added P\(_i\) at pH 7.0 and 6.6. D. Absolute force-velocity and power-load relationships of the myocyte in lane 2 above (B) expressing ~95% α-MyHC with each experimental condition. Isometric force decreased equally with addition of 5 mM P\(_i\) or decreasing the pH to 6.6 and fell further with 5 mM P\(_i\) at pH 6.6 all of which shifted their respective force-velocity relationships to the left. Leftward shifting of the force-velocity relationship resulted in less absolute power output at a given force. However, the decline in power was less than the fall in force in the presence of 5 mM P\(_i\) at either pH 7.0 or 6.6.
Figure 3.1). The absolute power output of α-MyHC myocyte preparations fell ~20% with 5mM P_i, ~40% at pH 6.6, and ~50% with 5mM P_i at pH 6.6, all significantly less than power output without additional P_i at pH 7.0. However, the fall in power with additional P_i at either pH 7.0 or 6.6 was less than was expected from the fall in force whereas the fall in power at pH 6.6 without added P_i was equivalent to the fall in force. The reason for less decline in power output than force with P_i (at pH 7.0 or 6.6) in α-MyHC myocytes is more easily seen with force-velocity relationships normalized to isometric force. Normalized α-MyHC myocyte force-velocity relationships (Figure 3.2) reveal an increase in the velocity of shortening occurring with addition of P_i while increased H^+ alone had no effect on loaded shortening velocity. The increase in loaded shortening velocity increased power at all loads greater than ~10% isometric force in all α-MyHC myocytes, with normalized peak power output being ~30% greater with 5mM P_i, unchanged at pH 6.6, and 16% greater with 5mM P_i at pH 6.6. Interestingly, the P_i-induced increase in loaded shortening velocity was attenuated in combination with elevated H^+.

In contrast to α-MyHC, β-MyHC myocyte absolute power generating capacity was not diminished by either 5mM P_i or pH 6.6, however, power did fall when applied in combination (Table 3.2). The normalized force-velocity relationships of β-MyHC myocytes (Figure 3.3) reveal increases in the velocity of shortening occurring with addition of P_i and H^+ alone and in combination. Moreover, these increases were much greater than those observed in α-MyHC myocytes with 5mM P_i producing a 73% increase, pH 6.6 a 43% increase, and 5mM P_i at pH 6.6 a 88% increase in peak normalized power output. These increases in loaded shortening velocity provided the basis for the preserved absolute power
Figure 3.2. Normalized force-velocity and power-load relationships from α-MyHC myocytes.

Force-velocity and normalized power-load curves shifted upward in response to added Pi while there was no effect with lower pH in α-MyHC myocytes (n = 9). Upward shifts indicating increases in loaded shortening velocity occurred with Pi and helped to maintain power generating capacity. Points are mean ± S.E. of the combination of all data points at similar forces.
generating capacity of β-MyHC myocytes. Also of interest, the P$_i$-induced increase in loaded shortening velocity was augmented by added H$^+$ in β-MyHC myocytes, which was opposite that seen in α-MyHC myocytes. Larger increases in loaded shortening velocity with metabolites in β-MyHC myocytes than α-MyHC myocytes resulted in similar power generating capacity. Normally, as with control conditions, α-MyHC myocytes generate more power than β-MyHC myocytes, however addition of P$_i$ or H$^+$ removed this difference resulting in similar power production.

Finally, the rate constant of force redevelopment ($k_{tr}$) was assessed in α- and β-MyHC myocytes with each experimental condition. P$_i$ addition increased $k_{tr}$ in α-MyHC myocytes at both pH 7.0 and 6.6, while lowered pH alone did not affect force redevelopment rates (Table 3.1). Similar alterations in $k_{tr}$ with P$_i$ and H$^+$ have previously been reported for α-MyHC myocytes (4, 58). On the other hand, β-MyHC myocyte force redevelopment rates were not statistically significantly different with P$_i$ or H$^+$ addition (Table 3.2) however there was a trend for P$_i$ addition at pH 7.0 to increase $k_{tr}$ ($p = 0.07$).

**DISCUSSION**

This study directly examined the effect of increases in P$_i$ and H$^+$ on cardiac myocyte force production, loaded shortening velocity, power output and rates of force development. Moreover, experiments utilized myocytes expressing predominantly either α-MyHC or β-MyHC to ascertain if there is a cardiac MyHC dependent response to ischemic metabolite
Figure 3.3. Normalized force-velocity and power-load relationships from β-MyHC myocytes.
Force-velocity and normalized power-load curves shifted upward in response to added $P_i$ while there was no effect with lower pH in β-MyHC myocytes ($n = 8$). Upward shifts indicating increases in loaded shortening velocity occurred with $P_i$ and $H^+$ helped to maintain power generating capacity. Points are mean ± S.E. of the combination of all data points at similar forces.
concentration. The main findings of this study were as follows: 1) maximal Ca\(^{2+}\) activated tension decreased comparably in \(\alpha\)-MyHC and \(\beta\)-MyHC myocytes with Pi and lowered pH both alone and together; 2) Pi addition increased loaded shortening velocity in both \(\alpha\)-MyHC and \(\beta\)-MyHC myocytes; 3) lowered pH had no effect on velocity of shortening in \(\alpha\)-MyHC myocytes but increased loaded shortening velocity in \(\beta\)-MyHC myocytes; and 4) power generating capacity of \(\alpha\)-MyHC myocytes fell to a greater extent with Pi and H\(^+\) than \(\beta\)-MyHC myocytes resulting in similar power output between \(\alpha\)-MyHC and \(\beta\)-MyHC myocytes during Ca\(^{2+}\) activations in the presence of Pi and lowered pH separately and together. From this it can be concluded that MyHC does not affect the force response to metabolite accumulation in cardiac myocytes, however MyHC is a determinant of the effect on loaded shortening and power output. The differential effects of these conditions on shortening velocity between \(\alpha\)- and \(\beta\)-MyHC myocytes may represent a mechanism whereby the heart compensates to maintain function during pathological conditions (repeated ischemia or congestive heart failure) by expressing the more tolerant MyHC isoform (\(\beta\)-MyHC).

**MyHC as a determinant of force-velocity and power-load properties of cardiac myocytes during simulated ischemic conditions.**

The inhibitory effects of ischemic metabolites on force production in muscle have been investigated extensively in skinned skeletal and cardiac preparations. Some of these studies have provided the possibility of an inverse relationship between preparation diameter and force decline (70, 139) which is thought to exist because of lower Pi accumulation from myofibrillar ATPases in thinner preparations during Ca\(^{2+}\) activation. Importantly though, this would indicate that the scale of ATPase activity may also be a determinant of force
Figure 3.4. Comparative plots of force and power production between α- and β-MyHC myocytes.

A. Effect of P\textsubscript{i} and decreased pH on myocyte force production in α-MyHC and β-MyHC myocytes. Values are scaled to the value at pH 7.0 with no added P\textsubscript{i}. Force declined similarly between myocyte populations expressing either α-MyHC or β-MyHC. B. Absolute power production between α-MyHC and β-MyHC myocytes. α-MyHC myocyte power production while greater than β-MyHC myocyte power with no added P\textsubscript{i} at pH 7.0, fell to comparable levels as β-MyHC myocytes with P\textsubscript{i} or H\textsuperscript{+} alone or in combination.
sensitivity to metabolites, meaning a more active ATPase will result in greater accumulation (at a given diameter). This is in agreement with some recent data showing more responsiveness in fast-twitch compared to slow-twitch skeletal muscle fibers (94, 103, 146). However, this idea remains controversial as studies have also reported greater metabolite sensitivity in slow-twitch fibers (23) or no difference (113) between fiber type force response. The disparity of these results may lie in experimental solutions or conditions, such as temperature as force sensitivity to metabolites is temperature dependent (23).

Nevertheless the potential difference between skeletal fiber-type sensitivity to metabolites suggests the potential for a MyHC-dependence of force response to Pi and H+ in cardiac muscle. In vertebrates, two MyHC isoforms are expressed in the myocardium, α-MyHC and β-MyHC, with considerable homology containing 93% identical amino acids (88), but are functionally distinct. For example, α-MyHC exhibits two to three times the actin-activated ATPase activity (82) and generates ~3 times more power than β-MyHC (51). However, analysis of differential force response to Pi and H+ with cardiac MyHC content has not been addressed to our knowledge. Comparison of results from some studies may offer potential support for a MyHC dependence of force response in cardiac muscle. For example, van der Velden et al. (150) reported a ~35% decline in force per decade of Pi in human donor and heart failure ventricular myocytes, both of which likely express β-MyHC primarily, while others have reported larger (45-65%) declines per decade in rat cardiac preparations expressing primarily α-MyHC (27, 58, 69). Yet direct comparison of these results may be errant as they were collected under different experimental conditions with preparations of varying size, which as discussed above, may be an important variable in the response to metabolite accumulation. Though, in regards to differential Pi effect due to size, our previous
work (58) and the van der Velden study (150) both utilized single ventricular cardiac myocytes and yet produced very different force response to 10 mM P_i, 35% and 65% declines in α-MyHC and β-MyHC myocytes, respectively. The reason for this difference is unclear at this time but may be the result of differential myofibrillar protein phosphorylation status as proposed (150), solutions used, myocyte preparation or myofilament protein expression. As for the response of cardiac myocytes to acidosis, Solaro et al. showed that neonatal myocardium is less sensitive to acidosis than adult myocardium (133). However, the authors concluded the change in sensitivity of force production observed was the result of a shift in troponin I isoform from the slow skeletal isoform in neonates to the cardiac isoform in adults, although heavy chain expression was not completely characterized. In this study an equivalent drop in force was observed between α-MyHC and β-MyHC myocytes with Ca^{2+}-activating solutions at lower pH or containing P_i implying cardiac MyHC independence to these conditions (Figure 3.4). This supports that ATPase rate, and consequent metabolite production, is not a major determinant of metabolite sensitivity of force at least in single myocyte preparations where the diameter is small and equivalent. Finally, the combined effect of P_i and H^+ on force production was comparable in α-MyHC and β-MyHC myocytes and while additive there was no synergistic effect (whole greater than the sum of the parts) in combination, in agreement with previous studies in cardiac muscle (69, 103, 150).

In this study P_i and H^+ affected loaded shortening differentially in myocytes expressing α-MyHC and β-MyHC. α-MyHC myocytes displayed (1) faster loaded shortening velocity with P_i, (2) no effect with H^+ itself, and (3) an attenuated P_i effect on velocity when applied in combination with H^+. Alternatively, β-MyHC myocytes (1)
exhibited greater increases in loaded shortening than α-MyHC myocytes with P_i, (2) were also faster with H^+ and (3) displayed faster loaded shortening when applied in combination than either alone (but not to a statistically significant level). It was this greater effect of P_i and H^+ on β-MyHC myocyte loaded shortening velocity that maintained the power generating capacity of the myocyte even with the fall in force. Maintenance of power generating capacity may be important functionally during the ejection phase of the cardiac cycle when the ventricle is shortening against a load and generating power. If the velocity of shortening is sustained at a given load, then the extent to which the myocytes shorten and consequently the ventricles contract during a heartbeat will remain similar, thereby alleviating any fall in ejection volume during ischemia. Under similar Ca^{2+} activated conditions α-MyHC myocytes can generate more power and thus shorten faster at a given load than β-MyHC myocytes. However, the difference in power capacity between α-MyHC and β-MyHC myocytes is diminished with increased P_i and/or H^+ to the point where essentially there is no difference in power generation between α-MyHC and β-MyHC myocytes with either P_i or H^+ alone or in combination (Figure 3.4). Furthermore, increases in metabolites to concentrations greater than employed in this study may actually result in β-MyHC myocytes maintaining their functional capacity while α-MyHC myocytes continue to decline. We were unable to test this idea as force production falls to levels that become experimentally limiting in our myocyte preparation.

Our earlier work (58) demonstrated an increase in loaded shortening velocity of cardiac myocytes with increased [P_i]; here we extend that work by reporting the effects of P_i addition and lowered pH individually and in combination, which commonly occurs during
ischemia *in vivo*, in α- and β-MyHC myocytes. The P\(_i\)-induced increase in loaded shortening velocity occurred in both α- and β-MyHC myocytes resulting in greater peak power output when normalized for the fall in isometric force. P\(_i\) addition is thought to decrease force production by shifting populations of cross-bridges from a force-generating state in which the actomyosin complex has ADP alone bound (AM-ADP) to a weakly or strongly bound lower-force generating state in which both ADP and P\(_i\) are bound (AM-ADP-Pi) (53). P\(_i\) addition also speeds the kinetics of transition through the force-generating steps leading to P\(_i\) release (4). We previously concluded that loaded shortening and power output at intermediate and high loads are determined by force-generating transitions coupled to P\(_i\) release and/or an isomerization that is in rapid equilibrium with P\(_i\) release from the actomyosin complex (58).

On the other hand, H\(^+\) alone had no effect in α-MyHC myocytes, but it attenuated the P\(_i\)-induced speeding of loaded shortening velocity when used in combination with P\(_i\). Our result of lowered pH having no effect on loaded shortening velocity contrasts a previous report where velocity decreased (120). The reason for the discrepancy between results is not clear but it may be due to preparation size as the trabeculae preparation used in that study had greater than 20 times cross-sectional area of our myocyte preparation, or differences in solution composition. Attenuation of the P\(_i\) effect by lower pH that occurred may be the result of H\(^+\) reducing the pool of strongly-bound cross-bridges by driving them to a weakly bound state, which has been postulated previously (69). Alternatively, it may be that H\(^+\) directly slows P\(_i\) release from the ATP binding cleft, which may also slow loaded velocity.

In contrast to α-MyHC myocytes, lowered pH did not attenuate the P\(_i\)-induced increase in loaded shortening velocity in β-MyHC myocytes; in fact loaded shortening was augmented toward faster velocities at the same load but this difference did not reach significance.
Augmentation of loaded shortening may be the consequence of H$^+$ speeding P$_i$ release in β-MyHC myocytes. Nevertheless there is a MyHC dependent difference in response to P$_i$ at a lower pH in cardiac myocytes and the sub-molecular mechanisms are unknown.

Rate of force development increases with P$_i$ in both cardiac myocytes (4, 58) and skeletal muscle fibers (92) while no change occurs with pH in skeletal muscle fibers during maximal Ca$^{2+}$ activation (91). Importantly, it is the rate of force development that likely determines how quickly the ventricles complete isovolumic contraction (phase 2 of the cardiac cycle) allowing the remaining time of systole for ejection (phase 3). α-MyHC myocytes develop force 2-3 times more quickly than do β-MyHC at maximal Ca$^{2+}$ activation (here and (40)), which likely allows for the ejection phase to commence earlier and last longer in working ventricles. In addition, α-MyHC myocytes appear to benefit from this P$_i$-induced increase in force development to a greater extent than β-MyHC myocytes. This may ultimately mean that even though α-MyHC myocyte loaded shortening is more greatly attenuated by ischemic metabolites global ventricular function may be maintained as faster isovolumic allows for more ejection time.

**Possible implications for impaired function in heart disease**

Myocardial tissue exposed to ischemic conditions has been shown to have greater relative expression of β-MyHC in adult rat cardiac myocytes than control tissues (167, 169). Expression of β-MyHC in myocytes reduces their power generating capacity (51) but in doing so these myocytes appear to become both more economical (123) and more resistant to the effects of ischemic metabolites (this study). Thus, β-MyHC appears to provide more
resistance to falls in energy charge as it produces force at a much smaller energy cost (123) and faster loaded shortening velocity with increased metabolites. In agreement with this, post-ischemic function was greater in hearts expressing some (~25%) β-MyHC as compared to control rat hearts (10). Theoretically, myocardium exposed to repeated bouts of ischemia may attempt to offset reductions in myocyte function occurring as a result of lowered [ATP] or free energy of ATP hydrolysis by expressing the more economical isoform of MyHC thus conserving ATP as less is consumed at the myofilaments. Decreased actomyosin activity and ATP conservation may also be important as a decrease in [ATP] leading to a decrease in ATPase activity, specifically ones associated with Ca\(^{2+}\)-extrusion (SR and sarcolemmal Ca\(^{2+}\) ATPases), is thought to be causal for apoptosis and more chronic dysfunction (as reviewed by (5, 12)). In addition to β-MyHC expression conceivably being protective to myocardium that is frequently made ischemic, increased β-MyHC expression also commonly occurs during the progression to heart failure experimentally and clinically (119, 167, 169). Thus, β-MyHC expression also appears to be important in the transition to a compensated heart in response to pathophysiological stimuli perhaps by preserving myocardial function during energetic challenge.
Chapter 4. PORCINE CARDIAC MYOCYTE POWER OUTPUT IS INCREASED FOLLOWING CHRONIC EXERCISE TRAINING

ABSTRACT

Chronic exercise training increases the functional capacity of the heart perhaps by increased myocyte contractile function, as has been observed in rodent exercise models. We examined whether cardiac myocyte function is enhanced following chronic exercise training in Yucatan miniature swine, whose heart characteristics are similar to humans. Animals were designated as either sedentary (SED), cage confined, or exercise trained (EX), underwent 16 to 20 weeks of progressive treadmill training. Exercise training efficacy was shown with significantly increased heart weight to body weight ratios, skeletal muscle citrate synthase activity, and exercise tolerance. Force-velocity properties were measured by attaching skinned cardiac myocytes between a force transducer and position motor and shortening velocities were measured over a range of loads during maximal Ca\(^{2+}\) activation. Myocytes from EX pigs (n = 9) had comparable force production but a ~30% increase in peak power output compared to SED myocytes (n = 8). Interestingly, EX myofibrillar samples also had higher baseline PKA-induced phosphorylation levels of myosin binding protein-C (MyBP-C) and cardiac troponin I (cTnI), which may contribute to the increase in power. Overall, these results suggest that enhanced power generating capacity of porcine cardiac myofibrils contributes to improved cardiac function following chronic exercise training.
INTRODUCTION

Chronic exercise is known to improve cardiac function as indexed by increases in maximal levels of cardiac output (CO) and stroke volume (SV) (125). While increased CO is an ultimate goal of exercise training, enhanced SV is a primary adaptation given the fact lower heart rates (HR) occur at a given workload following chronic exercise training (29). The increase in stroke volume is thought to be due, in part, to training-induced alterations of the intrinsic contractile function of the myocardium (36, 66, 95). Alterations of contractile function following exercise are accompanied by increases in force production due to myocyte hypertrophy (101), increased Ca\textsuperscript{2+} sensitivity of force (26), and possibly increased shortening velocities (163). There also have been a few studies that focused on the effects of exercise training on the entire force-velocity relationship as it is velocity of shortening under a load that is most applicable to in vivo ventricular function. Two studies utilizing a rat model of chronic exercise training reported increased loaded shortening velocities in myocardial preparations from trained animals (25, 95). However, alterations in loaded shortening have not been investigated in other animal models, specifically larger animal models such as swine that may more closely mimic human physiology.

The purpose of this study was to evaluate cardiomyocytes from a pig model of exercise training for mechanical changes intrinsic to the myofilaments. The use of a porcine model of exercise training to investigate physiological changes that occur in large mammals may be applicable to humans as pigs and humans have similar heart morphology and adaptive responses to chronic exercise with respect to maximal oxygen consumption (VO\textsubscript{2max}), SV, HR and skeletal muscle oxidation capacity (for review see (87)). This model
previously has been shown to produce increased exercise tolerance, moderate cardiac hypertrophy, increased coronary blood flow capacity, increased oxidative capacity of skeletal muscle and lower heart rates during exercise at submaximal intensities (77). These changes occurred without any apparent alterations in myofibrillar ATPase, Ca\(^{2+}\) regulatory systems, or the metabolic system (77). Since CO was sustained with lower HR at submaximal workloads, this implies increased SV. Increased SV could be the result of a number of things including less aortic resistance, greater ventricular volume, and/or increased myocardial contractility. The main objective of this study was to determine if cardiac myofibrillar contractility differs between sedentary (SED) and exercise trained (EX) pig myocytes to account, at least in part, for the increased SV. Contractile function was assessed in permeabilized cardiac myocyte preparations by measuring isometric force, loaded shortening velocity, power output and rate of force development during maximal Ca\(^{2+}\) activation. Another objective was to determine if alterations in cardiac myofibrillar proteins may account for any observed functional differences. Specifically, we examined relative myosin heavy chain (MyHC) isoform contents, relative cardiac troponin T (cTnT) isoform contents, and PKA-induced baseline phosphorylation of the myofibrillar proteins myosin binding protein-C (MyBP-C) and cardiac troponin I (TnI).

**METHODS**

**Animal Model**

Adult male miniature swine weighing 25-40 kg were obtained from the breeder (Charles River) and familiarized with treadmill exercise over a 1- to 2-wk period of time.
Treadmill performance tests were administered to each animal to evaluate exercise tolerance as previously described (77). Pigs were randomly assigned to SED or EX groups, with the EX group completing the 16- to 20-wk endurance training program described previously (77, 78). Pigs assigned to the SED group were restricted to their enclosures (2 x 4 m) and did not exercise. Treadmill performance tests were again completed at the end of 18-20 weeks to determine the effectiveness of the training protocol on exercise duration. The overall efficacy of the training program was determined by comparing the exercise tolerance (as reflected in the treadmill performance test), heart weight-to-body weight ratios, and skeletal muscle oxidative capacity of trained and control groups. At the time of sacrifice, samples were taken from the middle of deltoid muscles, frozen and stored at -70°C for citrate synthase analysis. Citrate synthase activity was measured from whole muscle homogenate using the spectrophotometric method of Srere (137). Animal care and use procedures complied with the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health and were approved by the University of Missouri Animal Care and Use Committee.

**Isolation of cardiac myocytes**

The heart was excised from the experimental animal following administration of a pre-anesthetic mixture of ketamine (35 mg/kg) and xylazine (2.25 mg/kg) and the general anesthesia thiopental (25 mg/kg). A piece (3 x 3 cm) of left ventricular free wall near the left anterior descending coronary artery was removed with one half quick frozen in liquid nitrogen for biochemical analyses and the other half placed in ice cold relaxing solution for myocyte experiments. The piece in relaxing solution was cut into smaller pieces (2-3 mm) and homogenized with a hand blender. The resultant slurry was transferred via pipette into
15 mL tubes that were centrifuged 75 sec at 165 x g after which the supernatant was discarded. The cells were suspended for 3 min in 0.5% ultra pure Triton X-100 (Pierce Chemical Co.) in relaxing solution. The skinned cells were washed twice with cold relaxing solution, centrifuged, and suspended in 10-15 ml of relaxing solution and kept on ice during the day of the experiment. Myocytes were used within 12 hours of isolation.

**Solutions**

Relaxing solution in which the ventricles were disrupted, skinned, and resuspended contained (in mmol/L): EGTA 2, MgCl$_2$ 5, ATP 4, imidazole 10, and KCl 100 at pH 7.0. Compositions of relaxing and activating solutions used in mechanical measurements were as follows (mmol/L): EGTA 7, free Mg$^{2+}$ 1, imidazole 20, MgATP 4, creatine phosphate 14.5, pH 7.0, Ca$^{2+}$ concentrations of $10^{-9}$ M (relaxing solution) and $10^{-4.5}$ M (maximal activating solution), and sufficient KCl to adjust ionic strength to 180 mM. The final concentrations of each metal, ligand and metal-ligand complex at 12°C were determined with the computer program of Fabiato (31). Immediately preceding activations, muscle preparations were immersed for 60 s in a solution of reduced Ca$^{2+}$-EGTA buffering capacity, identical to normal relaxing solution except that EGTA is reduced to 0.5 mM. This protocol resulted in more rapid steady state force development and helped preserve the striation pattern during activation.

**Experimental Apparatus, Mechanical measurements, and Data analysis.**

See Chapter 2.
**SDS-PAGE, Western blot and Autoradiography.**

After mechanical measurements, MHC isoform expression was determined for each myocyte preparation. The single myocyte was removed from the experimental apparatus, suspended in 8 µl of SDS sample buffer, and stored at -80°C for subsequent SDS-PAGE analysis. The gel electrophoresis procedure was similar to one previously described (93). The gels for SDS-PAGE were prepared with 3.5% acrylamide in the stacking gel and 12% acrylamide in the resolving gel. Samples were separated by SDS-PAGE at constant voltage (250 V) for 6.5 h. Gels were initially fixed in an acid-alcohol solution, followed by glutaraldehyde fixing. MHC isoforms were visualized by ultrasensitive silver staining, and gels were subsequently dried between mylar sheets.

Western blot analyses were completed as previously described (74). Briefly, serial dilutions (2, 1, 0.5 µg) of left ventricular homogenates were prepared in SDS sample buffer and separated by SDS-PAGE as described above except the runtime was reduced to 3 hours. Gels were placed on nitrocellulose membrane and transferred from the gels to nitrocellulose using a semidry blot apparatus for 45-60 min at constant current (100 mA). The nitrocellulose membranes then were placed in a blocking buffer (3% BSA in Tris-buffered saline plus Tween 20 (TTBS)) and rocked overnight at 4°C. The blocking buffer was removed, and membranes washed in TTBS. The primary cTnT antibody (Advanced Immunochemical) (cTnT 1:2,000 in 0.6% BSA in TTBS) was allowed to react with membranes for 1 h followed by washing in TTBS. Secondary antibody (S-adenosyl-L-methionine-IgG 1:2,500 in 0.3% BSA in TTBS) reacted for 1 h followed by three washes using TTBS. On completion of the final wash, membranes were coated 1 min with enhanced
chemiluminescent substrate (Amersham) that reacts with the secondary antibody. Membranes were placed between two pieces of clear acetate and exposed to photography film for ~1 min, followed by film development to detect relative amounts of cTnT protein isoforms. Relative amounts of each isoform were determined by measuring the areas under the peaks using QuantiScan (Biosoft) software and an Epson scanner.

To determine the PKA-induced phosphorylation status of the myofibrillar substrates, myofibrillar samples were incubated with the catalytic subunit of PKA in the presence of radiolabeled ATP, separated by SDS-PAGE, and visualized by autoradiography as described previously (73). Briefly, skinned cardiac myocytes (10 µg) were incubated with the catalytic subunit of PKA (3 to 5 µg/mL) and 50 µCi [γ-32P]ATP for 30 minutes. The reaction was stopped by the addition of electrophoresis sample buffer and heating at 95°C for 3 minutes. The samples were then separated by SDS-PAGE, silver stained, dried, and subsequently exposed to x-ray film for visualization. Individual radiolabeled protein bands were then excised from the gel and quantified using a scintillation counter (Packard 1900 TR). Stoichiometric cTnI phosphate incorporation was calculated using the following equation:

\[
\text{cpm cTnI}/[\text{cTnI}] \cdot [\text{P_i}]/\text{cpm total} = [\text{P_i}]/[\text{cTnI}],
\]

where all concentrations are in moles and cpm is counts per minute. MyBP-C phosphate incorporation was assessed in the same manner.

**Statistics**

Comparisons between sedentary and exercised samples for force, normalized and absolute power outputs, \(k_{tr}\), and all biochemical analysis were made using a Student t-test. \(p\)
RESULTS

Animal Model

Exercise trained miniature Yucatan swine had lower body weight (BW) than sedentary counterparts (~20%; \( p = 0.003 \)) with no difference in heart weight (HW) (Table 4.1). However, because of the reduction in body weight there was a ~25% increase in HW to BW ratio (HW:BW) \( (p = 0.005) \). In addition to increased HW:BW the efficacy of the training protocol was characterized by an increase in exercise tolerance, as indexed by an increase in time to exhaustion during treadmill performance tests, and increased deltoid muscle citrate synthase activity (Table 4.1). Taken together these central and peripheral markers of training provide confirmation that the treadmill training protocol was effective in producing a trained state.

Myocyte Studies

Ventricular hypertrophy often occurs in animals undergoing stringent training paradigms and is commonly due to the enlargement and elongation of individual myocytes (for review see (96)). Our myocyte preparations were not different in length, width or overall volume (Table 4.1). However, the possibility of enlargement or elongation is possible as a quantitative assessment of a large number of enzymatically isolated single myocytes was not performed in our study. Nevertheless, mechanical measurements are expressed relative to myocyte size when applicable to correct for any size-induced
variability. Myocyte preparation mechanical measurements are summarized in table 4.2.

Previous studies in cardiac myocytes have reported either no change (25) or increased

<table>
<thead>
<tr>
<th></th>
<th>Sedentary</th>
<th>Exercise Trained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Wt (kg)</td>
<td>48.0 ± 5.8</td>
<td>38.5 ± 2.5*</td>
</tr>
<tr>
<td>Heart Wt (g)</td>
<td>213.0 ± 24</td>
<td>214.0 ± 34</td>
</tr>
<tr>
<td>Heart Wt / Body Wt</td>
<td>4.44 ± 0.25</td>
<td>5.53 ± 0.62*</td>
</tr>
<tr>
<td>Duration of performance test,</td>
<td>25.2 ± 3.9</td>
<td>23.7 ± 2.8</td>
</tr>
<tr>
<td>initial (min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of performance test,</td>
<td>23.1 ± 3.3</td>
<td>37.3 ± 6.1*</td>
</tr>
<tr>
<td>final (min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate synthase activity</td>
<td>15.4 ± 3.8</td>
<td>23.0 ± 2.6*</td>
</tr>
<tr>
<td>(µmol·min⁻¹·mg protein⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myocyte length (µm)</td>
<td>142.1 ± 36.7</td>
<td>158.1 ± 25.8</td>
</tr>
<tr>
<td>Myocyte width (µm)</td>
<td>20.8 ± 5.7</td>
<td>24.6 ± 6.3</td>
</tr>
<tr>
<td>Myocyte volume (µg)</td>
<td>0.077 ± 0.03</td>
<td>0.073 ± 0.03</td>
</tr>
<tr>
<td>Myocyte SL, pCa 9.0 (µm)</td>
<td>2.46 ± 0.05</td>
<td>2.39 ± 0.08</td>
</tr>
<tr>
<td>Myocyte SL, pCa 4.5 (µm)</td>
<td>2.41 ± 0.08</td>
<td>2.33 ± 0.02</td>
</tr>
</tbody>
</table>

Values are means ± SD. * indicates p < 0.05 vs. Sedentary. SL, sarcomere length.
maximal force (163) following exercise training. In this study, we found no difference in absolute force production between myocytes from SED (28.6 ± 10 kN·m²) and EX (28.4 ± 13 kN·m²) animals at pCa 4.5.

The primary aim of the study was to assess differences in power output between myocytes from sedentary and exercise trained Yucatan miniature swine. We found peak absolute power output in myocytes from exercise trained animals to be ~57% higher than myocytes from sedentary animals (SED = 0.42 ± 0.2 µW/mg vs. EX = 0.66 ± 0.2 µW/mg, p = 0.02). Figure 4.1 shows a representative force-velocity and power-load relationship of skinned cardiac myocytes taken from both exercise trained and sedentary myocardium. The force-velocity relationship (shown in the top panel) displays similar maximal force production between myocytes, but the myocyte from exercise trained myocardium has less curvature of the force-velocity relationship resulting in faster rates of shortening at intermediate loads. The bottom panel shows the power-load relationship for these two preparations. The EX myocyte had higher power output at most loads with the greatest difference occurring at peak power output. The inset graph shows peak power output points from each SED and EX myocyte, with EX preparations exhibiting enhanced power generating capacity.

Another way to observe changes in velocity of shortening and power output is to normalize the data to the maximal isometric force of each preparation; this allows assessment of shortening velocity at a given load. Interestingly, the force-velocity relationship has
Table 4.2. Myocyte mechanical properties from sedentary and exercise trained miniature Yucatan swine.

<table>
<thead>
<tr>
<th></th>
<th>Maximum Force (kN·m(^{-2}))</th>
<th>(F_{\text{opt}}) (P/P(_{o}))</th>
<th>(V_{\text{opt}}) (ML·s(^{-1}))</th>
<th>(a/P_{o})</th>
<th>Peak absolute power output (pW)</th>
<th>Peak absolute power output (mW·mg(^{-1}))</th>
<th>Peak normalized power output (P/P(_{o})·ML·s(^{-1}))</th>
<th>(k_{p}) (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sedentary</strong></td>
<td>28.6 ± 10</td>
<td>0.14 ± 0.04</td>
<td>0.12 ± 0.04</td>
<td>0.022 ± 0.01</td>
<td>25.3 ± 16.0</td>
<td>0.42 ± 0.18</td>
<td>0.016 ± 0.004</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td><strong>Exercise</strong></td>
<td>28.4 ± 13</td>
<td>0.16 ± 0.03</td>
<td>0.15 ± 0.04</td>
<td>0.041 ± 0.02*</td>
<td>43.9 ± 17.1*</td>
<td>0.67 ± 0.20*</td>
<td>0.022 ± 0.004*</td>
<td>1.3 ± 0.5</td>
</tr>
</tbody>
</table>

Values are mean ± SD. Differences were considered significant at p < 0.05. \(P_{o}\), isometric force. \(F_{\text{opt}}\), force at which peak power occurs. \(V_{\text{opt}}\), velocity at peak power. ML, muscle length. * indicates p < 0.05 vs. sedentary value.
Figure 4.1. Representative absolute force-velocity and power-load relationships in myocyte preparations from sedentary and exercise trained pigs. 
A. Absolute force-velocity and power-load relationships of myocytes from (○) sedentary and (●) exercise trained pigs. Isometric force was similar between preparations but loaded shortening velocity was faster in the EX myocyte, resulting in greater peak power output. B. Plot of peak power data points for individual cells from each pig. SED myocytes displayed lower power values than EX myocytes. Bar indicates the mean ± S.D. for data points.
similar loaded shortening velocities at most high and low loads. However, shortening velocity between 10% and 25% relative force was significantly higher in myocytes from exercise trained pigs. These loads correspond to peak power output and are most physiologically relevant as they are thought to be the loads encountered by the heart in vivo. Normalized peak power output also was significantly higher in myocytes from EX pigs (SED 0.016 ± 0.003 P/P₀ ML·s⁻¹, EX 0.022 ± 0.005 P/P₀ ML·s⁻¹). A similar increase in normalized peak power output, as well as a similar decrease in curvature (a/P₀) of the force-velocity relationship, has been reported in rat cardiac myocytes following exercise training (25).

We also addressed the effects of exercise training on the rate of myocyte force development following a mechanical perturbation. The rate of force redevelopment was determined in the presence of maximal Ca²⁺ and is thought to be limited by the rate that cross-bridges activate the thin filament (141). An increase in the rate of force redevelopment would allow more rapid ventricular pressure development and thus more time to be spent in the ejection phase of the cardiac cycle. We found no difference in the rate constant of force redevelopment (kᵣ) between myocytes from sedentary and exercise trained animals (SED 1.6 ± 0.3 ML·s⁻¹; EX 1.3 ± 0.5 ML·s⁻¹), suggesting that the rate of force development independent of Ca²⁺ handling plays a minimal role in augmenting ventricular performance following exercise training.
Biochemical Analysis

MyHC expression was similar between SED and EX left ventricle homogenate samples with the $\beta$-MyHC isoform accounting for all MyHC content. However, some individual myocytes did contain detectable amounts of $\alpha$-MyHC protein indicating that a fraction of cells from the left ventricle expressed $\alpha$-MyHC. All myocytes used in mechanical measurements appeared to contain only $\beta$-MyHC based on SDS-PAGE analysis. In addition, because changes in cTnT isoform content have been implicated in functional changes that occur in a diabetic/dyslipidemic exercise pig model (74), we examined whether relative cTnT isoform contents were different between pig populations. Three distinct isoforms of cTnT were expressed (so named cTnT$_1$, cTnT$_2$, and cTnT$_3$ in accordance with each isoform’s SDS-PAGE migration pattern). Relative cTnT isoform content was similar between SED ($58 \pm 7\%$ cTnT$_1$, $29 \pm 5\%$ cTnT$_2$, and $13 \pm 4\%$ cTnT$_3$) and EX myocardium ($57 \pm 7\%$ cTnT$_1$, $30 \pm 5\%$ cTnT$_2$, and $13 \pm 5\%$ cTnT$_3$).

Basal PKA-induced phosphorylation of myofibrillar proteins was assessed using a back-phosphorylation assay (73). Autoradiograms following SDS-PAGE separation displayed phosphorylation of MyBP-C and cTnI. Stoichiometric analysis of phosphate incorporation in cTnI yielded measurements of $1.2 \pm 0.2$ and $0.7 \pm 0.2$ µmol of $P_i$ / µmol protein ($p < 0.05$) in SED and EX samples, respectively. Decreased $P_i$ incorporation indicates a higher baseline phosphorylation of this protein. $P_i$ incorporation into MyBP-C followed the same trend as cTnI with EX animals having less incorporation than the SED control (SED $2.2 \pm 0.8$ µmol of $P_i$ / µmol protein, EX $1.5 \pm 0.5$ µmol of $P_i$ / µmol protein),
however this difference in phosphorylation was not statistically significant ($p = 0.10$). The disparity between cTnI and MyBP-C may be that PKC is also known to phosphorylate MyBP-C at the same residues as PKA, but at different residues on cTnI. Analysis of PKC-induced phosphorylation could not be performed because of inadequate myocardial sample. The finding of higher baseline cTnI phosphorylation may contribute to increased loaded shortening and power output in EX myocytes since PKA phosphorylation has previously been shown to increase power output in rat myocytes (52).

**DISCUSSION**

**Effects of exercise on force-velocity properties and power output.**

The purpose of this study was to evaluate myocytes from a pig model of exercise training for mechanical changes intrinsic to the myofilaments. We found that myocytes obtained from exercise trained myocardium generated greater peak power output than sedentary counterparts despite producing similar isometric force when normalized to preparation size. This is important as it defines the difference in power output was a consequence of altered cross-bridge interaction kinetics of the myofilaments as opposed to hypertrophy of the cell to increase the quantity of myofilaments.

Absolute power generating capacity of a cardiac myocyte is determined by the number and rate of cycling cross-bridges. The number of cross-bridges strongly bound and in the force-generating state determines maximum force development. Conversely, at very low forces the muscle shortens at very high speeds, with the maximal velocity thought to be limited by the rate of detachment of cycling cross-bridges. It is this relationship between
Figure 4.2

A

Bands for an autoradiogram showing representative phosphate incorporation into MyBP-C and cardiac cTnI after PKA treatment in skinned cardiac myocytes. Lane 1 contains skinned cardiac myocytes obtained from two EX pigs, whereas lane 2 contains myocytes from two SED pigs.

B

Densitometric analysis of MyBP-C and cTnI bands yielded values indicating greater PKA-induced phosphorylation of cTnI from SED samples. Higher levels of phosphate incorporation indicates reduced levels of baseline phosphorylation in SED compared to EX.

<table>
<thead>
<tr>
<th></th>
<th>Myosin Binding Protein-C</th>
<th>Troponin I</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Exercise</strong></td>
<td>1.54 ± 0.5</td>
<td>0.71 ± 0.2</td>
</tr>
<tr>
<td><strong>Trained</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sedentary</strong></td>
<td>2.26 ± 0.8</td>
<td>1.22 ± 0.2*</td>
</tr>
</tbody>
</table>

**Figure 4.2. Effect of PKA-induced phosphorylation on SED and EX myocytes.**

A. Bands for an autoradiogram showing representative phosphate incorporation into MyBP-C and cardiac cTnI after PKA treatment in skinned cardiac myocytes. Lane 1 contains skinned cardiac myocytes obtained from two EX pigs, whereas lane 2 contains myocytes from two SED pigs. B. Densitometric analysis of MyBP-C and cTnI bands yielded values indicating greater PKA-induced phosphorylation of cTnI from SED samples. Higher levels of phosphate incorporation indicates reduced levels of baseline phosphorylation in SED compared to EX.
force and velocity that determine power output. In this study, force was unchanged yet velocity at intermediate loads (10 and 25% isometric) increased in cardiac myocytes from EX pigs, as compared to SED controls. This increase in loaded shortening velocity corresponded to an increase in peak power output. The increase in loaded shortening and power output can be the result of, as stated above, either an increase in the number of force-generating cross-bridges bound at loads around $F_{opt}$ or an increase in the rate of cycling cross-bridges. As there was no observed change in isometric tension between SED and EX myocytes, then it becomes likely that exercise training induced an increase in the rate of cycling of the cross-bridges at intermediate loads.

Previously it has been shown that phosphorylation of the myofibrillar proteins cTnI and MyBP-C by PKA produced a similar increase in absolute power in rat skinned cardiac myocytes (52). However, in that study there were increases in isometric tension and loaded shortening velocities over a greater range of loads following PKA-induced phosphorylation. Although the exact reasons for these differences are not known, they certainly may arise from differences in types of animals, phosphorylation by other kinases or variations in contractile protein isoform expression. Regarding the latter possibility, it may be that MyHC composition imparts dissimilar regulation of cross-bridge kinetics following PKA-induced phosphorylation, resulting in a reduction of loads where cross-bridge cycling is increased. In agreement with this, $\alpha$-MyHC ATPase activity and the rate cycling cross-bridges have been reported to be more responsive to $\beta$-adrenergic stimulation than preparations containing $\beta$-MyHC (60, 161). Regarding possible changes in contractile protein isoforms, it has been shown previously that a small increase in $\alpha$-MyHC protein expression in rat skinned cardiac
myocytes produced a significant increase in power output capacity (51). Additionally, some studies have reported increased α-MyHC content in rat cardiac myocytes following various exercise regimes such as progressive treadmill training (66), sprint training (171) and swimming (64, 106). While others have reported no change in MyHC isoform content after training (25, 96) including previous work on Yucatan miniature swine following the same training protocol (77). Our work is in agreement with the previous work in pigs in that we did not observe any change in MyHC expression in samples from ventricular homogenates. We did however detect α-MyHC in a small proportion of myocyte preparations but it was not detected in any myocyte preparations from which mechanical measurements were made. The fact that power output was greater in myocytes from exercise trained pigs with no change in MyHC content is in agreement with a recent study finding an increase in rat myocyte power output following treadmill training with no change in MyHC expression (25).

Recently, a correlation between myocardial cTnT isoform expression and ventricular fractional shortening was reported in a pig model of exercise in combination with diabetes/dyslipidemia (74). With diabetes cTnT content shifted toward greater expression of lower molecular weight cTnT isoforms (cTnT₂ and cTnT₃) when compared to control pigs, and moderate endurance exercise prevented the cTnT isoform shift and improved fractional shortening of the ventricle. In the present study, however, SED and EX myocardium had similar cTnT isoform contents, indicating cTnT isoform expression was not a contributor to the greater power output reported in EX myocytes.
β-adrenergic signaling pathway following exercise training.

Multiple studies have found increased left ventricular responsiveness to β-adrenergic stimulation following exercise training (48, 136). The means of increased responsiveness could be an increase in any part of the signaling pathway: catecholamines, β-adrenergic receptors, receptor G-proteins, or adenylate kinase expression/activity. Some of these have been reported not to be increased following exercise training (circulating catecholamines (111) and β-adrenergic receptor density(48)), while others do increase (β-adrenergic receptor Gs subunits or ratio to Gi (11, 48, 122) and basal and peak adenylate cyclase activities (11, 126)). Ultimately the end result of enhanced β-adrenergic stimulation is increased phosphorylation of intracellular target proteins at the plasma membrane (L-type Ca\(^{2+}\) channel) and myofibrillar apparatus (cTnI and MyBP-C). Phosphorylation of L-type Ca\(^{2+}\) channels leads to a greater magnitude of Ca\(^{2+}\) in the cell, however no increase in intracellular Ca\(^{2+}\) transients following exercise training has been observed in myocytes (79, 107, 163). Here we do report an increase in phosphorylation of the myofibrillar apparatus at cTnI with a trend for higher phosphorylation in MyBP-C as well. A difference in phosphorylation of the two proteins may signify PKC involvement as cTnI is phosphorylated by PKC, on a different site than PKA, while MyBP-C phosphorylation by PKC occurs on the same sites as those phosphorylated by PKA. This could not be distinguished experimentally.

In conclusion, we found peak power generating capacity to be greater in myocytes from chronically exercise trained pigs as compared to sedentary counterparts. The mechanisms creating this difference is not known, but a potential contributor may be greater PKA-induced phosphorylation of myofilament proteins cTnI and MyBP-C in EX myocytes.
Overall, the results suggest that changes in contractile properties intrinsic to individual myocytes may contribute, at least in part, to the enhanced ventricular function associated with exercise training.
Chapter 5.  DISCUSSION

5.1 Summary of Results

Myocardial performance depends on many factors including the architecture of the ventricles, aortic pressure, and the contractile state of the myocardium. Myocardial performance can be altered in times of stress whether it be acute stress such as ischemia or chronic stress such as endurance exercise training. My work has focused on the effects that ischemia and endurance training have on altering the contractile state of myofibrils, which is a key determinant of overall myocardial performance. I have determined the effects of metabolites that rise during ischemia (\(P_i\) and \(H^+\)) on the functional capacity of permeabilized myocyte preparations. Moreover, alterations in function were measured in myocytes expressing either \(\alpha\)- or \(\beta\)-MyHC to ascertain differences in ischemic tolerance, as isoform content is known to be altered in pathological states. It was found that \(P_i\) and \(H^+\) alone and together decreased power generating capacity of \(\alpha\)-MyHC while only in combination did they diminish \(\beta\)-MyHC myocyte power. The greater tolerance toward ischemic conditions was attributed to a \(P_i\) and \(H^+\) induced increase in the velocity of loaded shortening. In addition, \(P_i\)-induced changes in contractile properties of \(\alpha\)-MyHC myocytes were more thoroughly assessed to probe the states of the cross-bridge cycle states that are most important in determining power output. Power near isometric loads is most likely limited by force-generating transitions that limit isometric force (i.e., the balance between attachment and detachment of positively strained cross-bridges), while power at very low loads is most likely limited by the cross-bridge transitions that limit maximal shortening velocity (\(V_{\text{max}}\)) (i.e., detachment of compressively strained cross-bridges). Experiments here utilized \(P_i\) to
increase one of these processes (i.e., force-generating transition rates) without affecting the other process (i.e., detachment of compressively strained cross-bridges) in order to determine which is most important in determining power output at intermediate loads where muscles tend to operate in vivo. $P_i$ increased loaded shortening velocity over most of the load range without any effect on $V_{max}$, implying that cross-bridge steps associated with $P_i$ release (i.e., force-generating transitions) are the most important in determining power output over physiological relevant loads. Finally, a pig model of endurance exercise training was used to determine if changes intrinsic to the myocardial myofilaments were, in part, responsible for greater SV that occurs with exercise training. Increased peak power generating capacity was observed in myocytes from exercise trained animals as compared to sedentary controls. One potential mechanism for this change in contractile function was found to be an increase in PKA-induced phosphorylation of myofibrillar proteins. These results provide a molecular and cellular basis for functional changes observed acutely and chronically with stress. In addition, they provide insight into chemomechanical steps of the cross-bridge cycle that determine loaded shortening and power output of cardiac myocytes.

5.2 Determinants of myocyte power output

Muscle power is the product of force and velocity and it known to reach an optimum at intermediate forces or loads that the muscle is working against. One aspect of my work focused on defining the chemomechanical cross-bridge states that are most important in determining maximal power output (i.e., at intermediate load ranges). To begin this discussion, I will first discuss the force-velocity relationship and the factors thought to determine the slopes of curvature of the relationship. Next I will incorporate the 1957
Huxley model of muscle contraction (63) in an attempt to quantify how factors such as $P_i$, $H^+$, and phosphorylation of myofibrillar proteins alter force-velocity curvature and overall power by affecting rate constants of cross-bridge transitions. Then, I will provide discussion of specific chemomechanical cross-bridge states likely corresponding to the transitions that regulate power output over most loads.

The force-velocity relationship is well defined by a rectangular hyperbole with force decreasing with increased velocity. The fall in force with increased velocity is hypothesized to arise from two factors, one is decreased number of cross-bridges exerting positive force and the other is increased number of compressively strained cross-bridges that form as the muscle shortens and oppose normal force production. At high force the first of these processes (i.e., decreased number of cross-bridges exerting positive force) is thought to be most important and determines the slope of the force velocity curve near isometric force. At high velocities (low loads) the second process (i.e., increased number of cross-bridges exerting compressive force) is thought to be most important and determines the slope near $V_{\text{max}}$. The curvature of the force-velocity relationship (i.e., $a/P_o$) is determined by the ratio of these two slopes and, thus, its value indicates the relative contribution of these two processes.

A shift in force velocity curvature with $P_i$ addition, lowered pH, and myofibrillar phosphorylation may indicate which process, either number of force generating cross-bridges or detachment of compressed cross-bridges, are limiting power output over a wide range of relative loads. First, a reasonable assumption is that power output at very low loads is limited, like $V_{\text{max}}$, by the detachment rate of cross-bridges. On the other hand, at high loads
(i.e., near isometric force) where filament sliding is minimal, power output is most likely limited by cross-bridge attachment rates or transition rates from weakly-bound to strongly-bound force-generating cross-bridges. Conceptually, at these high loads, rates of cross-bridge attachment are predicted to limit power output by dictating how many force-generating cross-bridges interact with thin filaments that slide past cross-bridges at a constant velocity. For example, if force-generating transitions are relatively slow, then slow shortening rates are needed to accumulate enough force-generating cross-bridges to support a high load. Slower shortening rates would yield relatively low power output compared to a situation where faster attachment rates could accommodate higher shortening velocities. If indeed, power output at low loads is determined by cross-bridge detachment whereas power output at high loads is determined by cross-bridge attachment and force generation, the question arises as to where in the power-load curve does the limiting step shift from cross-bridge detachment to force-generating transitions. Determining this transition point would provide evidence regarding the chemomechanical steps that are most important in determining power output at intermediate loads where muscles operate in vivo. If for instance, power output is determined by force-generating transitions only at relatively high loads, then maximum power output is most likely regulated by cross-bridge detachment rates. If, on the other hand, the shift between force generating transitions and cross-bridge detachment rates occurs at very low loads (e.g., less than 20% isometric force), then kinetics of cross-bridge attachment and force generating transitions probably determine maximum power output. Addition of P_i increased the velocity of loaded shortening over most loads (15-100% isometric) making the relationship less curved and more shallow (i.e. a greater a/P_o) (Tables 2.2, 3.1, and 3.2). Since P_i release is intimately linked to force generation (53)
this indicates force-generating transitions are involved. These observations of changes in curvature at moderate to high loads appears to indicate force-generating transitions as the primary process determining optimum power.

An alternative, more quantitative method of determining the impact of $P_i$, $H^+$, and myofibrillar phosphorylation on force-velocity relationships is to fit my data to the Huxley model (63) of muscle contraction (equation 5.1). The model discusses cross-bridges in terms of force-generating and non-force generating populations. Furthermore, force-generating cross-bridges can be either positively strained, contributing to force production and muscle shortening, or negatively strained, opposing force production and shortening. Application of these terms to describe muscle contraction and the relationship between force ($P$) and velocity ($V$) is shown by the equation (63):

\[
(5.1) \quad P = \frac{f_i}{(f_i + g_i)} \left\{ (1 - \exp \left[ -(f_i + g_i) \frac{h}{V} \right] \right\} \left\{ 1 + \frac{1}{2} \left[ \frac{(f_i + g_i)}{g_2} \right]^2 \frac{V}{(f_i + g_i) h} \right\}
\]

In this equation $f_i$ is the rate constant of positively strained cross-bridge attachment, $g_i$ is the rate constant of positively strained cross-bridge detachment, $g_2$ is the rate constant of detachment of negatively strained cross-bridges, and $h$ is a constant related to the distance over which a cross bridge may attach. The interaction of these constants define the characteristics of the force-velocity relationship with the shape of curve between the two extreme points (isometric force and $V_{\text{max}}$) determined by the factors that regulate each of the extremes. In this model, isometric force is limited by the number of positively strained cross-bridges (i.e., force-generating) which can be increased or decreased by changing the rates of attachment ($f_i$) or detachment ($g_i$) in the following relationship:

\[
(5.2) \quad f_i/(f_i + g_i)
\]
The other end of the force-velocity relationship is \( V_{\text{max}} \) which is proposed to be limited by the amount of negatively strained cross-bridges and is defined by \( g_2 \). Negatively strained cross-bridges build up as the muscle moves at higher velocities and the filament sliding speed at which force of positively strained cross-bridges equals force of negatively strained cross-bridges is \( V_{\text{max}} \). Thus, curvature of the force-velocity relationship is determined by the interaction of force-generating transitions and detachment processes. The Huxley model defines curvature as:

\[
(f_1 + g_1)/g_2
\]

Here, high values of \((f_1 + g_1)\) relative to \(g_2\) decreases curvature. Conversely, increased \(g_2\) relative \((f_1 + g_1)\) increases curvature. The inverse nature of the constants \((f_1 + g_1)\) and \(g_2\) and how they alter force-velocity and power-load relationships is shown in Figure 5.1. Relative to a given relationship (middle force-velocity curve) changes in force-velocity curvature toward a more shallow curve are completed by either increasing \(f_1\) and \(g_1\) \((2(f_1 + g_1))\) or decreasing \(g_2\) \((0.5g_2)\). A shallower force-velocity relationship results in greater relative peak power. Alternatively, a more curved relationship is formed when \(f_1\) and \(g_1\) are decreased \((0.5(f_1 + g_1))\) or \(g_2\) is increased \((2g_2)\), resulting in less relative power.
Figure 5.1.
Absolute force (P) and velocity (V) data from each experiment with P\textsubscript{i} addition were modeled using Sigmaplot software to quantify rate constants to explain changes in curvature. The Huxley equation (Equation 5.1) was solved for the variables \((f_1 + g_1)\) and \(g_2\). The data yielded the following results (in s\(^{-1}\)):

**Table 5.1.**

<table>
<thead>
<tr>
<th></th>
<th>0 mM</th>
<th>2.5 mM</th>
<th>5 mM</th>
<th>10 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>((f_1 + g_1))</td>
<td>8.1 ± 1.1</td>
<td>9.6 ± 0.8*</td>
<td>10.1 ± 0.7*</td>
<td>10.9 ± 1.9*</td>
</tr>
<tr>
<td>(g_2)</td>
<td>147.8 ± 1.6</td>
<td>148.8 ± 0.7</td>
<td>148.7 ± 0.9</td>
<td>150.0 ± 1.3*</td>
</tr>
</tbody>
</table>

Increasing \([P_i]\) progressively increased the sum of the rate constants of attachment and detachment of positively strained cross-bridges (i.e., \(f_1 + g_1\)) without altering \(g_2\) (except with 10 mM compared to 0 mM). A larger \((f_1 + g_1)\) relative to \(g_2\) increases the rate of force-generating cross-bridge interaction according to this model, implying faster cycling of positively strained cross-bridges. This is consistent with a shallower slope as, there would be a greater probability of cross-bridge attachment and transition to force-generating states at a given speed of thin filament sliding. In addition, \(g_2\) was not changed or was slightly larger with increasing \([P_i]\), so it would have negligible effects on curvature. Importantly the effect of \(P_i\) on \(g_2\) was also assessed mechanically to provide an estimate of \(g_2\) for the model and provide further evidence of lack of a \(P_i\) effect. \(V_{\text{max}}\) was not altered by increasing \(P_i\) concentrations according to force-velocity relationships, however attaining \(V_{\text{max}}\) in this manner requires an extrapolation in a range of data that can have high variability. An alternative method is to measure the unloaded shortening velocity \((V_o)\) of muscle using a
series of slack steps. $V_o$ measurements are in agreement with $V_{max}$ from force-velocity curves and $g_2$ calculations from the Huxley model showing no effect of $P_i$ addition on $V_{max}$, which is thought to be limited by the rate of detachment of negatively strained cross-bridges.

Comparison of $\alpha$-MyHC and $\beta$-MyHC myocyte data with additional $P_i$ and $H^+$ alone and in combination using the Huxley model provided the following values:

Table 5.2.

<table>
<thead>
<tr>
<th></th>
<th>pH 7.0, 0 mM</th>
<th>pH 7.0, 5 mM</th>
<th>pH 6.6, 0 mM</th>
<th>pH 6.6, 5 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-MyHC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$(f_1 + g_1)$</td>
<td>7.8 ± 0.8</td>
<td>9.1 ± 1.0*</td>
<td>8.4 ± 1.2</td>
<td>8.8 ± 0.8*</td>
</tr>
<tr>
<td>$g_2$</td>
<td>147 ± 4</td>
<td>148 ± 2</td>
<td>147 ± 5</td>
<td>148 ± 3</td>
</tr>
<tr>
<td>$\beta$-MyHC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$(f_1 + g_1)$</td>
<td>2.7 ± 0.2</td>
<td>3.4 ± 0.3*</td>
<td>3.3 ± 0.5*</td>
<td>3.5 ± 0.5*</td>
</tr>
<tr>
<td>$g_2$</td>
<td>73 ± 2</td>
<td>75 ± 2</td>
<td>75 ± 1</td>
<td>74 ± 1</td>
</tr>
</tbody>
</table>

The values for $\alpha$-MyHC myocytes show an increase in the sum of the rate constants of positively strained cross-bridges with $P_i$ addition at either pH, but not with increased $[H^+]$ by itself. The values from the Huxley model are qualitatively similar to those reported in Table 2.1 for the effects of these conditions on absolute power. However, with this model there is not discrimination between the effect of 5 mM $P_i$ at pH 7.0 and pH 6.6. An explanation for this discrepancy may be that unlike with $P_i$ at pH 7.0 where an estimation of $g_2$ was made ($V_o$), the effect of a lower pH on $g_2$ was assumed to have no effect (from $V_{max}$ data extrapolated from force-velocity relationships). This assumption may be incorrect though as
$V_o$ has been reported to be decreased at lower pH values (120). A smaller $g_2$, as predicted from previous work (120), would contribute to a shallower force-velocity relationship because according to the model the ratio of $(f_1 + g_1)$ to $g_2$ would be increased.

For the β-MyHC myocyte calculations $g_2$ was assumed to be half of the value for α-MyHC myocytes. Since isometric force is similar between myocytes expressing α-and β-MyHC, then $f_i$ and $g_i$ must change proportionately with a shift from α-MyHC to β-MyHC. A nearly two-fold difference in $(f_i + g_i)$ between α-MyHC and β-MyHC myocytes is within the range of changes reported in mechanical perturbations associated with the constants (i.e., $k_i$) (40). Changes in $(f_i + g_i)$ were qualitatively similar to changes in peak normalized power in β-MyHC myocytes, with experimental conditions having a greater positive effect on $(f_i + g_i)$ than in α-MyHC myocytes (~17% increase in α-MyHC, ~25% increase in β-MyHC with 5mM $P_i$ at pH 7.0). In addition, an underestimation of the effect of lower pH alone and in combination with $P_i$ on $(f_i + g_i)$ may have occurred because of the assumption of no change in $g_2$ at lower pH.

Finally, myocyte force and velocity data from exercise trained and sedentary pigs yielded the following results:

Table 5.3.
In agreement with the previous models, an increase in the sum of the rate constants governing force-generating cross-bridges occurred with exercise training; this yielded a decrease in the curvature of the force-velocity relationship. Interestingly, \((f_1 + g_1)\) were smaller than reported for rat \(\beta\)-MyHC myocytes even though both are composed of \(\beta\)-MyHC. This is in agreement with \(k_{tr}\) and power measurements between the two species, with pig myocytes having ~2-fold lower \(k_{tr}\) and peak power output values. This modeling data implies an apparent increase in the rate of force-generating transitions associated with myofibrillar phosphorylation, consistent with previous reports (52, 60).

The model implies that processes associated with force generating transitions (i.e., rates of positively strained cross-bridges) are the most important in determining loaded shortening and power output at intermediate loads where muscles perform work. Assuming force-generating steps limit power at intermediate loads it becomes of interest which steps in the cross-bridge cycle are coupled to the changes in contractile properties. A major challenge of muscle physiology lies in defining chemomechanical states during contraction. The two state model provides a framework to base discussion for a more complex, multi-variant model (such as provided in the cross-bridge scheme (Figure 1.4)), which has been proposed to explain a multitude of muscle biochemical and mechanical data. Increases in

<table>
<thead>
<tr>
<th></th>
<th>SED</th>
<th>EX</th>
</tr>
</thead>
<tbody>
<tr>
<td>((f_1 + g_1))</td>
<td>2.02 ± 0.1</td>
<td>2.46 ± 0.2*</td>
</tr>
<tr>
<td>(g_2)</td>
<td>50.1 ± 0.4</td>
<td>50.2 ± 0.5</td>
</tr>
</tbody>
</table>
force-generating transitions (increased $f_i + g_i$) qualitatively describe the mechanical changes (e.g., changes in $k_{tr}$, power and force-velocity curvature) with P$_i$, H$^+$, and myofibrillar phosphorylation but it cannot describe the quantitative differences between these changes. For example, $k_{tr}$ and power both increased with progressive addition of P$_i$, but $k_{tr}$ increased to a much greater extent (~300% versus 30% at 10mM Pi, respectively). This discrepancy can be rectified by a multi-state model with additional steps regulating each of the processes separately.

The Huxley model predicts loaded shortening velocity and power output are determined by force-generating transitions. Steps corresponding to force-generating transitions include steps 4-6 of the cross-bridge cycle model. I propose the steps limiting loaded shortening to be the primary force-generating isomerization and P$_i$ release (steps 4 and 5). Changing the rates of these steps (i.e., 4 and 5) during shortening may greatly impact mechanics by allowing cross-bridges to cycle to force-generating states faster thus maintaining attached force-bearing cross-bridges at higher velocities (i.e., decreased curvature of the force-velocity relationship). Additionally, these steps have been reported to be sensitive to P$_i$ (17, 19, 20, 39, 53, 141, 147, 156) unlike the other force-generating step (step 6) which is insensitive to increased concentrations of P$_i$ because of an irreversible isomerization after P$_i$ release. Additional experiments have demonstrated the primary isomerization (step 4) and P$_i$ release (step 5) to be sensitive to changes in temperature (115, 155, 172) and length perturbations (i.e., strain) (62, 112, 116, 172). Steps limiting power are thought to be sensitive to temperature as an increase in temperature increases power to a much greater extent than force (~20 fold compared to ~3 fold, respectively) in skeletal
muscle fibers (23). Additionally, there is an inherent strain dependence of power as observed in the force-velocity relationship. Sensitivities to Pi, temperature and strain all provide evidence for the possibility that it is the primary isomerization coupled to Pi release that determines loaded shortening and power output in muscle and perturbations of these steps will affect velocity of loaded contractions.

\( k_{tr} \)

As mentioned above, \( k_{tr} \) was increased to a much greater extent than loaded shortening velocity by Pi addition, implying regulation by difference processes; however the Huxley model can not differentiate between an effect on \( k_{tr} \) and on power. A multi-state model though provides an attractive point of differentiation between the two mechanical processes. One possibility is that force development is limited by the cooperative activation of near-neighbor regulatory units on the thin filament by strongly binding cross-bridges (37, 39, 141) and addition of Pi increases the number of strongly binding non-force generating cross-bridges (A M ADP Pi), which has been reported in skeletal muscle with Pi (94, 108). Consistent with Pi increasing thin filament activation, like addition of strongly binding NEM modified cross-bridges (142), addition of Pi eliminated the slow phase of unloaded shortening following slack steps in skinned fast-twitch skeletal muscle fibers during submaximal Ca\(^{2+}\) activations (89). This evidence supports the proposal that steps regulating activation of the thin filament and force-generating step limit \( k_{tr} \).

*Force*
Another way the two-state model of Huxley does not accurately describe mechanical findings is force declines with metabolite addition. The model defines force as \( f_1/(f_1 + g_1) \) with \( f_1 \) and \( g_1 \) proportional over most positive attachment distances. Since \( (f_1 + g_1) \) was increased by metabolites (per the model), \( g_1 \) would have to increase to a larger extent than \( f_1 \) otherwise force would increase or stay the same if \( f_1 \) and \( g_1 \) increased in proportion. If one assumes isometric ATPase to estimate \( g_1 \), then there appears to be little effect of \( P_i \) on \( g_1 \), since \( P_i \) only decreases isometric ATPase to a very small degree (<5% per decade of \( P_i \)) and to a lesser extent than force (27). A multi-variant model of muscle contraction provides a better means of describing the declines in force with \( P_i \) and \( H^+ \) addition. \( P_i \) release is coupled to the primary force-generating state so an increase in \([P_i]\) can cause the reversal of the force-generating step (20, 53), leaving fewer cross-bridges to produce force. In addition, as \( P_i \) decreases ATPase rate less than force it can be surmised that force per cross-bridge may also be decreased. Similar to \( P_i \), reduction of force at lower pH may be the result of a shift in cross-bridge populations toward non-force generating states because a proton is thought to be lost during the force-generating transition (1). Lowering the pH would then effectively shift cross-bridge populations back to a pre-force generating state. However, this is not likely the only way in which \( H^+ \) decreases myofibrillar force because a reduction in force of rigor cross-bridges (32) and stiffness per cross-bridge (91) occur with decreased pH indicating force per cross-bridge is also declined. Therefore, the effect of pH on isometric force is most likely a combination of a decrease in the number cross-bridges and the force generated per cross-bridge. From this discussion it can be seen a more complex model of muscle contraction is needed to accurately describe mechanical changes with \( P_i \) and \( H^+ \).

5.3 MyHC dependent sensitivity to metabolites
Another interesting and novel finding presented here is a description of MyHC dependence of mechanics in response to ischemic metabolites (Figure 3.4 and Tables 3.1 and 3.2). Measurements of mechanical changes with ischemic metabolites in cardiac muscle have primarily been limited to isometric properties (i.e., isometric force and \( \text{Ca}^{2+} \)-sensitivity of force). Moreover, mechanical changes in response to ischemic metabolites have not been investigated with consideration to MyHC content, which is highly correlated to many dynamic processes (\( V_{\text{max}}, k_{tr} \) and power) (40, 72). I found isometric force and its response to ischemic metabolites was not different between MyHC myocyte populations. During isometric contraction, there is no shortening so cross-bridges are either detached or positively contribute to force generation so the cross-bridge cycle is likely limited by the attachment and detachment of positively strained bridges (\( f_i \) and \( g_i \), respectively; according to the two-state model). Because isometric force is similar between myocytes expressing \( \alpha \)-and \( \beta \)-MyHC, then \( f_i \) and \( g_i \) must change proportionately with a shift from \( \alpha \)-MyHC to \( \beta \)-MyHC. A proportional shift in these processes is not surprising as many mechanical changes thought to be associated with attachment and detachment rates are altered in a linear fashion with the shift from \( \alpha \)-MyHC to \( \beta \)-MyHC (\( V_{\text{max}} \) and \( k_{tr} \)) (40, 72). In contrast to the lack of MyHC dependence of isometric forces with ischemic metabolites, there was a striking difference in how MyHC affected power output response to ischemia metabolites. Absolute power output was more greatly reduced in \( \alpha \)-MyHC myocytes than in \( \beta \)-MyHC myocytes. This was the product of a greater \( P_i \)-induced increase in loaded shortening velocity and an exclusive \( H^+ \)-induced increase of loaded shortening velocity in \( \beta \)-MyHC myocytes. Others studies examining a potential MyHC dependent effect on dynamic contractile properties are limited.
For example, in skeletal muscle fibers no difference was observed in the P<sub>i</sub> effect on power generating capacity of fast-twitch and slow-twitch muscle fibers (23). However, in this study when temperature was increased toward physiological conditions (30° C), slow-twitch muscle fiber power generating capacity increased 20-fold, whereas fast-twitch power generating capacity increased ~7 fold, with no difference in force production between fiber types (23). A greater effect of temperature on muscle power may indicate steps limiting power generation have fiber-type sensitivity to temperature. This idea is in agreement with data comparing the effect of temperature on cross-bridge rate constants from fast- and slow-twitch muscle fibers (22, 155). Wang and Kawai measured a greater than 2 fold difference in temperature sensitivity of the force-generating step (step 4 here) between fast-twitch and slow-twitch muscle fibers (Q<sub>10</sub> = 3.3 and 6.7, respectively) (155). Further evidence suggests the force-generating step is more closely coupled to the P<sub>i</sub> release step in slow twitch skeletal muscle (same MyHC as cardiac β) and intermediate skeletal muscle (i.e., type IIa MyHC fiber) than in fast twitch skeletal muscle, with slow being greater than intermediate (22). More closely coupled steps would confer greater P<sub>i</sub> sensitivity to the primary force-generating step as well (22). Greater temperature dependence existing between fiber types in addition to changes in the coupling between the isomerization and P<sub>i</sub> release steps that provide a greater P<sub>i</sub> sensitivity for force-generating steps (22), also may provide a mechanism for the greater P<sub>i</sub>-dependence of loaded shortening to ischemic metabolites observed in β-MyHC compared to α-MyHC myocytes (Chapter 3).

5.4 Other experimental considerations
Force depression from P\textsubscript{i} addition is concentration dependent with the relationship fit well by a single declining exponential (Figure 2.1). An exponential relationship indicates small changes in [P\textsubscript{i}] at low initial levels have greater impact on force inhibition than do changes from higher initial concentrations. Utilization of enzyme-linked P\textsubscript{i} “mopping” systems to remove P\textsubscript{i} from solutions has shown force depression occurs with [P\textsubscript{i}] as low as 0.2 mM (109). This indicates that P\textsubscript{i} tonically inhibits force production in cardiac muscle even under normal conditions when [P\textsubscript{i}] is low (1-2mM (2)). Also, this may indicate that solutions used in this study may have missed this more linear region of depression as experimental solutions and myocyte preparations contain P\textsubscript{i}. To examine the relationship of lower concentrations of P\textsubscript{i} it is necessary to minimize the contaminant P\textsubscript{i} in solutions. The presence of significant P\textsubscript{i} contamination in our solutions or preparations was examined by the incorporation of sucrose phosphorylase (SP) and its substrates into relaxing and activating solutions while correcting for changes in ionic strength and balance. This enzyme and protocol have been shown previously to decrease [P\textsubscript{i}] to as low as 0.2 mM in skeletal muscle preparations (69, 109). Inclusion of SP in activating solutions had no effects on myocyte force production, loaded shortening velocity, or power output (Figure 5.2) indicating a relatively low level of P\textsubscript{i} contamination in experimental solutions, although in contrast to previous work in skeletal muscle preparations, this finding was not unexpected as skeletal preparations have upwards of 20-30 fold greater cross-sectional area and several fold greater length than do our myocyte preparations, meaning overall ATPase and PCr hydrolysis rates
Figure 5.2

- pCa 4.5  \( F_o = 9.18 \pm 3 \, \mu N \) (n = 4)
- pCa 4.5, with SP  \( F_o = 9.16 \pm 3 \, \mu N \) (n = 4)
will be higher. Thus, it is probable that contaminant [P_i] in our experiments are lower than those estimated in skeletal preparations (0.7 mM P_i), and possibly as low as 0-0.2mM.

Differences in P_i production due to preparation size yields the possibility of differential response of preparations to experimental conditions because of P_i accumulation. In fact, some studies have demonstrated an inverse relationship between preparation diameter and force decline (70, 139) which is thought to exist because of lower P_i accumulation from myofibrillar ATPases in thinner preparations during Ca^{2+} activation. Data showing force response to increasing [P_i] is plotted with preparation width indicated for each curve (Figure 5.3). Our preparations displayed limited variability in force response to P_i addition. The variability that does exist is most likely the result of random selection of solution order as a small amount of preparation deterioration (less than 20%) is common following multiple force-velocity determinations. Meaning, there may be a greater relative fall in isometric force with 10 mM P_i if it is the last condition examined as opposed to first. Nevertheless, preparation width did not correlate with force decline to 5 mM P_i (Figure 5.3) further indicating preparation size did not confound the results.

Application of P_i and H^+ in combination has been reported to produce a synergistic effect in skeletal muscle (102, 159). The reasoning for a greater effect in combination was the formation of a greater amount of the diprotonated form of P_i (P_i^{2-}) at a lower pH; the P_i^{2-} form of P_i is exclusively responsible for force declines in skeletal muscle (102). However, the results presented here and previously (69, 103) find that it is total [P_i] and not the P_i^{2-} form
Figure 5.3

Inorganic Pao_4 added (mM) vs.Normalized Force

Myocyte width (µm) vs. Normalized Force with 5mM Pao_4
that is responsible for force depression in cardiac muscle. Lack of synergy can be seen by examining the effects of P$_i$ and H$^+$ addition separately and in combination on isometric force production (Figure 5.4). The top graph in Figure 5.4 has force relative to the value obtained at pH 7.0 with no added P$_i$; force depression resulted from either P$_i$ or H$^+$ addition, however the greatest effect occurred when applied in combination. These results may suggest the P$_i^-$ form is responsible for the depressive effect as both increasing the [P$_i$] and lowering the pH generates a greater [P$_i^-$], with the greatest concentration occurring when in combination. If increased [P$_i^-$] is responsible then normalizing force to the isometric value at pH 7.0 of each [P$_i$] (bottom Figure 5.4) would show disparate results at the lower pH as a greater [P$_i^-$] would be present when applied in combination as opposed to just lower pH. This however is not observed, indicating effects of P$_i$ and H$^+$ on force production are independent and additive in cardiac muscle, unlike skeletal. This discrepancy has been attributed to an apparent fiber type difference in P$_i$ sensitivities (103).
The studies of this dissertation examined how acute changes in the ionic environment associated with ischemia as well as chronic stress levels induced by exercise training modulate myocyte function. The results indicated that P_i decreases force production, yet increases loaded shortening velocity in a [P_i] dependent manner in all myocytes, attenuating the decline in power of α-MyHC myocytes and ablating the decline in β-MyHC myocytes. Since P_i release is tightly coupled to force-generating states this supports the hypothesis that force-generating transitions are most important in determining loaded shortening velocity and power output generated by cardiac myofibrils. In addition, increased [H^+] also depressed force in cardiac myocytes but, interestingly, increased loaded shortening exclusively in β-MyHC myocytes. This suggests that β-MyHC myocytes are more resistant to declines in power output induced by ischemic metabolites. Finally, cardiomyocytes from exercise trained pig myocardium had increased power output compared to those from sedentary control pigs, and the increased power was associated with higher baseline PKA-induced phosphorylation levels of cardiac myofibrils from exercised trained pigs. Overall, these alterations in ionic milieu, MyHC content, and phosphorylation status after exercise training, all of which increased loaded shortening velocity, are put forth as potential mechanisms to aid the heart’s physiological response to stress, making it more tolerant to mediators of stress and more capable of meeting systemic demand.
LITERATURE CITED

15. **Chen C, Chen L, Fallon JT, Ma L, Li L, Bow L, Knibbs D, McKay R, Gillam LD, and Waters DD.** Functional and Structural Alterations With 24-Hour Myocardial
Hibernation and Recovery After Reperfusion: A Pig Model of Myocardial Hibernation. 


33. **Fenn WO.** A quantitative comparison between the energy liberated and the work performed by the isolated sartorius of the frog. *Journal of Physiology* 58: 175-203, 1923.


73. Korte FS, McDonald KS, Harris SP, and Moss RL. Loaded Shortening, Power Output, and Rate of Force Redevelopment Are Increased With Knockout of Cardiac Myosin Binding Protein-C. *Circulation Research* 93: 752-758, 2003.


110. Patel JR, Fitzsimons DP, Buck SH, Muthuchamy M, Wieczorek DF, and Moss RL. PKA accelerates rate of force development in murine skinned myocardium expressing


131. **Siemankowski RF, Wiseman MO, and White HD.** ADP dissociation from acto-S1 is sufficiently slow to limit unloaded shortening velocity in muscle. *Journal of Biological Chemistry* 260, 1985.


VITA

Aaron Curtis Hinken was born November 20, 1977 in Denver, CO. He attended public schools in Windsor, MO, Great Falls, MT, Helena, MT and graduated from Windsor High School (May, 1996). He received a B.H.S in nuclear medicine technology (August, 2000), and a Ph.D. in physiology (May, 2005) from the University of Missouri-Columbia. He will continue researching molecular regulation of cardiac muscle contraction and alterations with heart failure as a postdoctoral fellow at the University of Illinois-Chicago under the supervision of R. John Solaro.