

The role of myosin regulatory light chain phosphorylation in cardiac health and disease

by

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Declaration

A subsection of work contained in chapters 4 and 5 is published in the Journal of Biological Chemistry (1). All work presented in this thesis is my own. The contributions of others are mentioned in the acknowledgments and any methodological techniques that are included for clarity but were performed by others are clearly stated in the text. Work included in the appendices is solely the work of others and is included for clarity.

Abstract

In this thesis we examined the effect of myosin associated regulatory light chain (RLC) phosphorylation level on cardiac muscle, ensembles and single molecules. We measured the ability of RLC phosphorylation change in muscle to alter force, power and unloaded shortening. The ATPase rate of full length cardiac myosin was determined with a novel protocol using gelsolin capped actin, which allowed novel measurements of myosin ATPase with full length (filamentous) myosin in low ionic strength. Actin gliding assays determined the effects of RLC phosphorylation level on actin gliding velocities under high and negligible load. The lifetime of strongly bound actomyosin states and the displacement of single myosin molecules were examined using an optical trapping three bead assay. A quantitative Phos-tag SDS-PAGE protocol was used to assess RLC phosphorylation level in inherited (mutation) and acquired (infarct and heart failure) human and rat diseases.

Cardiac disorders in human and rat left ventricular myocardium correlated with increased RLC phosphorylation. RLC phosphorylation alters the ability of muscle to produce force, power and maximal unloaded shortening. Increased RLC phosphorylation accelerated the ATPase rate of cardiac myosin; reduced the lifetime of strongly bound actomyosin states and increased the displacement of actin by myosin. This data correlated with an increased ability of myosin with phosphorylated RLCs to translocate actin, under high and low load in the actin gliding assay.

Cardiac myosin with raised RLC phosphorylation can produce more force and power during shortening due to changes in ATPase cycle, lifetime of the strongly bound states and power stroke size under load. Therefore myosin can perform work on actin faster and produce a longer actin displacement with each cycle. Thus proving that RLC phosphorylation level alteration impacts systolic myocardial performance in human health and disease by altering both myosin mechanics and kinetics.

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Glossary of terms

ABD: actin binding domain
ADP: adenosine diphosphate
ADS: actomyosin dissolving solution
AF: atrial fibrillation
 a/P_0 : curvature of the force velocity relation
APS: ammonium persulfate
ATP: adenosine triphosphate
BDM: 2,3-butanedione monoxime
 β -ME: 2-mercaptoethanol
BFA: biotinylated F-actin
BSA: bovine serum albumin
C4: control animals four weeks post intervention
C20: control animals twenty weeks post intervention
CCD: charge-coupled device
CICR: calcium induced calcium release
CIP: calf intestinal phosphatase
CMI: chronic myocardial infarction
cMLCK: cardiac myosin light chain kinase
CSA: cross sectional area
DCM: dilated cardiomyopathy
 dK_{atp} : K_{atp} of detachment rate
DMSO: dimethyl sulfoxide
DS: dissolving solution
DTT: dithiothreitol
 dV_{max} : maximal detachment rate
 E_a : activation energy
EB: extraction buffer
ECL: electrochemiluminescence

EDTA: ethylenediaminetetraacetic acid

EGTA: 1,2-Di(2-aminoethoxy)ethane-N,N,N',N'-tetra-acetic acid

ELC: essential light chain

EtOH: ethanol

F_0 : peak isometric force

FAB: F-actin buffer

F-actin: filamentous actin

FV: force velocity

G-actin: globular actin

GLH: Glutathione

GOC: glucose oxidase and catalase mixture

HCM: hypertrophic cardiomyopathy

HDTA: 1,6-Diaminohexane-N,N,N',N'-tetraacetic acid

HeNe: helium neon laser

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HMM: heavy meromyosin

HS: high salt buffer

IDCM: idiopathic dilated cardiomyopathy

K_{atp} : ATP concentration that half maximal gliding velocity is observed

K_{atpase} : actin concentration at half maximal activation

KH: Krebs-Henseleit

KM: kinase mixture

LA: left atrium

LAD: left anterior descending

LDA: length dependent activation

LMM; light meromyosin

LV: left ventricle

LS: low salt buffer

MgATP: magnesium and ATP

MB: motility buffer

MHC: myosin heavy chain

MI: myocardial infarction

MI4: chronically infarcted animals four weeks post ligation

MI20: chronically infarcted animals twenty weeks post ligation

ML: muscle lengths

ML/s: Muscle lengths per second

MM: Michaelis-Menten

MOPS: 3-(N-morpholino)propanesulfonic acid

MW: molecular weight

MWM: molecular weight marker

MyBP-C: myosin binding protein c

NA: numerical aperture

NADH: nicotinamide adenine dinucleotide

NBB: neutravidin biotinylated beads

NBP: nucleotide binding pocket

NYHA: New York Heart Association

PBS: phosphate buffered saline

Pi: inorganic phosphate

PMSF: phenyl methyl sulfonyl fluoride

PP: peak power

PV: power velocity

Q_{10} : temperature coefficient of a 10-degree temperature change

RLC: regulatory light chain

RPM: revolutions per minute

S1: catalytic subunit of myosin

SAP: shrimp alkaline phosphatase

S1: subfragment 1

S2: subfragment 2

SDS-PAGE: sodium dodecyl sulphate poly acrylamide gel electrophoresis

SEM: standard error of the mean

SERCA: Sarcoplasmic/Endoplasmic Reticulum Ca^{2+} -ATPase

SL: sarcomere length

skMLCK: skeletal muscle myosin light chain kinase
smMLCK: smooth muscle myosin light chain kinase
SR: sarcoplasmic reticulum
SS: skinning solution
TEMED: tetramethylethylenedia
TES: N-tris[hydroxymethyl]ethyl-2-aminoethanesulfonic acid
TFP: trifluoperazine
Tm: tropomyosin
Tn: troponin
Tris: trisaminomethane
TRITC: tetramethylrhodamine
 t_c : duration of the full actomyosin cycle
 t_s : lifetime of the strongly bound actomyosin states
 V_{max} : maximal velocity
 V_{PP} : velocity at which peak power was observed
 X_0 : displacement produced by a power stroke
ZIPK: zipper interacting protein kinase

Chapter 1 – Introduction

1.1 The cycle of cardiac activity and muscle contraction

The mammalian heart is a dual circulatory pump, which moves blood around both the systemic and pulmonary circulatory systems. It is central in maintaining appropriate tissue perfusion of gases and metabolites in all areas of the body, and is therefore crucial for sustaining life. The mechanisms that govern the function of the heart as an efficient pump are still not fully understood. The efficiency of the pump is tantamount, as it will go through its full cardiac cycle many millions of times in an average life span. The cardiac cycle is defined in two phases, diastole and systole. The state in which the heart relaxes and perfuses its own tissue, through its coronary blood supply, is the diastolic phase. The mechanical work of heart muscle (myocardium) to actively pump blood is performed during the systolic phase.

Each cardiac cycle begins with the spontaneous depolarisation of a specialised set of cells in the sinoatrial node. The wave of depolarisation (action potential) spreads throughout the heart via its specialised conduction system, which includes the atrioventricular node and Purkinje fibres that innervate the ventricles. The action potential is spread between neighbouring cardiomyocytes by intercellular contacts (intercalated discs), which allows them to transmit both electrical excitation and force. The process of depolarisation via the spreading action potential is causative of muscular contraction (systole). It sets in motion a process termed, calcium induced calcium release (CICR). The process of CICR begins with a relatively small calcium release (triggered by the spreading action potential), which occurs through a voltage-gated L-type Ca^{2+} current (I_{Ca}), carried predominantly by the $\text{Ca}_v1.2$ pump and minimally the Na/Ca exchange pump (NCX) into the cell. I_{Ca} occurs in close proximity to the intracellular calcium stores of the sarcoplasmic reticulum (SR), which is covered in ryanodine receptors that bind calcium. Upon binding of calcium these receptors mediate a larger release of intracellular calcium from the SR (review on

ryanodine receptor activation (2)). The result of this explosive calcium release from the SR is the synchronous release of calcium to the myofilament made possible by the transverse tubular (t-tubular) networks arrangement with the contractile machinery (review (3)). For appropriate relaxation to occur (diastole) after activation the calcium concentration around the contractile machinery (cytosolic calcium) must be reduced. This occurs through several mechanisms: i) the sequestration of calcium back into the SR by the Ca-ATPase pump (SERCA) and its associated regulatory proteins; ii) the removal of cytosolic calcium to the outside of the cell by specialised calcium pumps and exchangers such as NCX and the sarcolemmal Ca-ATPase (PMCA); iii) removal of cytosolic calcium by the mitochondrial Ca uniporter (MCU) into the mitochondria of the cell. These intricate processes govern the activation state of the myofilament and are often affected in cardiac disease (for review see (4)).

The release of calcium from the sarcoplasmic reticulum, bathing the myofilament in calcium, triggers contraction by binding to the regulatory complex of proteins (troponin) that govern the activation state of the contractile machinery (this process is described in more detail in section 1.4). The two key proteins responsible for contraction are the molecular motor of the myocardium (myosin), which performs the mechanical work to shorten the myocardium, and the filamentous protein that it performs its mechanical work on (actin).

1.1.1 Origins of muscle research and the sliding filament hypothesis

The viscous protein responsible for contraction in muscle was first extracted in 1864 by Kühne who named it 'Myosin' (review (5)). This was the starting point of myosin and muscle research. The findings of Engelhardt and Lyubimov (1939), that myosin would convert chemical energy (ATP) into mechanical motion, fell in line with Lohmann's (1934) findings that ATP was most likely to be the energy source of muscle contraction. It was then discovered that these myosin molecules would

assemble to form filaments, which would interact with actin filaments in-vitro, proving actin and myosin to be the basic constituents needed for muscular contraction (6).

However, in striated muscle these proteins are organised into sarcomeres that are repeating units 2-3 μm long. Observations by Hugh Huxley of 'meridional periodicities of the muscle', inferred that one constituent of the sarcomere stayed at a constant length when the muscle was stretched. This constituent was later identified as the myosin containing thick filament (7, 8). Further electron microscopy showed that the sarcomere was made of two types of filament, the previously identified 1.6 μm long thick filaments (A-band), and 1 μm long thin filaments (I-band, containing actin) (9).

Independent observations by Hugh and Andrew Huxley showed that the A-band of the sarcomere stayed constant in length during contraction, whereas the I-band would shrink in size (10, 11). These independent observations lead to the proposal of the sliding filament hypothesis. This hypothesis proposed that the thick and thin filaments would slide past each other to create the observed shrinking of the I-band region. This was later strengthened by the observations that thick and thin filaments did indeed associate with each other (9).

1.1.2 The cross-bridge cycle

The evidence that muscle contraction was driven by cross-bridges came from electron microscopic visualisation of cross-bridges by Hugh Huxley (9). Shortly after which, the cross-bridge cycle was proposed (12). The first evidence of how the cross-bridge shape would change to produce the basis for movement was observed in insect flight muscle, where it was found that the angle of the myosin molecule could change, dependent on the state of the muscle (13). This later led to the proposal of a forward swinging cross-bridge mechanism by Huxley (14). The original cross-bridge cycle postulated by Lymn and Taylor, provided evidence for ATP hydrolysis in myosin in its un-bound state, and that addition of ATP caused bursts of

increased ATPase activity, which were almost stoichiometric to the abundance of myosin heads (15). These findings made it evident that myosin was the driver of cellular contractility. We now understand that the type of myosin determines the contractile parameters of the muscle. This is because the motor (myosin) determines the force production (force produced per cross-bridge) and the velocity of displacement (influenced by the kinetics of the cross-bridge cycle).

We can start by describing the cross-bridge cycle from rigor, which is the point where myosin is bound to actin at a 45-degree angle in the absence of ATP (1 in figure 1.1.2). Mg-ATP binding to myosin manifests in a rapid dissociation of myosin from actin, which is followed by ATP hydrolysis performed by the myosin ATPase to form adenosine diphosphate (ADP) and inorganic phosphate (Pi). At this juncture the myosin rebinds actin at a 90-degree angle with both ATP hydrolysis products still bound (2 in figure 1.1.2). This strongly bound actomyosin ADP + Pi state is likely responsible for force production in the cross-bridge cycle. There is still not a clear understanding of the nature of the force-generating step and how it is associated with product release. Broadly, Pi is released and the force generating power stroke occurs, which subsequently forms the force-bearing state in the cross-bridge cycle known as the actomyosin ADP state. ADP release then creates a conformational change in the myosin molecule, moving it back to a 45-degree angle in respect to actin. The ADP release step is often the rate-limiting step in the cross-bridge cycle. Upon ATP binding the whole cycle can begin again.

The structural mechanisms of the cross-bridge cycle have been reviewed in depth (16). We know that many of the states of the actomyosin cycle are determined by kinetic rates, which can be influenced; one of these influencers we believe to be the myosin associated protein the regulatory light chain (RLC), the topic of this thesis. I will further elaborate the role that RLC phosphorylation may play in regulation of the cross-bridge cycle in cardiac contraction in subsequent section.

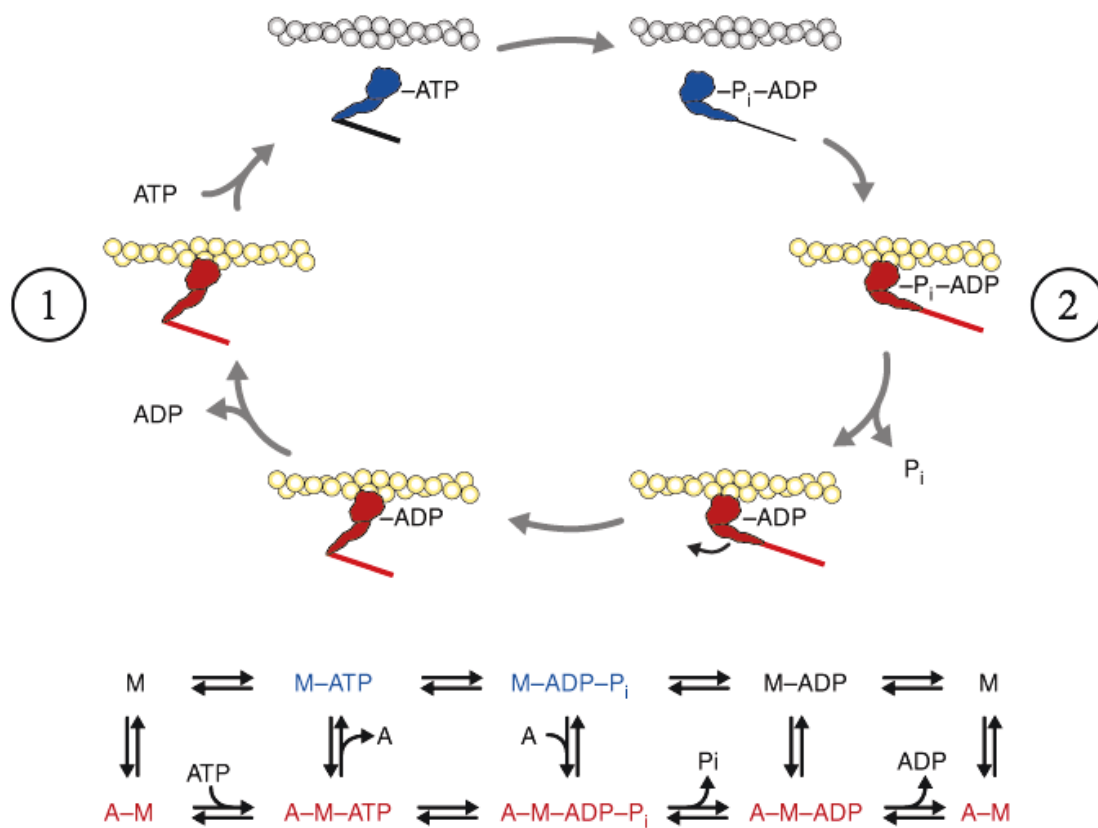


Figure 1.1.2: The cross-bridge cycle. **Top**, Schematic diagram showing the states of the actin-myosin ATPase cycle. Actin bound states are indicated in red and dissociated states in blue. **Bottom**, the actin-myosin ATPase cycle showing all possible reaction states between actin (A), myosin (M), bound nucleotide (ATP) and hydrolysis products (ADP and P_i). This figure was adapted from Murphy et.al. (17).

1.1.3 The sarcomere length-tension relationship

The force a sarcomere can produce is governed in part by the extent of myofilament (actin and myosin) overlap (18). Larger overlap regions allow for more myosin heads to be in the proximity of actin and available for cross-bridge cycling to produce the force of contraction. In skeletal muscle the length-tension relationship describes the way in which force is altered by myofilamentous overlap. For sarcomeres shortening below 2.0 μm , force decreases until it reaches zero, at a sarcomere length of about 1.6 μm in skeletal muscle. Between 2.0 μm and 2.2 μm , force plateaus at its maximal value; this region of the relationship is plateaued due to the number of myosin cross-bridges in reach of actin binding sites remaining constant. This occurs as the thin filament begins to move over the bare zone at the centre of the sarcomere, where no myosin heads are situated. Above 2.2 μm in length, the force produced by the sarcomere diminishes as the degree of myofilament overlap decreases with increasing sarcomere length. This manifests as a linear decrease in force as the sarcomere length is increased. This phenomenon occurs up to approximately 3.6 μm where no active force is produced anymore, as there is no myofilamentous overlap remaining (Reviewed in Shiels and White, 2008 (19)).

The length-tension relationships of skeletal and cardiac muscle do differ significantly (Figure 1.1.3). The length-tension relationship in cardiac muscle was first described by Julian and Sollins (1975) (20). The ascending limb of the cardiac length-tension relationship is steeper than that of skeletal muscle, even though the lengths of the filaments are equal. The reason this is believed to be true is thin filament cooperativity, which is an observable increase in contractility of cardiac muscle, not observed in skeletal muscle, due to increases in calcium sensitivity produced by increasing sarcomere length. The slope of the ascending limb in cardiac muscle is also adaptable, depending on the inotropic state of the tissue. This adds a layer of complexity to the classical relationship observed in skeletal muscle and is thoroughly

reviewed (19). The working range of vertebrate skeletal muscle seems to be in the region of optimal overlap approximately $2.2\ \mu\text{m} \pm 13\%$ (depending on muscle and species), extensively reviewed (21), whereas cardiac muscle is typically between $1.9\text{-}2.2\ \mu\text{m}$ (22).

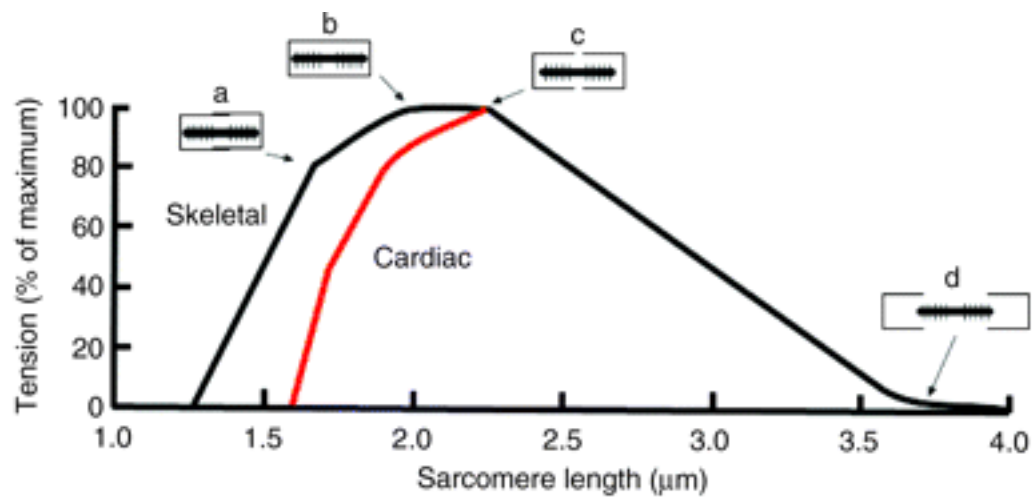


Figure 1.1.3: The length tension relationship. Illustration of the sarcomere length tension relationship of skeletal (black line) and cardiac (red line) muscles. Inset a, b, c, and d show the myofilament overlap at each point along the sarcomere length tension relationship (19).

1.1.4 The force-velocity relationship of muscle

The theory behind the force-velocity relationship was presented by A.V. Hill in his 1938 paper entitled “The Heat of Shortening and the Dynamic Constants of Muscle” (23). From his studies he derived the force-velocity relationship to describe the way in which force and velocity can be modelled mathematically. Sonnenblick was the first person to describe this relation in cardiac tissue using cat papillary muscle (24).

The force that can be generated by muscle is in part a function of the velocity at which it shortens. The faster muscle shortens, the more limited the time becomes for myosin to find an actin binding site and undergo a productive power-stroke. Therefore as shortening velocity increases the force that can be produced is reduced.

The force-velocity relationship defines the ability of muscle to produce force during shortening and can be measured using several types of muscle activation. One type of manoeuvre allows the muscle to shorten against a constant load. The force the muscle can produce during each shortening manoeuvre is plotted against the velocity of that manoeuvre, performed over a range of loads. Edman-style slack test measurements are used to assess the velocity at which muscle can shorten against no applied load, and defines the peak unloaded shortening velocity ($V_{\max}$) (25). Isometric activations are used to measure force with no shortening of the muscle (F_0) when it is held at a fixed length. $V_{\max}$ and F_0 were measured experimentally and provided the x and y intercepts of the force-velocity relations in many studies.

It is important to measure changes in the force-velocity relationship in cardiac tissue as this type of measurement gives the best *ex-vivo* measure of cardiac contractility. This is because during systole, cardiac muscle performs the bulk of its contractile work during muscle shortening. The inherent sensitivity of the assay to determine differences between experimental treatment groups makes it a useful tool for determining changes in myocardial contractility.

The force-velocity relationship in cardiac tissue is not constant as it is influenced by inotropy and pre-load in the intact organ, leading us to believe that RLC phosphorylation may be a modulator of the force-velocity relation. The greater the pre-load, the faster the muscle will shorten; generally in the context of our permeabilised tissues, this effect is linked to the length-tension relationship discussed above.

1.2 The cardiac sarcomere and its constituents

The functional unit of the contractile apparatus responsible for muscle shortening is the sarcomere. The sarcomeres of striated muscles are highly ordered structures, which we now know are comprised of many more proteins than just the myosin containing thick filament, and the actin containing thin filament (Figure 1.2.1) (26). There are also many more proteins that are involved in anchoring the sarcomere to the extracellular matrix, which are important for translating the changes in length of the sarcomere to the cardiomyocyte. There exists a detailed review that encompasses many of these proteins in the context of cardiomyopathy (27).

The sarcomere has distinctive regions that can be observed clearly and distinctly under the electron microscope. The Z-disks of the sarcomere define the lateral boundaries of a single sarcomere, and importantly provide the tether between actin of one sarcomere and that of the next (mediated by α -actinin layer structure). Z-disk associated proteins also play a key role in mechanotransduction of the sarcomere and are involved in the development of the hypertrophic response (reviewed herein (28)). Filamentous actin is connected to the Z-disk by its barbed end and interdigitates with the myosin containing thick filaments. The thick filaments lie in the region known as the sarcomeric A-band (anisotropic band). The A-band is the region in which the thick and thin filaments overlap, whereas the I-band (inotropic band) is

the region that contains largely only the thin filament, where there is no myofilamentous overlap with the thick filament. During muscle shortening it is the I-band region that reduces in size as the sarcomere shortens.

I will give a brief overview of the main constituents of the contractile machinery of the sarcomere directly involved in activation and force production. This will provide the context within which the myosin associated regulatory light chain operates. This will be broadly subdivided into thin filament proteins, structural proteins of the contractile machinery (titin and myosin binding protein-C), and thick filament proteins.

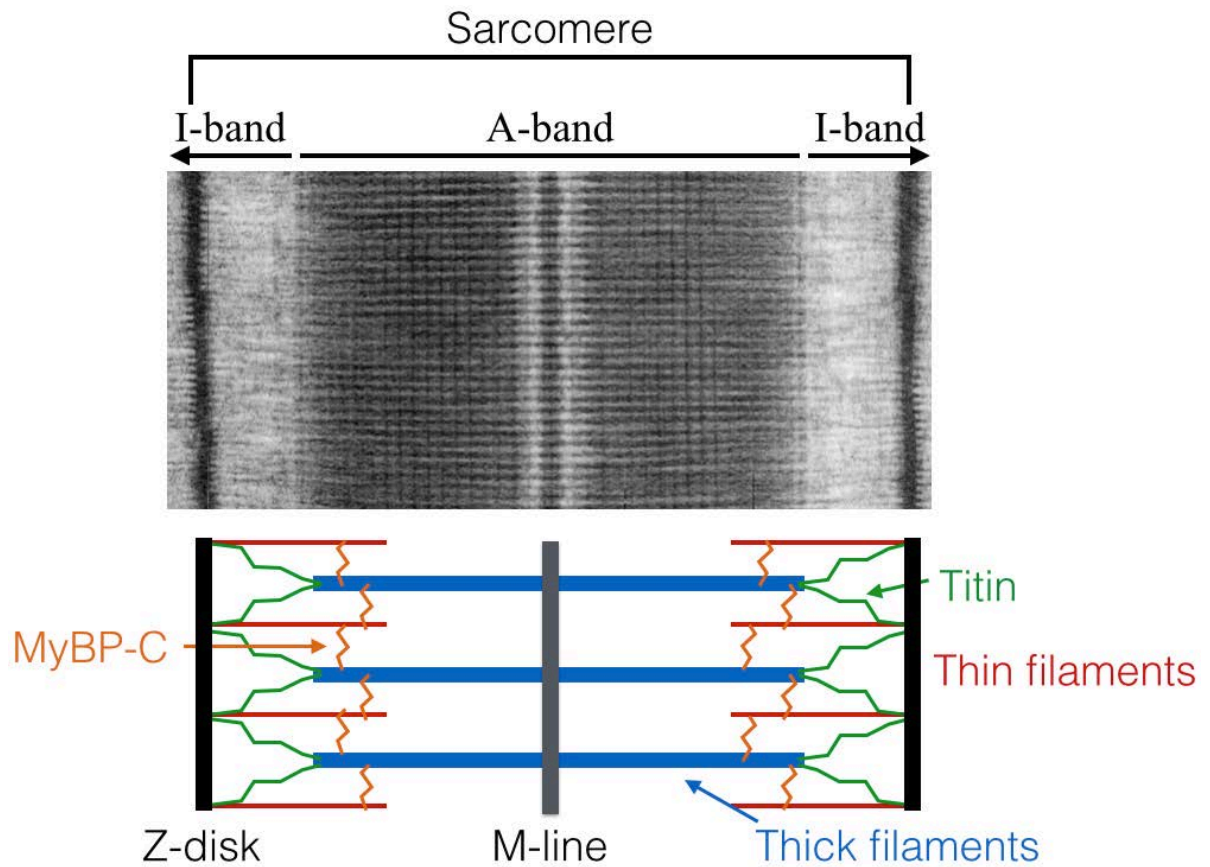


Figure 1.2.1: The Sarcomere. **Top,** Electron micrograph of a single cardiac sarcomere (provided by Pradeep Luther, Imperial College London). **Bottom,** Simplified depiction of the cardiac sarcomere indicating the positioning of the thick (myosin containing) and thin (actin containing) filaments within the structure of the sarcomere.

1.2.1 Thin filament proteins

The thin filaments of the cardiac sarcomere are made of actin filaments, which provide the binding sites for myosin to interact with, and perform its mechanical work (sarcomere shortening). As discussed below cardiac myosin is not switched 'on and off' by regulatory light chain phosphorylation like some other non-striated myosins. Instead the availability of the myosin binding site on actin is regulated by the calcium availability to the molecular 'on/off' switch, which consists of the troponin complex and tropomyosin.

1.2.1.1 Actin

Actin monomers (G-actin) polymerise to form the filamentous actin (F-actin) found in the thin filament. The actin molecules exist as three isoforms of 42 kDa proteins that have two lobes separated by a cleft centred on the bound ATP/ADP+Pi (29). The regulation of actin filament assembly and disassembly has been expertly reviewed previously, and will not be discussed in great depth herein (30). Importantly, the primary myosin binding site on actin involves interaction between two subdomains (1 and 3) of one actin, and a small interaction with the next actin molecule on the actin helix (31).

The filamentous actin forms a double helix of monomers, completing a turn every 72 nm (32). Actin filaments are very dynamic and there are many states an actin filament can occupy. There are also actin-binding molecules such as phalloidin, which can stabilise the filaments and are employed in experiments where actin concentrations are low to slow filament disassembly (29). The actin filament is polar, having a barbed end (+) and a pointed end (-). Actin filaments are orientated in the sarcomere in such a way that the barbed ends are all orientated towards the Z-disk and are bound there by α -actinin. The polarity of the actin filament determines the direction in which the myosin will face when interacting with the actin filament.

Cardiac myosin is a plus-end directed motor, which will pull the thin filament's barbed end towards the M-line in the sarcomere from both sides, creating sarcomere shortening.

1.2.1.2 Tropomyosin and the troponin complex

The availability of the myosin binding sites on actin are governed by the thin filament regulatory proteins of tropomyosin (Tm), and the troponin (Tn) complex of proteins.

Tn is a complex of three individual proteins that play distinct roles in thin filament activation (reviewed herein (33)). 1 Tn and 1 Tm control the availability of approximately 7 actin molecules to myosin binding. The separate units of the Tn complex are troponin-C (TnC) 18 kDa, troponin-I (TnI) 23 kDa and troponin-T (TnT) 35 kDa. TnC is the calcium binding subunit of the Tn complex that senses and binds calcium to activate the thin filament. TnI is the inhibitory subunit of the Tn complex, which prevents contraction in the absence of calcium binding to TnC. TnT binds both TnC and TnI and anchors the Tn complex to Tm.

Tm is a 284 amino acid protein that is negatively charged, weighs approximately 33 kDa and is an elongated coiled-coil dimer. Tm comes in three isoforms in cardiac muscle: the α , β and κ -Tm, with the predominating form in adult life of humans and mice being α -Tm (34, 35). Regulated striated muscle has continuous dimerised strands of Tm that are polymerised to span the entire actin filament by head to tail overlap. Each Tm dimer spans seven actin monomers, is approximately 42 nm in length, and wraps around the actin filament near the groove of the actin helix (36). Recently high resolution structures of the F-actin tropomyosin complex have been published (37).

The role of Tm in the sarcomere is as a regulator of thin filament activation. When the Tm is in its 'off' state the dimer lies outside the groove of the actin helix, blocking the myosin binding site on actin and sterically preventing actomyosin interactions (38,

39). Intracellular calcium release creates a conformational change in Tm via the troponin complex (discussed above). By this mechanism the Tm shifts into the groove of the actin helix revealing the myosin binding site on actin. Myosin binding, to actin, then leads to further exposure of myosin binding sites on adjacent actin molecules, and accelerates subsequent myosin binding rates (40, 41) (cooperative activation reviewed (42)). The removal of intracellular calcium reverses these processes and switches 'off' the thin filament, preventing myosin binding with actin.

1.2.2 Structural proteins of the sarcomere

These proteins interact across large regions of the sarcomere and regulate a plethora of processes concerning sarcomere assembly, protein turnover, and contractile regulation. They help provide tethers between regions of the sarcomere and the myosin containing thick filament.

Shown in figure 1.2.1 there are titin and myosin binding protein C (MyBP-C) proteins that are at the interface between the thick filament and its surrounding sarcomeric infrastructure (either thin filament or Z-disk).

1.2.2.1 Titin

Titin is a single giant polypeptide that spans the regions from the Z-disk to the M-band. This is where the proteins overlap with each other over successive half sarcomeres, and form a continuous polypeptide chain that runs the length of the myofibril. Different sections of titin play distinct roles in the development and regulation of the sarcomere. The A-band spanning region of titin may act as a molecular ruler as a guide for thick filament assembly and protein turnover (43-45). A spring like portion of the molecule is known to be the molecular spring of the sarcomere, which creates passive tension (43, 46, 47). The level of passive tension can be fine tuned by phosphorylation (through protein kinase A, C and G) and differential splicing of titin, which is known to be altered in cardiac disorders

(reviewed (48)). The extensible regions of the molecule act as molecular sensors of stretch, and are involved with accessory titin binding proteins in relaying this information to trigger the hypertrophic response (48). Titin is also involved with length dependent activation (LDA) whereby increases in sarcomere length (within a physiological range) result in increased calcium sensitivity of the myofilament (49-52).

1.2.2.2 Myosin binding protein-C

Cardiac MyBP-C is a protein that associates with the thick filament and plays a role in regulation of cardiac contraction (reviewed (53)). The regulation of contraction by MyBP-C is mediated through interactions it shares with myosin, actin, or both simultaneously. These interactions can be altered by phosphorylation of MyBP-C by protein kinase A (PKA), calcium calmodulin kinase 2 delta (CaMK2 δ) and less specifically by application of β -adrenergic agonists (54, 55). Unlike titin MyBP-C doesn't seem to be imperative for correct sarcomere assembly as MyBP-C knockout does not adversely affect sarcomere assembly (56). The effects on regulation of contraction mediated by MyBP-C phosphorylation are postulated to arise from changes to co-operative activation (53).

1.2.3 Thick filament proteins

The thick filament is the home to the mechanical motor (myosin) that utilises energy in the form of ATP to drive sarcomere shortening. As a definition, myosins are a diverse group of molecular motors that convert chemical energy, in the form of ATP, into mechanical motion. There are a large amount of identified myosins, which have been subdivided into different classes, based largely on the properties of their globular motor domains. Cardiac myosin is a part of the class II myosin subgroup. Class II myosins are conventional two-headed myosins, which form filaments, and consist of two heavy chains and four light chains (57). Broadly, class II myosins can be split into two categories: i) those that are controlled by regulatory light chain

(RLC) phosphorylation (smooth and non-muscle myosins); ii) those that display ATPase activity without RLC phosphorylation (cardiac and skeletal myosin) (58). From now I shall focus only on the striated cardiac muscle myosins.

1.2.3.1 Cardiac myosin

Cardiac myosin is expressed as two separate isoforms in the human myocardium. These are myosin heavy chain (MHC) alpha (α) and beta (β), which are expressed from the *MYH6* and *MYH7* genes respectively, and have molecular weights of approximately 200 kDa. These two different isoforms display different ATPase activities, where alpha cardiac myosin has a higher ATPase and actin affinity compared to the beta isoform (59). Notably, it is typical for smaller mammals to express a higher proportion of alpha MHC in their ventricular myocardium (mice and rats), whereas larger mammals predominantly express beta MHC in adulthood with low (5-10%) of alpha MHC expression (60). MHC expression can be affected in disease (pressure overload, diabetes, hypertrophy and heart failure), during development, and by hormonal control (61, 62).

Each MHC has two bound light chains, light chain one (LC-1 also known as the essential light chain, ELC, reviewed (63)); and light chain 2 (LC-2 also known as the regulatory light chain, RLC), which are approximately 27 kDa and 18 kDa respectively (64). Two myosin heavy chains with their bound light chains form a myosin molecule (Figure 1.2.3.1). Each myosin molecule could therefore be formed by either homodimers or heterodimers of MHC isoforms (α/α or β/β or α/β) (65).

The cardiac myosin molecule, like other class II myosin molecules, consist of three main domains; the tail, neck (or lever), and heads of the molecule. Proteolytic cleavage of the myosin molecules by trypsin initially creates two fragments (66). These fragments are the heavy meromyosin (HMM, molecular weight approximately 350,000) and the light meromyosin (LMM, molecular weight approximately 150,000).

The HMM consists of both the globular heads of the myosin, which possess the actin binding site, nucleotide binding pocket, and the neck of the motor, which binds the two light chains with a short section of tail, ensuring the molecule is still a dimer. The LMM contains the remainder of the coiled coil tails of the molecule, which enables the motor to form filaments (discussed below). The HMM can be further digested using papain to form two smaller subfragments known as subfragment 1 (S1) and subfragment 2 (S2) (67). S2 is the remainder of the tail section that was still present in the HMM molecule. The S1 fragments are single globular heads of the MHC with the entire neck region (and both light chains), which possess the ATPase and actin binding domains. The S1 fragment is the region containing the motor domain responsible for driving muscle contraction by actin binding and ATP hydrolysis.

Myosin molecules can self-assemble via their coiled coil tail domains into bi-polar thick filaments (< 0.3 M ionic strength), which is how they are found in the cardiac sarcomere (68). The centre of the thick filament is formed by the anti-parallel polymerisation of myosin, whereas the peripheral portions of the thick filament result from the parallel association of myosin molecules. The ability of myosin molecules to bind to each other in parallel and anti-parallel arrays allows the formation of these bi-polar filaments; where the heads are orientated towards the end of the filament, and the tails are orientated towards the centre. The tails of the myosin molecules form the backbone of the thick filament, leaving the heads to protrude from the surface. The heads protrude from the backbone every 14.3 nm in a helical arrangement that repeats every 43 nm (69). The resultant thick filament with this arrangement has a bare zone, which is lacking in myosin motor domains and has no ability to bind actin. This region is known as the M-line (Mittelscheibe in German, translated as 'middle disk' in English). The conformation of the myosin thick filament is important as it provides the directionality of muscle contraction. It ensures that the actin filaments

are drawn in from both sides towards the M-line to create sarcomeric shortening that translates into muscular contraction.

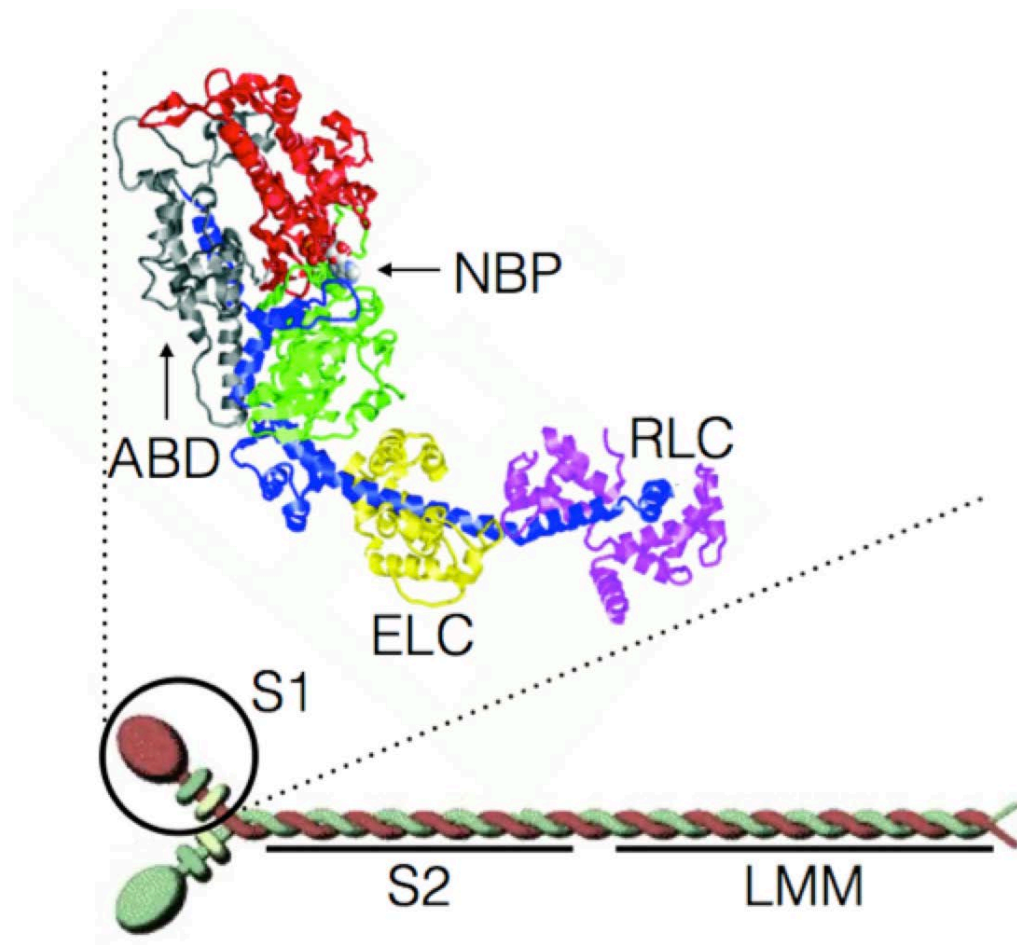


Figure 1.2.3.1: Cardiac myosin. Diagrammatic representation of a cardiac myosin molecule. Inset ribbon structure of the S1 (subunit 1) of the myosin molecule indicating the actin binding domain (ABD), nucleotide binding pocket (NBP) and both light chains bound to the molecule; the essential light chain (ELC) and regulatory light chain (RLC). Figure adapted from Craig et.al. (70) and Ruegg et.al. (71).

1.2.3.2 Cardiac RLC

The main theme of this thesis is to describe the effect of RLC phosphorylation level on cardiac myosin function. Specifically, how alterations to the extent of RLC phosphorylation can influence cardiac myosin mechanically and biochemically, and how this affects muscle contractility as a whole.

The RLC of myosin was first identified in 1969 and classified as a member of the EF-hand Ca^{2+} -binding protein superfamily due to its possession of a divalent cation-binding site (72-74). It is an 18 kDa protein that is bound to the neck region of myosin S1 at the second, more C-terminal of the two IQ motifs on the myosin molecule (Figure 1.2.3.1) (75). The N-terminus of the RLC is noncovalently bound to the myosin heavy chain at Asn-825 and Val-826 and the C-terminus wraps around the myosin heavy chain between Glu-808 and Val-826 (75). The RLC contains a divalent cation-binding site in its N-terminal region between amino acids 37 and 48, which can bind both Ca^{2+} and Mg^{2+} in the helix-loop-helix motif (Figure 1.4.3.2) (75). The binding of cations to RLC plays a role in altering the structural and contractile properties of myosin (75, 76). The structural data available for RLCs was obtained primarily with fast chicken skeletal myosin, and in more detail in scallop myosin (31, 77). So far there has been no crystal structure published of the cardiac RLC including the N-terminal 20 amino acids, which are home to the phosphorylation sites of the RLC. This is due to the regions inherent disorder making it difficult to resolve with crystallography.

It is important to note that not all mammalian species have the same identical RLCs in the adult left ventricle. Specifically, cardiac RLC has several isoforms in the human heart, which are distinct charge variants previously denoted as LC-2 and LC-2* (78, 79). The LC-2 isoform is the more highly expressed of the two at 70% of the total (78, 80). The LC-2 is also the more phosphorylated protein of the RLC isoforms in the

human heart; interestingly, disease does not seem to alter the expression levels of the two charge variants significantly (78). This means that the myosin molecules in human ventricular myocardium (with two heads and therefore two RLCs attached) can theoretically exist as nine different isoenzymes (LC-2/LC-2, LC-2/LC2* and LC2*/LC2* bound to either MHC α/α , α/β or β/β) (60). It must be noted there is little known about the relative abundances of these theoretical compositions of myosin molecules as we don't understand if certain RLC species preferentially bind IQ motifs of certain MHCs. However, this is not the same in rat myocardium, where to our current knowledge, only one isoform of RLC exists, and the myosin isoenzyme variety is instead solely altered by the combinations of alpha and beta myosin heavy chains (81). Additionally, it must be noted that there are further differences between human and rat RLC molecules. Human RLCs only possess one RLC phosphorylation site at serine-15, whereas two phosphorylation sites have been identified in the RLCs from mouse and rat myocardium to date (82). Regulation of RLC phosphorylation will be discussed in more detail below (Figure 1.2.3.2) (82).

In light of the evidence that RLC phosphorylation was dynamic in the myocardium (of rats), and that seemingly myosin light chain kinase (MLCK) activity could not account for all of the phosphorylation occurring, it was postulated that other kinases may also act on the cardiac RLC in-vivo (83). In relation to this point we now know of several MLCK isoforms that are expressed in cardiac tissues, these are: i) smooth muscle MLCK (smMLCK); ii) skeletal muscle MLCK (skMLCK); and iii) cardiac muscle MLCK (reviewed (84, 85)). Activation of these kinases is mediated via increases in cellular calcium. Broadly, MLCKs are inactive without the binding of Ca^{2+} /calmodulin. Binding of Ca^{2+} /calmodulin to the calmodulin-binding domain of the MLCK alleviates its autoinhibition, allowing the N-terminal tail of the RLC to interact with MLCK for phosphorylation (86). However, in the case of cMLCK there is conflicting evidence. Chan et al have reported activity of cMLCK without Ca^{2+} /calmodulin activation (87).

This was unexpected as cMLCK contains both the autoinhibitory and calmodulin binding domains characteristic of other MLCKs, and has a high affinity for Ca^{2+} /calmodulin (88, 89). Due to these conflicting results about cMLCK's dependency on Ca^{2+} /calmodulin binding, further studies need to be performed to clarify its regulation. In regard to which MLCKs play a physiological role in the phosphorylation of cardiac RLC, there is evidence that skeletal muscle MLCK (skMLCK) acts on cardiac RLC as it is detected in cardiac tissues (90). However, it is thought that the expression level of skMLCK is too low to maintain RLC phosphorylation (91). Ablation of skMLCK in cardiac tissue shows no reduction in RLC phosphorylation or cardiac abnormality phenotypes, strengthening the view that skMLCK activity may not be an essential kinase for cardiac RLC phosphorylation (91). Interestingly, smMLCK is also expressed in cardiac muscle cells, but at 10-20 fold lower concentration than cMLCK (87). Cardiac RLC is also a poor substrate for smMLCK, signifying that smMLCK would be inefficient at altering cardiac RLC phosphorylation (91). The role of smMLCK may be predominantly responsible for phosphorylation of the cytoplasmic non-muscle myosins within the cardiac cells, providing the reason for its presence in the myocardium (92). The loss of expression of the long isoform of smMLCK does not manifest in any changes to basal cardiac function, suggesting this may be correct (93). The predominant kinase responsible, and necessary, for basal cardiac RLC phosphorylation cMLCK was more recently identified in mice and humans (87, 88, 94, 95). The fact that cMLCK cardiac expression is at a level 10-20 times higher than smMLCK and skMLCK implicates it as the primary kinase responsible for the cardiac RLC (96). This assumption is backed up by the use of cMLCK knockout models, which observed that RLC phosphorylation is almost completely abolished by cMLCK knockout (87, 88, 94, 97). The regulation of RLC phosphorylation is also made more complex by the ability of an alternate kinase, zipper interacting protein kinase (ZIPK) to phosphorylate cardiac RLC (98). ZIPK activity is independent of the Ca^{2+} /calmodulin pathway and has been

shown to act both in cardiomyocytes, and the organ (98, 99). It is believed that ZIPK is not involved in upholding the basal RLC phosphorylation level in the heart (94), even though knockdown of ZIPK (by siRNA) in cardiomyocytes decreased RLC phosphorylation by 34% (98). The physiological role of ZIPK and its specificity for cardiac RLC has, as of yet, not been fully explored.

Control of RLC phosphorylation in the myocardium is a tug of war between the activity of kinases (cMLCK and ZIPK) and phosphatases. The phosphatases for controlling RLC dephosphorylation have already been identified. A study in cardiac muscle cells showed that cardiac RLC could be dephosphorylated by the phosphatase, type 1 phosphatase subunit-catalytic δ isoform (PP1C- δ) (100). An independent study showed that overexpression of a subunit for myosin phosphatase 2 (MYPT2), which complexes with PP1C- δ , caused increased PP1C- δ activity and resultant reduction in RLC phosphorylation that manifested with cardiac abnormalities (101).

Important questions pertaining to the regulation of RLC phosphorylation in normal physiological conditions, and importantly during disease, still need to be addressed. However, we do now know that basal RLC phosphorylation can be maintained by low activities of just cMLCK and PP1C- δ (95). Furthermore, how RLC phosphorylation level relates to the ability of RLC phosphorylation to influence myosin function is the main theme of this thesis and is elaborated below.

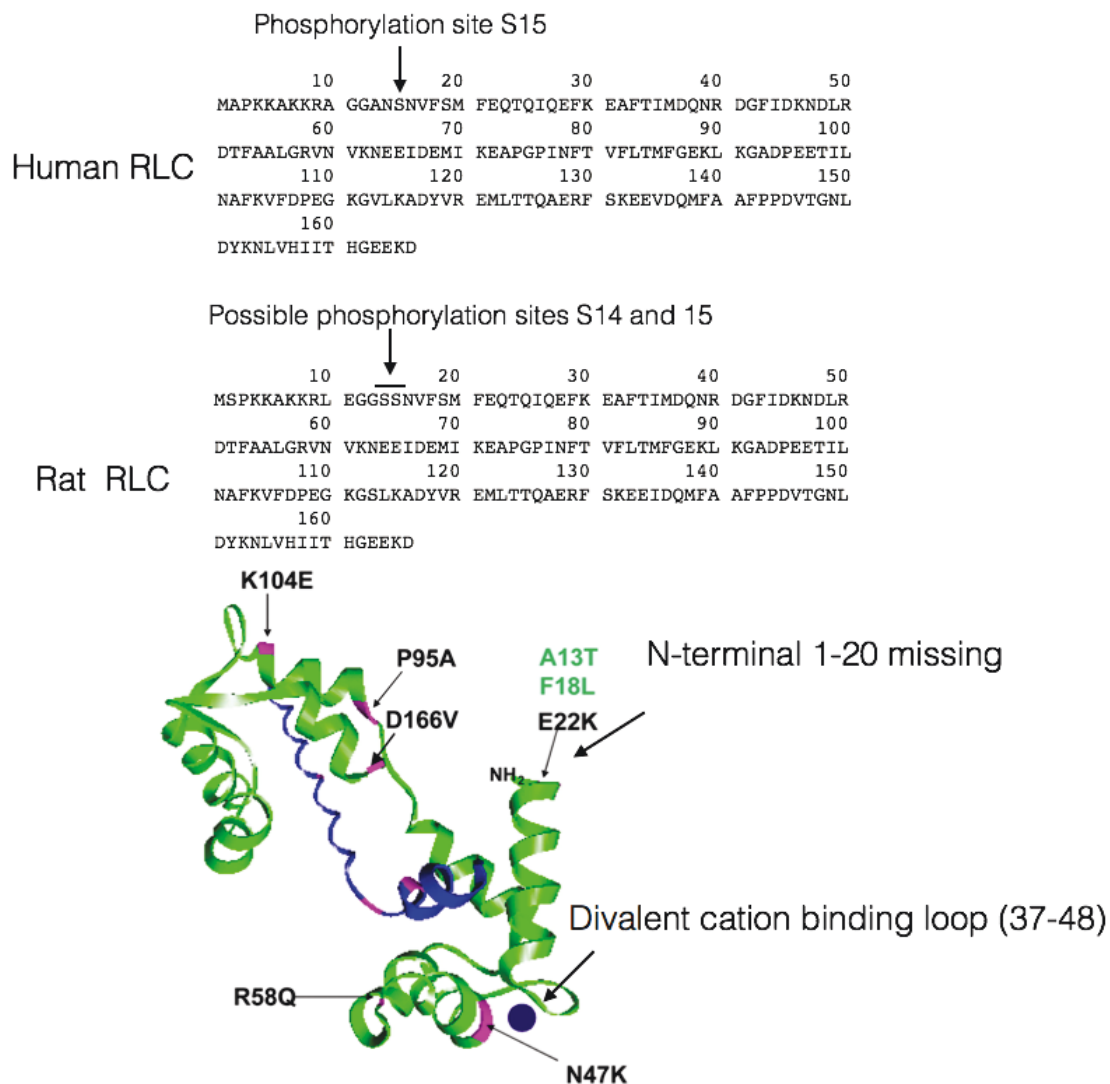


Figure 1.2.3.2: The RLC. **Top**, Amino acid sequences of human ventricular (*Homo Sapien*) regulatory light chain (UniProt Q14908) and rat (*Rattus norvegicus*) ventricular regulatory light chain (UniProt P08733) (proposed phosphorylation sites annotated for both species). **Bottom**, Schematic representation of human ventricular regulatory light chain indicating familial hypertrophic cardiomyopathy causing one-point missense mutations taken from Szczesna-Cordary et.al. 2005 (102). Notably there is no available proposed structure for amino acids 1-20, which contain the phosphorylation sites in mammalian cardiac RLCs. This is due to the inherent disorder of the N-terminal 1-20 region.

1.3 Regulation of myosin by RLC phosphorylation

Historically, the first evidence that RLC phosphorylation played a role in the activation of the actin activated ATPase of myosins in general came in 1975 in platelet myosin (now known as non-muscle myosin II A) (103). This finding was swiftly followed by near simultaneous publications showing the same to be true in smooth muscle myosin (104-107). The regulation of the activity of these myosins by RLC phosphorylation was an 'on/off' switch of the myosins ATPase. Therefore, the activity of the contractile system was controlled at the level of RLC phosphorylation, determined by the activity of Ca^{2+} /calmodulin regulated MLCKs, and myosin specific phosphatases (as discussed above). This phenomenon is not upheld in striated muscle myosins. Striated muscle myosin activities are regulated by the availability of myosin binding sites on actin, mediated by Ca^{2+} binding to the thin filament regulatory complex, as previously mentioned. This left a conundrum, does myosin RLC phosphorylation in striated muscle alter striated myosin function?

It was discovered that RLC phosphorylation did indeed modulate actin activated ATPases of cardiac myosin (108, 109); and that cardiac RLC phosphorylation was dynamic *in vivo* during 'physiological responses' of the myocardium (adrenaline infusion, cardiac pacing, exercise, and stretch) (110-115). However, it was not an 'on/off' switch for striated muscle function.

The resting cardiac RLC phosphorylation level was discovered to be between 0.3-0.4 mol Pi/ mol RLC (116), concomitant with the first purified myosin light chain kinase from myocardium (117). Building upon *in vitro* studies, it was shown *in vivo* that abolition of RLC phosphorylation (siRNA knockdown or transgenic expression of nonphosphorylatable RLCs) altered cardiac structure and led to hypertrophy, dilatation of cardiac chambers, and reduced systolic pressure (88, 118, 119). This inferred that RLC phosphorylation was indeed important for upholding a normal cardiac structure and function. Further *in vivo* work showed that RLC phosphorylation was raised during the hypertrophic response and aided contractile function post

infarction (120, 121); and that this held true for an aortic constriction model of hypertrophy (rat myocardium) and in acute ischemia (canine myocardium) (122, 123). Further *in vitro* experiments in muscle (cardiac myofibrils) described that enriched RLC phosphorylation correlated with a reduction in cross-bridge cycling (124). Studies in isolated fibre bundles (porcine ventricular myocardium) showed that enriching RLC phosphorylation increased the calcium sensitivity of the preparations and increased force development (only in limiting $[Ca^{2+}]$) (125-128). Structural x-ray diffraction studies postulated that RLC phosphorylation was causing a shift of myosin heads away from the thick filament backbone, towards the thin filament (129).

Interestingly, a spatial gradient of RLC phosphorylation throughout the ventricular myocardium has been observed, the inner myocardium has a lower phosphorylation level than the outer (90, 130). The balance between kinase and phosphatase activities in separate myocardial layers creates this gradient; it has been established that the activity of RLC phosphatases in the outer myocardial layers are lower than in the inner layers (131, 132). Differences in RLC phosphorylation levels across myocardial layers are postulated to reduce wall stress in the inner myocardium, whilst ensuring efficient contraction of the entire organ (90). This phenomenon creates another level of complexity on top of the molecular level effects of RLC phosphorylation, as the function of the whole organ must be considered.

Undoubtedly, appropriate RLC phosphorylation is needed for normal cardiac function. When this balance is disturbed it leads to changes in tissue structure, and changes in the function of individual myosin molecules, discussed below.

1.4 RLC phosphorylation in disease

Interestingly, there exists a spectrum of cardiac disorders that are either caused by, or result in changes to, cardiac RLC phosphorylation. Some of these are inherited diseases caused by mutations in the RLC or other sarcomeric protein genes. Some are acquired cardiac disorders, including but not limited to ischemic disease, pressure overload disorders, and heart failure. These diseases display varying magnitudes of RLC phosphorylation level change. Some show increased RLC phosphorylation, whilst others show diminished RLC phosphorylation. Enriched RLC phosphorylation by transgenic overexpression of phosphorylated RLC does not detrimentally alter cardiac physiology, and may be protective in reducing the hypertrophic response in diseased tissues (133). This infers that the changes to RLC phosphorylation that are causative of disease are linked to diminished RLC phosphorylation, not enriched phosphorylation. Increased RLC phosphorylation is more likely to be an adaptive response to a disease phenotype, which may however, become maladaptive later in disease progression.

There are many identified RLC mutations that can be broadly characterised as having two effects on RLC phosphorylation (Figure 1.2.3.2). 1) The K104E mutation in the cardiac RLC, which is not near the myosin RLC phosphorylation site, is typified by reduced RLC phosphorylation (not due to interference of phosphorylation by the kinase) (134). This phenomenon is also observed in other mutants, which are distant from the RLC phosphorylation site, such as the D166V and R58Q mutants (135-137). Interestingly, these mutants present with left ventricular hypertrophy. There is evidence that using ventricular porcine myosin to artificially increase the phosphorylation level of nonphosphorylatable RLCs, by replacement with recombinant pseudophosphorylated RLCs, (replacement of serine-15 with negatively charged aspartic acid to mimic the negative charge of serine phosphorylation) can partially alleviate the disease phenotype, (actin activated ATPase, actin gliding under load, calcium sensitivity but not isometric force) in the case of the D166V mutant

(138). 2) The E22K mutation shows completely ablated phosphorylation, and seemingly interferes with the ability of kinases to phosphorylate the usual serine 15 residue (139). In some of these mutants, cardiac diseases are indeed caused by an inability to phosphorylate the RLC appropriately (140, 141), rather than the RLC phosphorylation change being a side effect of the disease state. There is also evidence that mutation of other proteins that cause hypertrophic cardiomyopathies (HCM) display altered RLC phosphorylation levels. This includes the MHC R723G mutation, which has a reduced RLC phosphorylation and a phenotypic profile of left ventricular dysfunction, hypertrophic disease with possible outflow obstruction, sudden cardiac death, and heart failure (142). The R723G mutation was characterised as having a reduced maximum force generating capability, but unchanged calcium sensitivity, although, this was measured in end stage disease with significant myofibrillar loss and disarray (143). Another sarcomeric protein mutation this time of the ELC (A57G mutation), characterised by septal hypertrophy, showed increased RLC phosphorylation concomitant with reduced isometric force production, and increased calcium sensitivity in a background of sarcomeric disarray (144, 145). Interestingly, in the case of both of these mutants, which display similar contractile phenotypes (yet opposite RLC phosphorylation profiles), it was not established if the phenotypes were a direct result of the mutations, or were secondary responses to the mutations by the myocardium in end stage disease. This highlights that we still do not have a clear cause and effect relation between mutation, phenotype, and RLC phosphorylation levels in disease. This leaves us further questions: i) is RLC phosphorylation change adaptive or maladaptive in genetic disease; ii) does this paradigm shift later in end stage disease?

Acquired cardiac disorders can also affect RLC phosphorylation levels. Diabetic cardiomyopathies have been shown to exhibit reduced RLC phosphorylation concomitant with contractile function loss (146). Pressure overload phenotypes have

been shown to develop reduced RLC phosphorylation with impaired diastolic relaxation (97, 147). There is conflicting evidence on the effect of myocardial infarction (MI) on RLC phosphorylation. RLC phosphorylation has been shown to be stable in left ventricular myocardium up to 4 weeks post-infarct, whilst others suggest it can be acutely (within days) reduced post chronic myocardial infarction, then increase to higher levels as disease progresses (148-152). The differences in many of these measurements may, in part, be due to the time of tissue sampling in the disease timeline. Additionally, there is evidence for both RLC phosphorylation reduction and enrichment in end-stage heart failure, whereas there is only evidence for reduction in hypertension and dilated cardiomyopathies (153-158). Differences in the literature could be accounted for by area of myectomy sampling, as Berlin et.al. have shown RLC phosphorylation change to be chamber specific in chronic heart failure (159). Reduced RLC phosphorylation in end-stage heart failure is hypothesised to be compensatory. This is because it counteracts increased calcium sensitivity of the myofilaments created by other tissue alterations. However, not all types of heart failure seem to display this phenotype. We ourselves have observed increased RLC phosphorylation in a rat model of chronic myocardial infarction that is studied at two clinically relevant time-points (compensation and decompensation) during tissue remodelling post infarction (1).

This data again leaves us with specific questions: i) In acquired cardiovascular disease is RLC phosphorylation adaptive or maladaptive; ii) what governs the magnitude and level of RLC phosphorylation change?

1.5 Rationale of this study

Although certain aspects of RLC phosphorylation have been explored, there remain uncertainties concerning the role and mechanism by which RLC phosphorylation modulates cardiac myosin. Previously it has been hard to extract the specific effects of RLC phosphorylation from the effects of other sarcomeric protein modifications. This is in part due to the use of *in-vivo* and *in-vitro* techniques, which have likely altered other sarcomeric proteins during the course of the studies. Therefore, we have employed a method to influence RLC phosphorylation without affecting other proteins of the sarcomere. We have done this by expressing recombinant RLCs, phosphorylating them to different levels and exchanging them with native RLCs. This has been done in cardiac muscle strips (trabeculae) from rats to allow us to measure force and power produced in muscle strips during shortening, a set of parameters which have not been assessed systematically before. These parameters are important, as the mechanical work performed in systole occurs during muscle shortening. This shortening, combined with the ventricular pressure that it builds, functions to expel blood from the ventricles. The importance of RLC phosphorylation in ensuring the appropriate contractile function of the heart has been highlighted above, from these findings we believed that RLC phosphorylation likely plays a role in the force-velocity relation of myocardium.

As we discovered that the force-velocity relation of the myocardium was altered by RLC phosphorylation, we wanted to understand what was causing this effect. Therefore, we studied the effect of RLC phosphorylation in biochemical assays at the molecular level, namely on the ability of myosin to use MgATP and the ability of myosin to translocate actin under load as molecular ensembles. This was combined with single molecule assays to determine how ATP concentration can alter the duration of single myosin and actin binding events, and how RLC phosphorylation change influences the power stroke. The purpose of these experiments was to

improve the molecular understanding of RLC phosphorylation effects on cross-bridge kinetics and mechanics.

Additionally, it is still unclear how RLC phosphorylation is altered in the myocardium of individuals who have had acute or chronic myocardial infarctions. Therefore, we studied how RLC phosphorylation was affected post MI in a rat model of chronic MI. The intention was to understand how dynamic post-translational modifications may be altered during different phases of tissue remodelling in the progression towards heart failure. We have used human heart failure samples from patients who have either acquired or inherited forms of heart failure to validate our animal model data. This line of investigation allows us to integrate what we have learned about the mechanical, contractile and kinetic effects of RLC phosphorylation to observations of RLC phosphorylation level in cardiac diseases and disorders.

With these investigations we have added a new understanding to the existing literature. This has allowed us to better understand the role that RLC phosphorylation can play in health and disease.

We have observed that RLC phosphorylation level can alter power and force during muscle shortening, whilst also influencing the peak unloaded shortening rate of the muscle. These findings were explained by ensemble and single molecule experiments, which indicated that RLC phosphorylation level could alter the actomyosin cycle time, the duration of the strongly bound actomyosin states, influence myosin step size, and alter the ADP release rate.

1.6 Context of this thesis

The research displayed herein was performed in two laboratories with complementary expertise in muscle physiology, biophysics, and biochemistry. We began the project with the hypothesis that:

- RLC phosphorylation level is altered in diseases that are either inherited or acquired.
- RLC phosphorylation alters the ability of myocardium to produce force and power, manifesting in changes to the force-velocity relationship of cardiac muscle.
- Assuming that RLC phosphorylation alters the force-velocity relation, it does this through alterations in either, i) the cross-bridge mechanics; ii) the kinetics of ensembles and single myosin molecules; iii) or both.

To address these research aims we have assessed the effects of RLC phosphorylation at different complexity levels: permeabilised muscle, myosin ensembles, single molecules, and bulk biochemical assays of muscle proteins in solution.

Chapters 2 and 3 of this thesis outline in detail how these experiments have been designed and carried out. Chapters 4, 5, and 6 present the results of our biochemical and mechanical investigations into cardiac myosin RLC phosphorylation. We provide a discussion of this work in the context of previous experimentation and provide future directions in chapter 7.

2 - Methodologies for mechanical experimentation in cardiac tissue

2.1 Excision of cardiac tissue from rat

2.1.1 Trabecular Isolation

Female Sprague-Dawley rats weighing between 250-350 g were anaesthetised using isofluorane inhalation. Full anaesthesia was determined by observing a depression in the rate of respiration and the loss of the ability to balance and react to gentle inversion of the anaesthesia chamber. Animals would then immediately undergo cervical dislocation. The abdomen was opened by an incision posterior to the diaphragm. The incision was widened to the width of the abdomen, the diaphragm was then resected to open the thorax and expose the pericardial cavity. The heart was held between two fingers and lifted out of the pericardial cavity to gain access to the aortic arch. Fine scissors were used to excise the heart above the aortic arch to preserve the structure of the aorta for cannulation and retrograde perfusion.

Upon removal, the heart was plunged into ice-cold oxygenated (95%) Krebs-Henseleit (KH) solution (composition in mM: 119 NaCl, 4.7 KCl, 0.94 MgSO₄, 1 CaCl₂, 1.2 KH₂PO₄, 25 NaHCO₃, 11.5 glucose) incorporating 2,3-Butanedione Monoxime (BDM, 30mM) and Heparin (12 units/ml). BDM is a compound that reduces tissue hypercontracture during dissection by interfering (reversibly) with calcium regulation of contraction and calcium homeostasis in the cells (160). BDM has also previously been described as having phosphatase activity (160), although we find no evidence of this in our experiments. The heart was massaged in this solution to remove blood, preventing coagulation and hypercontracture.

The heart was then placed in a dissecting dish with a Sylgard base on the cooled stage of an upright dissection microscope. Excess free hanging fat was trimmed away and the aortic arch was removed to leave a single section of aorta leading into the left ventricle. Through this opening the heart was cannulated using Langendorff

apparatus. The tissue was subsequently retrogradely perfused with fresh oxygenated KH solution with BDM (30 mM). The flow rate of the apparatus was adjusted to prevent chamber bulging, usually at a rate of 1-5 mls per minute. The heart was removed when the solution ran clear of blood.

The heart was then pinned down in a Sylgard dish in fresh KH solution with BDM. The atria and then right ventricle were removed (by trimming along the septum within the line of the right atrium). Once the right atrium was removed the entire aorta was dissected away. The left ventricle was opened through the aortic outflow along the septum between right and left ventricle (LV) towards the apex of the heart. When this was achieved the LV was pinned back to access the endocardium of the LV.

Free hanging un-branched trabeculae of between 1-2 mm length and 100-300 μ m in width were excised. It was usual to have a yield of between 2 and 5 trabeculae from each cardiac preparation. These trabeculae were moved to a rectangular dissecting dish with fresh oxygenated KH solution with BDM. When all free hanging trabeculae were removed, aluminium T-clips were attached at either end. These clipped trabeculae were then moved to a small volume-dissecting dish with Sylgard and pinned down to a length just beyond slack (Figure 2.1.1).

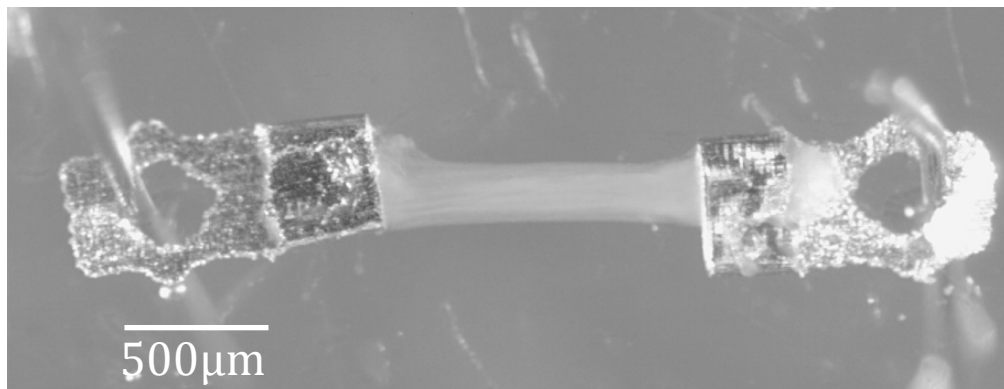


Figure 2.1.1: The trabeculae. Permeabilised trabecular preparation held in place at both ends using folded aluminium T-clips in a Sylgard coated dish.

2.1.2 Trabecular permeabilisation and storage

Once pinned down, trabeculae were chemically permeabilised using a 2% Triton X-100 incorporating Relaxing solution in which they were bathed for 30 minutes at 5°C (Composition mM: 60 TES, 8.66 MgCl₂, 20 EGTA, 5.43 Na₂ATP, 10 Glutathione, 33.71 potassium propionate at pH 7.4; potassium propionate was used to increase ionic strength as a counterion to NaCl). Chemical permeabilisation disrupted cellular membranes and the membranes of intracellular structures, such as the sarcoplasmic reticulum (SR) and mitochondria. This process was performed to allow direct access to the sarcomeric contractile machinery, whilst disrupting the ability of the tissue to produce its own ATP. It also disrupts the ability for calcium homeostasis, leaving the sarcomeric machinery energetically isolated and unable to become activated by its own calcium release. This permeabilisation is performed to allow isolation and preservation of the contractile machinery for mechanical experimentation.

Upon completion of permeabilisation the preparations were rinsed with several dish volumes of chilled relaxing solution to remove remaining Triton X-100. Chilled 50% relaxing solution containing 50% glycerol and which incorporated protease inhibitors (4 mg/l leupeptin, 10 mM PMSF and 50 mg/l trypsin inhibitor) were then added to the dish, which was transferred to a -20°C freezer for storage. Trabeculae were kept no longer than 3 days; typically experiments were conducted the day after sample preparation. Storage had no effect on trabecular function.

Prior to experimentation, trabeculae were rinsed with relaxing solution at 5°C to remove storage glycerol. Samples were kept at 5°C on the day of experimentation on the chilled stage of an upright microscope.

2.2 Experimental set-up of temperature jump apparatus

The experimental temperature jump (T-Jump) was used for all mechanical measurements of force and velocity in permeabilised trabeculae. The stage consisted of two platforms each with two pedestals that were coated with quartz sheets (Figure 2.2.1). The platforms could be individually heated and cooled by the flow of water through the platforms' core. The rear platforms (1 & 2) were cooled to 0.5°C whilst the front platforms (3 & 4) were held at 20°C. Attached to the front platform was a quartz trough that was engineered to be open ended with Teflon inserts to retain solution. This trough is used to set sarcomere length by laser light diffraction, and measure trabecular dimensions by light microscopy.

30 µl drops of solution were placed onto the quartz pedestals of these platforms and formed a bubble. The solution bubble remained a uniform shape when the same volume was applied to each platform, this was achievable as the platform dimensions were identical.

The trabeculae were suspended above these platforms in experimental solutions using two hooks. The experimental hooks were hand-made from 0.05 mm-thick stainless steel wire that had been sharpened and bent to form the hook. On one side of the preparation, the hook was attached to a silicon wafer that acted as a force-transducer (AE801; HJK Sensoren, Friedburg) to obtain measures of force, which were normalised to trabecular cross-sectional area. On the other side the hook was attached to a homemade motor that was able to move the hook with precision in rapid movements with amplitudes up to 250 µm.

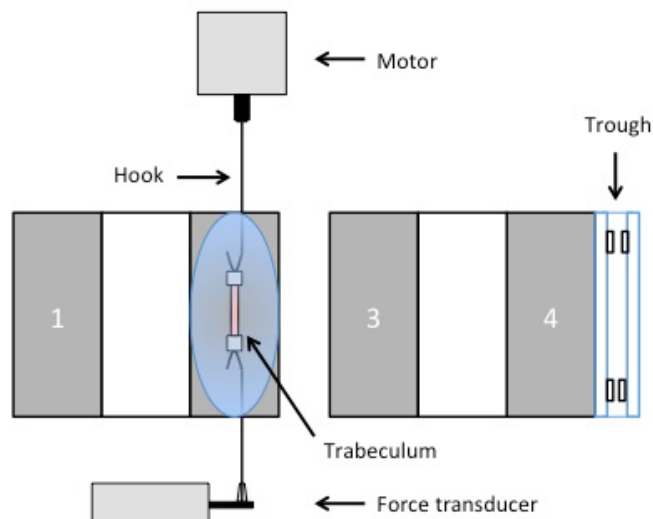
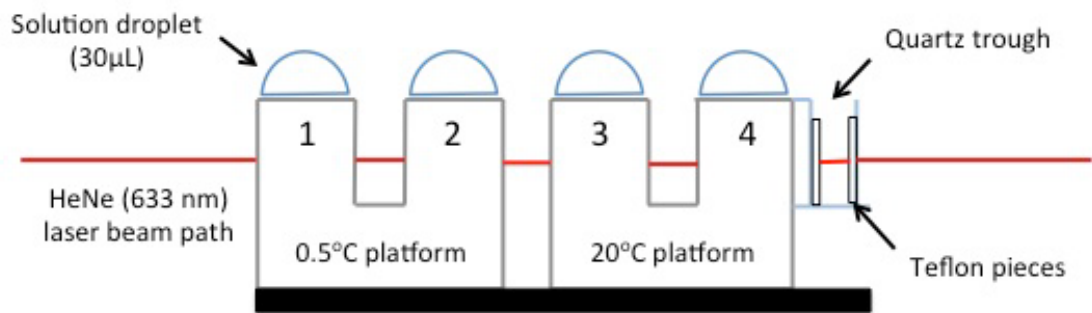


Figure 2.2.1: The temperature jump apparatus. Top, Side view of temperature (T-jump) experimental platform. Red line indicates beam path of HeNe laser for measuring sarcomere length when fibres are immersed in calcium free (relaxing) solution in the quartz trough. **Bottom,** Top down view of the same platform illustrating the hooks used to hold the trabeculae in droplets on top of each pedestal. The trabeculae were held above different pedestals by rapid sliding of the platform using a horizontal stepper motor.

2.3 Sarcomere length measurement and trabecular dimensions

Once trabeculae were attached to the experimental set-up by aluminium T-clips and fixed with shellac, they were submerged in a calcium free relaxing solution in the quartz trough. A Helium Neon (HeNe) 633 nm laser beam passed through the preparation to yield a diffraction pattern. This diffraction pattern is indicative of the regular order of sarcomeric proteins. The laser light diffraction produces a zero order band, a pair of symmetrical first order diffraction bands and often second order diffraction bands. These bands were visualised by projecting this diffraction pattern onto a paper screen at a fixed distance (k) from the point at which the laser light passed through the trabecular preparation. The sarcomere length was calculated by applying Bragg's law:

$$SL = k \lambda / d$$

Where sarcomere length (SL) was calculated by determining the wavelength of the incident laser light (λ), the distance between the specimen and the screen (k), and the distance between the first order diffraction bands on the screen (d).

Micromanipulators attached to the motor and force transducer housings were used to manually move the hooks and adjust the muscle to attain the desired sarcomere length at rest (2.1 μ m). Diffraction patterns in larger sections of cardiac tissue are more diffuse and harder to measure. This is because trabeculae scatter light to a greater extent than skeletal muscle cells, creating a more diffuse diffraction pattern. At the end of each experiment sarcomere length was re-measured to make sure a constant sarcomere length of 2.1 μ m was upheld.

Whilst submerged in the quartz trough, an up-right microscope was used to measure preparation length, width, and depth. Length was measured by using a stage micrometer (1 division = 10 μm), and translating the stage to visualise both ends of the trabecula. Width measurements were obtained with an eye-piece graticule (1 division = 19 μm) on the up-right microscope. Depth measurements were made with a fine-focusing dial with micrometer markings (1 division = 10 μm) on the microscope. The bottom and top of the preparations were focused in turn and thereby the distance in-between was measured. Cross-sectional area (CSA) was calculated by approximating the preparation as having an oval shape. Measurements of width and depth were performed at three points along the length of the preparation, and the following equation was used to calculate the samples CSA:

$$\text{CSA (m}^2\text{)} = (\text{Depth (m)} \times \text{Width (m)} \times \pi)/4$$

CSA was used to normalise force data obtained from mechanical experimentation to each preparation's cross-section. This allows comparisons to be made between preparations of differing dimensions.

2.4 Experimental protocols

2.4.1 Control of the experimental temperature jump apparatus

A tailor-made LabView programme was used to control the horizontal and vertical stepper motors for moving the experimental platform (Dr. Marco Caremani). There were two modes to this programme. The programme could be used to make fine adjustments to the platform's position to fine-tune the exact location of the preparation over the platform. Full activation cycles could also be performed where the preparation was moved over several platforms, and held in place for defined periods. This programme also integrated the controls for the other custom-built motor that was used to allow controlled shortening of the trabecula for force-velocity and slack test measurements. The movement of the platforms could be synchronised to the activity of the motor controlling trabecula length to achieve automatic and reproducible experimental protocols.

2.4.2 Slack test measurements

Once an appropriate trabecula had been mounted it was fixed in place by gluing the T-clips to the hooks using a paste of shellac dissolved in ethanol. It was important to do this to prevent the T-clips from falling off the hooks and to reduce mechanical vibrational noise from the slackening procedure. Prior to any slack test measurements, trabeculae underwent a full cycle of activation and relaxation to ensure the muscle was functional. Poorly functioning trabeculae could be identified, as they were either unable to relax fully after activation, manifesting in a 'passive' stiffness, or produced poor isometric force. Activation was achieved by the movement of the preparation through a series of solutions on top of the platforms as depicted in table 2.4.2.1. A representative force trace is shown in figure 2.4.2.2. Activation cycles began with the trabecula in a pre-activating solution on top of platform 1 (Table 2.4.2.1). The preparation was then moved to a low temperature

(2°C) activating solution and held for a few seconds to allow the diffusion of calcium and ATP into the core of the muscle. Then the preparation was moved to the next platform that held warm (20°C) activating solution where it would develop force until an isometric plateau was observed. At this point the muscle preparations were released rapidly between 8-14% of their initial lengths within 1-2 ms (Figure 2.4.2.3). These length changes were held for a second, allowing force to redevelop to isometric plateau at the lower sarcomere length. The trabecular preparations were then moved from activating a relaxing solution (without calcium), before the muscle was re-stretched to its original length. This was done to minimise tissue damage by re-stretching while the muscle was stiff, and still producing force. These cycles of activation were repeated for slackening lengths of 8, 10, 12 and 14% for all preparations.

Peak unloaded shortening velocity ($V_{\max}$) was measured from these manoeuvres. The time it took from the point denoted $T = 0$ (defined by the endpoint of the motor release) for the slack to be taken in was defined by the deviation of force from slack force ($F = 0$). The time it took for the slack of the release to be taken in (seconds) was then plotted as a function of the release length (normalised muscle lengths). This was done for all release lengths and fit by linear regression. The slope of the linear regression provided the $V_{\max}$ with the units of muscle lengths (ML) per second (ML/s).

	Relaxing (pH 7.1)	Pre-activating (pH 7.1)	Activating (pH 7.1)
TES	100	100	100
MgCl₂	7.8	6.8	6.5
Na₂ATP	5.7	5.7	5.7
EGTA	25	0.1	0
GLH	20	20	20
HDTA	0	24.9	0
CaEGTA	0	0	25

Table 2.4.2.1: Experimental solutions. All value concentrations in mM. The pH was set for the specific temperature that the solution was used for.

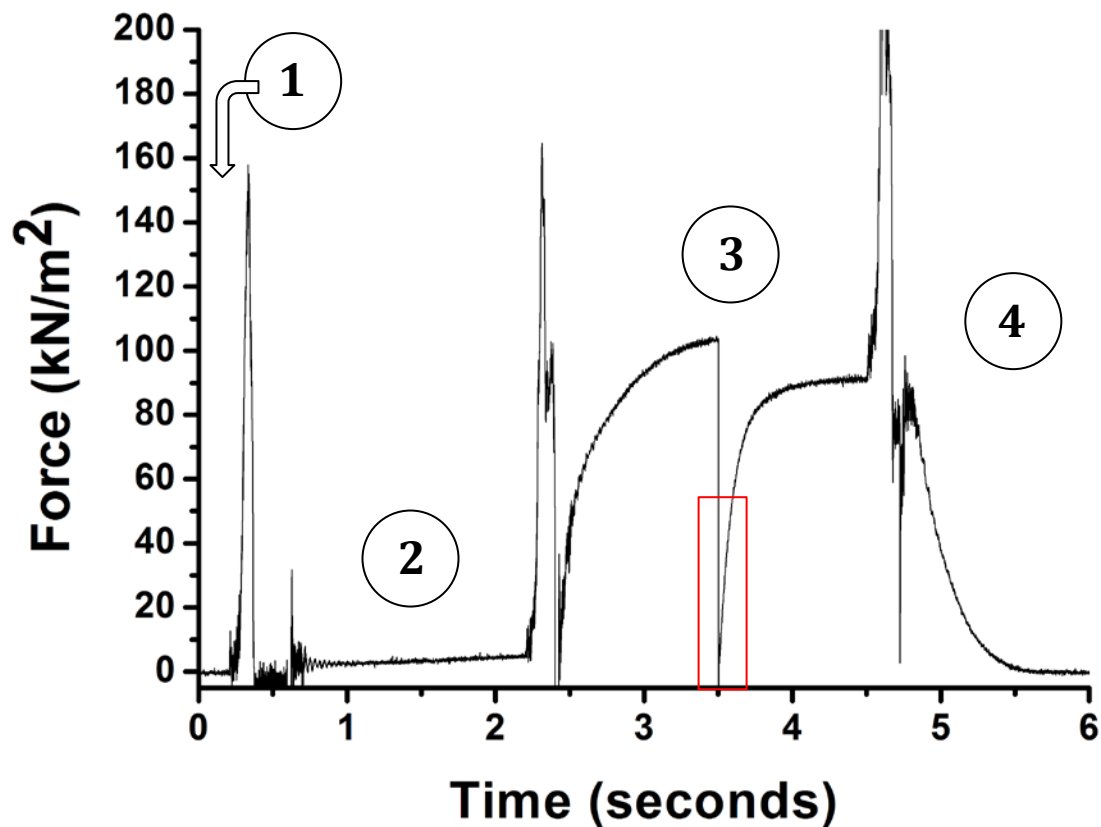


Figure 2.4.2.2: Trabecular activation raw force trace. Denotations of the platform number that the muscle preparation was held over are circled (Figure 2.2.1 for reference). The activation cycle began on platform 1 at 0.5°C in pre-activating solution. The preparation was then moved to platform 2 and held there for approximately 1.5 seconds in 0.5°C activating solution to allow the diffusion of ATP and calcium into the core of the sample. The preparation was then moved to platform 3 to 20°C activating solution where tension developed to an isometric plateau. The red box denotes the region where the mechanical manoeuvre was performed by releasing the muscle. The period following this allowed the preparation to re-develop isometric plateau at a lower sarcomere length. Four signified the movement of the preparation into 20°C relaxing solution where calcium is sequestered and the muscle is de-activated.

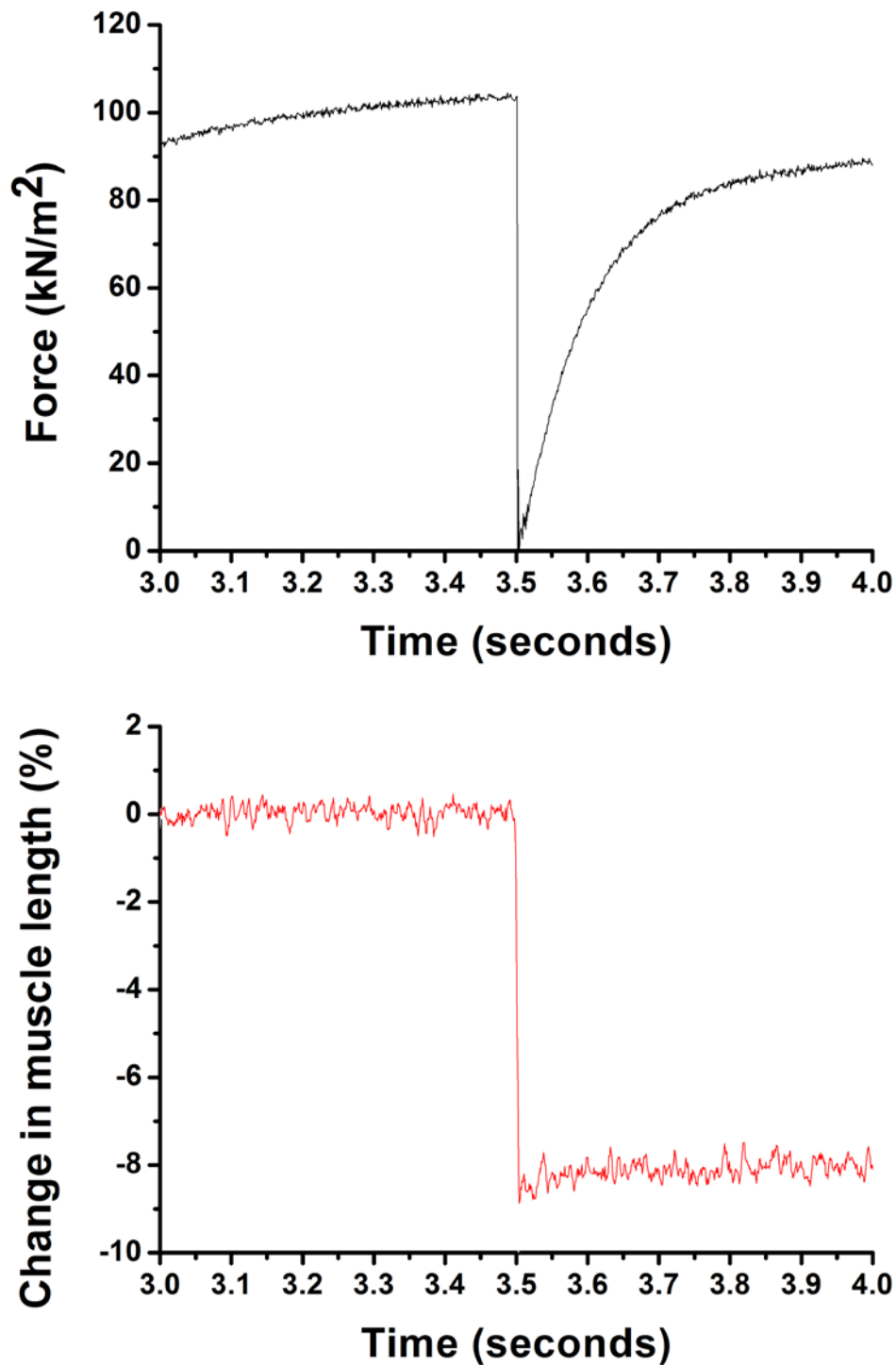


Figure 2.4.2.3: Slack test raw force trace. Enlarged traces of a slack test manoeuvre as highlighted by the red box in figure 2.6.2. **Top**, the force trace for the slack test manoeuvre. **Bottom**, the motor output trace showing the release length of the sample as a percentage of initial muscle length. The time for the motor to move from 0 to approximately -8% was measured to be 3 ms.

2.4.3 Force-velocity measurements

Once a preparation had been mounted and the Edman style slack tests had been performed the same exact trabecular preparation would undergo a series of force-velocity measurements. These manoeuvres were carried out until a full set of measurements had been obtained. Experimentation was cut off if the isometric force of the preparation dropped below 80% of the initial isometric force measured with the first activation cycle. A similar series of solution changes were applied to the preparations as had been performed for slack tests (Figure 2.4.2.2), but the motor releases of the muscle were different. Trabeculae were initially released between 2-3% of their initial length rapidly (~2 ms) releasing stored series elasticity. This initial release manoeuvre was identical in velocity to the slack manoeuvres, but to shorter release lengths, and was termed a motor 'step'. Once the initial rapid release had occurred a slower fixed velocity 'ramp' release was then performed, this release accounted for another 5-6% of initial muscle length and was performed at velocities between 0.1 and 1.5 muscle lengths per second (ML/s) (Figure 2.4.3.1). If an appropriate rapid release was performed before the fixed velocity release, a force plateau was observed during the slower ramp release (Figure 2.4.3.2). This was the force that could be maintained during a release of that specific velocity by the preparation. The forces observed over the range of release velocities (0.1-1.5 ML/s) were plotted to produce a force-velocity relation for each individual preparation.

For each trabecula the relationship between shortening velocity and the force during shortening was fit using a hyperbolic relation previously described by Hill (23). Excel solver was used to fit these relations and is demonstrated in figure 2.4.3.3.

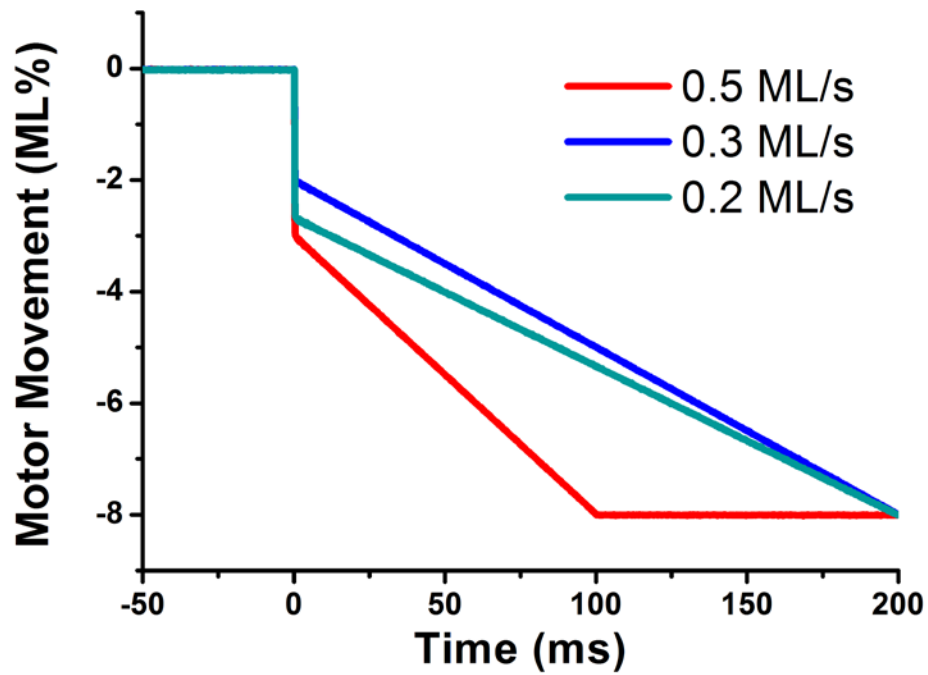


Figure 2.4.3.1: Motor input trace. Representative motor manoeuvres used to produce measurements of force during fixed velocity releases. All releases had an overall length of 8% of initial muscle length. The initial 'step' release and 'ramp' release lengths were adjusted to appropriately release any series elastic elements stored in the system from maintaining isometric plateau.

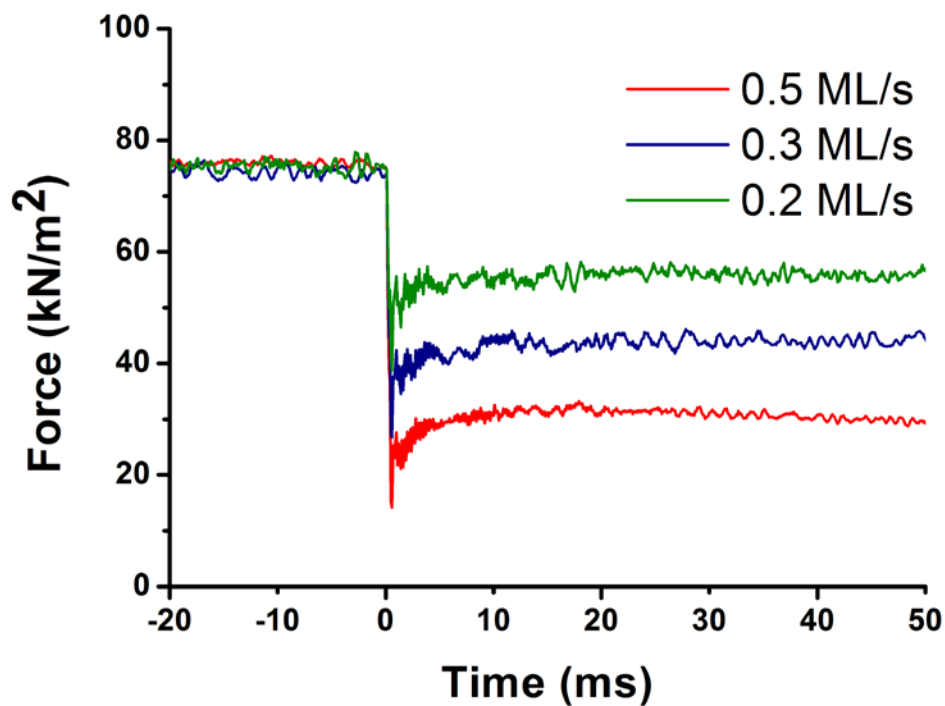


Figure 2.4.3.2: Force traces from force velocity measurements. Representative force traces from fixed velocity ramp manoeuvres shown in figure 2.3.4. Up to time = 0 the muscle was held at an isometric plateau, and then underwent a rapid 'step' release followed by a fixed velocity 'ramp' release, which began at $t = 0$. The force held at the specific 'ramp' release velocity was measured from the force plateau during the release.

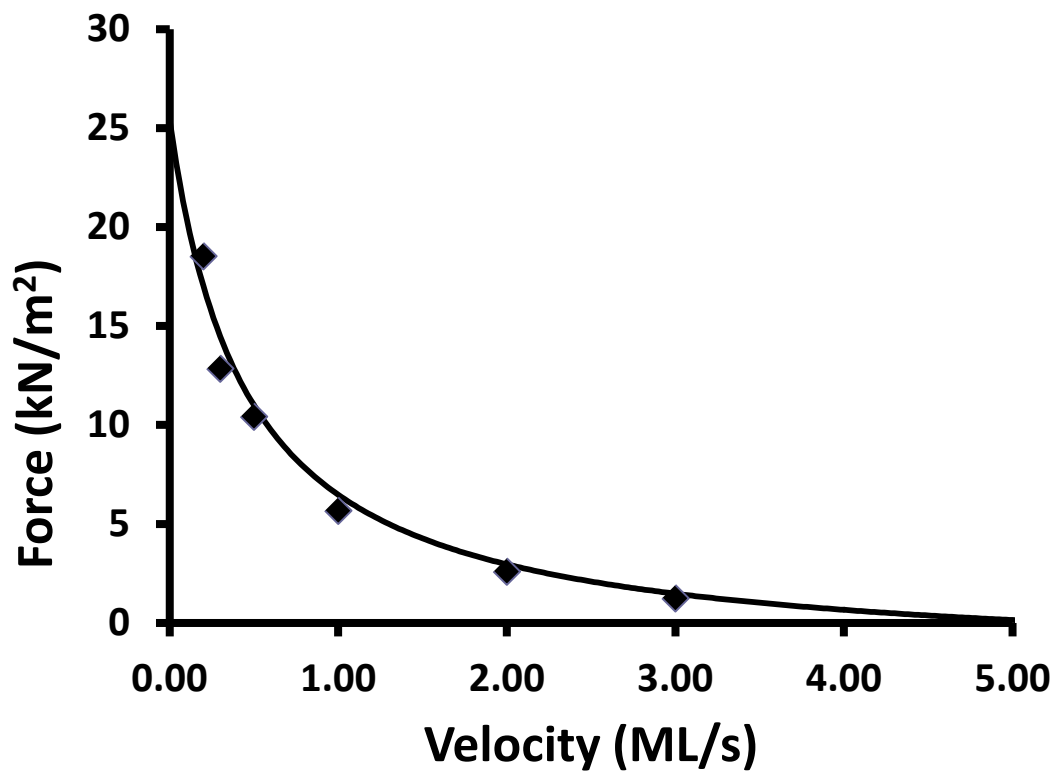


Figure 2.4.3.3: Force velocity relation. Plotted forces from a single trabeculum that had undergone many different velocity ‘ramp’ manoeuvres. The data set was fit using excel solver, applying the Hill equation, which was left un-constrained at both axis intercepts.

2.4.4 Data collection and analysis

Data was collected through a custom designed LabView programme (designed by Marco Caremani). For our experiments 2 channels were recorded, these channels gave the motor output voltage and the force transducer output voltage. Data was recorded for up to a minute. All signals were recorded at 10 kHz for the duration of the experiment. Data was exported to Excel 2007 for analysis. Data was plotted using Origin 8.5. Unless otherwise stated all data is expressed as a mean $\pm$ standard error of the mean (SEM). Statistical significance was assessed using two-way ANOVA (when making multiple comparisons) with post hoc pairwise multiple comparisons made using a Bonferroni adjusted t test method, where a cut off of $p < 0.05$ considered significant. This statistical analysis was applied when analysing all force velocity parameters measured between treatment groups.

2.5 Production and labelling of recombinant cardiac RLC

Recombinant cardiac RLC (cRLC) from *Rattus Norvegicus* was expressed in the laboratory of Malcolm Irving at King's College London under the guidance of Dr. Thomas Kampourakis. A large proportion of the cloning and expression was performed by Thomas Kampourakis, therefore this data is excluded from this thesis. The recombinant RLC was buffer exchanged to a labelling buffer (composed of: 0.1M NaCl and 0.1 M Sodium phosphate at pH 7.2) using PD-10 columns (GE Healthcare) and subsequently incubated with an NHS-Rhodamine ester 10 mg/ml in Dimethyl sulfoxide (DMSO) (Thermo Scientific). The dye reacts with the primary amine group of lysine residues, as shown in figure 2.5.1

Excess dye was removed using ZebaTM desalting spin columns (Thermo Scientific). The buffer was then exchanged using a PD-10 column to an 'exchange buffer' (5 mM ATP, 5 mM EGTA, 5 mM EDTA, 10 mM imidazole, 150 mM potassium propionate, 10 mM KH₂PO₄, 5 mM DTT, and 0.5 mM trifluoperazine (TFP) at pH 6.5). TFP is a phenothiazine that can be used to destabilise the interactions of RLC with its IQ motif allowing the exchange of light chains in solution, previously it has also been used to exchange the ELC (161). Protein concentration at this stage could no longer be ascertained with absorbance at 280 nm due to the inclusion of TFP, which masked absorbance at 280 nm. Therefore protein concentration was estimated using protein standards on 15% SDS-PAGE. Densitometric analysis using ImageJ software assessed cRLC band intensity. Spectral analysis was performed for absorbance at 555 nm of the protein dye conjugate, indicating a labelling ratio of approximately 1:4. Protein concentration at the end of labelling approximated to 0.6 mg/ml, which was a sufficient protein concentration for cRLC exchange in permeabilised trabeculae.

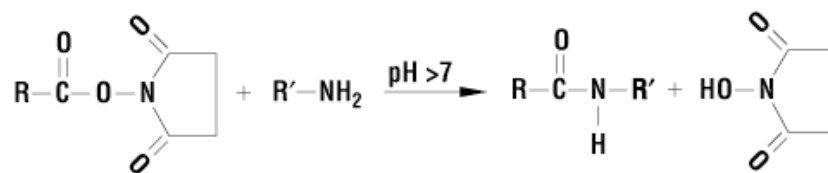
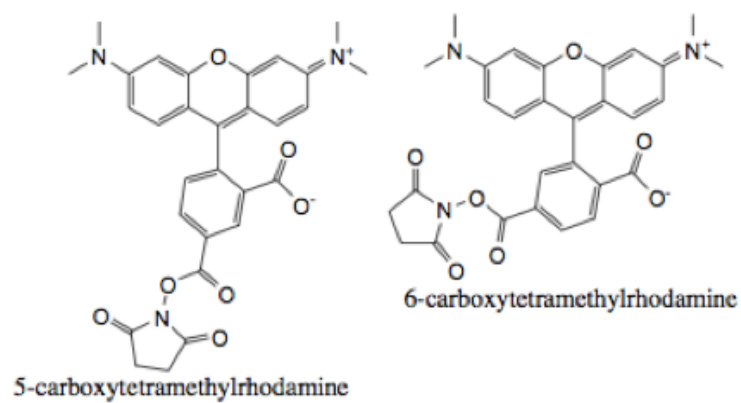


Figure 2.5.1: NHS-Rhodamine. **Top**, Depiction of the NHS-Rhodamine dye used for RLC labelling. **Bottom**, Simplified chemical reaction between the ester of the NHS-Rhodamine and primary amine group of lysine in the RLC protein (Taken from manufacturer handbook).

2.6 RLC exchange into permeabilised trabeculae

Trabeculae were permeabilised as previously described. Once samples were T-clipped they were mounted onto the experimental set-up and sarcomere length (SL) was set at 2.1 μm (by laser light diffraction) over a cooled 0.5°C platform in relaxing solution. 50 μl of approximately 30 μM RLC in exchange buffer incorporating 30 μM Troponin C (TnC), was added to the next platform held at 20°C. The trabecula would then be moved via the horizontal stepper motor to the platform with the RLC exchange solution. The trabecula was held there for 45 minutes (the RLC exchange solution was changed for fresh solution every 15 minutes). The RLC exchange solution incorporated TnC to replenish any TnC that was lost from trabeculae into the solution during exchange. Loss of TnC was characterised by performing exchanges without TnC replenishment. The observed effect was an impaired ability for trabecular relaxation after activation. This was typified by persistent post-activation resting tension in relaxing solution. When we added TnC to all of our exchange solutions the trabeculae relaxed appropriately after activation cycles.

RLC localisation and exchange efficiencies were assessed by the use of confocal microscopy by performing optical sectioning of trabecular samples (aided by Dr. Valentina Caorsi) that had undergone exchange with the Rhodamine labelled RLC. First z-stack images were acquired to assess specificity of RLC exchange. It was observed that with appropriate post-exchange washing, very little background fluorescence was observed in the regions of the sarcomeres that should not be occupied by RLC. This phenomenon is clearly observed in figure 2.6.1.1, where little background fluorescence is observed in the trabecular preparations in regions where the RLC should not be present.

RLC exchange efficiency was inferred by using a second round of exchange with unlabelled recombinant RLC to chase fluorescence out of the trabeculae. Exchange

efficiency was calculated by integrating the fluorescent intensity of the primary and secondary exchange fluorescences. The two fluorescent intensities could then be compared. This process was performed on 3 separate preparations (Figure 2.6.1.2). The second exchange washed out on average 50% of the fluorescent intensity created by the primary exchange, with the labelled recombinant RLC (Figure 2.6.1.3). This signified an exchange efficiency of approximately 50% when using this protocol. Previous experiments (not shown) used exchange solutions without TFP, which found very low 5-15% exchange efficiencies, which were not adequate for testing our hypothesis.

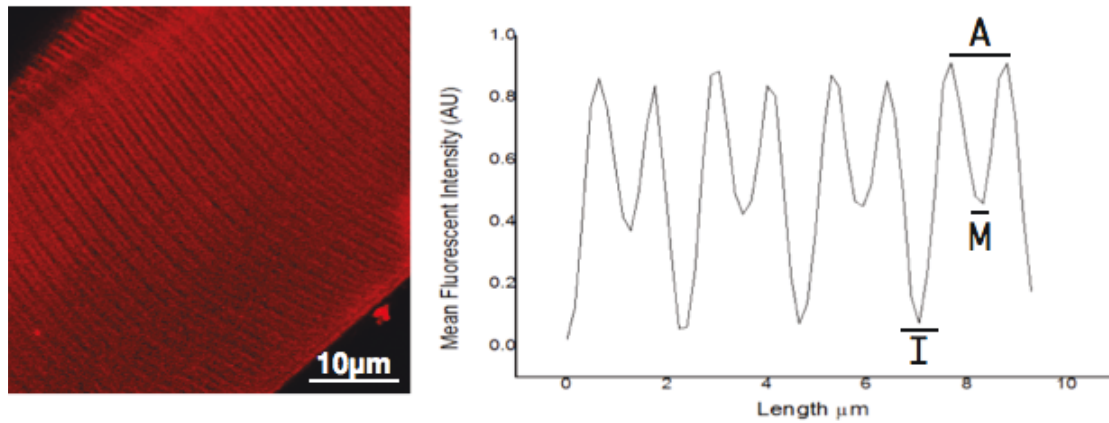


Figure 2.6.1.1: RLC exchange on myofibrils. **Left,** Confocal micrograph of myofibril after exchange with fluorescently labelled RLC. **Right,** Fluorescent intensity across the sarcomeres in arbitrary units. Fluorescent intensity was centred upon the myosin neck region containing A-band where the RLC should bind. This was ascertained by measurement of fluorescent region length at different sarcomere lengths. The region of labelling fluorescence did not alter in size during stretch indicating it to be the A-band. Notably there was dimming of fluorescent intensity in the M-line region where RLC is not present and a complete lack of fluorescence in the I-band regions where there is no myosin thick filament. The plot of mean fluorescence has been subtracted for background fluorescence by sampling areas outside of the tissue preparation. Excitation of the sample was at 543 nm and myosin backbone autofluorescence was below background fluorescence as this excitation wavelength. Wavelengths closer to 800 nm are needed to observe fluorescence due to the sarcomere (162).

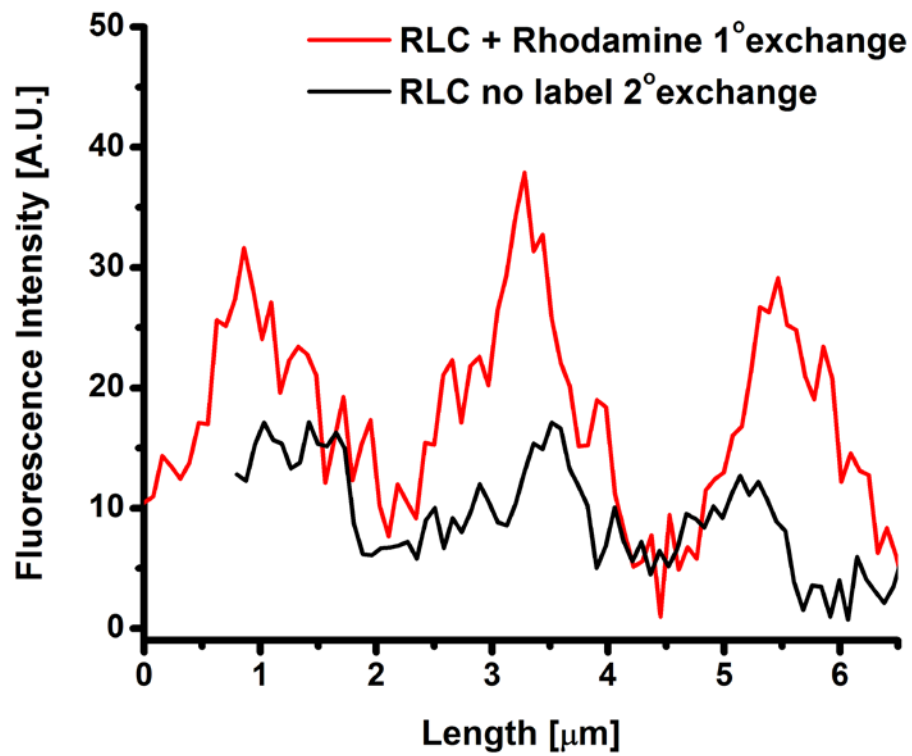


Figure 2.6.1.2: Chase exchange. Raw fluorescent intensity profiles of sarcomeres in trabecular tissue that had undergone a primary exchange with Rhodamine labelled RLC (Red plot). Black plot shows the reduction in fluorescent intensity across several sarcomeres after secondary exchange on the same preparation with unlabelled RLC.

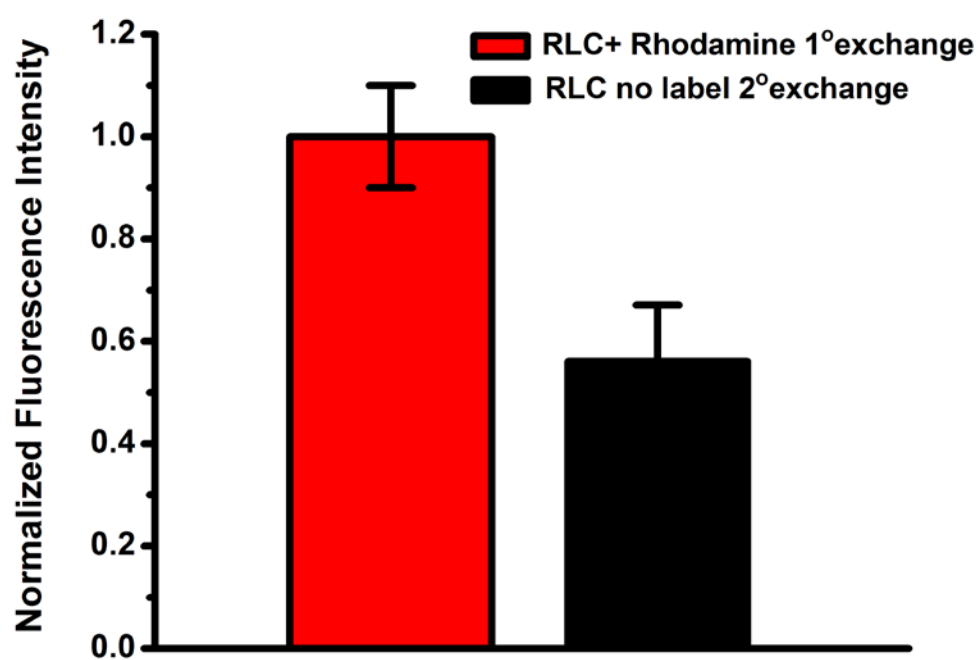


Figure 2.6.1.3: Exchange efficiency. Calculated fluorescent intensity changes observed after the 'chase out' secondary exchange with un-labelled RLC.

2.7 Phos-tag SDS-PAGE for determining RLC phosphorylation

To quantify protein phosphorylation levels, gel electrophoresis with Phos-tag was used (163). The Phos-tag method incorporates Phos-tag molecules (a manganese dependent phosphochelator) into a poly acrylamide gel. This phosphochelator transiently interacts with phosphoproteins travelling through the gel, slowing their motion, differentiating them from proteins that are not phosphorylated. Protein samples were diluted in protein loading buffer 2X (recipe: 2.5 mL 0.5 M Tris, pH 6.8, 4.0 mL 10% SDS, 2 mL Glycerol, 0.155 g DTT, 2 mg Bromophenol Blue, 1.5 mL H₂O) to approximately 5 µM in 15 µL aliquots and denatured by heating at 100°C for 5 minutes immediately before gel loading.

15% SDS polyacrylamide gels were cast. The gels incorporated the Phos-tag molecules, which differentiates phospho-species of the RLC by retarding the motion of phosphorylated proteins through the gel. The incorporation of this molecule into a standard SDS-PAGE allowed the direct measurement of phosphorylation in a single lane by densitometry.

0.75 mm gels were cast using a gel casting system (Bio-rad Mini-PROTEAN Tetra Cell). The constituents of the 15% resolving gel (Figure 2.7.1) were thoroughly mixed and subsequently polymerised with the addition of TEMED and APS. The mixtures were left to polymerise for 20 minutes with layers of dH₂O added on top of the hardening gels to ensure smooth and level polymerisation. Once the gels had become appropriately polymerised dH₂O was removed and the pre-mixed stacking gels (Figure 2.7.1) were added. Ten well combs were immediately inserted into the polymerising stacking gels to provide wells for protein loading. These mixtures were left for 20 minutes to solidify the comb was then delicately removed. The gels were run in 1X SDS running buffer (10X recipe: 60.6 g Tris base, 288 g Glycine, 20 g SDS and made up to 2 L with dH₂O). One well of the gel was always loaded with 15 µL of

molecular weight marker (Kaleidoscope, Bio-rad) for protein molecular weight estimation. Gels were run at 25 mA constant current for approximately 2 hours or whenever the 10 kDa molecular weight band of the molecular weight marker left the gel.

	15% Resolving gel (25 μm Phos-tag)	4% Stacking gel
dH₂O	3.46 mL	6.0 mL
40% Acrylamide/Bis (37.5:1)	3.75 mL	1.32 mL
0.5 M Tris-HCl pH 6.8	-	2.52 mL
1.5 M Tris-HCl pH 8.8	2.5 mL	-
10% SDS	100 μL	100 μL
10 mM MnCl₂ (H₂O)₄	50 μL	-
5.0 mM Phos-tag	50 μL	-
TEMED	14.3 μL	10 μL
10% APS	71.5 μL	50 μL
Total Volume	10 mL	10 mL

Figure 2.7.1: Phos-tag gel constituents. Gel casting ingredients for the Phos-tag incorporating SDS-PAGE for quantitative protein phosphorylation determination.

Post SDS-PAGE the gels were put into dH₂O for equilibration, after which a dry western blot was performed (iBlot, Invitrogen). The blot was run with 0.2 µm PVDF membranes for 6 minutes at 23 V. Post-blot, the membranes were submerged in blocking grade milk in blocking buffer (recipe: 100 µL Tween 20, 2 g blocking grade milk powder made to 200 ml in 1X PBS). The membranes were left for one hour at room temperature in blocking buffer and milk with gentle agitation. The membranes were then washed with phosphate buffered saline (PBS) three times for five minutes at room temperature with gentle agitation. Primary antibodies were then applied in blocking buffer without milk (blocking buffer 12 ml and 90 µl antibody making a 1:150 dilution (unfortunately no way of determining Ab concentration is provided by the supplier)). The antibodies used were specific to the cardiac RLC species (Monoclonal Antibody to Myosin F109 3E1, Enzo life sciences). The membranes were incubated for one hour at 4°C with gentle agitation in the primary antibodies. A series of three five-minute PBS washes were then performed and the membranes could then be immersed in secondary antibodies (Goat anti mouse IgG- HRP linked, Bio-rad, supplied in solution with no way of determining Ab concentration) in a 1:10000 dilution in blocking buffer without milk. This process was then followed by another short series of washes in PBS before the addition of the electrochemiluminescence (ECL) kit (Clarity Western ECL substrate, Bio-Rad) for five minutes. The membranes were then removed from the ECL substrate and imaged using a Kodak gel imager with an exposure time of 10 minutes. The membrane images were then thresholded and measured using ImageJ. Intensity peaks were used to calculate amount of protein in each phospho-state.

2.8 RLC phosphorylation enrichment and reduction of expressed RLC

Expressed cRLC was phosphorylated by incubation with a catalytic subunit of cardiac myosin light chain kinase (cMLCK), generously donated by Mathias Gautel (King's College London), and zipper interacting protein kinase (Invitrogen) at 37°C for 120 minutes in a kinase buffer (composition: 25 mM HEPES, 200 mM NaCl, 1 mM MgCl₂, 1 mM DTT, 5% glycerol and 200 µM ATP). Fresh kinase (20 nM final concentration) was added every 30 minutes to the reaction mixture until completion.

RLC phosphorylation was efficiently eradicated by incubation with shrimp alkaline phosphatase (Fermentas, no data to calculate concentration supplied) at a concentration of twenty units per one hundred microliter of 30 µM protein. The incubation was performed in kinase buffer without ATP for 60 minutes at room temperature. Phos-tag SDS-PAGE was used to determine final levels of phosphorylation in the protein before exchange.

Kinases and phosphatases were removed by column filtration using a molecular weight cut off column. Exclusion of kinases and phosphatases were tested by SDS-PAGE.

The phosphorylation and de-phosphorylation reactions were carried out on 4 separate occasions to determine the reproducibility of the protocol. Data for the separate types of reaction are shown in figure 2.8.1. Mixing the phosphorylated and de-phosphorylated RLC populations together in a 50:50 ratio made a control mimicking RLC population 'control' (Figure 2.8.1).

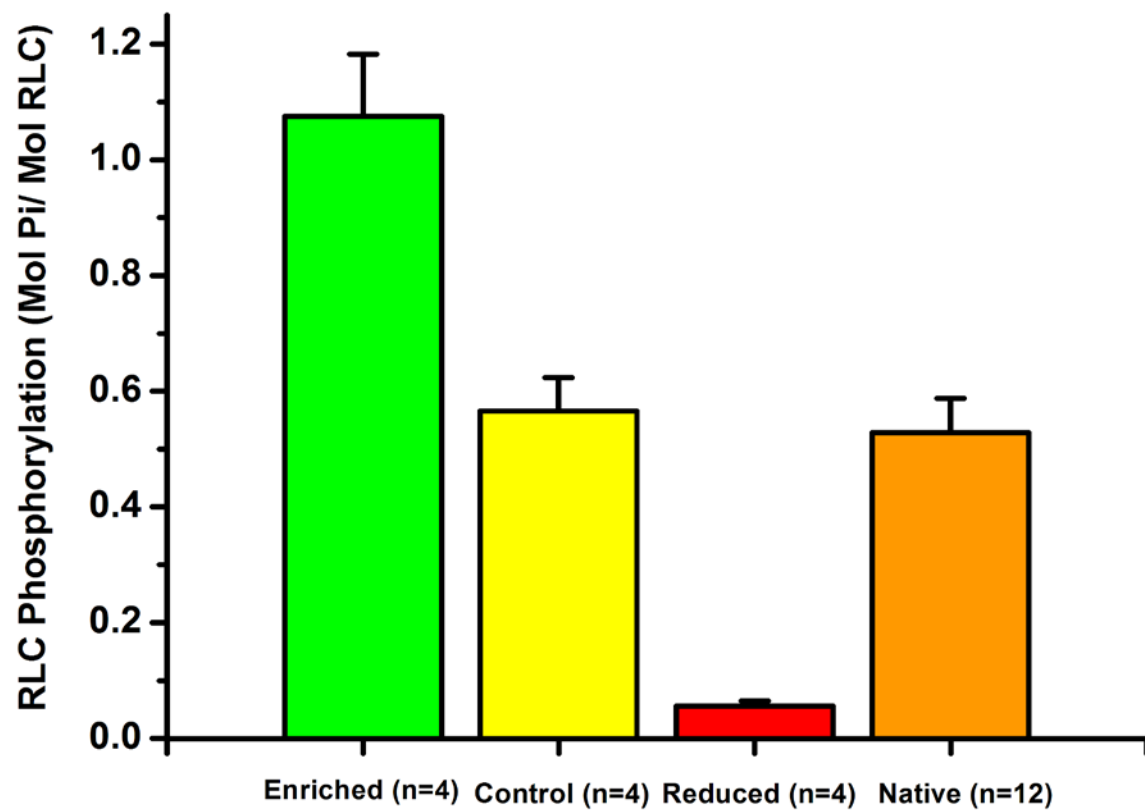


Figure 2.8.1: RLC phosphorylation of recombinant RLC. Plot of in-vitro recombinant *Rattus Norvegicus* RLC phosphorylation after kinase incubation (enriched) or phosphatase incubation (reduced). Mixing populations of enriched and reduced RLC species together in a 50:50 mixture produced 'control'. The 'native' RLC phosphorylation group was RLC from homogenised rat myocardium and showed the same level of RLC phosphorylation as the 'control' mixture.

3 – Ensemble and single molecule methodologies

3.1 Extraction of full-length cardiac myosin

Pig cardiac tissue was purchased as whole intact hearts (Pelfreeze, USA) and stored at -80°C . Pieces of left ventricular endocardium were mechanically powdered using a specially designed pestle and mortar that could be bathed in liquid nitrogen. Once the tissue had been powdered it could be stored at length at -80°C in vials containing 2 g of tissue each. Pig ventricular myosin was used for ensemble and single molecular experiments, as it is very similar to human myosin, with 98% sequence homology. Full-length myosin was used instead of HMM, in case HMM does not respond the same as full-length myosin to RLC phosphorylation. It is also difficult to make HMM, or indeed S1 myosin, without cleaving the RLC a α -chymotrypsin cleaves RLC (164).

The tissue purification protocol was a modified version that has previously been used for skeletal muscle myosin purification, and was adapted by Professor Emeritus Earl Homsher (UCLA) (165). It used 4 g of powdered left ventriculum, which was added to 40 mls of skinning solution (SS, composition: 25 mM Na_2EGTA , 50 mM MOPS pH 7.0, 6 mM Mg-acetate, 4 mM Acetic acid, 6 mM Na_2ATP , 2 mM DTT, 50 $\mu\text{g/ml}$ leupeptin, 7 $\mu\text{g/ml}$ pepstatin and 0.2 mM PMSF) at 4°C and stirred gently for 10 minutes. The solution was then spun at 12,000 G for 5 minutes. The pellet was taken up in SS again and washed a second time for 10 minutes, and then spun at 12,000 G for 5 minutes to further permeabilise the tissue and inhibit endogenous proteases. The supernatant was then discarded and the pellet was suspended in 40 mls of extraction buffer (EB, composition: 300 mM KCl, 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1 mM MgCl_2 , 5 mM K_2EGTA , 150 mM KH_2PO_4 , 2 mM DTT, 50 $\mu\text{g/ml}$ leupeptin, 7 $\mu\text{g/ml}$ pepstatin and 0.2 mM PMSF at pH 6.8). The extraction was performed on ice over 90 minutes with gentle stirring. The solution was then spun for 10 minutes at 12,000 G and the supernatant volume was measured and diluted with 20 volumes of chilled LS solution. After dilution the solution was left on ice for two hours and then centrifuged at 20,000

G for 20 minutes. The supernatant was subsequently discarded and the pellet was re-suspended in 10 mls of actomyosin dissolving solution (ADS, composition: 800 mM KCl, 10 mM Imidazole, 5 mM MgCl_2 , 2 mM 2-mercaptoethanol at pH 6.8). The ionic strength of the solution was then reduced by the addition of diluting solution (DS, composition: 40 mM MOPS, 5 mM MgCl_2 and 20 mM Na_2ATP at pH 6.8) to give a final ionic strength of 310 mM. The solution was then centrifuged at 440,000 G for 10 minutes; the supernatant was measured and diluted by 8 volumes of LS buffer and left on ice for 2 hours. The solution was then spun at 27,000 G for 10 minutes and the pellet was dissolved in 10 mls of HS solution. Following this, the protein was spin concentrated using Amicon ultra 100 K molecular weight cut off columns, which were spun at 4000 G until a 6-fold concentration was achieved. The concentrated protein was then diluted two fold by the addition of glycerol to make a final 50% glycerol solution that was stored at -20°C for no longer than 6 months. The purified protein was at a concentration between 3-6 μM in storage (Figure 3.1.1).

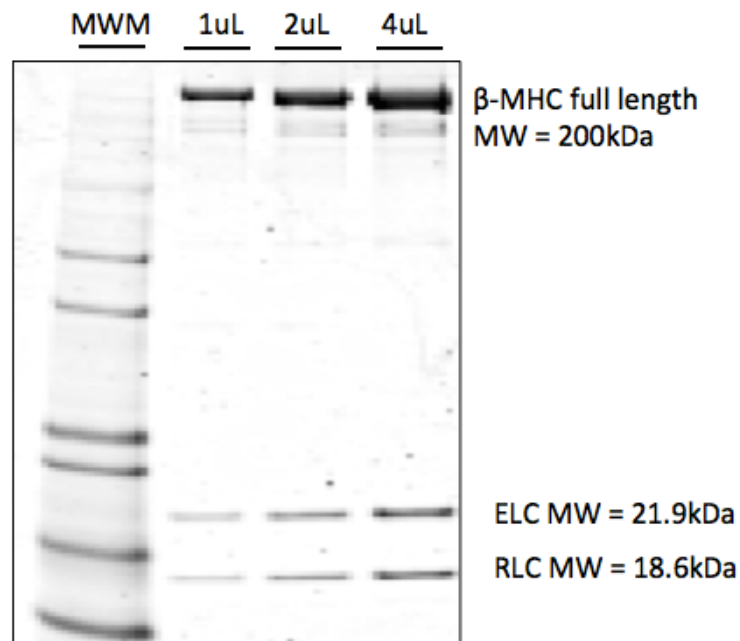


Figure 3.1.1: Extracted full-length porcine cardiac myosin. 4-12% Bis-Tris gel run at 200 V until loading buffer left the gel (~ 60 minutes). The molecular weight marker (MWM) was Mark12 (LifeTechnologies). Protein loaded was from the -20°C freezer post-glycerination and storage.

3.2 RLC phosphorylation enrichment and reduction when bound to porcine myosin from left ventricular extraction

Full-length porcine ventricular cardiac myosin stored at -20°C in high salt buffer (300 mM) and 50% glycerol was diluted 20 fold with a low salt buffer (LS, Composition: 1 mM DTT and 2 mM MgCl_2). Protein was then centrifuged at 100 K RPM in an ultra centrifuge using a TLA-100 rotor (Beckman) for 15 minutes. The protein was precipitated in this LS buffer and was present in the pellet of the centrifuged sample. The supernatant was discarded and the pellet was taken up in either a buffer for de-phosphorylation with calf intestinal phosphatase (CIP, Composition: 10 mM Tris, 300 mM NaCl, 1 mM DTT and 2 mM MgCl_2 pH 6.8), or a buffer for phosphorylation with smooth muscle myosin light chain kinase (smMLCK), called high salt buffer (HS, composition: 500 mM NaCl, 2 mM MgCl_2 , 5 mM 2-mercaptoethanol and 80 mM MOPS pH 7.1). To dephosphorylate the RLC bound to this myosin, 5 μl of CIP (50 units, New England Biolabs) was added to it in CIP buffer and left at room temperature for 30 minutes. This incubation protocol ensured that no phosphorylated light chains could be detected by gel electrophoresis using the Phos-tag method (Figure 3.2.1). Phosphorylation of the RLC was achieved by the addition of a kinase mixture (KM, addition of: 1 mM CaCl_2 , 5 mM MgCl_2 , 60 μM Calmodulin and 2 mM ATP) to the protein in HS buffer incorporating 0.8 μM full-length smMLCK (SF9 baculoviral expression by Feng Zhang) (166). The protein was left to incubate at room temperature for 30 minutes, after which fresh ATP and smMLCK was added and the mixture was left a further 30 minutes at room temperature (adapted from (166)). This protocol ensured that no un-phosphorylated RLC could be detected (Figure 3.2.1). After these incubations the protein mixtures could be re-precipitated with 20 volumes of LS buffer and spun at 100 K RPM to remove the kinase and phosphatases from the myosin component. The protein was then solubilised again using a HS buffer and the protein concentration was determined using the absorbance at 280 nm from a NanoDrop spectrophotometer, or by the Bradford method with a Cary 60 spectrophotometer (Agilent Technologies).

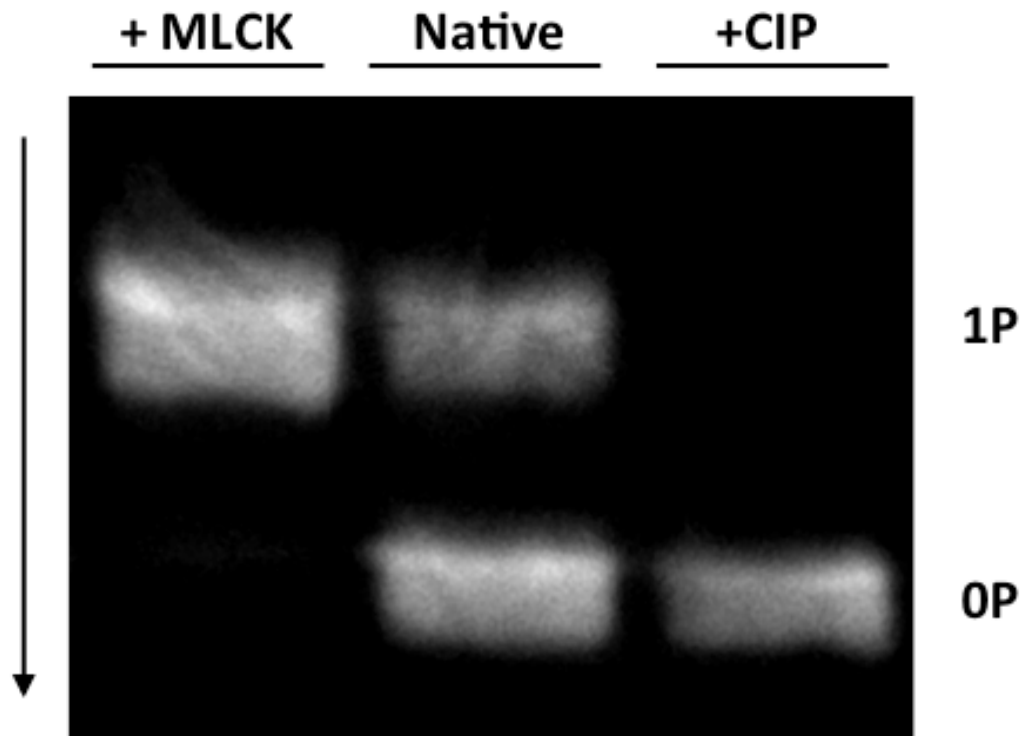


Figure 3.2.1: Phosphorylation of purified RLCs. Western blot of the myosin RLCs that had either been treated with smooth muscle myosin light chain kinase (smMLCK), calf intestinal phosphatase (CIP), or were untreated straight from the cardiac preparation (native). Arrow indicates the direction of migration of protein through the gel. The gel was 12% SDS-PAGE run at 100 mV for 2 hours.

3.3 Actin activated ATPase measurements with full-length myosin using the NADH coupled assay and Gelsolin capped actin

Actin activated ATPase measurements assess the ability of myosin to break down ATP in the presence of actin when in solution (review of the subject of measuring ATPases (167)). We wanted to understand if altering RLC phosphorylation level in turn altered the ability of filamentous full length cardiac myosin to hydrolyse ATP. This measurement is hard to perform as full length myosin in low ionic strength (which is needed for the assay) form filaments. Mixing filaments of myosin and actin in the ATPase assay leads to light scattering, which creates a non-linear fluorescence decay that cannot be used to assess ATPase rate. Therefore, we used gelsolin capped actin to reduce actin filament length, which prevents light scattering, and produces a linear fluorescence decay (figure 3.3.1). We proposed that RLC phosphorylation level increase would accelerate the steady-state ATPase of the myosin in its filamentous form. We measured this by the use of an NADH-coupled assay at 25°C (168, 169). This method assesses the rate at which ADP is produced by hydrolysis of ATP in solution, and manifests as a slope of fluorescence decrease over time:

1. $\text{ATP} \rightleftharpoons \text{ADP} + \text{P}_i$
2. $\text{ADP} + \text{phospho(enol)pyruvate} \rightleftharpoons \text{ATP} + \text{pyruvate}$
3. $\text{pyruvate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{lactate} + \text{NAD}^+$

Reaction 1 is catalysed by cardiac myosin full length filaments, reaction 2 by pyruvate kinase, and reaction 3 by lactate dehydrogenase. This protocol gives a proportional loss of NADH (measured by 340 nm absorbance loss) that is stoichiometric with the loss of ATP. This ensures that the myosin hydrolysis rate is

measured as long as each reaction has an equilibrium that is strongly in favour of the forward direction, and neither reactions 2, or 3 are rate limiting in the assay.

As mentioned previously, the above protocol was adapted for use with full-length myosin in low ionic strength (~10 mM) by the addition of gelsolin to create uniform F-actin lengths of approximately 900 nm assessed by electron microscopy (EM) (carried out by Dr. Neil Billington (NHLBI, NIH)). Gelsolin (Cytoskeleton) was mixed 1:200 with F-actin in actin buffer, with additional 120 μM CaCl_2 (FAB, composition: 2 mM MgCl_2 , 4 mM MOPS, 3 mM NaN_3 , 0.1 mM EGTA and 1 mM DTT at pH 7) and left at room temperature for 30 minutes. Myosin post-kinase/phosphatase treatment was diluted to 150 nM with LS buffer. The NADH coupled assay mix made as a 10X concentrate (10X EZ) was made up of 400 U/ml L-lactic dehydrogenase (Sigma), 2000 U/ml pyruvate kinase (Sigma), 2 mM NADH (Sigma) and 10 mM phospho(enol)pyruvate (Sigma). The final assay buffer conditions were 2 mM MOPS, 2 mM MgCl_2 , 0.1 mM DTT, 1 mM NaCl, 40 U/ml L-lactic dehydrogenase, 200 U/ml pyruvate kinase, 200 μM NADH and 1 mM phospho(enol)pyruvate, and the reaction was initiated by the addition of 2 mM ATP. The final myosin concentration in the assay was 15 nM molecules (30 nM heads) and the actin concentration was varied from 1-60 μM final concentration in the assay. The measurements were made in a Cary 60 spectrophotometer (Agilent Technologies) and the absorbance was measured at 340 nm for 10 minutes. Absorbance changes were plotted in Origin and fit with a linear regression to estimate the rate of absorbance change (Figure 3.3.1). The extinction coefficient used for NADH (ϵ) was $6220 \text{ M}^{-1}\text{cm}^{-1}$. All data is presented as a rate in seconds per myosin head against actin concentration and is fit with the Michaelis-Menten equation to obtain the kinetic constants of maximal actin activated ATPase rate (V_{max}), and the actin concentration at which half maximal ATPase is observed (K_{ATPase}), assuming 100% activity of the myosin in the solution.

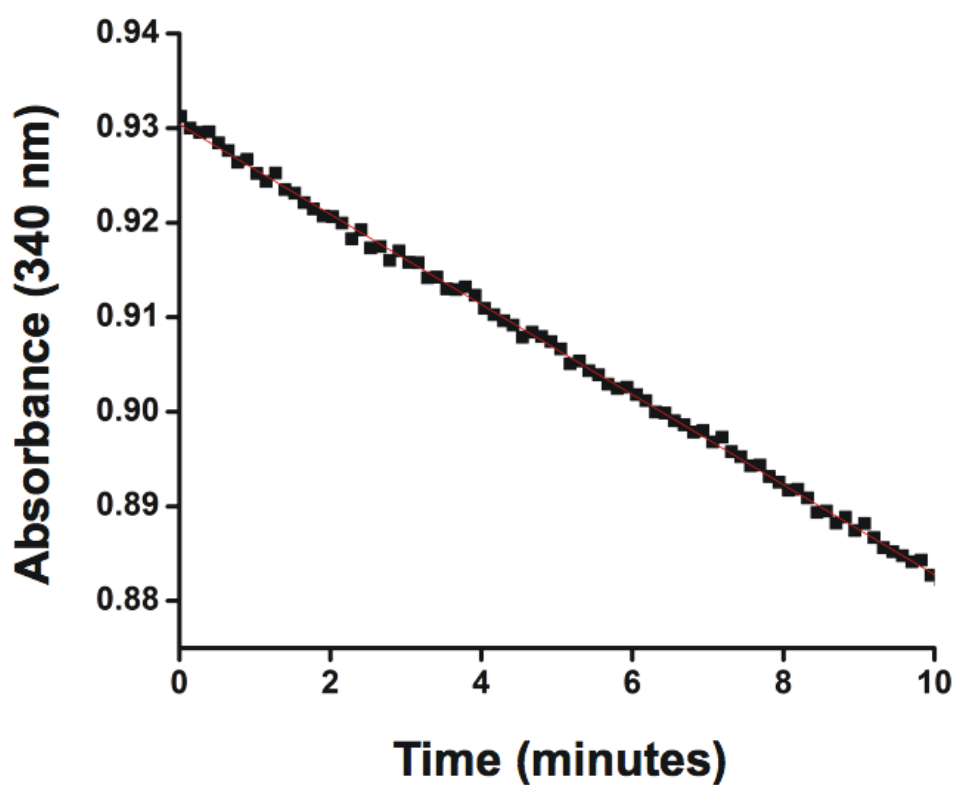


Figure 3.3.1: NADH absorption signal from gelsolin ATPase. Representative NADH absorption signal at 340 nm of a typical actin activated NADH linked ATPase assay with full-length cardiac myosin and gelsolin capped actin filaments.

3.4 Single molecule optical trapping with the three-bead assay

Optical trapping assays have been used on a plethora of molecules, many of which were molecular motors and come in many different experimental configurations (reviewed (170, 171)). The laser trapping technique works on the principal that a bead can be manipulated in solution by laser light. Therefore, we can hold an actin filament taught between two beads in solution, and allow a single myosin molecule on a third bead to interact with actin. Using this three-bead assay we can measure the displacement and duration of single myosin cross bridge interactions with actin. These observations allow us to assess if RLC phosphorylation level affects the displacement and lifetime of attachment created by a single power stroke.

3.4.1 Monomeric actin (G-actin) purification

Actin was purified from rabbit skeletal muscle and was provided by Dr. Feng Zhang (NHLBI, NIH) (172). G-actin was stored in liquid nitrogen and used to make fresh batches of F-actin weekly.

3.4.2 Biotinylated G-actin

Skeletal muscle biotinylated G-actin was purchased from Cytoskeleton Inc. (Catalog number AB07). The G-actin was modified to incorporate a covalently linked biotin that can bind random surface lysine residues via an activated ester group. The labelling stoichiometry was determined to be approximately 1 biotin per actin monomer. The biotinylated G-actin was determined to be > 99% pure and was supplied as a lyophilised powder that was stored for no longer than 6 months at 4°C. The biotinylated G-actin was reconstituted to 10 mg/ml with distilled H₂O, which would leave the protein in the following buffer: 5 mM Tris-HCl pH 8.0, 0.2 mM CaCl₂, 200 mM ATP, 5% sucrose and 1% Dextran.

3.4.3 Polymerisation of G-actin to F-actin

Actin exhibits an ATPase and requires ATP hydrolysis to polymerise (173, 174). During polymerisation G-actin hydrolyses its bound ATP to ADP + Pi, which occurs in two steps, firstly the cleavage of ATP, and then the slow release of Pi (174). Two forms of F-actin were polymerised for experimentation: i) G-actin was polymerised at a 90% to 10% ratio with biotinylated G-actin, ii) G-actin was polymerised on its own (without any biotinylated G-actin) to make F-actin with no incorporation of biotinylated actin monomers. The biotinylated G-actin mixtures were polymerised to a final concentration of 20 μ M in a salt containing polymerisation solution termed KMEI buffer (composition: 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 10 mM Imidazole and 20 mM ATP at pH 7.0) and left for 30 minutes at room temperature to polymerise. The G-actin that was un-biotinylated was polymerised by the addition of 100 mM KCl and 2 mM MgCl₂ was centrifuged at 70,000 G and then re-suspended in a low salt buffer for storage at -20°C (composition: 2 mM MgCl₂, 4 mM MOPS, 3 mM NaN₃ and 0.1 mM EGTA at pH 7.0).

3.4.4 Fluorescent labelling of biotinylated F-actin (BFA)

100 μ l volumes of 5 μ M fluorescently labelled BFA were prepared weekly for use on the optical trap. The BFA was labelled with Phalloidin-tetramethyl B isocyanate (TRITC-Phalloidin) with excitation at 540 nm and emission at 565 nm from the organism *Amanita Phalloides* purchased from Life Technologies (Catalogue No. R415), dissolved in EtOH. The BFA was suspended in a motility buffer (composition: 20 mM MOPS, 5 mM MgCl₂ and 0.1 mM EGTA at pH 7.4) and 10 μ M TRITC-Phalloidin. This mixture was left on ice in the dark for two hours to allow for complete labelling of the BFA.

3.4.5 Preparation of Neutravidin Biotinylated beads with TRITC-Rhodamine BSA

The NeutrAvidin biotin binding protein was used to cross-link biotin labelled polystyrene beads of 1 μm diameter (Life Technologies catalog No. F8769), with the biotin F-actin. 25 μl of 1 μm diameter beads were mixed with 25 μl of 5 mg/ml TRITC-BSA and 25 μl of 2.5 mg/ml Neutravidin, and left on ice for an hour. The beads were washed ten times with a motility buffer incorporating 1 mg/ml BSA whilst spinning at 12000 RPM between washes for 5 minutes at 4°C. Washing the beads in this buffer reduces non-specific binding and removes excess NeutrAvidin from the solution, which prevents the Biotin F-actin from cross-linking each other in the reaction chambers. Beads were washed approximately 4 times every 24 hours to further remove any NeutrAvidin that was liberated into the supernatant. Immediately before experimentation, the beads were sonicated for 10 minutes to make sure all beads were monodisperse in the reaction solution.

3.4.6 Preparation of reactive oxygen species scavenging system

The scavenging system used was a combination of glucose oxidase and catalase (GOC), which was mixed with glucose in the experimental mixture. The scavenging system neutralises reactive oxygen species by conversion of these species (such as hydrogen peroxide) to harmless by products (oxygen and water). Glucose Oxidase from *Aspergillus Niger* was purchased from Sigma Aldrich (G2133). 60 mg of glucose oxidase was mixed with 400 μl of distilled H_2O and 120 μl of well-shaken bovine catalase (Roche). The mixture needed to be thoroughly vortexed, and could then be centrifuged at 13,000 RPM for 5 minutes in an Eppendorff 5415D centrifuge. The supernatant was then removed using a 2 ml syringe and needle and pressed through a 0.2 μm pore filter tip to remove clumps from the solution. GOC was aliquoted and

stored at -20°C until needed for experimentation. 100X solutions of glucose were also pre-prepared by dissolving 400 mg of α -D-glucose in 1 ml of distilled H₂O to give a final stock concentration of 2.1 M, which was aliquoted and stored at -20°C.

3.4.7 Preparation of the optical trapping chamber

The optical trapping chamber consisted of two glass slides 24x60 mm No1 (130 μ m in thickness) from corning. They were assembled using double sided sellotape to glue the two slides together (Figure 3.4.7.1). Prior to chamber formation, one slide was coated in a thin film of 2 μ m diameter 'pedestal beads' that were suspended in 0.1% nitrocellulose in Amyl Acetate. Once the applied beads had dried the chambers were glued together as shown in figure 3.4.7.1. Once the chamber was assembled, 30 μ l of 0.2 nM full length cardiac myosin was pipetted into the chamber in a high salt solution and left for 1 minute to settle. Low surface densities of myosin were used to ensure that binding events were due to only one myosin molecule interacting with actin. On average at this loading concentration, 8-10 pedestals needed to be searched before one would be found that would show characteristic low variance binding events. Protein was added in high salt to ensure it was laid down as molecules and did not form filaments. The chamber was subsequently washed with three chamber volumes (approximately 60 μ l) of high salt buffer to remove any un-bound myosin, and then washed with three volumes of motility buffer with 1 mg/ml BSA. This was done to block the surfaces preventing either polystyrene bead or actin from binding the cover slip surfaces. The chamber was subsequently washed with motility buffer to remove any un-bound BSA. The chamber was then ready for the reaction mixture, which incorporated the labelled NBB, TRITC-phalloidin labelled actin, DTT, ATP, and a reactive oxygen species scavenging system incorporating glucose oxidase, catalase, and glucose. Each chamber was only used for 45-60 minutes before a new chamber was prepared. An example of the experimental configuration inside the chamber can be seen in figure 3.4.7.2.

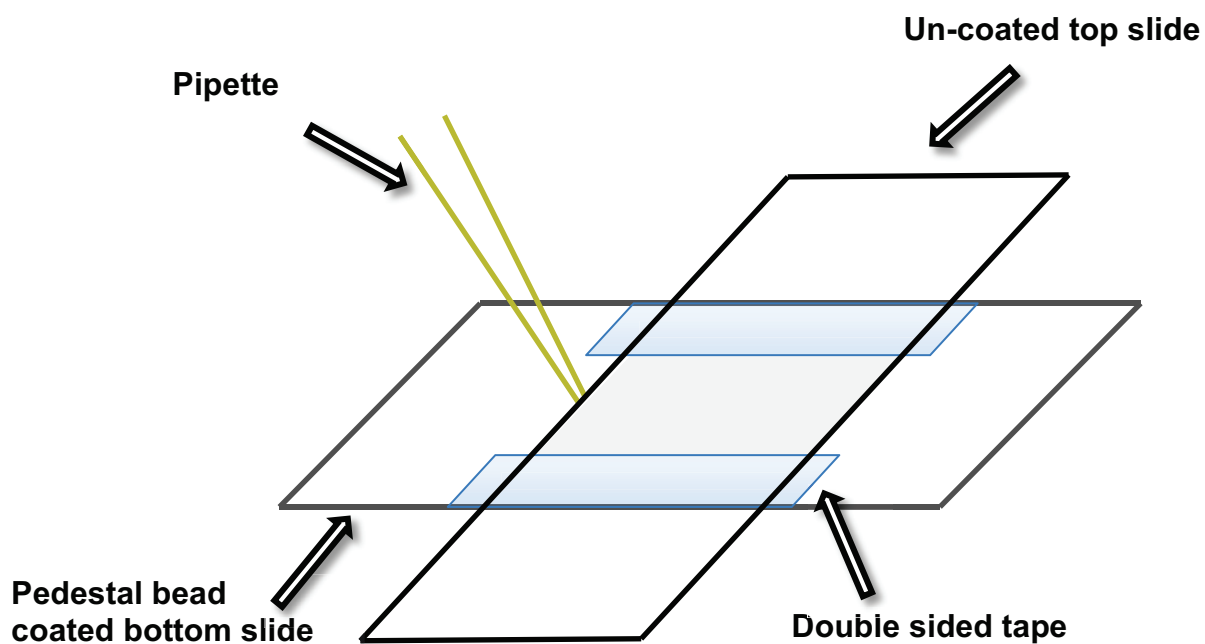


Figure 3.4.7.1: Schematic of the assembled optical trapping chamber. The chamber was open sided allowing for wicked flow of experimental solutions through the chamber. Once all chamber constituents were added, the chamber was sealed with varnish to prevent evaporation during experimentation.

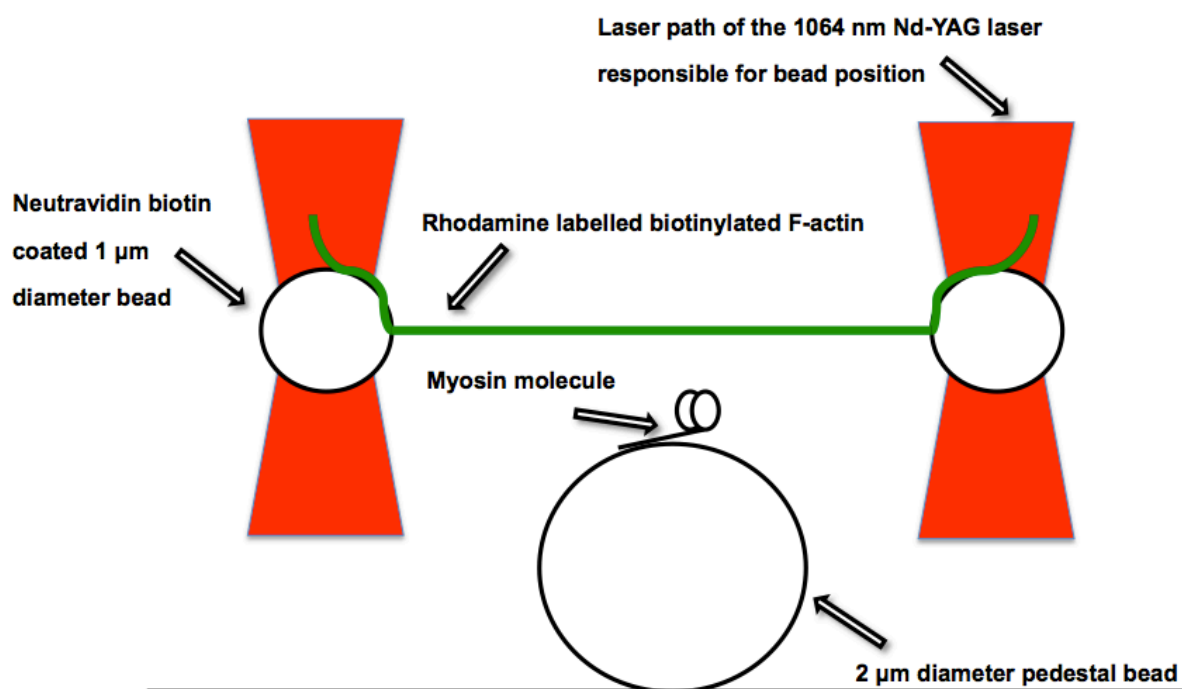


Figure 3.4.7.2: Schematic representation of the optical trapping three-bead assay. This representation shows the configuration of the dual beam optical trapping configuration during experimentation. Two small 1 μm diameter beads are held by the trapping gradient in solution with an actin molecule tethered between them (green) forming the bead actin bead assembly. This actin molecule is lowered towards the 2 μm diameter pedestal bead, which has a single molecule of myosin bound to it. Interactions between the myosin and actin are recorded as described below.

3.4.8 Optical gradient trap calibration

Due to the nature of the single laser optical gradient trap, the position of the bead within the focused laser beam was determined by the magnitude of the optical gradient, and that of the scattering force created by the incident photons of the laser (Reviewed (170)). The gradient force is largely determined by two things; i) how polarisable the trapped dielectric bead is and, ii) the optical intensity gradient at the focal point. For a stable trap to be achieved in three dimensions, the gradient component of force exerted on the bead to pull it into the focus of the laser must overcome the scattering component, which pushes the bead along the axis of the incident laser beam (175-177). Due to the diameter of the beads used, the current theory for the computation of trapping forces is incomplete. Therefore, calibrations must be made to determine force empirically by converting the voltage signals acquired from quadrant detectors into their equivalent forces. To do this calibrations of both trap stiffness and detector sensitivity were made, as previously described (178, 179).

A single 1 μm bead was trapped in each beam and brought to the approximate height above a pedestal needed to observe interactions. The voltage signals caused by Brownian motion of these beads in the solution are acquired for each bead for 5 seconds at a sampling rate of 20 kHz (effective sampling rate 10 kHz). We used a Lorentzian function to describe the power spectrum of the bead trapped in a harmonic potential under the influence of Brownian diffusion (178, 180). This Lorentzian function describes the roll off frequencies (f_c) dependence on the stiffness of the trap. The peak height of the power spectral density is dependent upon the viscous drag of the trapped bead, known as Stoke's coefficient. The Lorentzian function used to fit the power spectrum is equation 1:

$$S_v(f) = S_0 \left[\frac{1}{1 + \frac{f^2}{f_c^2}} \right] \quad (\text{Equation 1})$$

where:

$$S_0 = \frac{4\gamma k_B T}{C^2} \quad (\text{Equation 2})$$

Solving for C:

$$C = \sqrt{\frac{4\gamma k_B T}{S_0}} \quad (\text{Equation 3})$$

S_v = power spectrum (V^2/Hz), f = frequency (Hz), f_c = corner frequency (Hz, the point at which the power spectrum deviates from a linear relation), γ = viscous drag coefficient of the bead (Nsm^{-1}), k_B = Boltzmann's constant ($= 1.38 \times 10^{-23} \text{ JK}^{-1}$), T = temperature in Kelvin and C = the calibration constant for conversion of voltage to force (pNV^{-1}).

For frequencies where f is much smaller than f_c ($f \ll f_c$) the power spectrum can be described as a constant and horizontal (S_0) defined in equation 2. Rearranging this equation to solve for C, as in equation 3 allowed us to define the voltage to force conversion, in pN/V for each given trapped bead at a specific distance from the chamber surface. This could be done as we had measures of S_0 from the power spectral analysis, the viscous drag of the particle (γ) and the temperature (T), which was $296.15\text{K} = 23^\circ\text{C}$.

$$\gamma = 3\pi\eta d \quad (\text{Equation 4})$$

The Stoke's coefficient γ is described by the relationship of the diameter of the bead (d) = 1×10^{-6} m, and the dynamic viscosity of the solvent that the bead is suspended in, which in this case was modelled as water $\eta_{\text{Water}} = 0.001 \text{ Nsm}^{-2}$.

The stiffness (spring constant) α (Nm^{-1}) of the trap can also be calculated from the power spectrum using the following equation:

$$\alpha = 2\pi\gamma f_c \text{ (Equation 5)}$$

Now that the stiffness and force conversion of the trap and trapped bead are known, we used Hooke's law to calculate the displacement of the bead from the trap during an interaction χ (nm):

$$F = -\alpha\chi \text{ (Equation 6)}$$

F (pN) is the converted force of the interaction calculated from multiplying the raw voltage from the photodiode with the force conversion constant C . α is the pre-calculated trap stiffness (Nm^{-1}). We could then solve for the displacement χ (nm).

3.4.9 Analysis and limitations of optical gradient trap data

When bead actin bead assemblies (Figure 3.4.7.2) were oscillated by the acousto-optic deflector, (100 Hz and 30 mV amplitude in a sinusoidal waveform) (AOD,

Figure 3.4.9) myosin attachments to actin were defined by a drop in variance of the sensor bead output voltage. This drop in variance is expected when myosin binds actin due to the increased stiffness of the system when binding occurs. To this end, a running mean variance analysis was used to define significant variance changes, which were defined as binding events (181-183). The variance analysis used a rolling mean calculated over a time window of 100 data points (collected at 10 kHz = 1 ms) to calculate both mean and variance. Variance histograms were plot from this analysis, and variance was used to bin the data. Two distinct Gaussian distributions were found in traces containing binding events. One Gaussian with low variance (attached) and one Gaussian with higher variance (detached). From this data the cut-off for a binding event could be set. A three-point variance analysis was used. Once the variance in the bead trace reached values corresponding to the isthmus between the low and high variances (with at least one of those points having a mean variance the same as the mean of the low variance Gaussian), the section of the trace was scored as an attachment. When the variance increased to the mean of the higher variance Gaussian, this signified the end of an attachment. Custom-made LabView virtual instruments (VIs) (Luca Melli NHLBI, NIH) were used to perform this analysis and extract threshold levels for each individual trace recorded. The LabView VI's were then used to measure attachment durations by counting how long these variances stayed within the 'attached' variance state. LabView was also used to zero the bead positions in the x-direction and measure the raw voltage displacement from zero to measure the displacement of the actomyosin binding events. All raw data was then converted to corresponding forces (pN) and displacements (nm) as described above.

This type of oscillatory method doesn't allow the direct measurement of force produced by each actomyosin interaction. This is because using a sinusoidal oscillatory pattern creates a variable loading rate, which is applied to the myosin, due

to the variable oscillatory rate of a sinusoid. To measure force directly in the optical trap it is best to have no oscillation of the bead actin bead assembly. The theory that we observe actomyosin interactions arising from single binding events, works on the statistical principal that the concentration of myosin, added to the chamber, reduces the likelihood that more than one myosin molecule would be attached to any single pedestal bead. This point is proven by the need to probe at least 8 pedestals before one is found that exhibits actomyosin binding characteristics. Using single molecules in the optical trap we do not appreciate the true orientation of myosin in respect to actin as would be found in the sarcomere. Therefore, we may not observe regulatory characteristics associated with filamentous myosin.

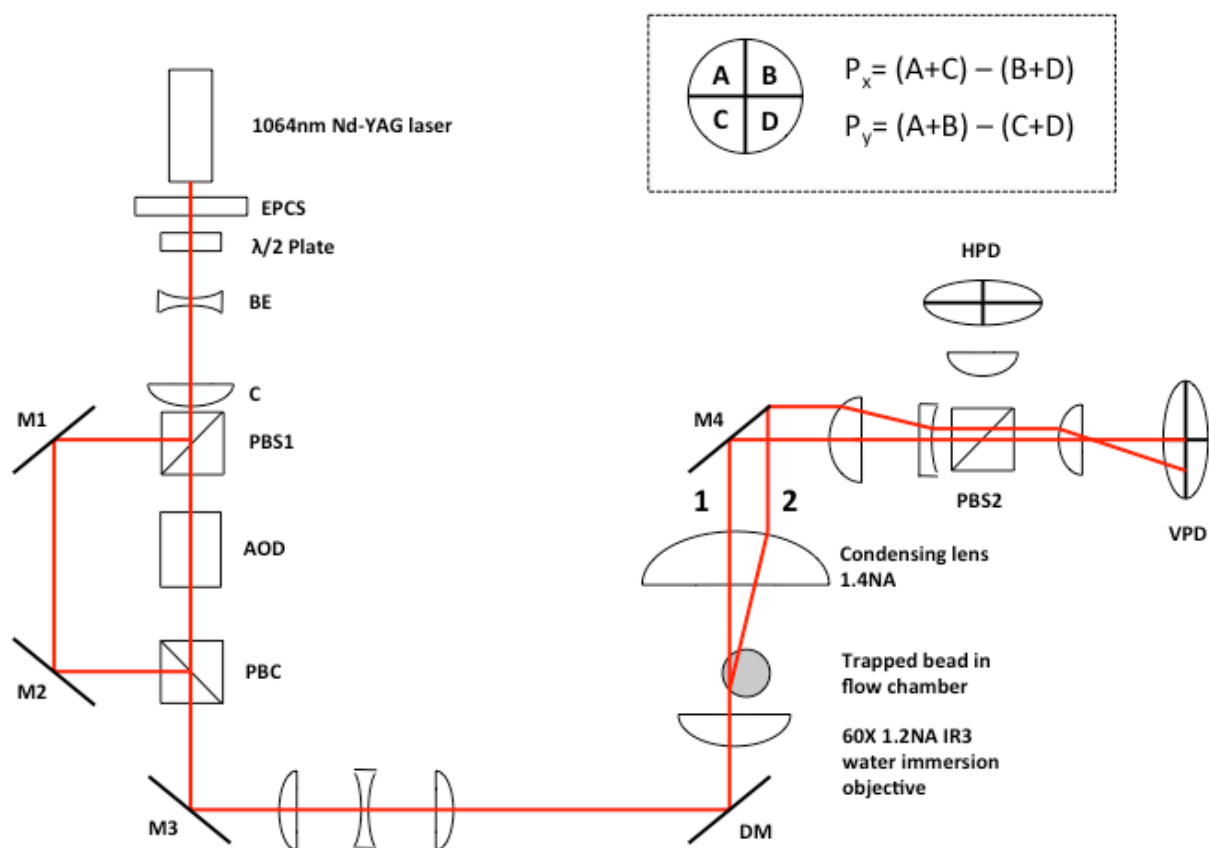


Figure 3.4.9: Simplified light path diagram for the optical gradient trap used for the three-bead assay as previously described (179). This diagram shows a simplified system where we only depict one of the trapped beads in the chamber. EPCS = electronic pedal controlled shutter, BE = Beam expander, C = Condenser, PBS = Polarising beam splitter, AOD = Acousto-optic deflector, PBC = Polarising beam combiner, DM = dichroic mirror, HPD = Horizontal positional detector, VPD = vertical position detector and M = mirror.

3.5 Actin gliding assay

The actin gliding assay provides a simple eloquent model system using the two purified proteins necessary for contraction, myosin and actin (184). The peak gliding velocity of actin, when propelled by myosin, is a function of the power stroke displacement, and the rate limiting step of the actomyosin cycle in the assay. When there is negligible load applied to the assay, ADP release is the rate limiting step (185). Using this technique allows us to make comparisons with our previous measures of unloaded shortening velocity, as this is also limited by ADP release (186).

3.5.1 Gliding assay chamber preparation

Much like the chamber preparation for the optical gradient trap, the chambers were built from glass slides (Figure 3.5.1). However, in the gliding assay, single slides were used and a smaller coverslip was stuck to it with double-sided sellotape. The coverslip was coated in a thin film of 1% nitrocellulose (187). Myosin was pre-spun in high ionic strength (>300 mM) with un-labelled stoichiometric amounts of F-actin with excess ATP at 480,000 G for 15 minutes in a Beckman TL100 ultracentrifuge. This process sedimented damaged myosin heads (dead heads), that bind actin but do not hydrolyse ATP, which contribute to poor quality of movement in the gliding assay (188). The myosin was then applied in high ionic strength solutions (approximately 300 mM NaCl) to the chamber to lay the myosin down as monomers. The chambers were then washed through with several volumes of high ionic strength buffer (HS buffer, composition: 500 mM NaCl, 5 mM β -ME and 80 mM MOPS at pH 7) to remove any unbound myosin. Then low ionic strength MB of 1 mg/ml BSA was washed through the chamber, and left for one minute to block non-specific binding sites on the chamber surface. The BSA was washed out using MB, then a MB with sheared un-labelled F-actin was washed through the chamber. Actin was sheared by

repeated aspiration through a needle to break the actin filaments down. This process was performed to enhance the gliding of actin by blocking any remaining dead heads that were not eradicated by the actin spin down (189). The un-labelled actin was then washed out with MB, with 1 mM ATP, so that only actin filaments that were irreversibly bound to myosin were retained in the chamber. The chamber was then washed with three volumes of MB to remove any remaining ATP before the labelled actin was added to the surface. TRITC-actin was added at a concentration of 20 nM in MB and left to settle for 20-30 seconds before the chamber was washed of any un-bound actin. The final reaction mixture was then added, which was prepared to have a constant ionic strength of 60 mM incorporating a reactive oxygen species scavenger; appropriate buffering agents, methylcellulose, and ATP. Frames from video recordings of the assay can be seen in figure 3.5.1. A recent review of this technique describes the assay in more depth (190). Methylcellulose is used to prevent the 'wobbling' of actin filaments that aren't completely bound to the surface of the chamber by myosin interactions. This occurs as methylcellulose is viscous, it prevents long axis motion, by increasing the viscous drag in the solution, preventing the actin from floating away from the surface. This aids the precision of tracking the filaments during an assay.

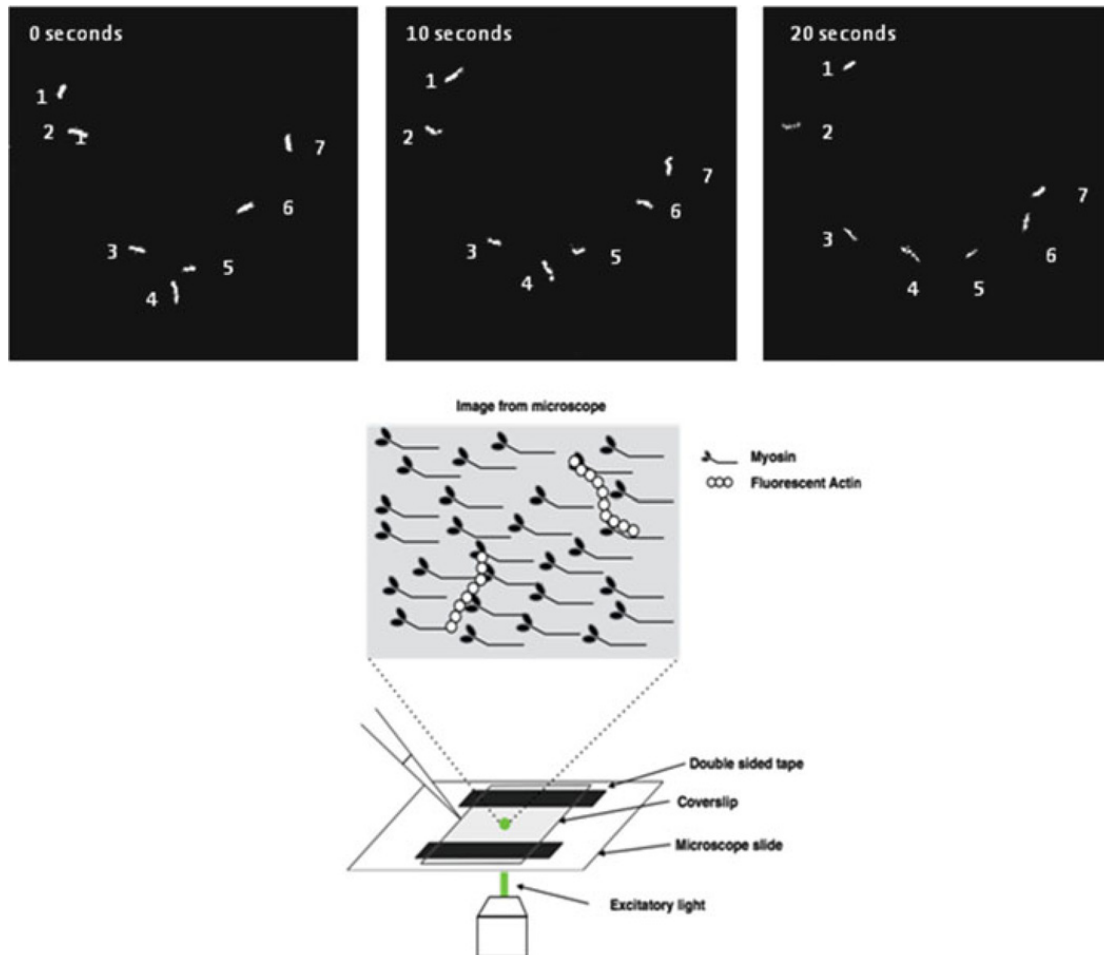


Figure 3.5.1: Schematic representation of the actin gliding assay chamber.

Top: Three Sequential frames taken over 20 seconds of an actin gliding assay showing seven actin filaments moving over a myosin coated surface. **Bottom:** Schematic of the assay flow chamber. Reproduced from our own publication (190).

3.5.2 Gliding assay recording

The chambers were kept at 30°C by an objective heater attached to an Olympus IX51 inverted microscope using an oil immersion lens. The temperature of the flow cell was set using a thermistor probe inserted into a dummy chamber to pre-set the temperature for the assay. The TRITC-actin was imaged using a filter set for collecting the emission of the Rhodamine dye with a high magnification 100X 1.3 numerical aperture (NA) objective. Fluorescence was detected with a charge-coupled device camera (CCD), which was displayed in real time on black and white TV monitors and was recorded onto VHS tapes for analysis and digitisation. A Hamamatsu Argus-20 was used to subtract the background fluorescence and to average frames of the recordings. Mean velocities of the individual actin filaments were calculated using a specialised tracking system (Cell Track System by Motion Analysis). This hardware and software was used to plot the centroids of the actin filaments in each frame of video captured. The device then connected these centroids sequentially over each frame to estimate an average velocity for each filament within the field of view. The mean velocities were calculated with the standard deviation of the means for each filament. A specialised filtering programme (written by Earl Homsher, UCLA) was used to filter out filaments that were unmoving, or displayed poor movement, by identifying filaments that had negligible velocity or had large standard deviations of their mean speeds, implicating stop start motion rather than smooth motion of actin gliding. Averages were made of individual filament speeds to produce population mean velocities ($\mu\text{m/s}$) and standard deviations.

3.6 Actin gliding assay protocols

3.6.1 Myosin concentration versus velocity

Actin gliding velocities were measured as a function of myosin concentration in the experimental chamber. Assessing the concentration of applied protein needed for the assay to reach maximal sliding velocity can be used as a surrogate, qualitative measure of myosin duty cycle (191). It is also prudent to test the surface density of the myosin needed to maintain peak gliding velocity so that for each gliding assay performed you can ensure that you reach this concentration for reliable peak actin gliding assessment. Myosin stock concentration was measured by spectrophotometry, and myosin was diluted to final assay concentration immediately before experimentation. Myosin concentrations from 40-400 nM were added to the assay chamber and left to incubate for 1 minute before washing. All myosin was added in high ionic strength (600 mM) buffer to ensure that it was not filamentous, but deposited as single two headed molecules. All experiments were performed at 30°C at pH 7.1 in 60 mM ionic strength final buffer with 2 mM MgATP. This assay was used to investigate the ability of RLC phosphorylation abundance to modulate acting gliding velocity under different surface densities of myosin molecules.

3.6.2 The effect of temperature on gliding velocity

Actin gliding velocities were measured as a function of experimental temperature. Measurements were made at 17, 20, 25, 30, 35 and 37°C. This was done to ascertain if the actin gliding velocity results observed by altering RLC phosphorylation, at non-physiological temperature, were upheld at physiological temperatures. Measuring actin gliding velocities over a range of temperatures allowed analysis by Arrhenious plots. These plots provide Q_{10} and activation energies (E_a) for a range of temperatures. This allows us to assess if RLC phosphorylation level affected the Q_{10} or E_a of the myosin. Motility buffers for the

actin gliding assay were adjusted so that the pH for each chamber at a specific temperature was pH 7.1 and the ionic strength was kept constant at 60 mM. All measurements were made in 2 mM MgATP, and at surface myosin concentrations of 200 nM. As sliding velocities increased with temperature the data acquisition was accelerated to more accurately track filaments that were moving at a faster velocity. Data acquisition was set at a frame for every 1 μm that the actin filaments would move. This meant that when the filaments moved a 1 $\mu\text{m/s}$, data acquisition was set to one frame per second. Data were sampled at the beginning and end of each experiment to ensure that velocity was unaffected by duration of assay.

3.6.3 ATP concentration versus velocity

Actin gliding assay velocities were measured as a function of MgATP concentration. This data can be plot by Michaelis-Menten kinetics to predict the peak gliding velocity at infinite MgATP. This allowed us to determine peak gliding velocity by an independent method to ascertain if we get a similar result to the value from the myosin concentration actin gliding assay. All experiments were performed at 30°C in pH 7.1 buffers with ionic strengths of 60 mM. MgATP concentration in the final assay buffer was varied between 0.01 mM and 2 mM. Precise MgATP concentrations were ensured by serial dilution of 100 mM ATP.

3.6.4 Actin gliding assay under load with α -actinin

Actin gliding velocities were assessed as a function of α -actinin concentration added to the assay chamber. α -actinin is a cytoskeletal actin binding protein and the addition of this molecule to the assay chamber adds load to the actin gliding assay. This is due to the interaction between α -actinin bound to the surface of the chamber and the actin gliding across the chamber surface propelled by the myosin. This assay allows the measurement of relative changes in the ability of myosin to produce 'isometric force' (192). We can use this technique to assess if there are relative

changes to isometric force, produced by myosin with different phosphorylation abundances. This allows us to check if the force changes we observe in skinned muscle are conserved at the level of the myosin ensemble.

All α -actinin assays were performed with 200 nM myosin at 30°C at pH 7.1 in 60 mM ionic strength buffer with 2 mM MgATP. α -actinin was mixed with myosin before addition to the experimental chamber. α -actinin was mixed with 200 nM myosin in concentrations between 0.5-5.0 μ g/ml pre-deposition in the experimental chamber. Once the α -actinin and myosin was sufficiently mixed, it was deposited in the assay chamber in high salt (600 mM) ionic strength buffer and incubated for 1 minute. The assay chamber was then washed of the myosin and α -actinin mixture with 3 volumes of 600 mM ionic strength buffer before the addition of actin and the final assay buffer.

4 – RLC phosphorylation studies

4.1 Measuring the abundance of regulatory light chain (RLC) phosphorylation

RLC phosphorylation abundance from the myocardium of several mammalian species was measured using the Phos-tag method (Chapter 2.7). We used this technique to measure phosphorylation abundance in rat left ventricular myocardium in healthy and diseased states. This method was also applied to measure RLC phosphorylation change in both pig and human myocardium, which possess the same level of RLC phosphorylation. It can be applied to small tissue volumes (10-20 mg) and therefore we were able to measure the RLC phosphorylation abundance in biopsies from diseased human left ventricular myocardium. The human tissue samples were kindly donated by Professor Steven Marston, Imperial College London, from a tissue bank held at the Hammersmith Hospital supplied by Professor Christobal Dos Remedios, University of Sydney. All animal procedures and perioperative management were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health under assurance number A5634-01. Ethical approval for human tissue samples was obtained from The Brompton, Harefield and NHLI, London and St Vincent's Hospital, Sydney. The investigation conformed to the principles outlined in the Declaration of Helsinki.

The use of highly specific antibodies, with ECL imaging, removed the chance of measuring other RLC isoforms, which allowed the use of small (< 20 mg) tissue samples. The ECL imaging was linear over the range of protein concentrations used for PAGE and gel imaging exposure times. Blot images were assessed for over exposure to make sure we were in the linear range for ECL imaging. Using Image J, band intensities were plotted and integrated to accurately determine the band area density for each lane on a Western blot. The ratio of the intensities between the

phosphorylated and un-phosphorylated bands gave the ratio or percentage of molecules that are phosphorylated in the sample, and the distribution of phosphorylation between the two available sites:

$$\text{Ratio: Mol Pi/mol RLC} = \frac{(\text{Intensity of 1P band} + (\text{intensity of 2P band} \times 2))}{(\text{Intensity of 2P} + \text{1P} + \text{0P bands})}$$

For the analyses of blot data, one-way analysis of variance was used to test our experimental hypotheses. Appropriate post hoc pairwise multiple comparisons were made using a Bonferroni adjusted *t* test method. Statistical significance was met when $p < 0.05$.

4.2 Rat RLC phosphorylation abundance assessment from left ventricular homogenate

RLC phosphorylation abundance was measured in rat left ventricular myocardial homogenate. Rat cardiac tissue has a predominant alpha cardiac myosin background in healthy adult animals, different from human and pig that predominate with beta cardiac myosin in the adult left ventricle.

We show that rat RLCs have multiple phosphorylation states, as the RLC molecules can be doubly phosphorylated as had been observed previously in mice (82). Figure 4.2.1 shows a representative Western blot of rat cardiac RLC showing four distinct bands on the Phos-tag Western blot. The observed four bands are explained in terms of the four-phosphorylation states, namely 0P, 1P Serine-14, 1P serine-15 and 2P. Scruggs et.al. 2010 observed that the RLC could be singly phosphorylated at either the serine-14 or serine-15 residue (82). When RLC was phosphorylated singly, 20% of the time this was at serine-14 and 80% of the time it was at serine-15 (82). We observe two bands in place of a single band for the 1P state with the same stoichiometry. Assuming rat and mouse have similar phosphorylation stoichiometries the Phos-tag gel method may allow us to distinguish the phosphorylation residue, whilst providing a value of the phosphorylation level of the protein. Additionally, the 2P band is in the same proportion of the total RLC, as has been measured by Scruggs et al. for doubly phosphorylated RLC, using mass spectrometry (3%)(82). As rat RLC is neither de-amidated nor has any expressed charge variants, this explanation for the fourth band in the Phos-tag gels is the most likely. Thus rat RLC phosphorylation abundance was measured to be 0.31 ± 0.05 mol Pi/mol RLC from n=12 different individuals.

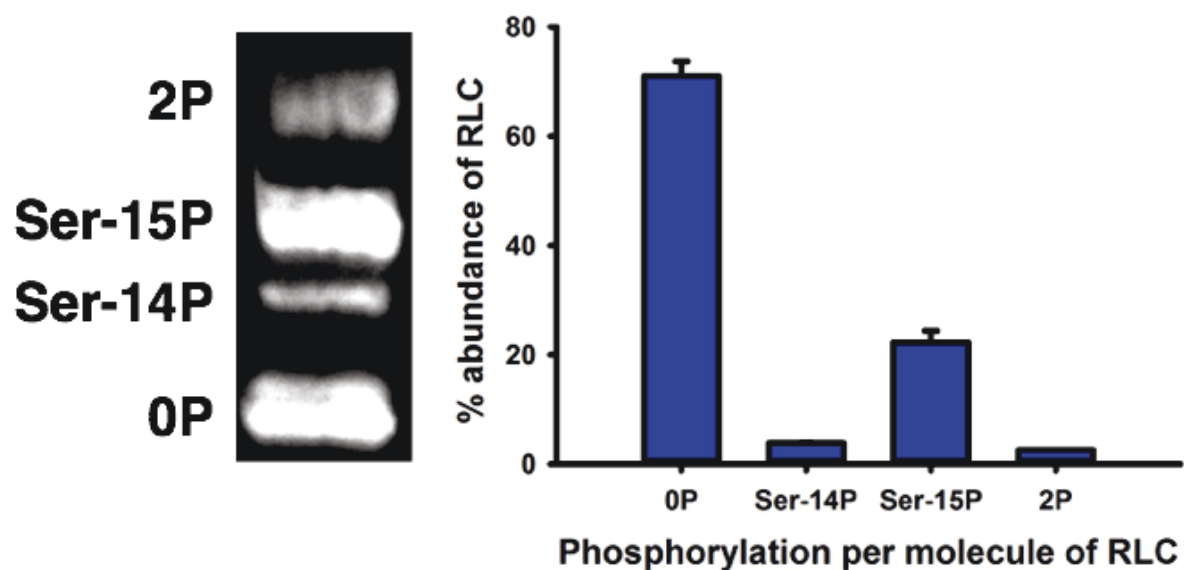


Figure 4.2.1: Rat RLC phosphorylation abundance. **Top left,** Representative Western blot of the RLC phosphorylation species found in rat left ventricular homogenate. The blot is overexposed for clarity to show the low intensity 2P and Ser-14P bands. The blot shows four distinct bands representing 4 different phosphorylation states. **Top right,** Distribution of RLC molecules in control wild-type rat (Sprague-Dawley) left ventricular myocardium as a function of phosphorylation level for each site. Data are from 5 separate samples, with 5 repeat measurements on separate gels. **Bottom,** Data are presented as mean $\pm$ SEM where SEM is calculated from the average of the measurements for each of the 5 hearts.

4.3 RLC phosphorylation change in a rat model of chronic myocardial infarction

Adult male Sprague-Dawley rats underwent irreversible left anterior descending (LAD) coronary artery ligation. This procedure left the animals with a chronic apical infarction confined to the left ventricle. Tissue upstream of the ligation, in the well-perfused myocardium of the left ventricle was sampled for Phos-tag analysis, at two time points after ligation. The tissues were sampled at 4-weeks post infarct, when the animals were considered to be displaying compensatory hypertrophic disease, and 20-weeks post-infarct, when a decompensated heart failure phenotype had been established (Appendix A (1)). The design of the study was to determine the time-course of protein alterations following myocardial infarction in the remaining perfused tissue of the left ventricle. Animal surgery was provided by Dr. Markus Sikkell and Dr. Alexander Lyon, and our in-vivo measurements of cardiac function in the animal model have been previously published (Appendix A (1, 193)).

It must be noted that there is no evidence to suggest a difference between the response of male and female rats to MI alone (194, 195). However, there are gender differences in regard to MI and induced hypertension post-MI, where females display concentric hypertrophy, whilst males display eccentric hypertrophy (196). We do not know conclusively if the results we observe herein would be conserved in female animals, therefore it would be prudent to repeat the experiments in the future with a cohort of female rats.

Control animals that were age-matched with animals that had undergone the surgical ligation were sacrificed at the same time-points as the ligated cohort. Age-matched control animals at four weeks after intervention (C4) had an RLC phosphorylation abundance of 0.30 ± 0.04 mol Pi/mol RLC (Figure 4.3.1). The myocardial infarcted animals at four weeks post intervention (MI4) had a phosphorylation abundance of

0.45 ± 0.02 mol Pi/mol RLC, a 50% increase above the C4 cohort. The age-matched control animals at twenty weeks post intervention (C20) had an RLC phosphorylation abundance of 0.31 ± 0.02 mol Pi/mol RLC, which was not significantly different from C4. Myocardial infarcted animals at twenty weeks post intervention (MI20) had an RLC phosphorylation abundance of 0.52 ± 0.04 mol Pi/mol RLC, a 68% increase on C20 animals. T-tests were used to compare treatment versus control within time-points and found significant differences between C and MI animals at both four weeks and twenty weeks post infarct with $p < 0.01$ and $p < 0.0005$ respectively.

Dr. O'Neal Copeland has measured the consequence of MI on MyBP-C and TnI phosphorylation in Professor Steven Marston's laboratory. This data has been omitted from the thesis as the work is not my own. However, it was found that TnI phosphorylation was unaltered by MI. MyBP-C phosphorylation was reduced from 2.9 ± 0.1 (C4) to 2.4 ± 0.1 mol Pi/mol MyBP-C (MI4), $p < 0.05$. This finding was reversed later in disease where MI20 animals displayed an increased MyBP-C phosphorylation level of 3.5 ± 0.4 in comparison to C20 of 2.5 ± 0.4 mol Pi/mol MyBP-C $p < 0.05$.

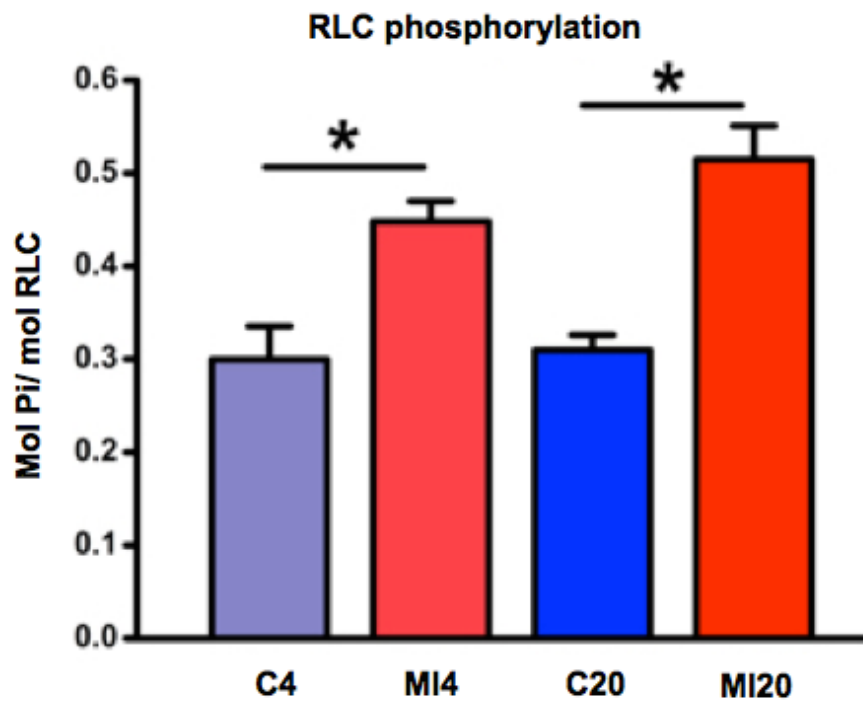


Figure 4.3.1: Rat RLC phosphorylation abundance MI study. Bar plot of RLC abundance in an age-matched cohort of animals that either had no intervention or were chronically infarcted by left anterior descending artery ligation. * Indicated a significant effect within age range $p < 0.01$. One way ANOVA was used with post hoc pairwise multiple comparisons made using Bonferroni adjusted t tests.

4.4 Assessment of RLC phosphorylation in a model of chronic myocardial infarction (CMI)

4.4.1 Assessment of RLC phosphorylation sites four weeks post-MI

Four weeks after surgical ligation the MI4 and C4 animals had RLC phosphorylation abundances of 0.45 ± 0.02 mol Pi/mol RLC and 0.30 ± 0.04 mol Pi/mol RLC. Assessing the individual phosphorylation sites (Figure 4.4.1), the percentage of protein that was unphosphorylated was $62 \pm 2\%$ in MI4 animals and $71 \pm 3\%$ in the C4 cohort ($p < 0.005$). 13% less protein in the MI4 group was completely unphosphorylated compared to the C4 cohort. Single 1P serine-14 phosphorylation site was occupied in $10 \pm 1\%$ of the MI4 molecules and $4 \pm 1\%$ of the C4 cohort. This was a 2.4-fold increase in the MI4 cohort compared to the C4 cohort. Single serine-15 phosphorylation was observed in $24 \pm 1\%$ of the RLC molecules from the MI4 cohort and $21 \pm 2\%$ in the C4 cohort. Phosphorylation of serine-15 was not altered by infarction at four weeks. The percentage of molecules that were doubly phosphorylated in the MI4 cohort were $5 \pm 1\%$ compared to $3 \pm 1\%$ in C4 molecules. This difference was not statistically significant. All data is displayed in figure 4.4.2.2.

4.4.2 Assessment of RLC phosphorylation sites twenty weeks post-MI

Twenty weeks post surgical ligation MI20 and C20 cohorts had RLC phosphorylation abundances of 0.52 ± 0.04 mol Pi/mol RLC and 0.31 ± 0.02 mol Pi/mol RLC, respectively. Assessing the individual phosphorylation sites (Figure 4.4.2.1), the percentage of protein that was un-phosphorylated was $48 \pm 2\%$ in the MI4W cohort and $78 \pm 4\%$ in the C20 cohort ($p < 0.0005$). The abundance of RLC molecules that were unphosphorylated was 38% lower in the MI20 cohort when compared to the C20 cohort. The percentage of RLC molecules singly phosphorylated at serine-14

was unaffected by MI, MI20 at $6 \pm 1\%$ and C20 at $6 \pm 1\%$ of the total RLCs. The percentage of RLC molecules phosphorylated singly at serine-15 in the MI20 cohort was $28 \pm 2\%$ and $14 \pm 3\%$ in the C20 cohort ($p < 0.0005$). The MI20 cohort had a 2-fold higher abundance of molecules with single serine-15 phosphorylation than the C20 cohort. The proportions of RLC molecules doubly phosphorylated were shown to be $4 \pm 1\%$ of the MI20 cohort and $2 \pm 1\%$ of the C20 cohort ($p < 0.05$). The MI20 cohort had a 2-fold greater proportion of RLC molecule doubly phosphorylated than the C20 cohort. All data is tabulated in table 4.4.2.2.

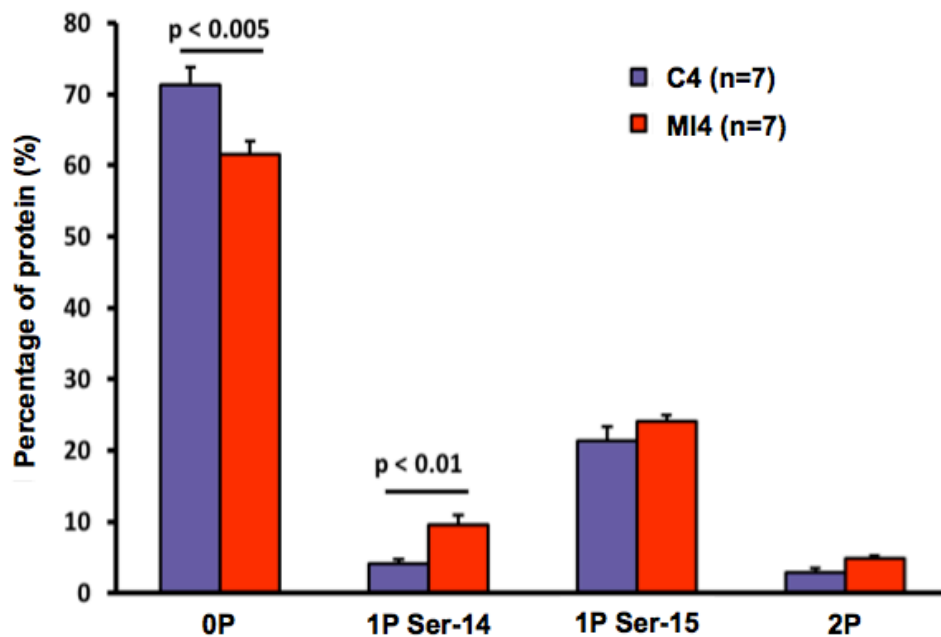


Figure 4.4.1: RLC levels at four weeks. Abundance of RLC molecules occupying the different phosphorylation states of the RLC from rat left ventricular myocardium in the C4 (n=7) and MI4 (n=8) cohorts. All data is plot as mean $\pm$ SEM. Any significant differences between phosphorylation site abundances are denoted with their corresponding p values in the diagram: n refers to the number of animals in each group.

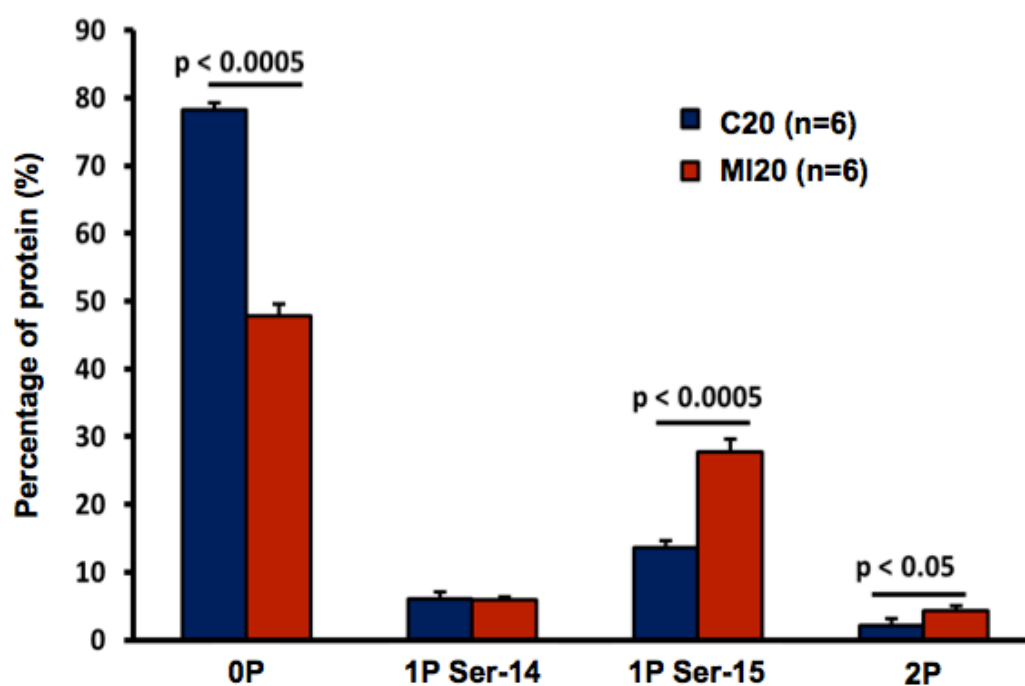


Figure 4.4.2.1: RLC levels at twenty weeks. Abundance of RLC molecules occupying the different phosphorylation states of the RLC from rat left ventricular myocardium in the MI20 (n=6) and C20 (n=6) cohorts. All data is plot as mean $\pm$ SEM. Any significant differences between phosphorylation site abundances are denoted with their corresponding p values in the diagram.

	C4 (n=7)	MI4 (n=8)	C20 (n=6)	MI20 (n=6)
2P	3 ± 0.5	5 ± 1.1	2 ± 0.8*	4 ± 0.7
1P Serine-15	21 ± 2.0	24 ± 0.9	14 ± 2.8*	28 ± 1.9
1P Serine-14	4 ± 0.6*	10 ± 1.4	6 ± 0.9	6 ± 0.5
0P	71 ± 2.6*	62 ± 1.9	78 ± 3.9*	59 ± 1.8

Table 4.4.2.2: Combined phosphorylation abundance from C and MI cohorts. All data is displayed as percentage of protein ± SEM. *Denotes significantly different within age-group where $p < 0.05$.

4.5 RLC phosphorylation assessment in porcine left ventricular myocardium and human control donor tissues

We were unable to use human or larger mammalian cardiac preparations for isolating trabeculae for permeabilised mechanics due to the difficulties in procuring fresh tissue on a weekly basis. Therefore, we used the best possible model available for mechanical experimentation, which was the Sprague-Dawley rat. We were unable to obtain enough human myocardium to extract myosin for ensemble and single molecule experiments. However, single pig hearts provided enough tissue for bulk protein, ensemble, and single molecule experiments. Myosin could be extracted from frozen pig hearts. As Pig beta cardiac myosin is closer in sequence (*Sus Scrofa*: P79293 shares 98% sequence similarity with *Homo Sapiens*: P12883) to human beta myosin than rat beta myosin (*Rattus Norvegicus*: P02564 97% amino acid sequence similarity with human) we decided to use pig myosin. Pig myosin is a good model in lieu of human left ventricular myocardium as they share similar levels of MHC isoform expression (reviewed (197)), different to rat cardiac myosin, which predominantly has alpha MHC in adulthood (198). Therefore, we chose pig myosin extracted from the left ventricular myocardium to perform bulk protein, ensemble, and single molecule assays.

4.5.1 Pig myocardial RLC phosphorylation level

What isn't yet known, is whether pig and human RLCs have the same amount of phosphorylation sites; and if the basal level of RLC phosphorylation in the left ventricular myocardium is the same. The study of both human and pig RLC phosphorylation will indicate the extent to which porcine heart could be used as a proxy for the human heart in the evaluation of the effects of RLC phosphorylation level change.

The RLCs of porcine left ventricular myocardium have two phosphorylation states as indicated in figure 4.5.1. The band that migrated more swiftly through the gel is the band that contains unphosphorylated protein. The band that migrated more slowly in the gel contains the phosphorylated protein that has been slowed by the Phos-tag molecule, which was impregnated into the poly-acrylamide gel.

Measuring RLC phosphorylation from the left ventricular myocardium of 5 separate healthy pig hearts indicated a basal phosphorylation level of 0.44 ± 0.7 mol Pi/mol. The difference in the number of phosphorylation sites between pig and rat is noteworthy and is discussed in more detail in chapter 7. Previously, pig left ventricular RLC phosphorylation has been assessed by mass spectrometry, finding that control animals had a phosphorylation level of 0.11 ± 0.01 mol Pi/mol RLC (199). This is significantly different to our findings of 0.44 ± 0.7 mol Pi /mol RLC. It is not clear which layer of the myocardium was sampled for protein analysis by mass spectrometry. This may be important to the basal RLC phosphorylation level and may be the reason for different values (90). Another reason for the discrepancy in the findings could be the experimental protocols carried out on the individuals before euthanasia. In the Peng et.al. study (199) there was a lengthy period (90 minutes) of high sedative and anesthesia (isofluorane 5%) use before euthanasia, which is known to depress systolic left ventricular function above 1.5% and may affect protein modifications in the short term (200).

4.5.2 Human myocardial RLC phosphorylation level

Homogenised human left ventricular samples from 4 human donor individuals were used to determine basal RLC phosphorylation in human myocardium. The individuals were of varying ages and genders as indicated in table 4.5.2.

The combined average RLC phosphorylation abundance from human donor tissues was 0.40 ± 0.04 mol Pi/mol RLC. Therefore pig (0.44 ± 0.7 mol Pi /mol RLC) and

human (0.4 ± 0.04 mol Pi /mol RLC) left ventricular tissues have statistically indistinguishable basal RLC phosphorylation levels. Notably, both pigs and humans have a single phosphorylation site on the Phos-tag gels, unlike the rat that displays two.

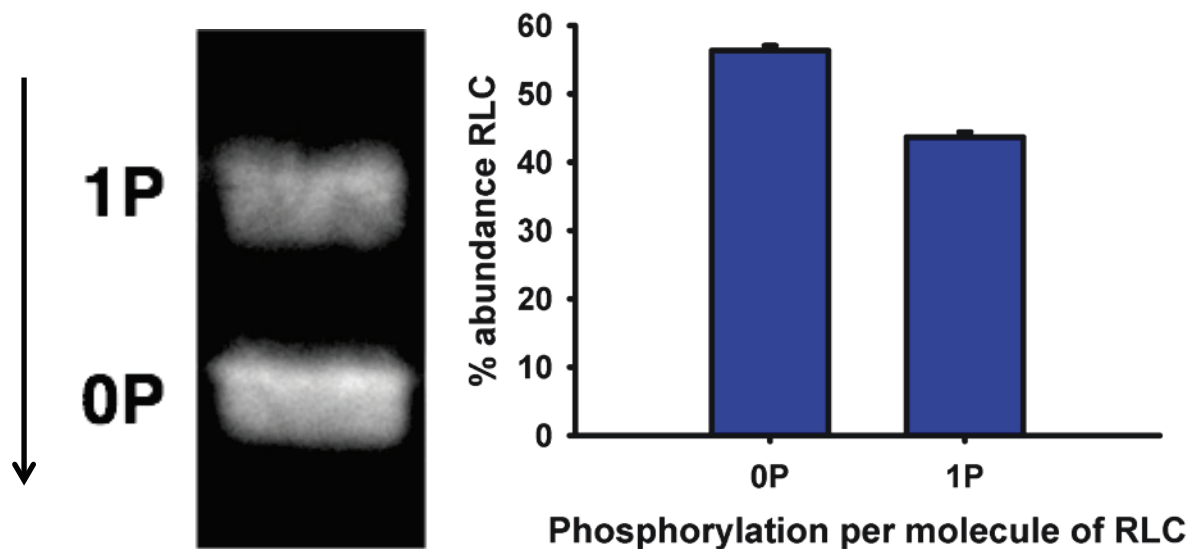


Figure 4.5.1: Pig myocardial RLC phosphorylation. **Left,** shows a representative Western blot for porcine left ventricular RLC. The arrow denotes the direction of travel of the protein through the gel. The two bands are denoted with the phosphorylation state of the molecules in each state. 1P = RLC molecules that have a single phosphorylation. 0P = RLC molecules that have no phosphorylation. **Right,** Distribution of RLC phosphorylation in pig left ventricular myocardium. Data represents the average from 5 pig hearts that have been repeatedly run on 5 separate gels. All data is presented as mean $\pm$ SEM where the SEM is calculated from $n = 5$ separate pig hearts.

Phosphorylation abundance in human RLC	
(mol Pi/ mol RLC)	
Female 25	0.40 ± 0.01
Male 23	0.39 ± 0.01
Female 59	0.35 ± 0.04
Male 53	0.45 ± 0.06

Table 4.5.2: Human healthy donor RLC phosphorylation. Phosphorylation abundance of 4 'healthy' donor individuals. Each donor was sampled four times to account for individual donor variance. All data is presented as mean ± SEM.

4.6 RLC phosphorylation abundance in human inherited and acquired cardiac disorders

To better understand if, and how, RLC phosphorylation level is altered in a range of cardiac disorders we obtained samples of human myocardium from a variety of sources. These samples were used to assess if RLC phosphorylation level was altered during cardiac disease that was not linked to RLC mutations, which are known to alter RLC phosphorylation level (reviewed (201)).

We assessed RLC phosphorylation abundance in human myocardial samples, which had identifiable inherited cardiac disease from explanted hearts. The donors had identifiable inherited cardiovascular disease, due only to sarcomeric protein mutations (Table 4.6.1). 10-20 mg of myocardium was obtained from each individual donor heart. The samples were split into approximately four or five individual pieces, which were each individually prepared for electrophoresis (this was the protocol used for all human tissue samples). Unfortunately, due to the size of the samples the specific region of myocardial layer was not determined. This may be an important factor for RLC phosphorylation level measurements (90).

Tissues were donated from a variety of disease causing mutations. The specifics of these tissues are described below:

A sample from a patient with a myosin binding protein-C (MyBP-C) mutation causing an amino acid substitution (R502W) was donated. This mutation was associated with an increased risk of hypertrophic disease and a chance of sudden cardiac death (202). The tissue from the MyBP-C R502W donor showed an RLC phosphorylation abundance of 0.50 ± 0.03 mol Pi/mol RLC.

Another sample was obtained from a donor with a troponin I (TnI) mutation K36Q. The K36Q mutation is known to cause dilated cardiomyopathies (DCM) rather than hypertrophic disease and reduces calcium sensitivity of the thin filament by 2 fold (203). The TnI K36Q myocardium had an RLC phosphorylation abundance of 0.51 ± 0.04 mol Pi/mol RLC.

Another patient with hypertrophic cardiomyopathy, caused in this case by a troponin T (TnT) K273N mutation (204), was also sampled and showed an RLC phosphorylation abundance of 0.61 ± 0.01 mol Pi/mol RLC. This mutation was shown to display heightened calcium sensitivity (204).

Two patients were sampled with MYH7 gene mutations in the human beta cardiac myosin (K847E and R719Q). The K874E mutation is relatively understudied, but seems to present as a hypertrophic disorder. The R719Q mutation is also a mutation that displays phenotypic hypertrophy, but seems to be a 'benign' defect (205). The K847E mutation showed an RLC phosphorylation level of 0.45 ± 0.1 mol Pi/mol RLC, not significantly different from control donor tissue. The R719Q mutation showed an average RLC phosphorylation abundance of 0.50 ± 0.1 mol Pi/mol RLC.

Averaging the RLC phosphorylation abundance across all the inherited disease types gave an overall RLC phosphorylation abundance of 0.51 ± 0.03 mol Pi/mol RLC, which was significantly different from control at 0.40 ± 0.04 where $p < 0.05$. However, there was no correlation between the severity of the disease phenotype, and the extent of RLC phosphorylation level change. The number of samples collected was insufficient to detect any correlation between RLC phosphorylation abundance and specific mutant phenotypes. However, both hypertrophic and dilated phenotype mutations showed a trend towards increased RLC phosphorylation with Phos-tag analysis.

Additionally myectomy tissues were sampled from human donor samples, which had no identifiable mutation, but had been diagnosed with heart failure of different degrees of severity (Table 4.6.2). Overall, in all myectomy samples with non-inherited heart failure, RLC phosphorylation abundance was raised, although to varying degrees. The average RLC abundance from all samples combined was 0.56 ± 0.03 mol Pi/mol RLC, which was 36% higher than the average from control tissues ($p < 0.01$).

Mutation (n=1)	Gender and age	Phenotype	RLC phosphorylation (mol Pi/mol RLC)	% change compared to control
MyBP-C3 (R502W)	M23	HCM	0.50 ± 0.03	+ 25%
TnI (K36Q)	M15	DCM (ischemic)	0.51 ± 0.04	+ 28%
TnT (K273N)	M26	HCM (hypocontractile)	0.62 ± 0.01	+ 55%
MYH7 (K847E)	F20	HCM	0.45 ± 0.1	+ 13%
MYH7 (R719Q)	F27	HCM	0.50 ± 0.1	+ 25%
Average	-	-	0.51 ± 0.03	+ 28%

Table 4.6.1: Human sarcomeric protein mutant RLC phosphorylation. Myectomy sample details from cardiac donor tissues with identified inherited cardiac sarcomere protein gene mutations were tested for RLC phosphorylation abundance. HCM = hypertrophic cardiomyopathy; DCM = dilated cardiomyopathy. MYH7 = human beta cardiac myosin heavy chain gene; MyBP-C3 = human myosin binding protein-C gene; TnI = troponin I and TnT = troponin T. All RLC phosphorylation data are stated as mean ± SEM where the SEM was calculated from 5 repeated runs of the same myectomy sample.

Heart failure Sample:	Gender and age	Clinical notes	RLC phosphorylation	% change compared to control
FC	M30	IDCM	0.44 ± 0.02	+ 10%
FF	F52	LV/LA dilatation with atrial flutter	0.71 ± 0.05	+ 78%
FG	M52	AF	0.50 ± 0.03	+ 25%
FH	F34	IDCM	0.58 ± 0.05	+ 45%
Average	-	-	0.56 ± 0.03	+ 40%

Table 4.6.2: Human acquired cardiovascular disease RLC phosphorylation.

Myectomy sample details from cardiac donor with acquired cardiovascular disease but with no known inherited mutations. The first column denotes the database name of the sample. IDCM = idiopathic dilated cardiomyopathy; LV = left ventricular; LA = left atrial; AF = atrial fibrillation. All RLC phosphorylation measurements are stated as mean $\pm$ SEM where SEM was calculated from 5 repeat measurements on 5 separate gels from a single myectomy sample.

4.7 RLC phosphorylation abundance in clinically defined human heart failure

We measured the RLC phosphorylation abundance in diseased human donor left ventricular myocardium that had been clinically diagnosed with acquired heart failure. All biopsies were collected, rapidly frozen, and stored after removal in liquid nitrogen. Tissues were selected from donors that had been clinically diagnosed with heart failure in two subsets of New York Heart Association (NYHA) classification of heart failures. These were defined as either NYHA I-II or NYHA III-IV defined heart failure. These donor tissues were from patients with heart failure of varying acquired disease causes, other cardiovascular morbidities and protein mutations summarised in tables 4.6.1 and 4.6.2. These measurements were performed to ascertain if worsening heart failure correlated with a change in RLC phosphorylation abundance, irrespective of the cause of the disease. As previously stated, control donor hearts of a variety of ages and both genders were assessed to have an RLC phosphorylation abundance of 0.41 ± 0.04 mol Pi/ mol RLC. Individuals classified in NYHA I-II heart failure had an average RLC phosphorylation abundance of 0.47 ± 0.05 mol Pi/mol RLC, not statistically different from control myocardium. Individuals classified in NYHA categories III-IV had an RLC phosphorylation abundance of 0.60 ± 0.03 mol Pi/mol RLC, which was 50% higher than the RLC abundance in healthy control tissues ($p < 0.05$) assessed by ANOVA and adjusted T-test.

5 – Muscle studies

5.1 The effect of RLC exchange on the force-velocity (FV) relations of permeabilised trabeculae of rat hearts

To test the specific effect of altering RLC phosphorylation in isolation of other sarcomeric protein alterations, we partially exchanged recombinant RLCs (with altered phosphorylation levels) with native RLCs. Force velocity measurements give a measure of the contractile function of tissue during shortening, which is the physiological state of muscle during systole in the heart. From these relationships; measures of peak power, velocity at peak power, isometric force and, unloaded shortening velocity can be obtained. These contractile parameters eloquently assess the effect of RLC phosphorylation level alteration on myocardial contractility, during physiological shortening.

As described in chapter 2 the RLCs of trabecular preparations were partially exchanged with recombinant proteins with known phosphorylation levels. These trabeculae underwent a series of activations by temperature jump to measure isometric force (F_0), force maintained at different shortening velocities, peak unloaded shortening velocities (V_{max}), peak power (PP) and velocity at peak power (V_{PP}). All force measurements were performed at sarcomere lengths of $2.1\ \mu\text{m}$ at 20°C in saturating calcium conditions ($32\ \mu\text{M}$ free calcium).

Control exchange experiments were performed to understand the effect of the exchange procedure on the contractile output of the tissue. Simulating exchange conditions without addition of RLC to replace any that was depleted during exchange interfered with the mechanics of the preparation and was not a viable control. TnC was also lost during exchange; to preserve appropriate relaxation post activation TnC was added to the exchange buffer as described in chapter 2. Therefore, RLCs with similar phosphorylation level to native RLCs were exchanged into permeabilised trabecular preparations to control for the effect of the exchange procedure. Force-

velocity relations from preparations that had undergone exchange and those that had not are shown in figure 5.1.1. Figure 5.1.1 shows the force-velocity relationships for both the un-exchanged (un-treated trabeculae) and the trabeculae that underwent exchange with RLCs of similar phosphorylation abundance (control exchange).

The process of exchange had little effect on the ability of the trabecular preparations to produce F_0 , $V_{\max}$, PP or V_{PP} when the exchanged RLC species had the same phosphorylation abundance as the native RLCs. These parameters are summarised in table 5.1.2.

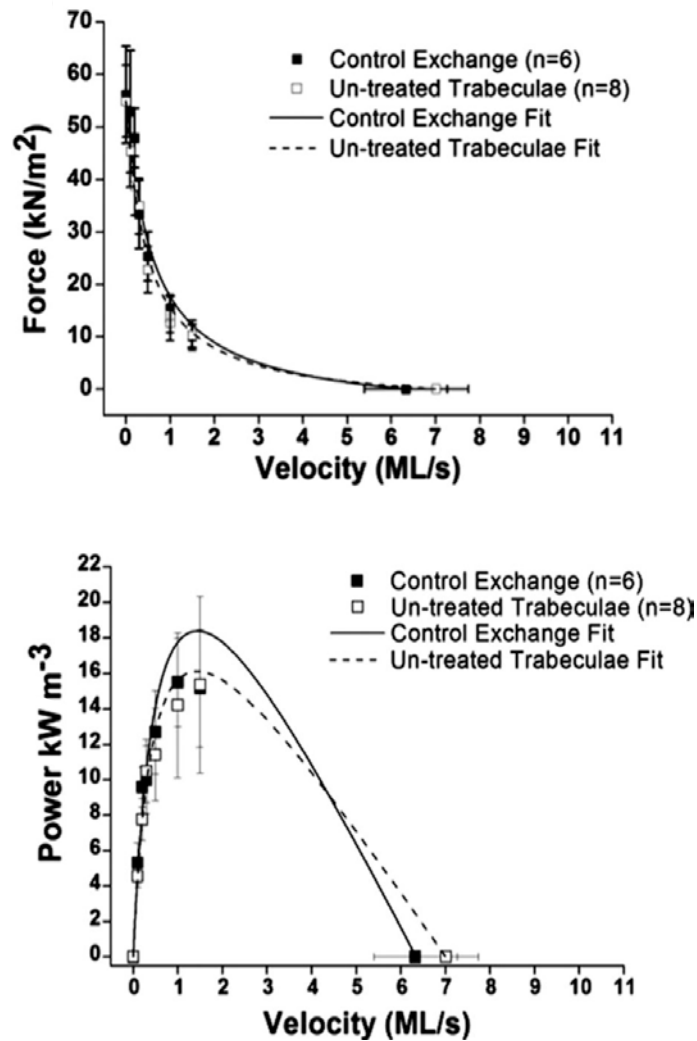


Figure 5.1.1: FV of control and control exchange. Top, Force-velocity (FV) relations of un-treated (open squares) and control exchanged trabeculae (closed squares). All data are shown as mean $\pm$ standard error of the mean (SEM). The n-number for each treatment group is stated in the plot legend. FV data are fitted with rectangular hyperbolas; untreated trabeculae (dotted lines) and control-exchanged trabeculae (black lines). **Bottom,** Power-velocity relations calculated from the fitted FV relations. These graphs are re-produced from our publication Toepfer et.al. 2013 (1).

	Enriched (n=8)	Control (n=6)	Un-treated (n=8)	De-Phos (n=5)
Peak Isometric Force (kN/m²)	81.9 ± 10 [¶]	79.9 ± 11	86.2 ± 9 [¶]	22.3 ± 7
V_{max} (ML/s)	8.85 ± 0.7 [¶]	6.33 ± 0.9	7.01 ± 0.7	5.34 ± 0.4
a/Po	0.16 ± 0.06	0.1 ± 0.02	0.04 ± 0.01	0.07 ± 0.02
Power (kWm⁻³)	38.9 ± 8 ^{¶§}	17 ± 3	14.7 ± 4	5.13 ± 3
Velocity at peak power (ML/s)	1.97 ± 0.2 ^{¶§}	1.36 ± 0.1	1.03 ± 0.2	0.9 ± 0.2

Table 5.1.2: FV parameters of RLC exchange. Parameters obtained from force-velocity relations in separate RLC phosphorylation treatment groups. [¶]Denotes the value is different from de-phosphorylated (p < 0.001). [§]Denotes value is different from native (p < 0.05).

5.2 The effect of altered RLC phosphorylation on cardiac trabecular force-velocity relations

As described in chapter 2, recombinant RLC was phosphorylated to different degrees by the use of kinases in-vitro, which were subsequently removed. These RLCs were then used to exchange with native RLCs in permeabilised trabeculae to alter the trabecular RLCs phosphorylation abundance. This procedure ensures that only RLC phosphorylation is affected as no kinases or phosphatases come into contact with the tissue eliminating the risk of altering the phosphorylation state of other sarcomeric proteins. RLC species that were either completely dephosphorylated or highly phosphorylated were exchanged into our trabecular preparations. These trabeculae were then used to measure F_0 forces maintained during different shortening manoeuvres, V_{max} , PP, and V_{PP} . These FV relations were then plotted alongside that of the control exchanged trabeculae (Figure 5.2.1). Exchange of de-phosphorylated RLC species into cardiac trabeculae decreased the ability of the trabeculae to produce F_0 . De-phosphorylated RLC exchange treated trabeculae generated $22 \pm 7 \text{ kN/m}^2$ of force whilst enriched RLC exchanged trabeculae created an F_0 of $82 \pm 10 \text{ kN/m}^2$ ($p < 0.001$). V_{max} was also affected by RLC phosphorylation abundance. The de-phosphorylated exchange trabeculae had a V_{max} of 5 ± 0.4 muscle lengths per second (ML/s) whilst the enriched phosphorylation exchanged trabeculae had a V_{max} of $9 \pm 0.7 \text{ ML/s}$ ($p < 0.001$). PP was affected by the degree of RLC phosphorylation abundance. Phosphorylated RLC exchange trabeculae (Enriched) created a PP of $39 \pm 8 \text{ kWm}^{-3}$, whilst de-phosphorylated exchanged trabeculae (Reduced) showed a PP of $5 \pm 3 \text{ kWm}^{-3}$ ($p < 0.001$). V_{PP} was shifted to higher velocities by enriching RLC phosphorylation $2 \pm 0.2 \text{ ML/s}$, which was different from native trabeculae $1 \pm 0.2 \text{ ML/s}$ and de-phosphorylated (Reduced) trabeculae $1 \pm 0.2 \text{ ML/s}$ ($p < 0.05$). The curvature of the force-velocity fit (a/P_0) was statistically unaffected by differing RLC phosphorylation abundances.

Increasing RLC phosphorylation in the trabeculae enhanced the ability of muscle to produce force, power and shorten under negligible load. Reducing RLC phosphorylation from native levels reduced the ability of the muscle to produce force, power, and shorten.

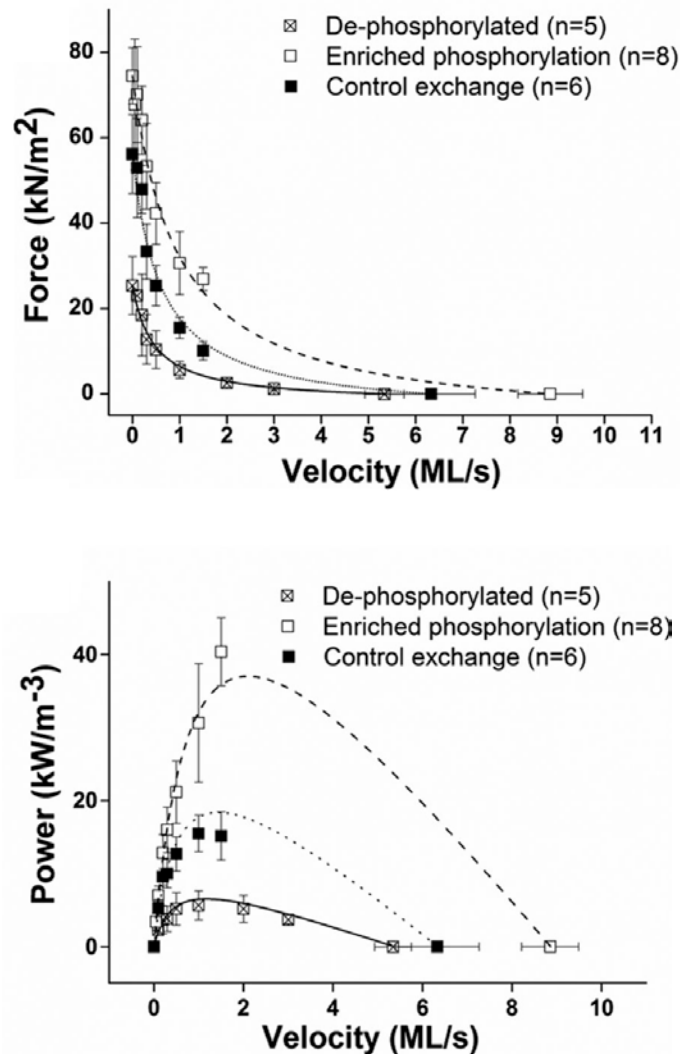


Figure 5.2.1: FV relations from RLC exchange. **Top,** FV relations comparing the effect of enriching and reducing phosphorylation by using RLC exchange. **Bottom,** Power-velocity relations calculated from the FV data showing the effect of RLC phosphorylation on the ability of the trabeculae to produce power. All data are presented as mean $\pm$ SEM and n. The number of trabeculae used in each group is indicated for each treatment group in the plot legend. These graphs are reproduced from our own publication Toepfer et.al. 2013 (1)

Mechanical parameters that were affected by RLC phosphorylation change showed sensitivity to the degree of RLC phosphorylation (Figure 5.2.2). This was evident for power, V_{PP} , and V_{max} . The titration of RLC phosphorylation on these three mechanical parameters showed a linear relationship, which in each case had a positive correlation with RLC phosphorylation abundance increase (from de-phosphorylated). This data adds further information about the effects of RLC phosphorylation level change previously assessed by Scruggs et.al. (140), which found a reduction in the power produced at the organ level, in response to RLC phosphorylation reduction by non-phosphorylatable RLCs in a transgenic mouse model. We also find reductions in power at the muscle level with reduced RLC phosphorylation (Table 5.1.2). It must however be noted that to date our data is the first that has observed the effects of RLC phosphorylation level independently of other sarcomeric protein modifications. As is seen in the Scruggs et.al. experiments, other sarcomeric protein modifications are altered during the development of the transgenic model (140). They try to normalise these other protein modification changes, however we cannot be certain that only MyBP-C and TnI are affected in the model, which they bring back to control levels using dobutamine. We are in agreement with Scruggs et.al. that RLC phosphorylation reductions are correlated with reduced peak isometric force (Table 5.1.2).

In summation, our trabecular data shows a clear dose dependence of RLC phosphorylation on contractile parameters of permeabilised cardiac muscle. To our knowledge this is the first time that this effect has been shown by force-velocity experimentation during physiological muscle shortening in isolation from other sarcomeric, tissue or organ changes confounding the results.

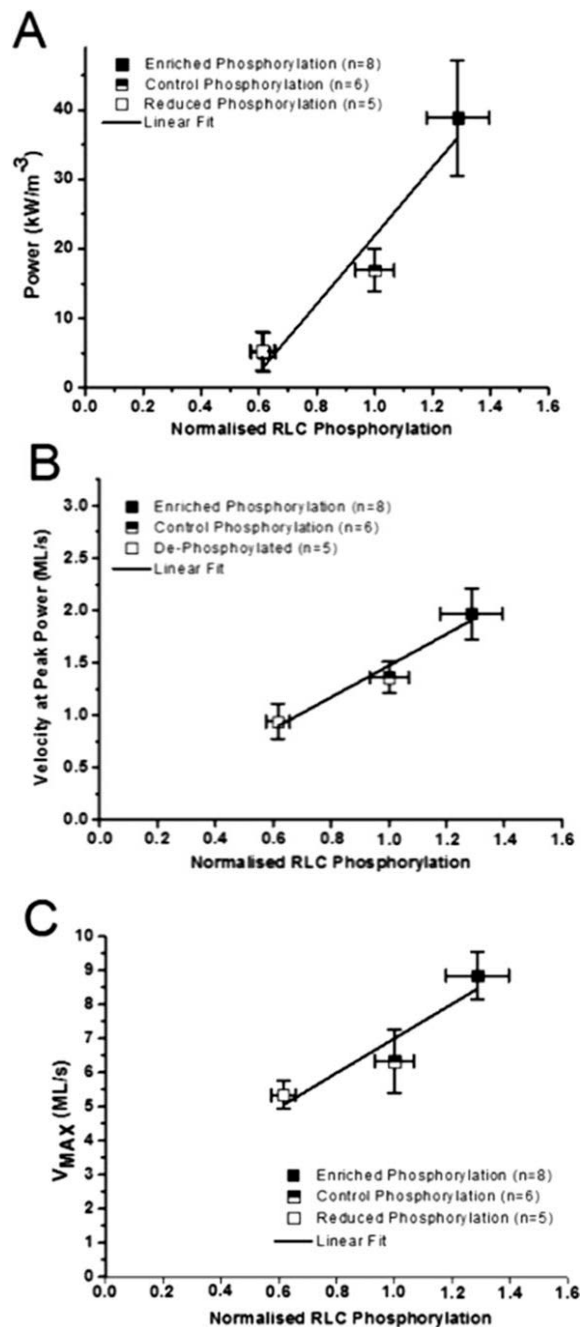


Figure 5.2.2: FV Parameters effected by RLC phosphorylation.

Parameters that showed sensitivity to RLC phosphorylation change were plotted as a function of RLC phosphorylation: **A)** Peak power, **B)** Velocity at peak power, **C)** Peak unloaded shortening velocity. All data is plot as mean $\pm$ SEM for both RLC phosphorylation and each individual parameter. All data sets were fit by linear regression. These graphs are reproduced from our own publication (1).

5.3 Force-velocity relations in a rat model of chronic myocardial infarction (CMI)

FV relations with measurements of F_0 , V_{max} , PP, V_{PP} and a/P_0 were created from a rat chronic myocardial infarct (MI) model described previously (1, 193)(See Appendix A). Trabeculae had been selected from non-infarcted regions of the myocardium upstream of the coronary ligation. Force-velocity measurements have not been made in this context previously.

Each time-point post-ligation had a corresponding un-operated cohort of controls, which were age-matched (C4 and C20). All animals from a specific time point post-ligation were sacrificed within a few days of each other and all experiments were performed within a week. Force-velocity relations were carried out in both saturating (32 μ M) free calcium and physiological sub-maximal free calcium (1 μ M). Otherwise, all experiments were performed as described for RLC studies at 20°C by temperature jump activation in chapter 2.

Notably, at both time points infarcted animals showed enriched RLC phosphorylation compared control. MI4 showed a 50% increase in RLC phosphorylation compared to control. MI20 showed a 68% increase in RLC phosphorylation compared to control.

With these experiments we wanted to investigate the relationship between enriched RLC phosphorylation at both MI4 and MI20, and the mechanical output of the trabeculae, within the context of other disease adaptations occurring concomitantly. We hypothesised, that increased RLC phosphorylation observed in the model would create sarcomeric contractile compensation that was independent from tissue hypertrophy and disease state.

5.3.1 Force- velocity relationships four weeks post-CMI in saturating 32 μ M calcium

Assessing muscle mechanics in saturating 32 μ M calcium allows us to measure the maximal output of the muscle. This is because the calcium dependent 'on/off' switch of thin filament regulation does not inhibit myosin interactions with actin in saturating calcium. Therefore, we get a measure of the un-inhibited output of the contractile machinery working at full capacity that is not affected by the calcium sensitivity of the myofilaments. Due to this we hypothesised that changes to muscle mechanics in saturating calcium could not in totality be created by changes in calcium sensitivity.

Four weeks post-operatively MI4 trabeculae showed enhanced contractile output compared to C4 in 32 μ M calcium (Table 5.3.1.1 and figure 5.3.1.2). F_0 was altered post-ligation with MI4 animals creating 72 ± 11 kN/m² of force, whilst C4 created significantly less force at 55 ± 7 kN/m² ($p < 0.02$). PP was significantly altered with MI4 animals creating an average 33 ± 8 kWm⁻³ and C4 creating 18 ± 4 kWm⁻³ ($p < 0.02$). a/P_0 was significantly affected as MI4 displayed 0.13 ± 0.03 and C4 at 0.05 ± 0.01 ($p < 0.02$). V_{PP} and V_{max} were statistically unaffected by infarction (Table 5.3.1.1) in 32 μ M free calcium.

This led us to believe that indeed the effects of MI on the contractile machinery created a level of sarcomeric contractile compensation. The fact that this was observable at maximal 32 μ M calcium activation meant it was not entirely an effect of calcium sensitivity.

5.3.2 Force- velocity relationships four weeks post-CMI in limiting 1 μ M calcium

We wanted to understand whether this compensation had a component that was due to calcium sensitivity changes. Therefore, we measured the contractile parameters of

the tissues in limiting sub-maximal (1 μ M) calcium activation. In this calcium range the thin filament is only partially activated, therefore, changes in calcium sensitivity created by myofilament proteins would manifest as a changes in the contractile characteristics of the muscle in limiting calcium.

Contractile parameters calculated from FV relationships of MI4 animals were also altered when compared to C4 cohorts in sub-maximal 'physiological' 1 μ M free $[Ca^{2+}]$ (Figure 5.3.2.1). MI4 cohorts produced a greater F_0 of 51 ± 8 kN/m² versus C4 21 ± 4 kN/m² ($p < 0.02$). Interestingly MI affected V_{max} in sub-maximal free calcium conditions (in contrast to our results in saturating calcium conditions) as MI4 animals produced a V_{max} of 5.1 ± 0.6 ML/s compared to C4 at 2.4 ± 0.3 ML/s ($p < 0.02$). We may observe an effect here due to an effect of calcium sensitivity on V_{max} , which was masked by using saturating calcium. PP was affected by MI as MI4 cohorts displayed a PP of 16 ± 2 kWm⁻³, whilst C4 produced PP of 9 ± 2 kWm⁻³ ($p < 0.02$). a/P_0 was also affected by MI treatment as MI4 showed 0.32 ± 0.2 versus C4 of 1 ± 0.2 ($p < 0.02$). V_{PP} was unaffected by MI in sub-maximal free calcium concentrations (Table 5.3.1.1).

These results proved that there seemed to be calcium sensitivity effects observable due to MI. This was because there were evident changes in V_{max} in sub-maximal activations that were not observed during maximal activation at 32 μ M calcium. This inferred that changes RLC phosphorylation (with the other modifications due to disease) were creating a compensatory phenotype at the sarcomeric level. This was leading to increased muscle function at both maximal and sub-maximal activation levels.

5.3.3 The effect of free $[Ca^{2+}]$ concentration on mechanical output of four weeks post-CMI cohorts

Table 5.3.1.1 summarises the effect of free $[Ca^{2+}]$ on C4 and MI4 contractile outputs. In all instances the parameters for MI4 and C4 cohorts at 1 μM $[Ca^{2+}]$ are different from those same measurements in 32 μM free $[Ca^{2+}]$. When comparing V_{max} at 32 μM free $[Ca^{2+}]$ between C4 and MI4 cohorts we observe no statistical change. However, in 1 μM free $[Ca^{2+}]$ we observe an increase in V_{max} of the MI4 cohort when compared to the C4 cohort. Thus, when the trabecular preparations are maximally activated, there is no effect of MI on V_{max} , but in a physiological range of free calcium the MI cohort shows a 2.4-fold increased V_{max} in comparison to C4. This signifies that the MI effect on V_{max} is a calcium sensitivity effect that is not observed in maximal calcium activation. The same effect of calcium sensitivity is observed for the V_{PP} from the trabecular preparations.

The magnitude of some MI effects on parameters from mechanical experimentation is altered by free calcium during activation. F_0 was 31% higher in MI4 animals in 32 μM $[Ca^{2+}]$ when compared to C4. F_0 was 2.4-fold greater in MI4 trabeculae at 1 μM free $[Ca^{2+}]$ when compared to C4. The magnitude of change between the F_0 in 1 μM free $[Ca^{2+}]$ was 3.9 fold higher than the change to F_0 in saturating 32 μM $[Ca^{2+}]$ between MI4 and C4. These parameters are also influenced by MI and free $[Ca^{2+}]$.

Interestingly, the magnitude of the effect of MI on both V_{PP} and PP is not altered by $[Ca^{2+}]$; in both instances the parameters were altered to the same extent when comparing $[Ca^{2+}]$.

All of these effects of MI on contractility were observed even with evident tissue fibrosis contractile material loss (Appendix B figure 4 and 5). This infers that the contractile gain observed post-MI in early disease is due to compensation at the sarcomeric level. To date there have been no other measurements of the effect of MI

on permeabilised muscle force-velocity relations distant from the infarct site. Noticeably, the ejection fraction of the entire organ at MI4 is still negatively impacted. This phenomenon is likely due to large infarct region in the left ventricle that leaves compliance reducing ejection fraction. This can be attenuated using collagen matrices to reduce scar tissue compliance and salvage ejection fraction (206).

Table 5.3.1.1: Parameters from FV and PV relations of AMC4W and MI4W cohorts in 1 μ M and 32 μ M free calcium.

	C4 32 μM (n=12)	MI4 32 μM (n=8)	C20 1 μM (n=9)	MI20 1 μM (n=6)
F₀ (kN/m²)	54.9 $\pm$ 6.84	71.8 $\pm$ 10.6	66.3 $\pm$ 5.51	62.1 $\pm$ 9.30
V_{max} (ML/s)	9.80 $\pm$ 0.75	8.49 $\pm$ 1.05	7.21 $\pm$ 0.58	4.77 $\pm$ 0.44 *
V_{PP} (ML/s)	1.73 $\pm$ 0.17	1.91 $\pm$ 0.26	1.17 $\pm$ 0.10	0.79 $\pm$ 0.08 *
PP (kWm⁻³)	17.9 $\pm$ 4.28	33.3 $\pm$ 7.89 *	14.0 $\pm$ 6.84	7.51 $\pm$ 1.44 *
a/P₀	0.05 $\pm$ 0.01	0.13 $\pm$ 0.03 *	0.05 $\pm$ 0.01	0.06 $\pm$ 0.01

*Significant effect of Ca-level treatment, $p \leq 0.001$

†Significant effect of MI treatment, $p < 0.02$

‡Significant interaction (Ca x MI), $p < 0.05$

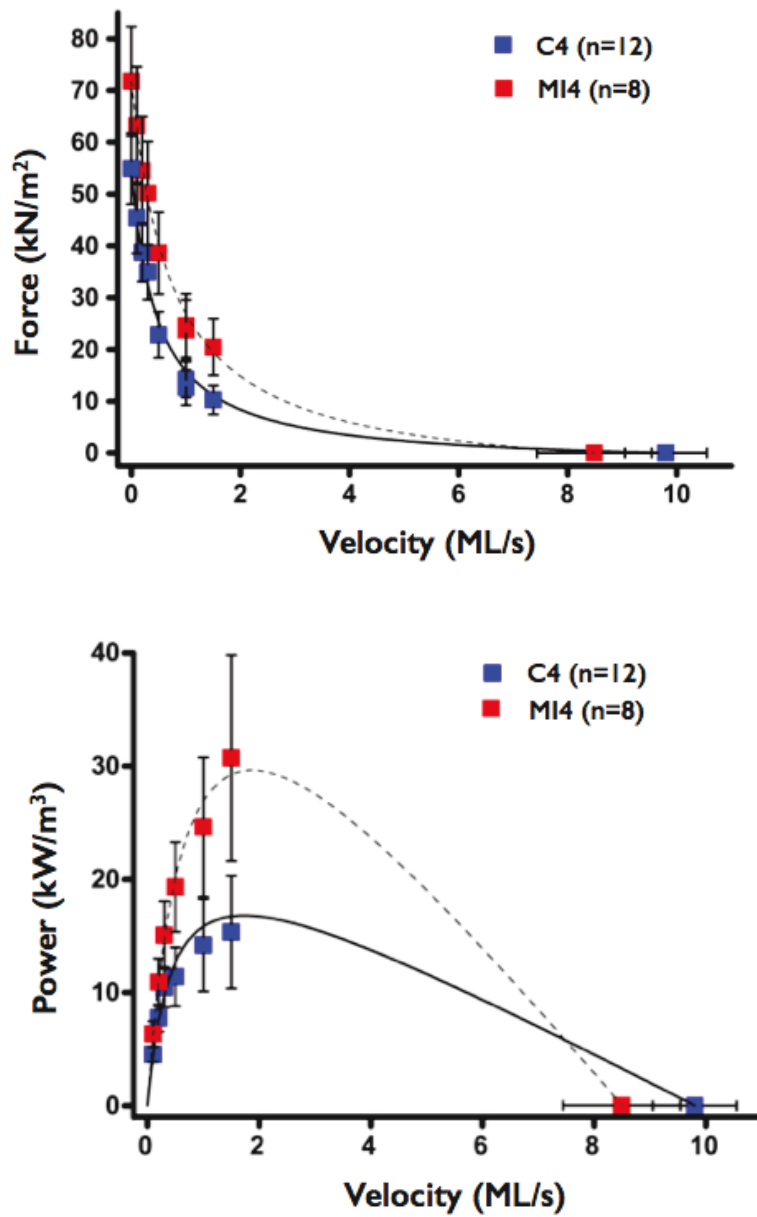


Figure 5.3.1.2: FV & PV four-week 32 μM $[\text{Ca}^{2+}]$. **Top**, Force-velocity relations from MI4 and C4 trabecular preparations in 32 μM free calcium. **Bottom**, Power-velocity relations calculated from the FV relationships of MI4 and C4 cohorts. All data were plotted as mean $\pm$ SEM.

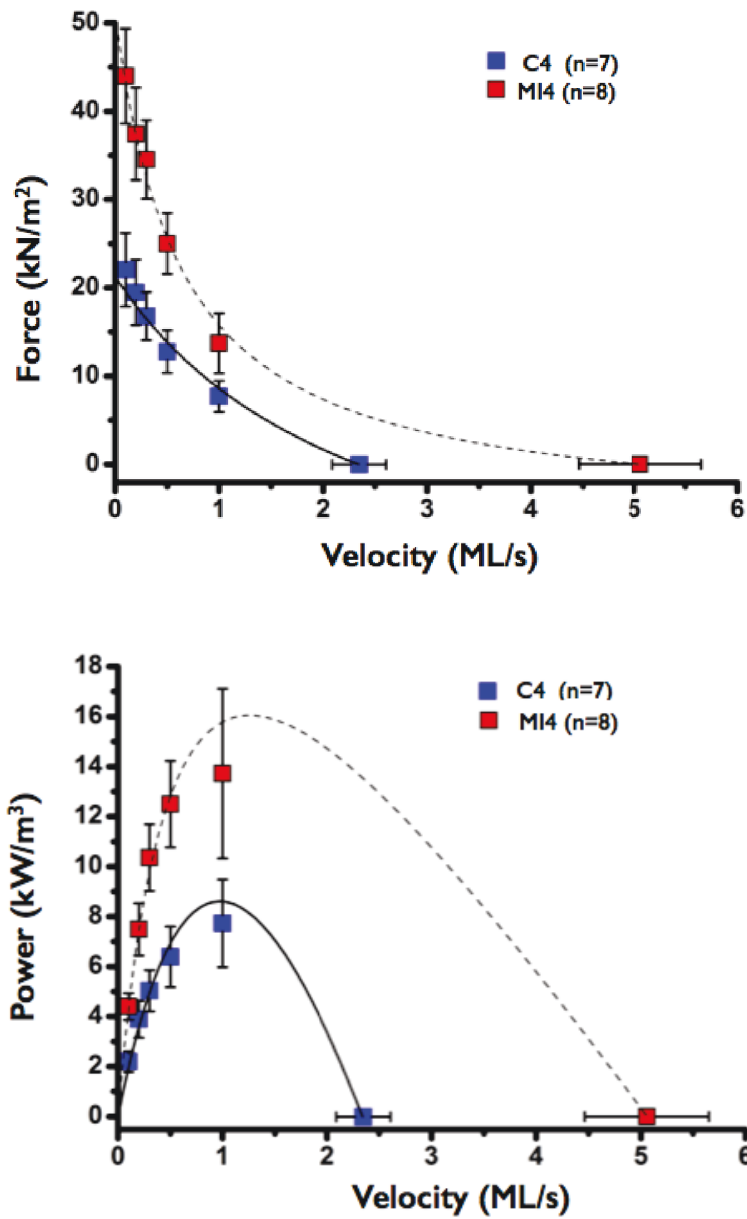


Figure 5.3.2.1: FV & PV four-week 1 μM $[\text{Ca}^{2+}]$. **Top**, Force-velocity relation from MI4 and C4 trabecular preparations in 1 μM free $[\text{Ca}^{2+}]$. **Bottom**, calculated power-velocity relations from MI4 and C4 animals. All data were plotted as mean $\pm$ SEM.

5.3.4 Force-velocity relationships twenty weeks post-CMI in saturating 32 μ M calcium

Having observed increased contractility of the trabeculae from MI4 animals, we wanted to see if this contractile compensation was conserved into the decompensated state (Appendix A). RLC phosphorylation was raised at MI20 in comparison to C20 so we hypothesised that contractility of the muscle would still be raised in compensation to the infarct.

We observed, twenty weeks post-operatively in 32 μ M calcium, infarcted animals (MI20) produced similar F_0 and a/P_0 parameters when compared to un-operated controls (C20) (Table 5.3.4.1 and figure 5.3.4.2). However, MI affected V_{max} , the MI20 cohort produced a significantly reduced V_{max} of 4.8 ± 0.4 ML/s in comparison to C20 of 7.2 ± 0.6 ML/s ($p < 0.02$). V_{PP} was affected by MI as the MI20 cohort displayed a reduced V_{PP} of 0.8 ± 0.1 ML/s versus C20 of 1.2 ± 0.1 ($p < 0.02$). PP was also affected where MI20 animals displayed a reduced peak power of 7.5 ± 1.4 kWm⁻³ compared to C20, PP of 14 ± 6.8 kWm⁻³ ($p < 0.02$). These findings inferred a loss of contractile function during shortening, where the isometric force production was not significantly affected. This meant that the compensation observed in the MI4 cohort was not conserved in later disease.

Interestingly, MyBP-C phosphorylation was also altered at this stage of disease. MI20 shows a MyBP-C phosphorylation level of 3.5 ± 0.4 mol Pi/mol MyBP-C, where C20 has a phosphorylation level of 2.5 ± 0.4 mol Pi/mol MyBP-C ($p < 0.05$). Increased MyBP-C phosphorylation is thought to increase the ability myosin to propel actin, because its binding to actin is reduced facilitating filament sliding (207). These findings all insinuate that contractile gain should still be evident at MI20. However, the reductions in functional contractile output during shortening may not be surprising when viewed in the context of the muscle structure at MI20. At MI20 there is

significant contractile loss and tissue disruption (Appendix B). It is likely that at this stage of disease the overall tissue disruption blunts any sarcomeric compensation due to post-translational modifications.

5.3.5 Force-velocity relationships twenty weeks post-CMI in limiting 1 μ M calcium

Following on from this we wanted to understand whether the effects at maximal calcium activation were conserved at physiological calcium activation. We hypothesised that the post-translational modifications of RLC and MyBP-C may help to compensate for the tissue disruption at physiological calcium (1 μ M)(as observed at MI4), because at physiological calcium there is only partial activation of the myocardium. Partial activation can be fine tuned by changes in myofilament calcium sensitivity, which may be significantly altered by phosphorylation change of MyBP-C and RLC.

Experimentation at sub-maximal physiological 1 μ M free calcium conditions (Figure 5.3.5.1) showed similar F_0 , V_{PP} , and a/P_0 for both MI20 and C20 cohorts (Table 5.3.4.1). Interestingly, V_{max} of MI20 animals was raised at 2.4 ± 0.8 ML/s in comparison to C4 at 2.1 ± 0.24 ML/s ($p < 0.02$). However the MI20 animals displayed lower PP 4.2 ± 0.6 kWm⁻³ compared to C20 of 5.9 ± 0.5 kWm⁻³ ($p < 0.02$).

These results infer that at physiological calcium, the tissue function of the MI20 cohort was not as depressed in comparison to full activation in 32 μ M calcium. This may be, in part, due to the effects of RLC and MyBP-C phosphorylation on muscle mechanics. Theoretically this effect is likely mediated through the roles of these proteins to alter the contractile properties of the myofilament via a calcium sensitivity shift (discussed further in chapter 7). However, these post-translational modifications do not compensate fully for the disease state. This is likely due to the severe tissue disruption in this end stage disease phenotype (Appendix B).

Table 5.3.4.1: Parameters from FV and PV relations of C20 and MI20 cohorts in 1 μ M and 32 μ M free calcium.

	C20 32 μM (n=12)	MI20 32 μM (n=8)	C20 1 μM (n=9)	MI20 1 μM (n=8)
F₀ (kN/m²)	66.3 $\pm$ 5.51	62.1 $\pm$ 9.30	29.5 $\pm$ 2.57*	21.8 $\pm$ 3.47*
V_{max} (ML/s)	7.21 $\pm$ 0.58	4.77 $\pm$ 0.44†‡	2.08 $\pm$ 0.24*	2.39 $\pm$ 0.79*†
V_{PP} (ML/s)	1.17 $\pm$ 0.10	0.79 $\pm$ 0.08‡	0.66 $\pm$ 0.06*	0.69 $\pm$ 0.15*
PP (kWm⁻³)	14.0 $\pm$ 6.84	7.51 $\pm$ 1.44†	5.93 $\pm$ 0.49*	4.21 $\pm$ 0.56*†
a/P₀	0.05 $\pm$ 0.01	0.05 $\pm$ 0.08	0.39 $\pm$ 0.11*	0.32 $\pm$ 0.11*

*significant effect of Ca-level treatment, $p \leq 0.001$

†significant effect of MI treatment, $p < 0.02$

‡significant interaction (Ca x MI), $p < 0.05$

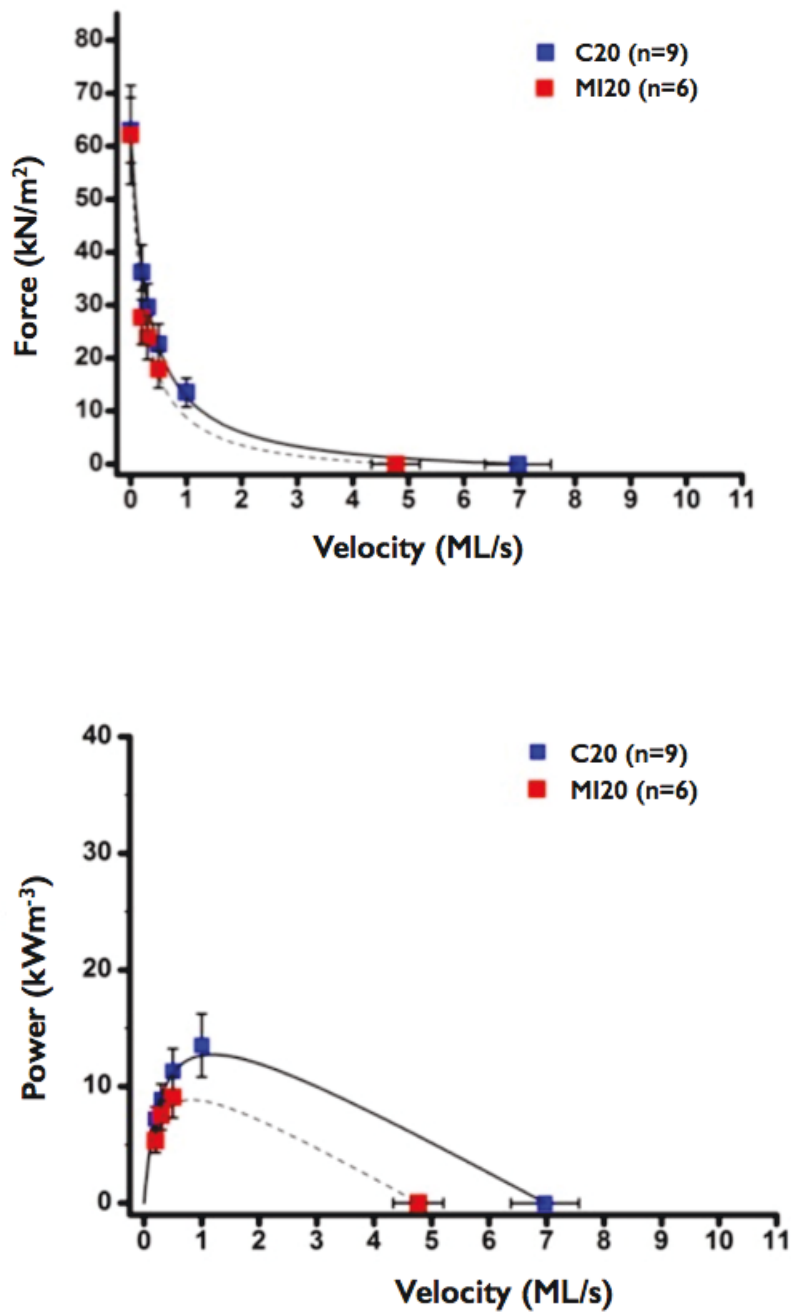


Figure 5.3.4.2: FV & PV twenty-week 32 μ M $[Ca^{2+}]$. **Top**, Force-velocity relation from MI20 and C20 trabecular preparations in 32 μ M free $[Ca^{2+}]$. **Bottom**, Power-velocity relations from the force-velocity measurements from MI20 and C20 animals. All data were plotted as mean $\pm$ SEM.

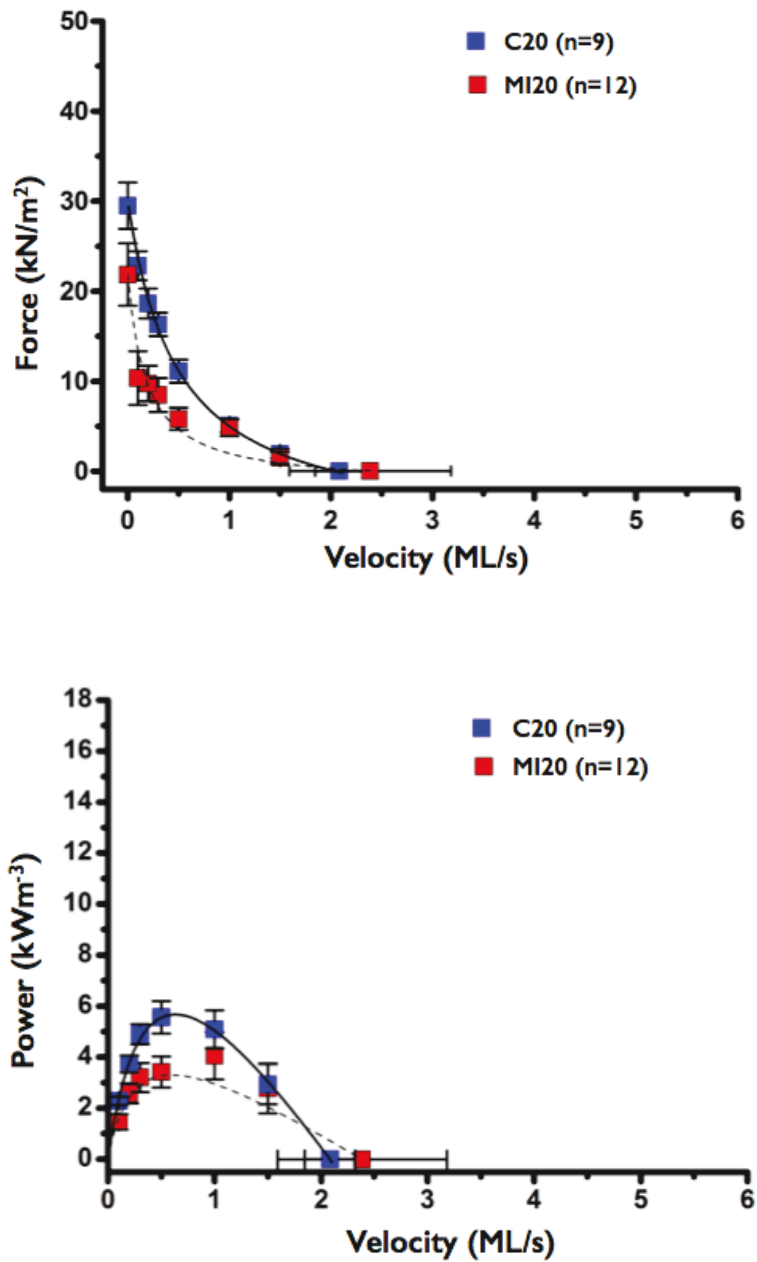


Figure 5.3.5.1: FV & PV twenty-week 1 μM $[\text{Ca}^{2+}]$ **Top**, Force-velocity relation from MI20 and C20 trabecular preparations in 1 μM free $[\text{Ca}^{2+}]$. **Bottom**, Power-velocity relations from the force-velocity measurements from MI20 and C20 animals. All data were plotted as mean $\pm$ SEM.

6 – Ensemble and single molecule experiments

6.1 Alteration of phosphorylation of RLCs on purified full-length pig cardiac myosin

Full-length myosin was purified from pig left ventricle with both light chains bound. After purification the RLC phosphorylation was 0.41 mol Pi/ mol of RLC assessed by quantitative specialised Phos-tag polyacrylamide gels. The phosphorylation abundance of RLC from porcine left ventricles frozen in liquid nitrogen immediately after sacrifice was indistinguishable at 0.42 mol Pi/ mol RLC from purified protein.

RLC bound to full-length porcine myosin was phosphorylated using smooth muscle myosin light chain kinase (MLCK) to efficiently phosphorylate 99.6% of RLCs (Table 6.1.1). A non-specific phosphatase, calf intestinal phosphatase (CIP), was used to fully de-phosphorylate all purified molecules in the samples of molecules treated (Table 6.1.1).

In each experiment in this chapter we assessed the effect of 4 separate phosphorylation levels of RLCs on the assay outcome. These were: i) completely phosphorylation RLCs (100P); ii) completely unphosphorylated RLCs (0P); iii) a 50:50 mixture of myosin with either fully phosphorylated or fully unphosphorylated RLCs (50P); iv) control phosphorylation, which was the phosphorylation of RLCs natively in the myocardium (40P).

It must be noted that the 50P mixture is a mixture of myosin, which have either both RLCs of the myosin molecule, or neither of the RLCs of the myosin molecule phosphorylated. Whereas, the 40P mixture is the population of phosphorylation seen in the myocardium, where, assuming equal probability of phosphorylating any RLC in the whole tissue, we have a population of 16% of the molecules with both RLCs phosphorylated.

	1P	0P	Mol Pi/ Mol RLC	N
RLC phosphorylation in tissue (porcine)	42%	58%	0.42 ± 0.02	3
RLC phosphorylation in purified samples (porcine)	41%	59%	0.41 ± 0.03	3
Enriched RLC phosphorylation with MLCK	99.6%	0.4%	0.99 ± 0.01	2
Reduced phosphorylation with CIP	0%	100%	0	2

Table 6.1.1: Porcine RLC phosphorylation alteration. Values obtained from blots of RLC species using the Phos-tag method. Percentages indicate the amount of RLC as a percentage of total in each lane that is in the phosphorylated (1P) or non-phosphorylated (0P) state. n values correspond to tissues or molecules from separate hearts. Each sample (n number in the table above) was sampled by gel electrophoresis 5 times and averaged.

6.2 The effect of myosin concentration and RLC phosphorylation on actin gliding

The propulsion of actin along a bed of myosin is determined by the swing of the lever arm, and the rate of exit from the load bearing states, which in the case of the actin gliding assay, is ADP release limited (185, 208). As the myosins we test have a fixed lever arm length it is unlikely that this is causing a change in peak actin gliding velocity. Therefore, a change in the rate of ADP release is likely the cause of any change we could observe with this assay. Interestingly, the peak unloaded shortening velocity of muscle is also ADP release limited (186, 209). We know from our muscle studies that peak unloaded shortening velocity can be altered by RLC phosphorylation. Using the actin gliding assay we can test whether this is true for pig myosin. This also allows us to assess if the effect of altering peak unloaded shortening velocity is due to a kinetic change in the ADP release rate of the myosin. We hypothesise that ADP release is altered by the RLC phosphorylation level of the myosin, which is why we observe changes in peak unloaded shortening in muscle.

Assessing the dependence of actin gliding velocity on [myosin] allows us to make sure we reach the [myosin] threshold for peak actin gliding in our varied actin gliding assay protocols. The measurement of the [myosin] necessary for peak gliding gives us a qualitative measure of the duty ratio of the motor, which allows us to measure the effect of RLC phosphorylation on the duty ratio of the myosin (191, 210). This principle relies on the knowledge that for an actin filament to attain peak gliding velocity, at least one myosin molecule must be interacting with it at any given moment (211, 212).

Actin gliding velocity was measured as a function of myosin concentration (μM) and the phosphorylation status of the bound RLCs at 30°C in 60 mM ionic strength buffer (Table 6.2.1). All myosin was laid down as two headed single molecules in high salt

(600 mM). Figure 6.2.2 shows both the effect of altering myosin concentration and RLC phosphorylation level on the sliding velocity of un-regulated actin along the myosin coated surface.

Data from chambers with less than 0.1 μM myosin added were fit with a simple linear regression, experiments where surface myosin was above 0.1 μM were pooled and averaged and the average velocity was plotted for the points above 0.1 μM (green horizontal line). Figure 6.2.2 shows that above approximately 0.1 μM myosin concentration sliding velocity is at its peak value. Therefore, for all experiments assessing maximal sliding velocity, 0.2 μM myosin was used to be sure the critical myosin concentration for maximal sliding velocity was present.

Notably, 0P and 40P RLC myosin species exhibited indistinguishable maximal sliding velocities above the threshold of 0.1 μM myosin at $0.78 \pm 0.02 \mu\text{m/s}$ and $0.82 \pm 0.03 \mu\text{m/s}$. 100P myosins exhibited raised maximal sliding velocities of $1.26 \pm 0.02 \mu\text{m/s}$ above 0.1 μM myosin. This value was significantly raised from 0P and 40P ($p < 0.0001$) by Bonferroni adjusted T-test. P50 myosin had a maximal sliding velocity of $0.98 \pm 0.02 \mu\text{m/s}$. This was distinct from 0P and 40P myosins $p < 0.001$, and also from 100P myosins $p < 0.0001$.

The actin gliding velocities observed in our 40P myosin populations are in the range of expected velocities for beta cardiac myosins, 0.5-1.5 $\mu\text{m/s}$, depending on assay conditions (213, 214). In summation, RLC phosphorylation level altered the ability of myosin molecules to translocate actin. This effect is likely created by a change in the ADP release rate, where increasing RLC phosphorylation accelerates ADP release. Interestingly, this effect seems to be dependent on both RLCs of a given myosin molecule being phosphorylated, proven by the similarity between 0P and 40P velocities, whilst 50P actin gliding velocity is the mean of 0P and 100P.

		100P	50P	40P	0P
Velocity	($\mu\text{m/s}$)	$1.26 \pm 0.02^{\S\P}$	$0.98 \pm 0.02^{\S}$	$0.82 \pm 0.03^{\P}$	$0.78 \pm 0.02^{\P}$
[Myosin] > 0.1 μM					

§ Signifies different from control $p < 0.0001$

¶ Signifies different from 50/50 $p < 0.0001$

Table 6.2.1: Actin-gliding velocities above 0.1 μM myosin concentration at different RLC phosphorylation abundances. All data are stated as mean $\pm$ SEM where each mean is $N > 800$ filaments.

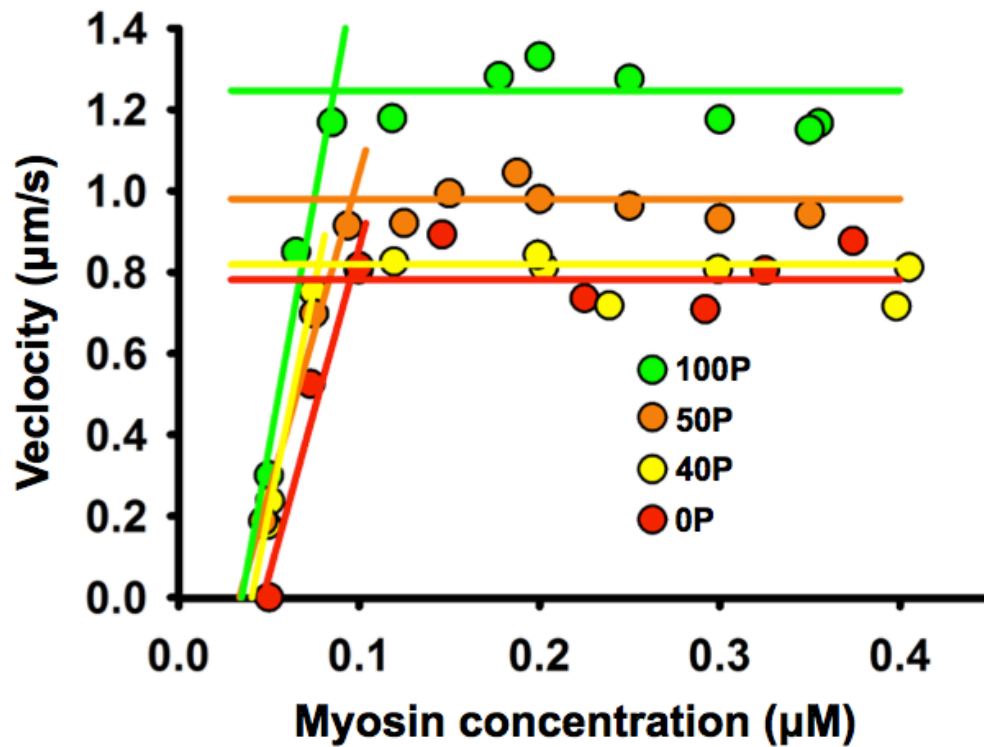


Figure 6.2.2: Actin-gliding velocity versus [myosin] Actin gliding assay velocities ($\mu\text{m/s}$) plot as a function of myosin concentration (μM) and RLC phosphorylation states as a percentage at 30°C in 60 mM ionic strength buffer. Each data point is the average of $N > 200$ filaments that were tracked and averaged. Data below 0.1 μM myosin were fit by simple linear regression. Fit of data above 0.1 μM myosin is the average of all velocities above 0.1 μM myosin. Error bars were omitted, as they were smaller than data symbols. Experimental scatter is due to variance of actin velocities between separate experimental chambers.

6.3 The effect of [ATP] and RLC phosphorylation abundance on actin gliding velocity

Measuring the ability of myosin to translocate actin as a function of [ATP] allows the use of a fitting procedure, which provides us two different parameters. It allows us a second method of assessing changes in actin gliding velocity created by changes in ADP release rate at high [ATP]. At submaximal [ATP] the rate of actin gliding is determined by the rate of ATP binding to the molecule. This allows us to determine the qualitative effect of RLC phosphorylation on ATP binding kinetics. Therefore, we can assess whether the fitting procedure predicts the same peak actin gliding velocities we measured in 6.2; and whether ATP binding kinetics is altered by RLC phosphorylation.

The velocity at which actin was translocated by myosin was measured as a function of both [MgATP] and RLC phosphorylation abundance at 30°C in 60 mM ionic strength buffer (Table 6.3.1 and Figure 6.3.2).

Relations were plot and fit using an unconstrained two parameter rectangular hyperbola. From these fitting procedures values for [MgATP] at which half maximal velocity is observed (K_{atp}) and maximal sliding velocity in $\mu\text{m/s}$ (V_{max}) were extracted.

Both 40P and 0P species had similar predicted V_{max} values of 0.90 $\mu\text{m/s}$ and 0.86 $\mu\text{m/s}$ respectively. Whereas 100P had a predicted V_{max} of 1.26 $\mu\text{m/s}$. 50P myosin populations had an intermediate predicted V_{max} of 1.05 $\mu\text{m/s}$.

K_{atp} of 40P and 0P myosin populations showed indistinguishable values of 0.10 mM. 100P myosins had a K_{atp} of 0.06 mM and 50P myosins had an intermediate K_{atp} of 0.08 mM. Statistical testing indicated no significant differences between the K_{atp} of any of the phosphorylation states. It is likely that the differences were too marginal to assess using the actin gliding assay.

The $V_{\max}$ of sliding velocities predicted by the fit of the gliding data were indistinguishable from the peak sliding velocities from the [myosin] gliding assay above 0.1 μM myosin for each RLC phosphorylation abundance group. However, we did not have enough statistical power to assess if ATP binding kinetics are altered by RLC phosphorylation.

	100P	50P	40P	0P
K_{atp}	0.06 ± 0.01	0.10 ± 0.02	0.10 ± 0.02	0.08 ± 0.01
V_{max} (μm/s)	1.26 ± 0.06 [§]	1.05 ± 0.05	0.90 ± 0.04 [§]	0.86 ± 0.03 [§]

§ Different from value of 50/50 where p < 0.01

Table 6.3.1: Values for actin-gliding velocity versus [MgATP]. Values for the [MgATP] at half maximal velocity (K_{atp}) and maximal sliding velocity predicted by the hyperbolic fit (V_{max}). All data are stated as mean ± SEM.

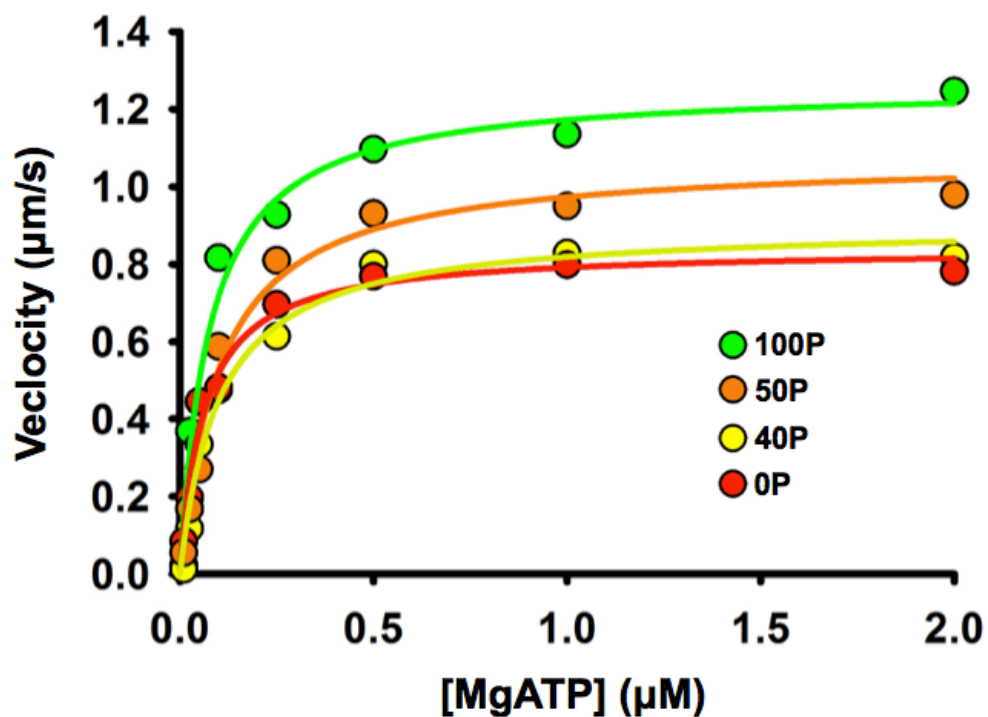


Figure 6.3.2: Actin-gliding velocity versus [MgATP]. Actin gliding assay velocities ($\mu\text{m/s}$), plotted as a function of MgATP concentration (μM) and RLC phosphorylation level. All measurements were made at 30°C in 60 mM ionic strength buffer. Each point is the average of $N > 400$ (from three separate experimental chambers) filaments that have been tracked and averaged. Error bars are omitted, as they are smaller than the data points displayed. All data were fitted with a two parameter rectangular hyperbola that has been left unconstrained.

6.4 The effect of temperature and RLC phosphorylation abundance on actin gliding velocity

Most actin gliding assays were performed at 30°C as the assay is most reproducible at this temperature, and higher temperatures are less easy to maintain accurately. Faster gliding created by higher temperatures shears actin faster, leading to a heterogeneous population of actin filament lengths. This occurs very soon after the assay is initiated, and is a problem because gliding velocity is altered by actin filament length. However, we wanted to understand if the effects of RLC phosphorylation on actin gliding velocity were conserved at physiological temperatures.

We measured the effect of temperature and RLC phosphorylation level on sliding velocities of cardiac myosins. This was done over a range of temperatures from 17-37°C in 60 mM ionic strength buffer corrected to be at pH 7.1 for every experimental temperature (Table 6.4.1).

Plots were not fitted, however lines were added between points to add clarity to the relationship between temperature and actin gliding velocity (Figure 6.4.2). Figure 6.4.2 emphasises the change in velocity of actin gliding as a function of temperature. Temperature had a modest effect on actin gliding velocity independent of phosphorylation abundance. Comparing velocity of motility at 30°C with velocities at 37°C showed that a change in temperature (7°C) caused a 3-fold increase in velocity, independent of phosphorylation abundance of the RLCs. At 37°C, 100P showed actin gliding velocities of $3.6 \pm 0.02 \mu\text{m/s}$ in comparison to 0P and 40P, which showed gliding velocities of $2.7 \pm 0.03 \mu\text{m/s}$ and $2.6 \pm 0.03 \mu\text{m/s}$ respectively. 50P myosins had an intermediate actin gliding velocity at 37°C of $3.1 \pm 0.06 \mu\text{m/s}$. Therefore, increases in actin sliding velocity observed at 30°C as a function of [myosin] (Figure 6.2.2) and [ATP] (Figure 6.3.2) are upheld at physiological

temperatures (Figure 6.4.2 top). When plotting the normalised velocity (for each RLC phosphorylation abundance) against temperature (Figure 6.4.2 bottom) we observed that the level of RLC phosphorylation had no effect on the magnitude of gliding velocity response to temperature. This is evident as all phosphorylation abundances overlap when normalising to peak velocity at 37°C and comparing within temperature groups. This infers that RLC phosphorylation does not alter cardiac myosins' response to temperature change.

Velocity ($\mu\text{m/s}$) $\pm$ SEM						
	17°C	20°C	25°C	30°C	35°C	37°C
100P	0.07 $\pm$ 0.002	0.14 $\pm$ 0.003	0.45 $\pm$ 0.005	1.25 $\pm$ 0.02	2.17 $\pm$ 0.02	3.59 $\pm$ 0.02
50P	0.06 $\pm$ 0.002	0.10 $\pm$ 0.003	0.32 $\pm$ 0.001	0.98 $\pm$ 0.02	2.03 $\pm$ 0.02	3.09 $\pm$ 0.06
40P	0.04 $\pm$ 0.002	0.09 $\pm$ 0.002	0.30 $\pm$ 0.006	0.82 $\pm$ 0.02	1.64 $\pm$ 0.01	2.59 $\pm$ 0.03
0P	0.04 $\pm$ 0.002	0.07 $\pm$ 0.002	0.26 $\pm$ 0.09	0.78 $\pm$ 0.03	1.56 $\pm$ 0.03	2.68 $\pm$ 0.03

Table 6.4.1: Values of actin-gliding velocity versus temperature. Sliding velocities of actin over differently phosphorylated myosin RLC species at a range of temperatures in 2 mM ATP and an ionic strength of 60 mM.

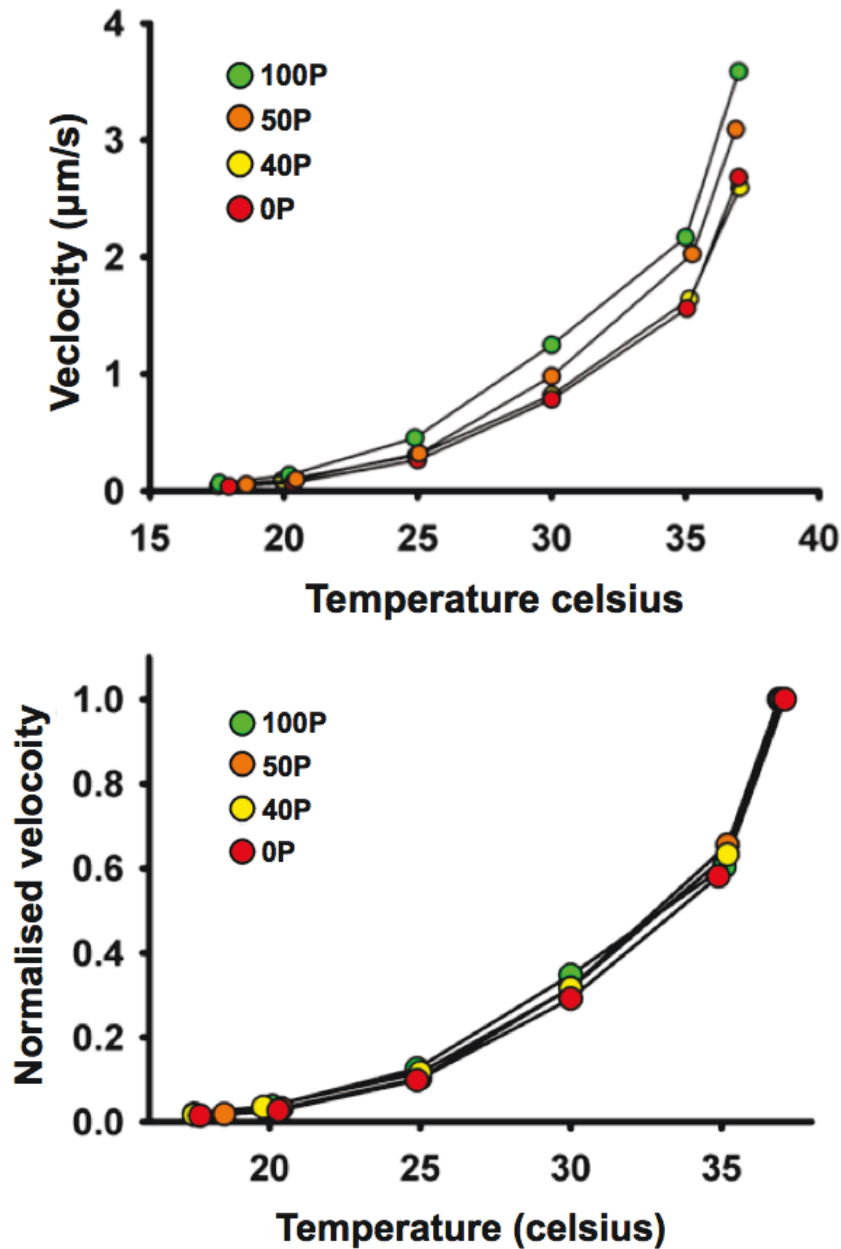


Figure 6.4.2: Actin gliding velocity versus temperature. Actin gliding velocity as a function of assay temperature and RLC phosphorylation. **Top**, Velocity of actin filaments ($\mu\text{m/s}$) as a function of temperature. **Bottom**, Same data plot as normalised actin sliding velocity (normalised against peak velocity at 37°C). All data points consist of $N > 200$ tracked actin filaments. Error bars are not displayed, as they are smaller than the data points in both instances.

6.5 Arrhenius plots of actin gliding data

It has been established with skeletal muscle myosins that there are changes in the mechanical and biochemical properties of these preparations between different temperature ranges 10-25°C and 25-35°C (215, 216). Lower temperatures display greater temperature sensitivities than higher temperatures; we wanted to see if this was true for cardiac myosin too. Changes in these temperature sensitivities manifest as changes of the Q_{10} of the myosin. The Q_{10} is a temperature coefficient that describes the temperature dependence of a given parameter, which in our case is actin gliding velocity. By plotting the effect of temperature on myosin gliding velocity as Arrhenious plots we can use linear regression to measure Q_{10} and the kinetic energy needed to start the reaction (also known as the activation energy, E_a). This allows us to assess if RLC phosphorylation level alters this relation.

Therefore, actin gliding velocities were expressed as the logarithm of velocity (Log V) versus the reciprocal of the temperature (in degrees Kelvin) multiplied by 1000 ($(1/K)*1000$). These plots were fitted with two separate unconstrained linear regressions including three temperature points each (Table 6.5.1). These two separate regressions encompassed three lower experimental temperatures between 17-27°C and the upper temperatures of 27-37°C. The intercepts of the linear regressions were used to estimate the break point signifying the point at which the activation energies of the reaction began to differ. All four phosphorylation abundances were plotted (Figure 6.5.2) and fit showing the two regressions converging on average at 27°C. All Arrhenius plots are combined showing the different RLC phosphorylation abundances (Figure 6.5.3).

The activation energies of the reactions at low (17-27°C) and high temperature (27-37°C) were calculated using the following equation:

$$E_a = \text{slope} \times 2.303 \times R$$

Where E_a is the activation energy in kilo Joules per mol (kJmol^{-1}), slope is the slope of the fit from the Arrhenius plot and R is the gas constant, which has a value of 8.314 Joules per Kelvin per mol ($\text{JK}^{-1}\text{mol}^{-1}$).

Activation energies for the reactions in low temperature and high temperature conditions were not affected by phosphorylation abundance of the regulatory light chain (Table 6.5.1). However, comparing E_a between temperatures within phosphorylation treatment groups showed significant differences (Table 6.5.1). Low temperature activation energies averaged at 192 kJmol^{-1} whereas high temperature activation energies averaged at 122 kJmol^{-1} .

The temperature coefficient for the low and high temperature groups were calculated using the following equation, previously described by Botinelli et.al. (217):

$$Q_{10} = (E_a/R \times 2.303 \times 1000 \times ((1/T-1)-(1/T+10)))$$

The Q_{10} of the sliding velocities was not affected by RLC phosphorylation abundance but was significantly altered between low and high temperatures (Table 6.5.1).

Normalising all velocities to maximal gliding velocity of actin over 40P myosin at each individual assay temperature gave further information about the effect of RLC phosphorylation on actin gliding velocity (Figure 6.5.4). As temperature increased towards physiological 37°C the similarity of velocities in all treatment groups converged. At $30\text{--}35^\circ\text{C}$ the velocities of 0P and 40P myosins had indeed overlapped. This suggests that the differences in velocity observed between 0P myosin and 40P myosin is not upheld at more physiological temperatures.

Another feature of this fitting indicated that when the normalised values for each RLC phosphorylation level was fitted with an unconstrained linear regression, the 0P and 100P treatment groups had opposing relationships. The 100P myosins had a negative slope towards higher temperatures. The opposite was true for the 0P

myosins, which had a positive slope. Interestingly, if the 100P and 0P values at each temperature were averaged together, the values became identical to the velocities from the 50P myosins, signified by the brown fit and points in figure 6.5.4, which were indistinguishable from the orange plots of the 50P myosins.

The data in figure 6.5.4 adds further supportive evidence for a hypothesis, which insinuates, in the actin gliding assay, effects of RLC phosphorylation level are contingent on both RLCs of the myosin molecule possessing RLC phosphorylation. If this phenomenon holds true in the sarcomere of the cardiac myocardium, then populations of myosin with one RLC phosphorylated could be modulated by the addition of P_i to the other head of the myosin molecule dimer to alter the mechanical capabilities of the dimer. It could be that there are effects of single headed RLC phosphorylation but they are below the sensitivity of the assays that we have employed.

		17-27 °C		27-37°C	
	Convergence	E _a	Q ₁₀	E _a	Q ₁₀
	Temperature (°C)				
100P	26.9	189 ± 0.9 [§]	13.4 [¶]	112 ± 25	4.2
50P	27.7	193 ± 7.7 [§]	13.9 [¶]	124 ± 18	6.9
40P	26.4	188 ± 12 [§]	13.2 [¶]	123 ± 18	4.9
0P	27.3	199 ± 3.6 [§]	15.4 [¶]	131 ± 25	7.7

Table 6.5.1: Parameters calculated from Arrhenius plots of actin gliding assay data. All data is expressed as the mean ± SEM. E_a = activation energy and Q₁₀ = the temperature coefficient for the rate of change of the reaction with a 10°C temperature change.

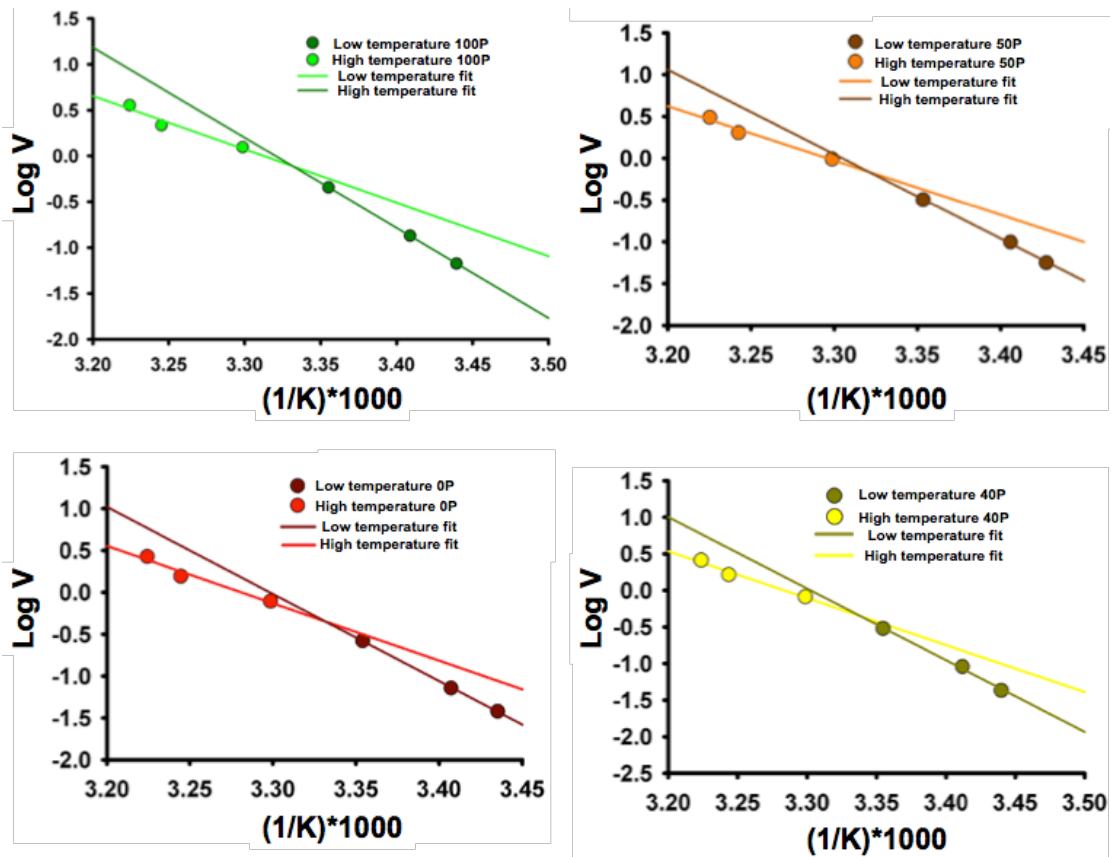


Figure 6.5.2: Arrhenius plots for different phosphorylation levels. Each point is $N > 200$ and no error bars are plotted as they are insignificant in size compared to the data points. Fits were left unconstrained and made between three points at low temperature (17-27°C) and high temperature (27-37°C).

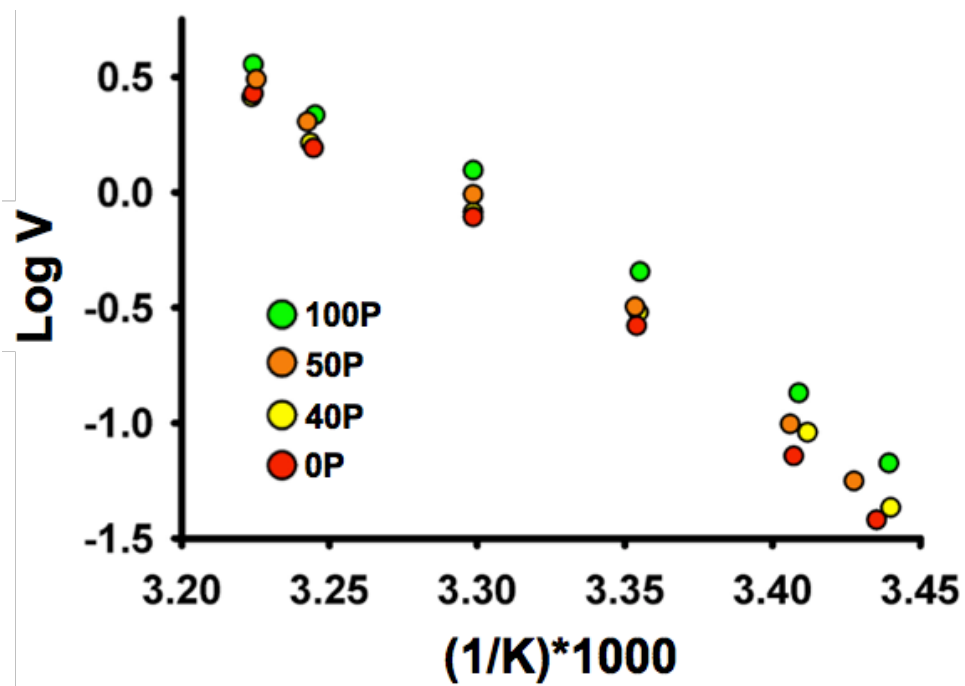


Figure 6.5.3 Combined Arrhenius plots of gliding velocity as a function of temperature for all phosphorylation levels. Regressions have been omitted to visualize individual data points. All data points are means of $N > 200$ filaments each.

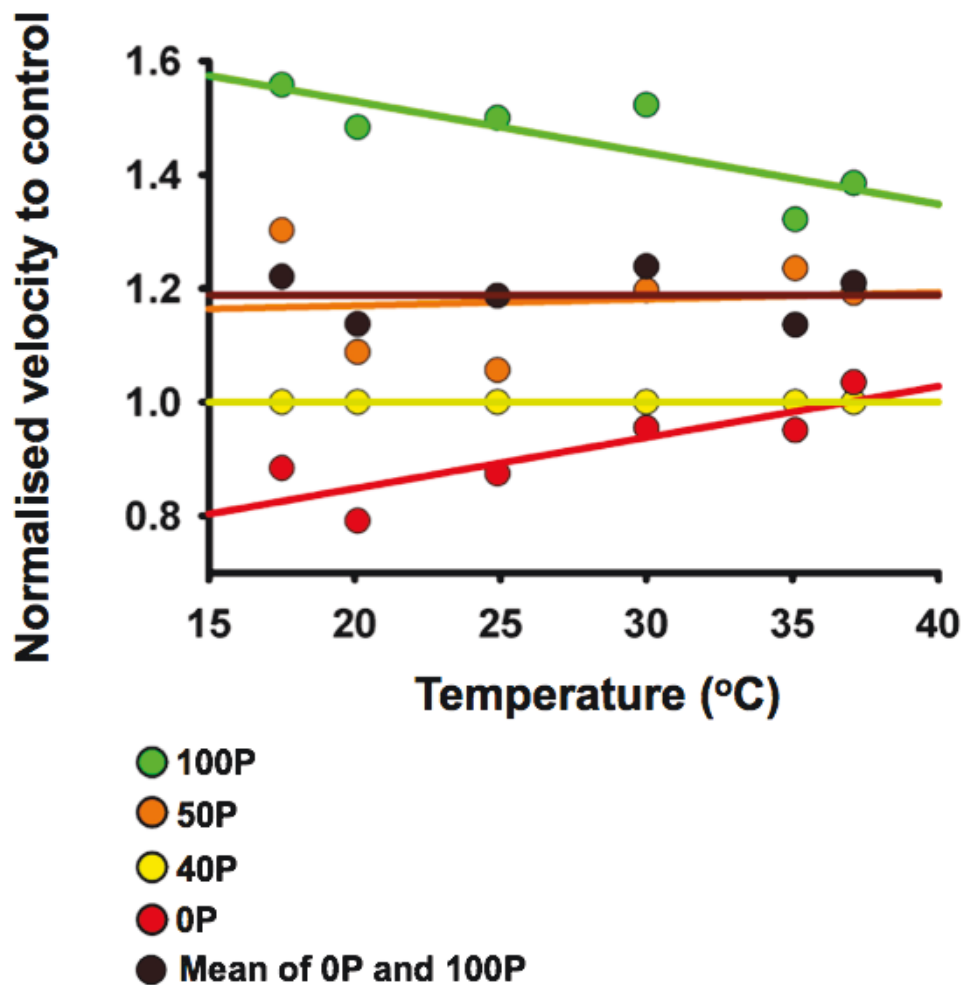


Figure 6.5.4: Normalised actin gliding velocity versus temperature. Normalised velocity to 40P myosin velocity as a function of temperature. Calculated mean of 0P and 100P is displayed in brown where the values for 0P and 100P were averaged with each other to produce the calculated mean of the two independent populations together. All fits are simple unconstrained linear regressions. $N > 200$ filaments for each data point. Error bars are omitted, as they are smaller than individual data points.

6.6 The effect of applied load and RLC phosphorylation abundance on actin gliding velocity

Measuring actin gliding velocities under load allows for the qualitative assessment of a myosin population's ability to produce force to counteract viscoelastic drag. The viscoelastic drag is created by transient binding of α -actinin (bound to the surface of the assay chamber) to the actin filaments gliding over myosin, impeding actins motion. The use of α -actinin to study the loaded mechanochemistry of myosins has been thoroughly reviewed (192).

Actin gliding velocities were measured as a function of RLC phosphorylation and α -actinin concentration. All assays were performed in 2 mM ATP at 30°C in a 60 mM ionic strength assay buffer at pH 7.1.

The relations produced by altering [α -actinin] showed a sigmoidal relation with lower levels of RLC phosphorylation abundance (Figure 6.6.1). As the phosphorylation abundance of the RLCs became greater the sigmoidal relation became flatter and eventually could not be fit with a sigmoidal relationship and was instead best described by linear regression. This phenomenon inferred that lower RLC phosphorylation levels had a load sensitive region where the actin gliding velocities are more sensitive to the incremental applied viscoelastic load of the α -actinin.

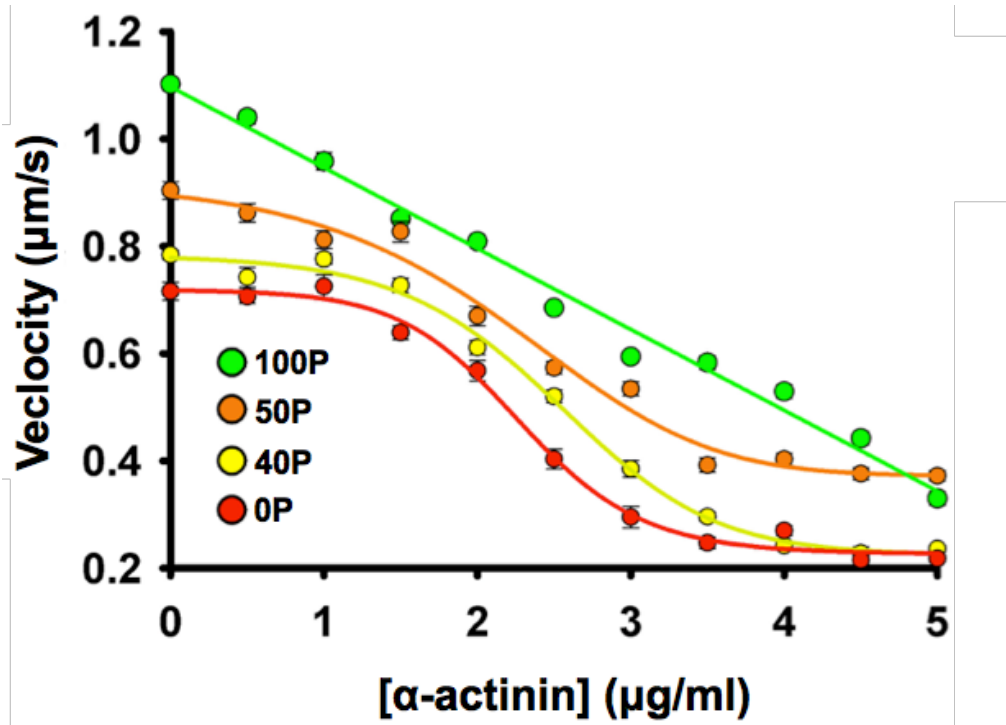


Figure 6.6.1: Effect of α -actinin concentration on actin gliding velocity of full-length pig cardiac myosin with differently phosphorylated RLC species. Fits are arbitrary sigmoidal relations to demonstrate the effect of α -actinin on actin gliding velocity. All plotted points are $N > 200$ with mean $\pm$ SEM displayed.

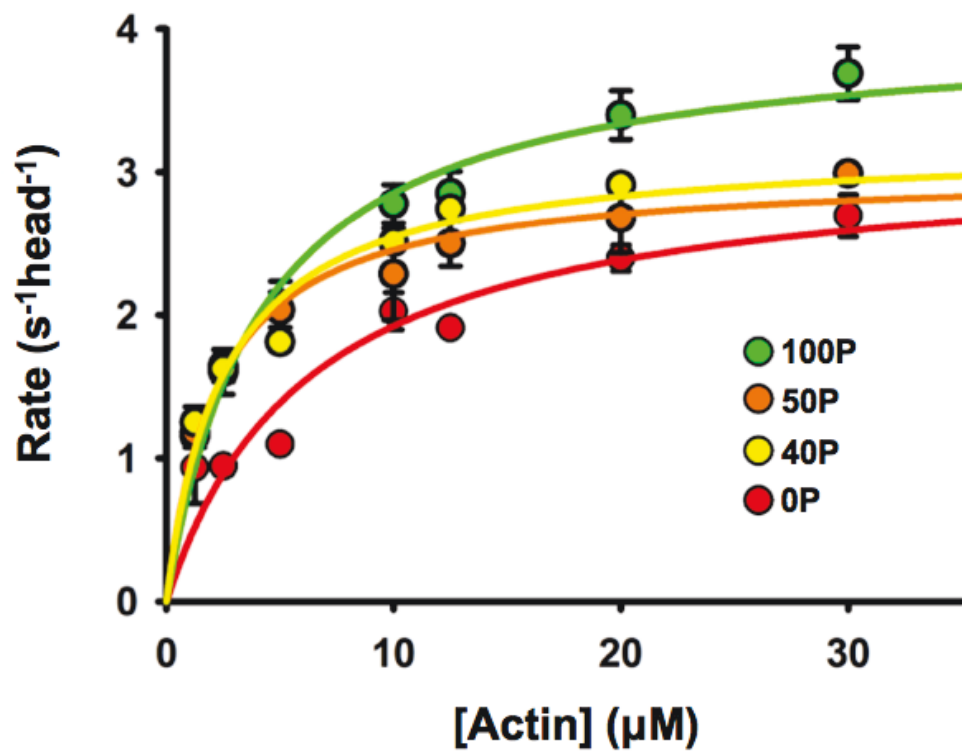
6.7 Gelsolin augmented ATPase measurements of porcine full-length cardiac myosin with altered RLC phosphorylation abundances

Making measurements of actin activated ATPase rates allows one to assess if RLC phosphorylation level change alters myosin's ability to utilise ATP in the presence of its substrate actin. ATPase measurements made over a range of actin concentrations allows us to qualitatively assess changes to the rate of Pi release in limiting [actin], and the nucleotide (ATP) hydrolysis rate at saturating [actin] (218). These distinctions are assessed classically as the two parameters of the fitting regime for the ATPase data. These parameters are termed: i) maximal ATPase rate ($V_{\max}$) assessing changes in the rate of ATP hydrolysis; ii) the rate of ATP hydrolysis at half maximal [actin] termed (K_{atpase}), which provides a qualitative assessment of Pi release (218). We proposed that RLC phosphorylation would influence the maximal ATPase rate of cardiac myosin; but only when both heads of the myosin dimer were phosphorylated in keeping with our measurements using the actin gliding assay.

Actin activated ATPase rates at 2 mM MgATP were measured by spectrophotometry using the NADH linked ATPase assay as described in chapter 3. The rate of ATP hydrolysis was calculated from the slope of the absorbance change and is stated as a rate per second per myosin head. The ATPase rate is assessed as a function of RLC phosphorylation abundance in the samples defined by Phos-tag gel electrophoresis. ATPase assays were performed at 25°C in a 10 mM ionic strength final buffer. Actin was capped with gelsolin at a stoichiometry of one gelsolin molecule per 200 actin monomers. Gelsolin was used to shorten F-actin to prevent large protein aggregates from forming during the assay. Actin concentrations were varied between 1 and 30 μM actin. Absorbance data was recorded over 10 minutes and sampled once every 10 seconds. The data were plotted as rate of ATP

hydrolysis as a function of actin concentration. All data were fit with Michaelis-Menten kinetic equation that predicted a maximal ATPase rate ($V_{\max}$ in $\text{s}^{-1}\text{head}^{-1}$) and actin concentration at which the rate of hydrolysis was half maximal (K_{atpase} in μM). This was performed for four different RLC phosphorylation abundances and stoichiometries (Figure 6.7.1). 100P RLC associated myosins had a $V_{\max}$ of 3.4 ± 0.2 and a K_{atpase} of 4.8 ± 0.9 . All other treatment groups of 0P, 50P and 40P had almost identical $V_{\max}$ parameters of 2.5 ± 0.1 , 2.6 ± 0.1 and 2.7 ± 0.2 . The $V_{\max}$ of 100P myosin samples was significantly different from all other groups where $p < 0.05$ (all ATPase data was assessed by one-way anova and post-hoc Bonferroni adjusted t-test). The K_{atpase} measurements from the Michaelis-Menten fits were 2.4 ± 0.3 , 2.8 ± 0.5 and 3.3 ± 0.9 for 0P, 50P and 40P treated samples respectively. Statistical significance was established between the K_{atpase} of 100P and 0P treated myosins where $p < 0.05$. There were no other statistically significant findings between treatment groups.

These findings insinuate that the hydrolysis step of the myosin ATPase cycle is accelerated by RLC phosphorylation, proven by the observable changes in $V_{\max}$ of the ATPase rate. Interestingly, there is evidence to support the finding that the Pi release rate is also altered by RLC phosphorylation. Evidenced by the differences observed in the K_{atpase} measurement between 0P and 100P myosins. It seems that the assay was not sensitive enough to detect a difference between 50P and 100P or 0P when assessing $V_{\max}$ and K_{atpase} . Therefore, we have no evidence to support the assumption that this is a phenomenon dependent on both heads of the myosin possessing RLCs that are phosphorylated. Further experiments need to be performed to test this hypothesis fully.



	$V_{\max}$ ($\text{s}^{-1}\text{head}^{-1}$)	K_{atp} (μM)
100P	4.0 ± 0.2	4.1 ± 0.7
50P	3.0 ± 0.1	2.3 ± 0.3
40P	3.2 ± 0.2	2.5 ± 0.6
0P	3.1 ± 0.4	6.3 ± 0.2

Figure 6.7.1: Effect of RLC phosphorylation on actin activated ATPase rate of full-length cardiac myosin at 25°C. Below: Table indicating the maximal rate of ATP hydrolysis from the Michaelis-Menten fits ($V_{\max}$) and the actin concentration at which the ATP hydrolysis rate is half of $V_{\max}$ (K_{atp}).

6.8 [ATP] and RLC phosphorylation abundance dependence of the myosin detachment rate with full-length cardiac myosin from pig

We have observed changes in the ability of muscle to produce power and shorten under varying loads, mediated by RLC phosphorylation level alteration. The parametric changes to muscle mechanics could be the result of changes to duration of actomyosin attachment, power stroke displacement, alteration of the strongly bound state duration, and the ability of molecules to produce force (reviewed (219)). The way one can assess many attributes of myosin motors is by watching the way they function in isolation. For this reason we use the three bead optical trap, we probe the function of individual myosin molecules and the effect that RLC phosphorylation has on their function. We propose that in order to observe changes in power production and unloaded shortening velocity in myocardium, we will observe changes in the way the single myosin molecules operate. Therefore, we hypothesise that the single myosin molecules biochemical and mechanical characteristics are altered by RLC phosphorylation to mediate this change. Therefore, we measured the attachment durations of single binding events of myosins to actin. This was done to determine the lifetime of the strongly bound actomyosin states (t_s). Binding durations were measured over a range of MgATP concentrations (1, 10, and 50 μ M) to determine the effect of RLC phosphorylation and MgATP concentration on attachment duration. Binding duration was measured from the beginning to the end of an actomyosin binding event. Binding events were signified by a reduction in signal variance in the sensor bead. The return of high variance sensor bead signal signified actomyosin detachment. Each attachment duration plot was fitted with a two parameter exponential decay to assess rate of detachment of myosin from actin determined by [MgATP] and RLC phosphorylation abundance. Each attachment duration plot was binned to maximise the R^2 of the hyperbolic fits at each [ATP]. As this assay deals with full length myosin molecules (two heads and therefore two

RLCs), the groups consist of either fully phosphorylated RLCs (100P) or completely de-phosphorylated RLCs (0P). As we are interrogating the interactions of single myosins with actin, we do not use 50P or 40P populations, because we will end up with myosins with unknown RLC phosphorylation level and stoichiometry. In the future, it would be fruitful to engineer single headed cardiac myosin molecules to interrogate the effect of double versus single headed phosphorylated myosins.

At an ATP concentration of 1 μM , 0P myosin had a detachment rate of $13 \pm 1 \text{ s}^{-1}$ and 100P myosin had a detachment rate of $11 \pm 1 \text{ s}^{-1}$ (Figure 6.8.1). At an ATP concentration of 10 μM , 0P myosin had a detachment rate of $46 \pm 2 \text{ s}^{-1}$ and 100P myosin had a detachment rate of $67 \pm 3 \text{ s}^{-1}$ (Figure 6.8.2). At an ATP concentration of 50 μM , 0P myosin had a detachment rate of $51 \pm 4 \text{ s}^{-1}$ and 100P myosin had a detachment rate of $93 \pm 10 \text{ s}^{-1}$ (Figure 6.8.3). These detachment rates were plotted for each treatment group of RLC phosphorylation as a function of [ATP] (Figure 6.8.4). The data points were fitted with the Michaelis-Menten (MM) equation, which gave values for V_{max} of detachment rate (dV_{max}) and K_{atp} of detachment rate (dK_{atp}). 0P myosin had a dV_{max} predicted by the MM fit of $56 \pm 3 \text{ s}^{-1}$ and 100P myosin had a dV_{max} of $105 \pm 5 \text{ s}^{-1}$. 0P myosin had a dK_{atp} of 3 ± 0.7 and 100P myosin had a dK_{atp} of 6 ± 1 .

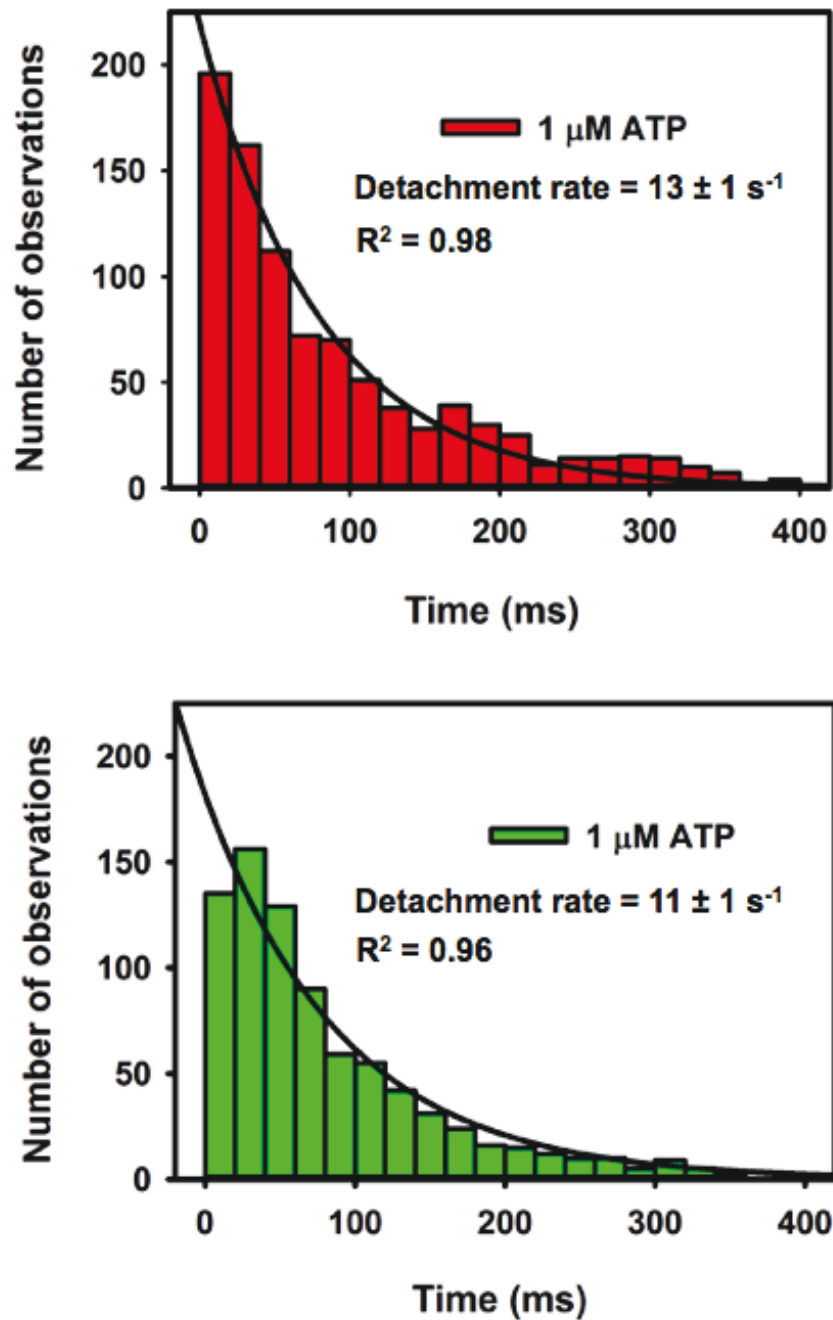


Figure 6.8.1: Attachment duration plots at 1 μM ATP. Top, Attachment duration plot for 0P myosin with 1 μM ATP at 22°C. **Bottom,** Attachment duration plot for 100P myosin with 1 μM ATP at 22°C. All data were fitted with a two parameter exponential decay. Numeric data is displayed as mean $\pm$ SEM. $n = 5$ myosin molecules for each plot.

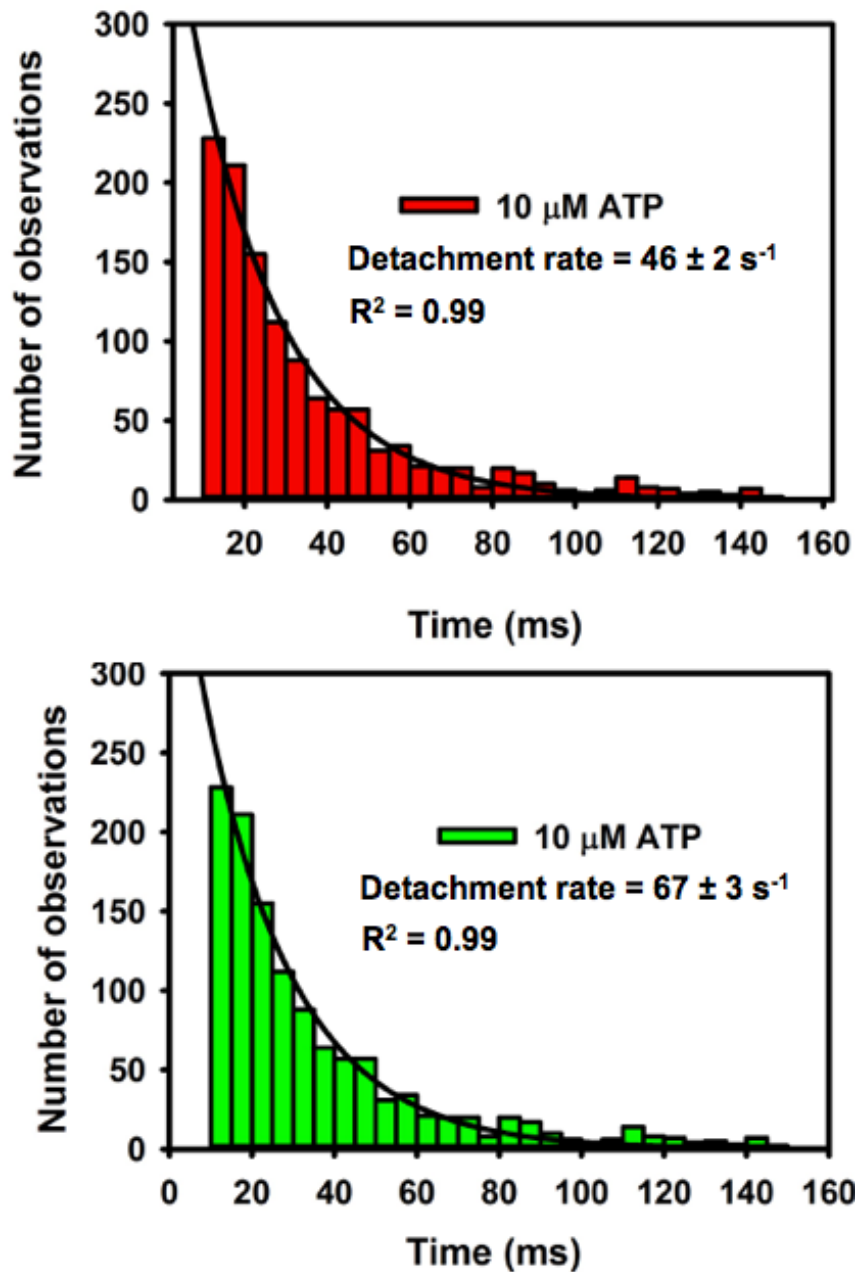


Figure 6.8.2: Attachment duration plots at 10 μM ATP **Top**, Attachment duration plot for 0P myosin with 10 μM ATP at 22°C. **Bottom**, Attachment duration plot for 100P myosin with 10 μM ATP at 22°C. All data were fitted with a two parameter exponential decay. Numeric data is displayed as mean $\pm$ SEM. $n = 5$ myosin molecules for each plot.

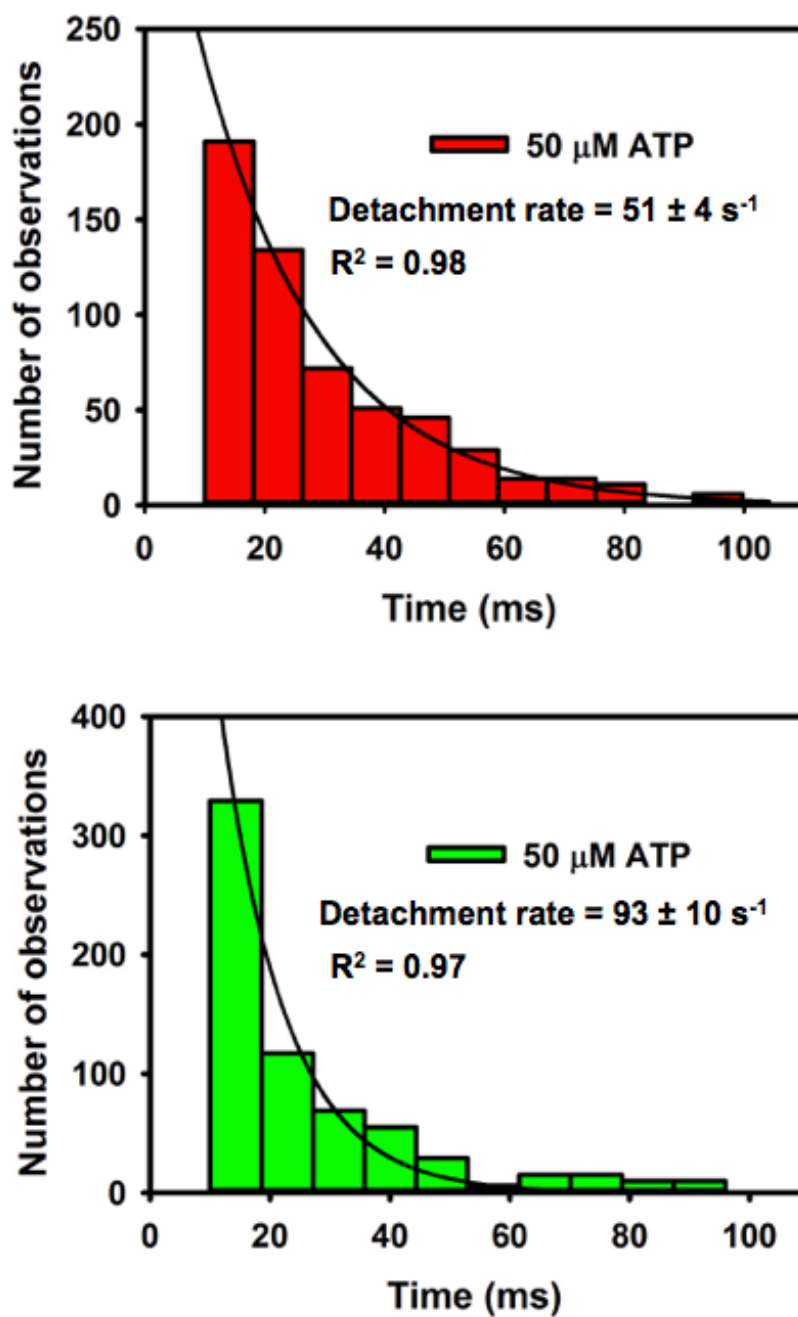


Figure 6.8.3: Attachment duration plots at 50 μM ATP. **Top,** Attachment duration plot for 0P myosin with 50 μM ATP at 22°C. **Bottom,** Attachment duration plot for 100P myosin with 50 μM ATP at 22°C. All data were fitted with a two parameter exponential decay. Numeric data is displayed as mean $\pm$ SEM. $n = 5$ myosin molecules for each plot.

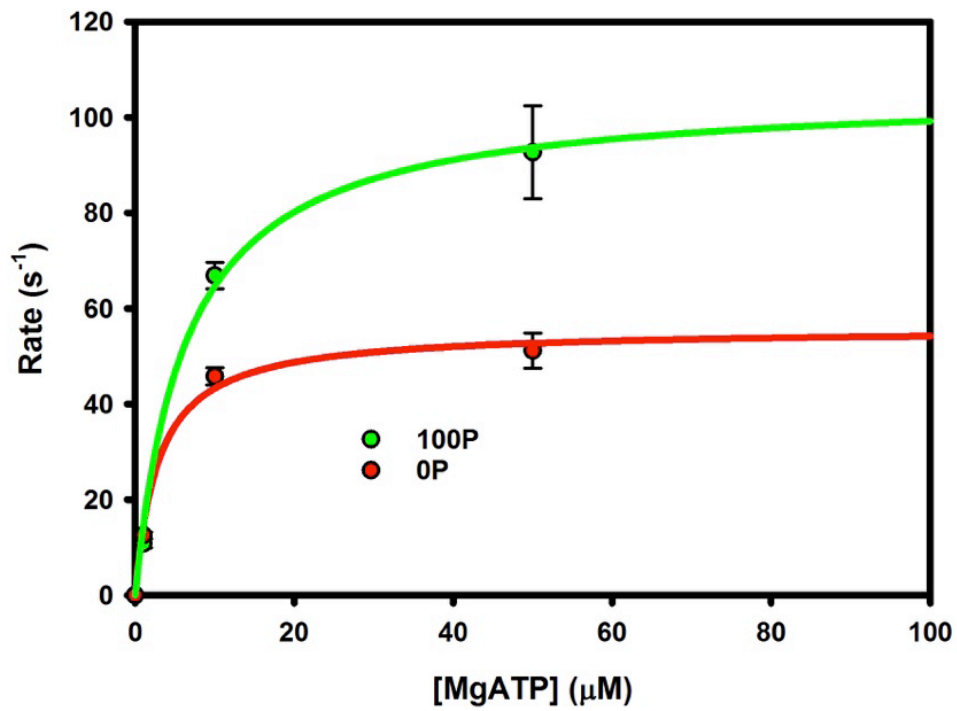


Figure 6.8.4: Plot of detachment rate of myosin from actin in varying [MgATP] as determined by the optical trap. **Green:** 100P myosins. **Red:** 0P myosins. All data are displayed as mean $\pm$ SEM. $n = 5$ myosins for each data point. Data were fitted by a Michaelis-Menten relation ($\text{Rate} = dV_{\text{max}} [\text{MgATP}] / dK_{\text{atp}} + [\text{MgATP}]$).

6.9 Determination of cardiac myosin step displacement with altered RLC phosphorylation

The displacement created by individual myosin molecules can be assessed using the optical trapping three bead assay. Performing this assay under loaded conditions allows us to assess the effect of RLC phosphorylation level on myosin molecule mechanics in a load dependent manner. This load is created by the sinusoidal oscillation of the actin filament over a myosin molecule bound to a pedestal bead. Assessing the ability of the myosin to produce its power stroke displacement under load is not necessarily assessing a change in myosin step size. This is because the RLC phosphorylation level cannot affect the myosin lever arm length, which is constant in cardiac myosin. Therefore, any changes in power stroke displacement under load are likely due to changes in lever arm stiffness, allowing the molecule to exert a larger power stroke displacement against an imposed load. This assay provides a single molecule approach for assessing the effect of load on the mechanical output of single myosin molecules. This data provides us with reductionist insight into our previous measurements in isolated muscle. We hypothesise that we will observe an increased power stroke displacement with increased RLC phosphorylation signifying that the myosin lever arm stiffness is altered by RLC phosphorylation.

Attachment displacements of individual binding events from single myosin molecules were measured at varying [MgATP] (1 μ M, 10 μ M, and 50 μ M) during motor bead oscillation. Centring the oscillatory sinus about the peak of sinusoidal oscillatory velocity, and measuring the amplitude of low variance binding events assessed displacement. Data were fitted with a single Gaussian relationship to the displacement extremities as previously described (220). The displacement shift of the peak of the Gaussian from zero describes the displacement the myosin motor

creates (X_0). In our experiments we observed a single lobed Gaussian characteristic of previous optical trapping experiments under load with this trap configuration (166, 221).

Individual displacement histograms were plotted for all [MgATP] and for 100P or 0P myosin. Fitting each individual Gaussian determined the direction of the power stroke in relation to the sensor bead. If a single pedestal had below 100 interactions the data were not used to determine displacement due to uncertainty of actin bead pair orientation. This method allowed interactions from multiple actin bead pairs (of previously un-known polarity) to be aligned and pooled into a single histogram. [MgATP] had no effect on myosin displacement when comparing between [MgATP] for each myosin phosphorylation level. Therefore, all measurements were combined into single histograms for either 100P or 0P myosin.

Displacement histograms for 0P myosin molecules were created from 8 separate myosins that showed 1300 interactions during experimentation (Figure 6.9.1). Interaction displacement was calculated for each pedestal probed and all pedestals were aligned and pooled. Displacement for 0P myosin was measured to be 4.5 ± 1.4 nm, where the fit of the histogram had an R^2 of 0.96.

Displacement histograms for 100P myosin molecules were created from 6 separate myosins with 1000 attachment observations split between them (Figure 6.9.2). The 100P myosin interactions were treated in the same way as the data from 0P myosin. Displacement for 100P myosin was 8.8 ± 0.9 nm, where the fit of the histogram had an R^2 of 0.96. Using a Student's t-test it was found that the difference between the power stroke displacements of 100P (8.8 ± 0.9 nm) and 0P (4.5 ± 1.4) were significant where $p < 0.005$. This inferred that the stiffness of the lever arm is likely altered by RLC phosphorylation allowing phosphorylated myosin to produce a greater lever arm swing under load.

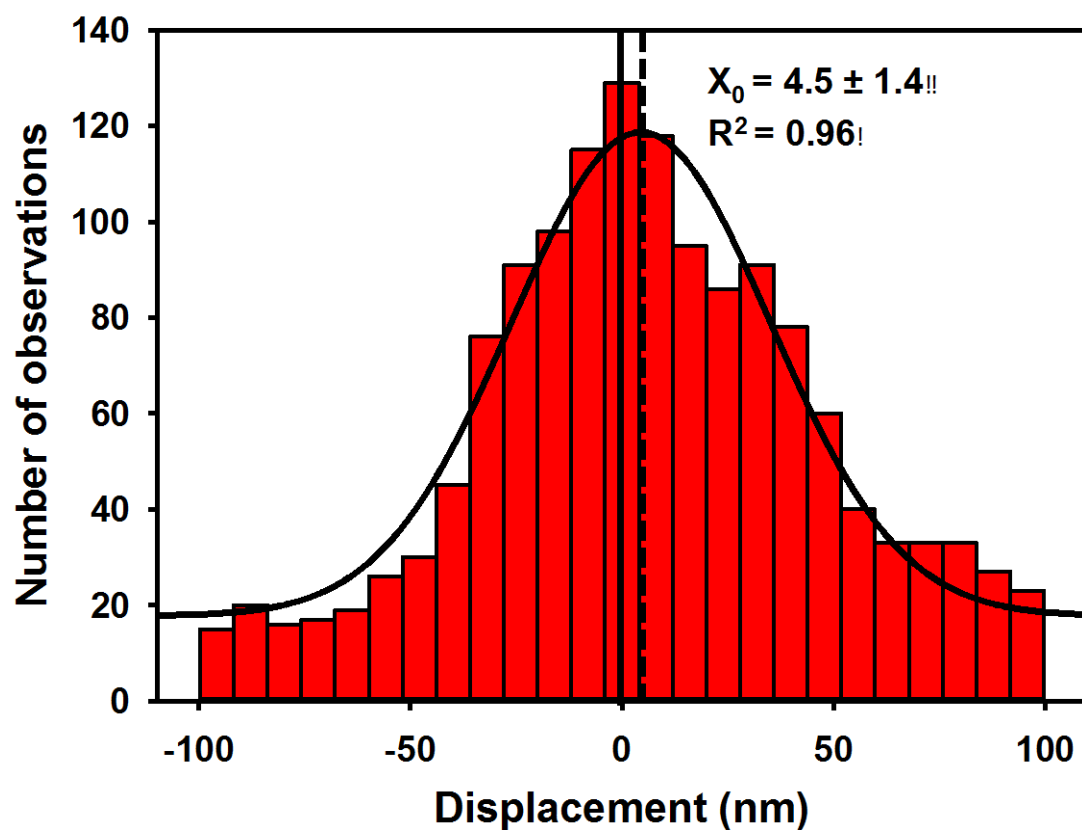


Figure 6.9.1: Displacement (X_0) histogram for 0P myosin fit by a 4 parameter Gaussian with ± 100 nm motor bead oscillation. Number of myosin molecules was 8, the number of observed interactions = 1300. Solid black line indicates where the peak of the Gaussian would be if there were no power stroke displacement. Dotted line indicates the centre of the Gaussian created by power stroke displacement. Data are displayed as mean $\pm$ SEM.

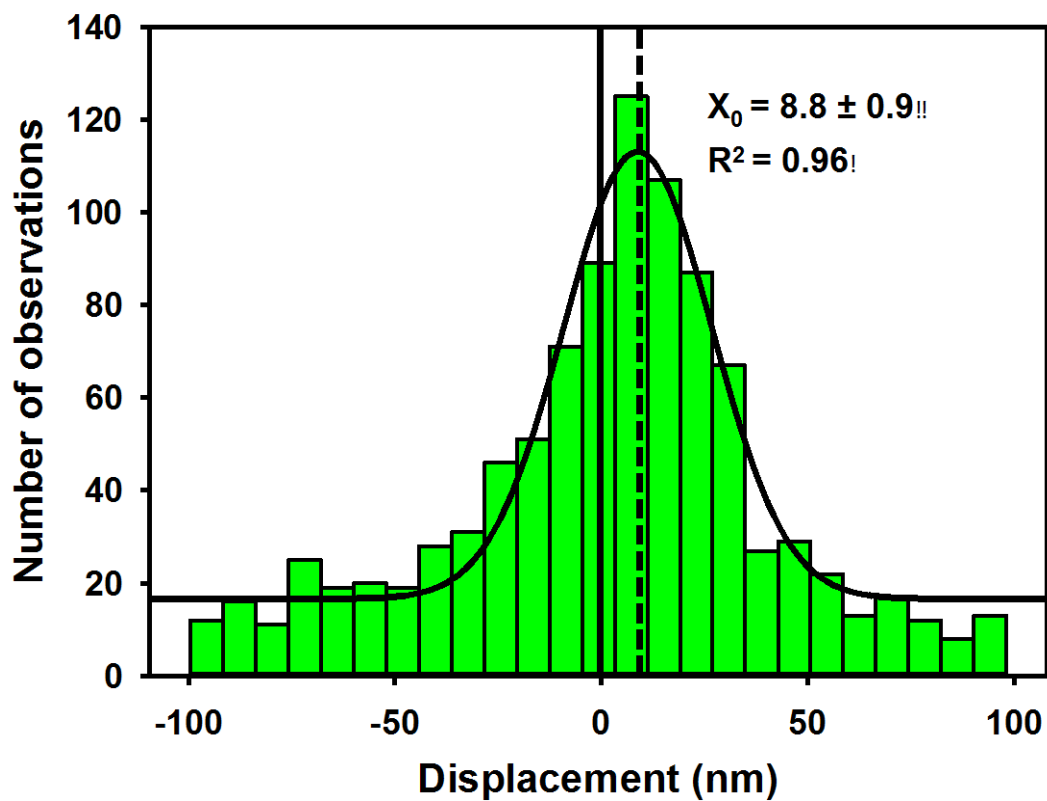


Figure 6.9.2: Displacement (X_0) histogram for 100P myosin fit by a 4 parameter Gaussian with ± 100 nM motor bead oscillation. Number of myosin molecules was 6, the number of observed interactions = 1000. Solid black line indicates where the peak of the Gaussian would be if there were no power stroke displacement. Dotted line indicates the centre of the Gaussian created by power stroke displacement. Data are displayed as mean $\pm$ SEM.

7 - Discussion

7.1 RLC phosphorylation level in health and disease

The phosphorylation abundance of the RLC in left ventricular myocardium is dependent on the mammalian species. Mouse and rat cardiac RLC have multiple phosphorylation sites (serine-14 and 15), whereas human and pig cardiac RLC can only be phosphorylated at one residue (serine-15), observed by mass spectrometry (82).

It is a new finding that rat RLCs have the ability to become bisphosphorylated. This bisphosphorylation has previously been observed in mouse cardiac RLCs, which are phosphorylated at both serine 14 and 15 residues (82). Rat RLC phosphorylation displays four bands on a Western blot after the use of Phos-tag augmented polyacrylamide gel electrophoresis. Analysis of the individual band densities revealed that the abundances of the four bands corresponded to the phosphorylation states observed previously in mouse by mass spectrometry (82). The four bands on the Phos-tag Western blots correspond to four distinct phosphorylation states as observed previously (82), zero phosphorylation, one phosphorylation at serine-14, one phosphorylation at serine-15, and double phosphorylation of the RLC at both serine-14 and 15. Our antibody was specific for only ventricular cardiac RLCs. The antibody recognises amino acids 45-59 of the RLCs, which are not conserved in non-muscle or smooth muscle RLCs. For this reason we are unlikely to detect RLCs that are not cardiac RLCs. We have only used tissue from the left ventricle for these studies so we have no contamination of atrial RLCs. Therefore, it seems that the Phos-tag method in this instance is able to separate proteins that are singly phosphorylated at separate phosphorylation sites. Knowing this we could calculate the molar ratio of phosphorylation in the myocardium of healthy donor rat to be ~ 0.3 mol Pi/ mol RLC, which is very similar to previously reported values in wild-type mice (82).

In larger mammalian (pig and human) myocardium there is only single phosphorylation of the RLC. In pig and human left ventricular myocardium from healthy donors we observe a basal phosphorylation level of 0.44 mol Pi/ mol RLC and 0.4 mol Pi/ mol RLC respectively, similar to previous measurements (222). Although the human and pig RLCs have only single phosphorylation sites the molar ratio of phosphorylation is greater than in rats (0.3 mol Pi/mol RLC). The difference in phosphorylation abundance in the two different mammalian systems is due to the amount of protein that is completely un-phosphorylated. In rat left ventricular myocardium approximately 70% of the RLC molecules have no phosphorylation. Pig and human left ventricular myocardium that were studied had only 56 – 60% of the RLC with no phosphorylation. This observation raises important questions about the way in which RLC phosphorylation may be used as a modulator of cardiac performance in these different mammalian systems. Explanations for the differences in basal RLC phosphorylation could be due to many factors. Of course, it is well established that the myosin heavy chain isoform of rodents is predominantly α -MHC, whereas pig and human myocardium has a predominance of the slower β -MHC isoform (223). The differences could be linked to the underlying differences in cardiac physiology between the mammalian species such as heart rate or gross cardiac morphology.

Human samples used for Phos-tag analysis could not be selected for specific left ventricular region due to the limited availability of tissue. For this reason we cannot rule out that the RLC phosphorylation abundance differences between human and rat myocardium are not due to differences in the tissue layer that was sampled from the left ventricle (90). Pig myocardium could be extracted from the mid left ventricular myocardium as had been done with rat myocardium indicating that RLC phosphorylation level in the myocardium of the left ventricle in rat and pig were different.

We determined the effect of cardiac disease on the phosphorylation abundance of the RLC in the myocardium of both human and rats to understand if the response of the two mammalian systems to cardiac insult was the same. We employed a method of left anterior descending (LAD) coronary artery ligation to produce chronic left ventricular apical myocardial infarcts in a cohort of adult rats (1, 193). We studied the RLC phosphorylation abundance of the mid left ventricular wall distant from the infarct zone at two time points after ligation. The first time point was four weeks post ligation when adaptive hypertrophy was observed. The second time point was twenty weeks into disease progression when the animals started to display classic signs of heart failure and cardiac de-compensation, typified by impaired diastolic relaxation, oedema, and left ventricular dilatation (See Appendix A). At four weeks post-ligation the RLC phosphorylation was 50% greater than in an age-matched control cohort (Figure 4.3.1). This rise in phosphorylation was typified by a substantial increase in the phosphorylation of the serine-14 phosphorylation site (Figure 4.4.1). Interestingly, twenty weeks post infarct the RLC phosphorylation abundance was further raised (68%) when compared to control. The increased phosphorylation abundance at twenty weeks was typified by significant increases of phosphorylation of single serine-15 and double phosphorylation of the RLC. Sole serine-14 phosphorylation of the RLC was not different from control at twenty weeks. It would be of interest to understand why the RLC phosphorylation is raised at both stages during disease progression, yet why the raised RLC phosphorylation shifts from a predominant increase in serine-14 to an increase in serine-15 and double phosphorylation. One possibility is that a specific kinase phosphorylates the serine-14 residue early in disease and another is activated later in disease to phosphorylate the serine-15 site predominantly. It is of course possible that the RLC molecules that had raised phosphorylation solely at serine-14 then become phosphorylated at serine-15 and therefore manifest as a raised double RLC phosphorylation at twenty weeks and show no change to sole serine-14 phosphorylation. This is the first time that the RLC

phosphorylation change has been assessed in a site-specific manner during disease progression.

Previous studies have measured RLC phosphorylation in acquired cardiac disorders (148, 158, 199, 224). The results from these studies have varied: a similar technique of LAD ligation was applied to mice that were sacrificed 3 days post infarction and were found to have RLC phosphorylation abundance in the non-infarcted myocardium that was 20% lower than control (148). Another study indicated the RLC phosphorylation abundance was reduced in post-MI myocardium at eight weeks and had normalised by sixteen weeks (158). A study of acute myocardial infarction in pig showed a slight reduction in RLC phosphorylation within 90 minutes of occlusion (199). Taken together these data suggest that the RLC phosphorylation change is dynamic during disease progression, and the phosphorylation abundance response may be different in the specific myocardial layers (epicardium or endocardium) (90). Many factors may influence the RLC phosphorylation level post MI. The size and placement of infarct may produce different levels of protein phosphorylation adaption post-MI. It is also believed that the RLC phosphorylation can be dependent on myocardial layer, which may respond differently to MI (151). Importantly the changes to RLC phosphorylation level have not been assessed later in disease, during disease progression, in the uninfarcted myocardium of the left ventricle. This may be the main reason why our measurements may be different to previous studies of RLC phosphorylation level in disease.

Additionally, we studied the phosphorylation abundance change from human patient left ventricular myectomy samples with varying cardiac disorders. We found a similar trend in RLC phosphorylation abundance change in human disease and in our rat model of myocardial infarction. Specifically, if the cardiac disorder was linked to a sarcomeric mutation (excluding RLC mutants) the RLC phosphorylation abundance was raised (Figure 4.6.1). All mutants displayed a raised RLC phosphorylation

abundance, ranging from the MYH7 (K847E) only raised 13% and a TnT mutant (K273N), displaying an RLC phosphorylation abundance raised by 55%, compared to control donor myocardium. The average increase in the RLC phosphorylation abundance in the mutant samples was 28%. It is not clear what the effect of these mutations is on the organ in isolation from the tissue adaptation that occurs alongside the disease. Therefore, it is hard to understand why RLC phosphorylation may be raised in these mutants. Further study is needed to explore the relationship between mutations in the sarcomeric proteins and the phosphorylation consequences to better understand the disease pathophysiology. The same is observed in a variety of human donor tissues from left ventricular myectomy samples that have different forms of acquired cardiac disorders (Figure 4.6.2). All patients have clinically defined heart failure. However, not all have the same cardiac disorders. What is abundantly clear is that in the individuals for whom it has been studied, RLC phosphorylation abundance is increased in comparison to normal donor tissues. The RLC phosphorylation abundance is raised by 40% on average in the diseased heart failure human donor samples. With these findings we establish a trend that in both human and rat mammalian models of cardiac disease we observe enrichment of RLC phosphorylation level in comparison to the basal levels in control cohorts. This does not hold true for all cardiac disorders that affect RLC phosphorylation as discussed below.

Interestingly, the raised RLC phosphorylation observed in many disease models and states suggests an adaptive response of the myocardium to the insults. The mechanism of how RLC phosphorylation level is raised is still elusive, in disease however, it is likely a shift in the balance between kinase and phosphatase activity (95). Raised RLC phosphorylation has been previously thought to be cardioprotective in certain cardiac disorders, and diminish or alleviate the hypertrophic response (133). Therefore, enrichment of RLC phosphorylation is likely to be a compensatory

adaptation in these disease states. Reduced RLC phosphorylation through mutation of the RLCs is causative of cardiac disease as the mutations interfere with RLC phosphorylation (139-141). There is evidence that other subsets of cardiac disorders show a reduction in RLC phosphorylation during diseases, which are not directly linked to RLC mutations inhibiting phosphorylation (97, 143, 146, 147). These mutations are more likely responsible for creating a signalling response in the myocardium that leads to alterations of cardiac RLC phosphorylation. Specifically, a cardiac MHC gene mutation (MYH7) R723G, which has been known in early disease to lead to increased isometric tension in the muscle and hypertrophy shows reduced RLC phosphorylation (142, 143, 225). The R723G mutant displays increased myocardial function, which may explain why myocardial adaptation is typified by decreased RLC phosphorylation abundance to counteract an increased ability of the myocardium to produce force. This means that RLC phosphorylation abundance can be adapted upward or downward to counteract a gain or loss of myocardial contractility. However, it must be noted that RLC phosphorylation can also be prevented by mutations in the RLC (139). This means that not all RLC phosphorylation abundance changes in disease are necessarily adaptive. More importantly, we still do not know why RLC phosphorylation in disease is sometimes maladaptive, and sometimes adaptive. Although, it is thought that RLC phosphorylation can help to normalise wall stress during systole (90). In the case of progressive chronic cardiac disease initial RLC phosphorylation abundance change may be adaptive but becomes maladaptive later in disease progression.

RLC phosphorylation level studies provided the framework for our mechanical measurements of RLC phosphorylation level change in permeabilised muscle (trabeculae from rat) and isolated molecules from pig. It defined the phosphorylation level changes that can be observed in disease (+50% in some cases and nearly 0% total phosphorylation in other cases) so we could mimic these RLC phosphorylation

levels in-vivo and in-vitro. This is done to understand the effects these RLC phosphorylation level changes produce to alter the contractile output of muscle and the basic biochemical function of myosin molecules.

7.2 The ability of myosin regulatory light chain phosphorylation abundance to alter trabecular muscle mechanics in the rat

To understand how altered RLC phosphorylation abundance affects muscle mechanics of isolated cardiac trabeculae, we compared the response to that of trabeculae of native (approximately 0.3 mol pi/ mol RLC) phosphorylation. We used isolated permeabilised rat cardiac trabeculae and exchanged recombinant RLC into the tissue that had either enriched or reduced RLC phosphorylation abundance (Figure 2.8.1).

We found that increasing RLC phosphorylation by approximately 50%, compared to control phosphorylation in rat trabecula, enhanced the ability of the tissue to produce force, power, and shorten under no load (Figure 5.1.2). Enriching RLC phosphorylation by 50% doubled power and raised the peak unloaded shortening velocity by 40% in comparison to control phosphorylation abundance. Conversely, reducing RLC phosphorylation by approximately 40% reduced the ability of the muscle to produce isometric force (4-fold), power (3-fold), and shorten under minimal load (-40%). Many contractile parameters even showed a phosphorylation dose dependence on their value (Figure 5.2.2). The ability of cardiac tissue to produce force and power has rarely been studied before. These measurements assess the ability of the cardiac tissues to function whilst the muscle shortens, which is the predominant physiological state of the myocardium during systole. By using the technique of RLC exchange we could be confident that only RLC phosphorylation was affected by our treatment. This isolated the effect of RLC phosphorylation from

other sarcomeric protein modifications that can often happen along side RLC phosphorylation through common signalling pathways.

Altering the ability of muscle to shorten under no load is correlated with changes to the ADP release rate of the actomyosin complex (209). ADP release is rate limiting for the cross-bridge cycle, and shortening of the muscle is dependent on the rate of ADP release. This infers that RLC phosphorylation may be altering the ADP release rate of the actomyosin complex, which our ensemble and single molecule experiments provide further evidence for. We further discuss this phenomenon below in relation to our other experimental data where we have further evidence that the ADP release rate may indeed be altered by RLC phosphorylation abundance.

In the context of our RLC phosphorylation studies that found (approx. 50%) enrichment of RLC phosphorylation abundance in cardiac disorders, we now understand that this increase in RLC phosphorylation abundance is modulating contractile function, increasing force and power during physiological shortening in systole. Assuming that ADP release is strain sensitive in cardiac myosin then acceleration of ADP release under strain by RLC phosphorylation abundance change may help to account for this phenomenon (226). We discuss this further in section 7.5.

Additionally, our data helps understand the basis for hypertrophy and eventual pump function decline in mutants of the RLC that cannot become phosphorylated (139-141). If mechanical work (power output during shortening) is insufficient to supply the circulatory needs, then the organ must adapt. This adaptation manifests as hypertrophy, which may be compensatory to the loss of systolic function due to RLC phosphorylation reduction. Evidently this response is unsustainable for the length of the organism's natural life span and precipitates further cardiac disorder and eventual failure (139, 140). Summarising, RLC phosphorylation abundance change

may be compensatory to the disease phenotype, but seemingly cannot prevent disease progression, and may be maladaptive later in disease.

Our mechanical trabecular data add to the existing knowledge describing the effects of altering RLC phosphorylation abundance in cardiac tissue. The current understanding of RLC phosphorylation in striated muscle myosin is a mechanism that allows fine-tuning of the myosin head proximity to the thin filament. This is due to the addition or removal of the negative charges of phosphate (129, 227-229). In regard to this it may also be true that phosphorylation of the RLC could increase the stiffness of the myosin neck region, possibly increasing the myosin duty cycle (230). Mechanical studies have mostly assessed the effect of RLC phosphorylation on both calcium sensitivity of force production and isometric force (135, 137, 141, 191). The experiments shown here address for the first time the way in which RLC phosphorylation alters the ability of the myocardium to perform mechanical work during muscle shortening.

Care should be taken when applying our mechanical results in rat myocardium to larger mammalian species such as pig or human, as human ventricular myocardium has two variants of the RLC. These are distinct charge variants denoted as LC-2 and LC-2* (78). The LC-2 is more highly expressed (70:30) and is the more highly phosphorylated of the two (78, 80). Therefore, the human ventricular tissue has three different myosin isoenzymes (LC-2/ LC-2, LC-2/ LC-2*, and LC-2*/LC2* with β -MHC). Whereas, rat ventricular RLC comes in one form, and the three ventricular myosin isoenzymes are formed by the RLC and the two isoforms of myosin (α -MHC and β -MHC) (81). No studies have been performed to understand the ability of RLC phosphorylation abundance to modify cardiac muscle performance in a β -MHC background. Additionally, pig and human RLCs have only one phosphorylation site at serine-15, whereas rat and mouse cardiac RLCs have two phosphorylation sites serine-14 and 15 (82, 140, 231). As of yet there are no studies that have been

performed to understand whether there are site-specific phosphorylation effects in the murine models and how they compare to larger mammalian models.

Together, our results demonstrated the ability of RLC phosphorylation abundance to alter work performed by cardiac muscle under load whilst suggesting that ADP release rate may be altered and form part of the explanation of this effect as ADP release is strain sensitive (226). There is, as of yet, no prevailing mechanism that explains the ability of the myocardium to alter force production under load due to RLC phosphorylation. It is also not yet understood if these alterations to RLC phosphorylation abundance in disease manifest mechanically as we have predicted (under load) in our trabecular exchange system.

7.3 The effect of chronic myocardial infarction (MI) with raised RLC phosphorylation on the ability of myocardium to produce power and force during compensation and decompensation

We investigated the effect of MI on the ability of cardiac muscle to produce force and power during shortening. The purpose of this experiment was to understand the way in which RLC phosphorylation abundance change (increase of approximately 50% observed in the MI model) influenced the muscle mechanics of the myocardium in the two classical phases of tissue remodelling post-MI (Appendix A, data provided by the laboratories of Dr. Kenneth Macleod and Dr. Alex Lyon). Our earlier work has shown that a 50% increase in RLC phosphorylation abundance increases the ability of the myocardium to produce power significantly, however, does this change translate into the diseased state where many other alterations and modifications are occurring alongside RLC phosphorylation?

Four weeks after MI the remaining well perfused myocardium of the left ventricle produced more power and force in both saturating free $[Ca^{2+}]$ and limiting physiological 1 μM free $[Ca^{2+}]$ than the control un-infarcted cohort (Figure 5.3.1.1). Interestingly, the power produced by the MI cohort at four weeks was double that produced in the control cohort in both calcium abundances. The same near doubling of power output was observed when we enriched RLC phosphorylation by 50% in isolation in our exchange experiments in saturating $[Ca^{2+}]$ (Table 5.1.2). Four weeks post-MI the raised RLC phosphorylation does correlate with an increased contractile function of the myocardium. This effect is not confounded by tissue hypertrophy or increased sarcomeric content in the tissue as all force and power measurements are normalised to tissue volume (provided by the Dr. Luther laboratory and shown by electron microscopy that sarcomeric content was not increased per unit volume of myocardium, Appendix B). This effect is not conserved at twenty weeks post-MI where the infarcted cohort has reduced power in saturating and limiting free $[Ca^{2+}]$ (Table 5.3.4.1). This is contradictory to our previous finding that RLC phosphorylation abundance increase correlated with increased force and power production. However, in this model at twenty weeks post-MI there is large-scale tissue disruption not observed at four weeks post-MI. This was typified by large-scale disruption to sarcomeric structure and surrounding tissue with evident mitochondrial abnormalities observed by electron microscopy (Appendix B as provided by the laboratory of Dr. Pradeep Luther). The effect of RLC phosphorylation on permeabilised muscle mechanics can be classified as adaptive post-MI at four weeks but cannot overcome large-scale tissue disruption and eventual contractile decline later in disease progression.

It would be of interest to understand if enriching RLC phosphorylation further post-MI at four weeks experimentally or therapeutically could alleviate the hypertrophic response, or if reduction in RLC phosphorylation would accelerate disease

progression (133). Further investigations need to be made to understand if RLC phosphorylation enrichment in the myocardium is truly adaptive or maladaptive in respect to disease progression. It is likely that this may depend on the disease model. There is evidence to support the hypothesis that later in disease RLC phosphorylation abundance enrichment is maladaptive as it may contribute to diastolic abnormalities due to its effect on calcium sensitivity (125-127, 232). It is well established that calcium homeostasis begins to become deregulated leading to higher cytosolic free $[Ca^{2+}]$ during diastole (233-236). If this is indeed the case and RLC phosphorylation increases calcium sensitivity of the myofilaments, then enriched RLC phosphorylation may contribute to impaired diastolic relaxation, exacerbating disease. Therefore, in individuals with enriched RLC phosphorylation and impaired diastolic relaxation, reduction of RLC phosphorylation could be therapeutically useful to restore diastolic function. This reduction in RLC phosphorylation would likely need to be a fine adjustment, as ablation of RLC phosphorylation could be detrimental to systolic function (Table 5.3.1.1). This could be performed by small molecule activation of phosphatases specific to the RLC or inhibition of kinases of the RLC. Other therapies could aid this by targeting the main phenotype of diastolic insufficiency by adapting calcium homeostasis by modulating calcium reuptake via SERCA (237).

7.4 The effect of RLC phosphorylation abundance on pig myosin in actin gliding velocity and low ionic strength ATPase assays

We were able to successfully fully phosphorylate and fully de-phosphorylate the RLCs of full-length cardiac myosin isolated from pig left ventricular myocardium. The use of the Ca^{2+} /Calmodulin regulated smooth muscle MLCK (231) attained near 100% phosphorylation of in-vitro isolated myosin molecules within an hour of

incubation at room temperature (Figure 3.2.1). Complete eradication of RLC phosphorylation could be achieved with these same molecules by incubation with a non-specific alkaline phosphatase from calf-intestine (CIP). Phosphorylation abundance was maintained for at least 48 hours after incubation with either kinase or phosphatase. Post-incubation kinases and phosphatases were easily removed by ultracentrifugation to purify the myosin from the kinase/phosphatase.

We measured the actin gliding velocity as a function of myosin concentration and myosin RLC phosphorylation abundance (Figure 6.2.2). An actin filament moves at its maximal velocity as long as there is a high enough surface density of myosin to ensure that one myosin head is interacting with actin at a given time (211, 212). For this reason a qualitative assessment of myosin duty ratio can be taken from the concentration of surface myosin needed to propel the actin at peak gliding velocity (191, 210). We observed that all myosin, irrespective of RLC phosphorylation abundance, attained peak actin gliding motility near the same myosin concentration threshold, indicating no change in duty cycle duration (approximately 0.85 μM for 100P myosin and 1.0 μM for 0P myosin). We found that myosin with both RLCs phosphorylated (100P) attained a higher peak actin gliding velocity than myosins that had lower RLC phosphorylation abundances (1.3 $\mu\text{m/s}$) (Figure 6.2.1). The RLC phosphorylation abundance was shown to impact the ability of the myosin to translocate actin, as myosins with eradicated RLC phosphorylation could only achieve a maximal gliding velocity of 0.8 $\mu\text{m/s}$. Myosins that had an RLC phosphorylation abundance of 50% made of a mixture of molecules with both RLCs of a single molecule phosphorylated (50%), and no RLCs phosphorylated (50%) had an intermediary peak actin gliding velocity of 1.0 $\mu\text{m/s}$. Interestingly, myosin that had native phosphorylation abundance (40%) showed actin gliding velocities indistinguishable from myosins that had no RLC phosphorylation (0.8 $\mu\text{m/s}$). This evidence supports a hypothesis that the percentage of myosin molecules with RLC

phosphorylation of both heads of the myosin is the determinant of altering actin gliding velocity. It is likely that the myosin RLC phosphorylation abundance in the native myosin (40%) is fairly evenly distributed across all the molecules, meaning proportionally far fewer molecules would have both RLCs phosphorylated. When we investigate a population of myosins that have almost identical RLC phosphorylation but have a higher proportion that are doubly phosphorylated myosin molecules, we observe a heightened actin gliding velocity, as indicated by the 50P myosin population.

The ability of cardiac myosin to translocate actin is limited by the ADP release step of the actomyosin cycle and is therefore detachment limited (185, 208, 238). These gliding velocity findings add further evidence to support the fact that RLC phosphorylation may be accelerating the ADP release rate in cardiac myosin. It is unclear as to why this would necessarily be a phenomenon that would be dependent on both heads of the myosin molecule possessing a phosphorylated RLC. This phenomenon could be explained by a mechanism of co-operativity between the two heads, which links dual RLC phosphorylation of the myosin molecule to changes in the ADP release rate of the head bound to actin. Further studies are needed to better define this relationship with single headed myosin molecule constructs or molecules that have only one head of the myosin possessing RLC phosphorylation (239, 240).

Further to this we investigated the effect of [MgATP] on the ability of cardiac myosin to translocate actin as a function of its phosphorylation abundance (Figure 6.3.2). We found no differences in the [ATP] that produced half maximal actin gliding velocity (K_{atp}) between phosphorylation groups. However, peak actin gliding velocities predicted from Michaelis-Menten kinetics (V_{max}) were substantially altered by RLC phosphorylation abundance (Figure 6.3.1). The 100P myosin had a predicted maximal sliding velocity of 1.3 $\mu\text{m/s}$, which was the exact same velocity measured in the actin gliding assay at 2 mM [ATP] in high protein concentration. The same was

applicable for all other myosin RLC phosphorylation abundances measured. This result reiterated our previous finding that peak sliding velocity is indeed affected by RLC phosphorylation, but in this case it was predicted by Michaelis-Menten kinetics. Interestingly, the phenomenon of double-headed phosphorylation was again apparent as 40P myosin had a similar predicted $V_{\max}$ to 0P myosins.

Following on from this we assessed the effect of temperature on the ability of the myosin to translocate actin in the actin-gliding assay as a function of RLC phosphorylation abundance (Figure 6.4.2). We found that the alteration of peak gliding velocity due to RLC phosphorylation change was upheld at physiological temperature (37°C). The peak gliding velocity of each RLC phosphorylation abundance group was approximately 3-fold greater at 37°C than at 30°C. By normalising the actin gliding velocity from each RLC phosphorylation abundance group we found that there was no affect of RLC phosphorylation abundance on the response of the myosin to temperature change. Arrhenius plots were used to calculate the activation energy and Q_{10} for each RLC phosphorylation abundance at low (17-27°C) and high (27-37°C) temperatures (Figure 6.5.2). Indeed, a well-known break point in the Arrhenious plot was seen at ~17°C. The activation energies and Q_{10} were not altered by RLC phosphorylation abundance but by temperature range (Figure 6.5.1). Lower temperatures showed higher activation energies and higher Q_{10} values from the myosin when compared to higher temperature acting gliding velocities, as had previously been observed in a variety of skeletal muscle isoforms (216). By normalising the velocities of all RLC phosphorylation abundances to 40P we discovered that the 0P myosins overlapped in actin gliding velocities at temperatures above 30°C. This is why we observe actin-gliding velocities determined by [myosin] and [ATP] on 40P and 0P myosins to be similar (Figure 6.5.4). It is not clear why actin gliding velocities between 17°C and 30°C for 0P and 40P myosin are different but above 30°C they are indistinguishable as we had discovered in our

[myosin] and [ATP] gliding assays. This effect warrants further investigation as it suggests that ADP release is faster in 40P myosin compared to 0P myosin at low (17-30°C) temperatures, but closer to physiological temperatures this is no longer true. Similar observations with myosin ATPase have been made with slow and fast skeletal myosins where isoform specific differences in ATPase became more similar at physiological temperatures (241). It would be interesting to understand if there is any structural significance to this phenomenon, or whether it is an inherent property in the actin gliding assay. Interestingly, in our normalised plot, calculating the average of the 0P and 100P relation we could mathematically predict the normalised velocity of the 50P treatment group. This phenomenon is also common to our other plots [myosin] and [actin]. This infers, that indeed in the actin gliding assay, the gliding velocity can be titrated by double headed phosphorylation, and adheres to a linear relation.

As motors in muscle often function under load, we wanted to understand the effect of applying a frictional viscoelastic load to the actin gliding assay to see how it would affect the ability of the myosin with different RLC phosphorylation abundances to translocate actin (Figure 6.6.1). In this assay the ability of the myosin to translocate actin is limited by both attachment and detachment kinetics of myosin from actin (192). The frictional loading assay can provide qualitative measures of the isometric force the myosin can produce by assessing the concentration of α -actinin that stalls the motion of the actin (242). However, this measurement assumes that the relationship of α -actinin to velocity is linear, which we do not observe with the cardiac myosin at all phosphorylation levels. Therefore, we make no calculation of the pseudo isometric force measured as concentration of α -actinin needed to prevent actin sliding (K_r). What we do observe is a marked slowing of actin filament velocity in myosin populations that have lower RLC phosphorylation abundances when comparing RLC phosphorylation abundance groups. This implies that the myosin

populations with lower RLC phosphorylation abundances are more affected by applied load and are less able to overcome this load and maintain sliding velocity. This phenomenon is linked to the ability of the myosin to produce force to overcome the viscoelastic drag of the transient α -actinin molecular interactions with actin.

Our measurements of actin-activated myosin ATPase in low ionic strength buffer indicate that RLC phosphorylation abundance can affect the ATPase rate of full-length cardiac myosin (Figure 6.7.1). The predicted peak rate of actin activated ATPase rate with infinite actin produces a rate of $4 \text{ s}^{-1}\text{head}^{-1}$ for myosin populations with fully phosphorylated RLCs. This corresponds to a mean cycle time of 250 ms. This is significantly slower than has been reported in the literature previously (10s^{-1}) and is probably due to the use of filamentous myosin and not single molecules in solution. For any thick filaments only a certain proportion of heads will be in the vicinity of an appropriate actin binding site. Whereas, with myosin molecules in solution there will be a myosin, or actin concentration for which they are able to bind freely to any actin sites they encounter and are not constrained in a filament (219). The peak rate of ATP hydrolysis in myosin that have either 50P, 40P, or 0P phosphorylation levels all share an ATPase rate of approximately $3 \text{ s}^{-1}\text{head}^{-1}$ corresponding to a mean cycle time of 330 ms. The similarity of maximal ATPase rate with myosin below maximal RLC phosphorylation may infer a dependence on high RLC phosphorylation before a change in mean cycle time can be appreciated. This is interesting, as reducing RLC phosphorylation in our muscle preparations did not significantly decrease the unloaded shortening velocity either. However, increasing RLC phosphorylation abundance in muscle significantly increased unloaded shortening velocity. It has been shown that the ATPase rate of myosin in limiting [actin] is rate limited by P_i release (218). Whereas the ability of muscle to shorten under no applied load was limited by ADP release (186, 209), which has been postulated for quite some time (187). This could implicate that both the rates of

Pi release and ADP release can be modulated by RLC phosphorylation in a similar fashion.

The actin concentration that gave half maximal ATPase rate (K_{atpase}) was altered when comparing myosin populations with RLC phosphorylation and those with no phosphorylation. Myosins with RLCs that had no phosphorylation had a higher 50% and 2-fold K_{atpase} compared to fully phosphorylated and intermediate phosphorylated RLC abundances respectively. It is not clear why the K_{atpase} of intermediate (40-50%) phosphorylated RLCs is lower than that of myosins with fully phosphorylated RLCs. The K_{atpase} values are very low in the ATPase assay as we are working with a low duty ratio motor in its filamentous form. It is likely that the myosin filaments keep actin attached to their surface, producing a very low K_{atpase} . Theoretically, as we are working with a filamentous ensemble, this makes the assay function as if the molecules have a high duty ratio. However, this is due to the effect of the ensemble in keeping the actin available to the myosin. The mathematically predicted K_{atpase} is lower in the intermediate phosphorylation groups due to the lower maximal rate of ATP hydrolysis than was observed with myosin with fully phosphorylated RLCs. This implies that maximally actin activated ATPase rates are only raised at high levels of RLC phosphorylation, whereas K_{atpase} is influenced by any presence in phosphorylation of the RLCs. The increase in K_{atpase} of the myosin with completely de-phosphorylated RLCs could be due to a reduced ability of the myosin to bind to actin (129). Hence at high saturating [actin] the same maximal rate of ATP hydrolysis is observed. It has also been postulated that in saturating [actin] the ATPase rate could be limited by the hydrolysis step and not Pi release in solution (218). The maximal ATPase rate of myosin is raised in the fully phosphorylated RLC myosin populations, which may indicate that high RLC phosphorylation abundance may alter the rate limiting hydrolysis step as well as Pi release rate at submaximal [actin]. Although it must be noted that this data came from experiments using skeletal

myosin S1 and not cardiac myosin filaments. The structural mechanism that explains this phenomenon is not yet understood and could be partially explained by the aforementioned myosin head reorientation with RLC phosphorylation (129).

7.5 The effect of RLC phosphorylation on attachment duration and power stroke displacement on pig cardiac myosin in the optical laser trap, three-bead assay

We had previously observed increases in both power and unloaded shortening velocity in muscle experiments. Unloaded shortening velocity can be altered in two ways; either by altering the attachment duration of the myosin, or by altering the displacement that the power stroke can produce. The ability of myosin ensembles in muscle to produce power can be altered by the ability of each molecule to produce force, by the strongly bound state time and the cycle time of the molecule (reviewed (219)). As previously described, we were able to produce fully phosphorylated and fully de-phosphorylated RLCs bound to pig full-length cardiac myosin. These preparations were used in the optical trap three-bead assay to determine myosin power stroke displacement and attachment lifetime with actin.

The actin bead pair was oscillated back and forth ~100 nm in a sinusoidal waveform at 100 Hz above a myosin coated pedestal bead. When interactions between the myosin and actin occurred there was an appreciable drop in oscillatory amplitude due to the stiffness of the cross-bridge attachment, which manifested as a drop in signal variance. The drop in signal variance was due to the reduction of bead pair compliance because of myosin binding, increasing system stiffness signifying the beginning of an attachment. The end of an attachment was defined by the variance returning to a pre-attachment state, i.e high system compliance due to the

progression away from strongly bound actomyosin states/ actomyosin detachment. The attachment duration was determined by measuring the duration of the low variance signal for each binding event. From this data the displacement of each interaction was calculated.

Attachment durations were measured under varying [ATP] from 1 – 50 μM . 50 μM ATP was the upper threshold of [ATP], as binding events became too short to reliably define at higher [ATP]. In low [ATP] the detachment of the actomyosin complex is rate-limited by ATP binding. We have shown herein that the detachment rate of myosin can be altered by RLC phosphorylation. Using Michaelis-Menten analysis to describe the detachment rate of myosin from actin at varying [ATP], we find a maximal detachment rate of 105 s^{-1} for myosin with full RLC phosphorylation abundance, and 56 s^{-1} for myosin with no RLC phosphorylation (Figure 6.8.4). As the maximal detachment rate is not [ATP] limited the rate limiting step of the detachment at maximal detachment is likely to be ADP release, or a structural change following ADP release. Thus it is likely that the ADP release rate is altered by RLC phosphorylation abundance as we had previously shown in our actin gliding assay results. We have previously calculated the mean cycle time for phosphorylated myosin as 250 ms (t_c) from the ATPase rate. Now we have measured the attachment duration of the strongly bound states (t_s) in saturating [ATP] to be approximately 10 ms in duration. We can therefore calculate the duty cycle of the myosin as:

Duty cycle = duration of strongly bound states (t_s)/ duration of full cycle (t_c)

This predicts that for myosins with fully phosphorylated RLCs we observe a duty cycle of 0.04 or 4% of the entire cycle. For myosin with de-phosphorylated RLCs the duty cycle was calculated to be 0.05 or 5% of the entire cycle. Therefore, it seems the duty cycles of the myosin are not altered by RLC phosphorylation abundance, as was inferred from the [myosin] actin gliding velocity data. However, entire cycle time and attachment duration were altered by RLC phosphorylation.

Additionally, we measured the individual displacements of the actomyosin interactions in the optical trap, as power and shortening produced by muscle can be altered by displacement of the myosin powerstroke. We determined that the displacement of the myosin in the optical trap under load could be altered in length by myosin RLC phosphorylation abundance. The unitary step size of a cardiac myosin molecule with 0P phosphorylation was calculated to be 4.5 ± 1.4 nm consistent with previous findings for myosin II molecules under load (220, 243), which are validated by structural studies (Figure 6.9.1) (31). The step size of the myosin molecules with 100P RLCs was calculated to be 8.8 ± 0.9 nm (Figure 6.9.2). This is a 50% larger step size than that of myosin with 0P RLCs, but still within previously measured ranges of myosin II step sizes (4-11 nm) (244, 245). The phosphorylation dependence of the cardiac myosin step size has not, to our knowledge, been observed previously. However, this result may explain why the literature reports varying step sizes for class II myosin molecules (246).

Velocity in the actin gliding assay can be mathematically predicted by knowing the t_s and the displacement (X_0) produced by the myosin as $\text{Velocity} = X_0 / t_s$ assuming the assay velocity is detachment limited. For myosin populations with phosphorylated RLCs, calculations for 25°C predict an actin gliding velocity of 800 ± 100 nm/s, which is close to our actual sliding velocities at 24.8°C of 600 nm/s. For myosin populations

with OP RLCs, calculations predict a gliding velocity of 220 ± 70 nm/s, which is in good agreement with our experimental data of 270 nm/s at 24.9°C. It is interesting however that the predicted actin gliding velocities calculated from parameters measured in the optical trap are similar to the actin gliding velocities. The velocity in the actin-gliding assay is produced under minimal load. Whereas the parameters measured in the optical trap are assessed when the molecule is actively being pulled upon by the oscillations for the actin bead pair.

The optical trap data suggest that phosphorylation of the RLC of cardiac myosin can fine-tune two aspects of the motor function. It can alter the ability of the motor to detach from actin in a phosphorylation dependent manner, by altering t_s , possibly by accelerating the ADP release rate when in saturating [ATP], and alter the unitary step size of the motor (X_0). It is not fully clear how the unitary step size is altered in a phosphorylation dependent manner. It could be linked to the stiffness of the molecule neck region (light chain binding domain). It is hypothesised that the addition of negative charge in the neck region could increase molecular stiffness as has been observed in other muscle myosins (230, 247). It must be noted that this change in step size is measured by a technique where the motor is put under load by bead oscillations and is measured on single myosin molecules not filaments. Functional predictions would suggest that a 2 IQ motif motor can have a maximal step size (due to its neck length) of approximately 9 nm (220, 248, 249). This is not often observed as many experiments are conducted under varying applied loads, which may prevent the complete step size (defined from the actomyosin structures). Therefore, RLC phosphorylation increasing motor neck region stiffness in a loaded assay may aid the molecule to produce its full swing length, which manifests in a closer to predicted maximal step size of the motor.

7.6 Comparisons of results from mechanical, ensemble, and single molecule experiments

We observe two ways of measuring the duty ratio of the cardiac myosin. We have assessed this parameter by [myosin] concentration-titrated actin-gliding assay, which provided a pseudo measurement to compare duty cycle of the RLC phosphorylation populations. We used measurements of the full ATPase cycle (t_c) from the NADH ATPase assay and measurements of the duration of the strongly bound states (t_s) from optical trapping to calculate the duty cycle. Both methods found no change in the duty cycle of the myosin when RLC phosphorylation abundance was altered, despite both parameters of the duty cycle being altered by RLC phosphorylation.

In cardiac muscle we were able to measure the ability of preparations with different RLC phosphorylation abundances to produce force and power under load (during shortening). We found that the muscle preparations with higher RLC phosphorylation had an increased capacity to produce force and power over a range of loads. It is well documented that the ability of muscle to shorten is likely governed by the ADP release rate, and that this rate is likely to be strain dependent (by Nyitrai and Geeves(250)) and more recently (226). The ability of muscle to produce power is governed by a few processes that relate to the force and displacement of individual cross-bridges and the proportion of cross-bridges in strongly bound states (219). We used the optical trap assay with applied load. In this assay it was shown that actomyosin detachment was faster in myosins that had phosphorylated RLCs versus those that had no RLC phosphorylation. Concomitant with this, we observed that the step size of the myosin could be altered by RLC phosphorylation abundance change. Myosins with fully phosphorylated RLCs were able to displace the actin filaments 50% further than those that had no RLC phosphorylation. By applying load with α -actinin in the actin gliding assay we found that myosin with phosphorylated RLCs

were able to oppose the viscoelastic drag of the actin/ α -actinin interaction, inferring more force per molecule. Using some of these parameters we were able to predict the actin gliding assay velocity for these myosins. These ensemble and single molecule experiments complimented our observations of changes to shortening velocity, force, and power in muscle. In respect to the optical trap and motility data it was surprising we could predict gliding velocity, as the actin-gliding assay was not performed under loaded conditions. This could mean that the myosin step size and strongly bound lifetime are little affected by load.

As changes to attachment lifetime and gliding velocity are often limited by ADP release, assuming a strain dependence of ADP release linked to myosin stiffness in the model put forth by Smith and Geeves (251), we may expect that RLC phosphorylation increases the stiffness of the myosin neck region. This may be the reason for the increased displacement of the myosin when under load in the optical trap (230, 247). Further experimentation is needed to determine the stiffness of the myosin molecule with and without RLC phosphorylation to clarify this point.

With the data presented here it is likely that the mechanism for increased force and power in muscle during shortening is in part due to the ability of the myosin to detach faster, displace actin further, and produce more force per molecule when RLC phosphorylation abundance of the RLC is raised. Therefore, RLC phosphorylation is likely to alter both the kinetics and the mechanics of cardiac myosin function. This may all happen alongside other previously observed effects of RLC phosphorylation: the myosin head proximity to the thin filament, which come into account when studying filamentous myosin (129), and the possible increases of myosin lever arm stiffness (230).

7.7 Future directions

Further experimentation should be performed to assess if altering RLC phosphorylation could alleviate contractile dysfunction in acquired or inherited cardiac disorders. A better temporal understanding of how RLC phosphorylation change accompanies many of these disease states and progressions will be needed to adequately answer this question. In the long term it would be of interest to find a way to specifically influence the RLC phosphorylation inside cardiac cells non-invasively to alter RLC phosphorylation therapeutically. So far, studies have begun to use expression of a phosphomimetic (serine to aspartic acid substitution at amino acid 15) RLC to alleviate a hypertrophic cardiomyopathy phenotype in the RLC D166V mutation (252). This data shows the merit of altering RLC phosphorylation to salvage disease in the instance where disease is caused by reduced RLC phosphorylation. These types of experiments should be extended to other cardiac disorders, which are known to display deficiencies in systolic function, to ascertain if RLC phosphorylation can salvage the disease phenotype. This being said, it may not be the best course of action in the treatment of human disease to express constitutively phosphorylated RLCs, even transiently by adenoviral transfection of the myocardium. This is because we do not know the long-term effects of enriching RLC phosphorylation and how it may alter the kinetics of relaxation, and therefore diastolic function. The best way forward therapeutically may be to use small molecule activators or inhibitors of myosin to correct for contractile gain or loss, which is detrimentally associated with disease progression. One such molecule already exists, omecamtiv mecarbil (OM), a myosin activator (253). This molecule is being developed to treat heart failures that present phenotypically with systolic dysfunction (254, 255). OM functions by altering the weak to strong actomyosin binding transition, increasing the myosins' duty cycle and therefore increasing its ensemble force and power output (253). It does this by directly binding the converter domain and

nucleotide binding pocket of myosin (253). This type of intervention likely provides the best modality for therapeutic intervention for these types of cardiovascular disorders. This is because the effect of the intervention is transient and can be fine tuned to the degree of systolic insufficiency. The problem that often arises is that there is more than just systolic insufficiency in heart failure; there can also be diastolic disturbances. We need to be able to treat all of the problems associated with the term 'heart failure' that are of varying modality. To try and compensate for diastolic disturbances the calcium regulatory systems in the cardiomyocyte are beginning to be targeted. Specifically, the sarcoplasmic calcium reuptake mechanism mediated via SERCA2a (reviewed(256)). Upregulation of SERCA2a expression, in heart failure, has been shown to increase systolic function, as well as normalising calcium homeostasis in the cardiomyocytes (256).

To better understand the clinical use of RLC phosphorylation as a therapeutic target in heart failure created by MHC or RLC mutations; we should use a reductionist approach to first understand the affect the mutation has on the mechanics and kinetics of the myosin motor. Recently, for the first time, cardiac myosins with HCM and DCM causing mutations have been able to be expressed and purified using mouse C2C12 cells (257-260). This means that these mutations can now be studied in isolation in the myosin molecule. This paves the way for testing if alterations to RLC phosphorylation level could aid the phenotype of these mutations *in vitro*. If we are able to rescue some of the mechanical or kinetic parameters affecting the myosin it would be fruitful to test this phenomenon *in vivo*, such as has recently be done with the RLC D166V mutation (252). The next step should to determine a way to alter RLC phosphorylation transiently to aid these disease phenotypes. An approach could be to find small molecule inhibitors, and activators for RLC kinases and phosphatases, so we can influence RLC phosphorylation through multiple mechanisms.

In regard to acquired heart failure, more needs to be done to determine if compensating for the disease phenotypes with RLC phosphorylation level change is a fruitful paradigm. Using methods, such as constitutive phosphorylation of the RLC in models of disease could be one way of addressing this situation. It would then be prudent to analyse in myocardial muscle how this is affecting tissue function by analysing muscle mechanics, tissue morphology, and biochemical alterations to other sarcomeric proteins.

Mechanical experimentation using cardiac trabeculae should be extended to measure the calcium sensitivity change associated with RLC phosphorylation. This would be of interest as many assays have only studied this effect when altering the phosphorylation of the RLC with promiscuous kinases that are known to alter other proteins phosphorylation levels. Further to our force and power measurements in muscle, it would be interesting to know if RLC phosphorylation changes the efficiency of the muscle. This could be assessed using an assay incorporating a molecular sensor for hydrolysis products whilst activating muscle (261, 262). If RLC phosphorylation increases the energy demand of the contractile apparatus, it may be detrimental in some heart failures where the cardiomyocytes are already showing disturbed energetics.

Further work should be performed to directly measure if there is a change of myosin stiffness associated with RLC phosphorylation level as is inferred by our displacement measurements in the optical trap. Pulling on bound cross bridges in the optical trap whilst in low [ATP] could be used to measure cross-bridge stiffness. It is also of interest to directly and quantitatively measure the force produced by cardiac myosin in the optical trap, to measure the effect of RLC phosphorylation abundance on force, as we have only provided qualitative evidence that this is the case with our α -actinin loaded actin gliding assay experiments. This can be done by further measurements in the optical trap with no actin bead pair oscillations. Measurements

with no actin bead pair oscillations should be made to see if RLC phosphorylation abundance alters the cross-bridges' ability to displace actin with no applied load. If displacement is not affected under minimal load it further strengthens the argument that the displacement change observed with changing RLC phosphorylation abundance is due to a stiffness change under oscillatory/loaded conditions.

Many of the answers to the remaining mechanical and kinetic effects of RLC phosphorylation will shed light on how to try and influence phosphorylation in disease. It will be important to address these questions in a multitude of ways to be sure our observations are indeed correct. Assuming RLC phosphorylation change does alter myocardial function in the way we believe it to, and that we can alter its phosphorylation therapeutically, it may become another powerful tool in treating many forms of cardiac diseases caused by inherited or acquired abnormalities.

8 – References

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Appendices

Appendix A - Characterisation of the rat myocardial infarct model

Prior and post sacrifice animals underwent a series of diagnostic tests to characterise heart function and other biometric markers. Heart weight measurements ascertained that at both four and twenty weeks post infarct heart weight was significantly increased when compared to age matched control (Figure 1A). Normalising heart weight with the tibial length allowed for a closer examination of heart hypertrophy in comparison to animal size (Figure 1B). These measurements further re-iterated that animals post-ligation at both four and twenty weeks displayed significantly increased heart weight. Measures of the left ventricular internal diameter in diastole (LVIDd) found that infarcted animals at both four and twenty weeks showed increased LVID when compared to age-matched controls (Figure 1C). Measurements of ejection fraction were made on all cohorts and showed that the ejection fraction was significantly reduced by infarction at both four and twenty weeks post-ligation (Figure 1D). Interestingly ejection fraction was further decreased when comparing four and twenty week infarcted cohorts from ~40% at four weeks to ~25% by twenty weeks.

There was evident hypertrophy in the model at four weeks post infarct (Figure 2A). This hypertrophy was not sustained when comparing to body weight of the animal at twenty weeks (Figure 2A). This is likely due to the large increase in body weight of the animals at twenty weeks (Figure 2B). Normalising heart weight to tibial length indicated that indeed increased heart weight is observed when correcting for animal size (Figure 2C). This indicated that the likely cause for body weight increase was oedema in the model at twenty weeks. Planimetry of both epicardial and endocardial layers in the mid left ventricle indicated that the scar that was formed post ligation was established by four weeks and was stable between four and twenty weeks (Figure 2D)

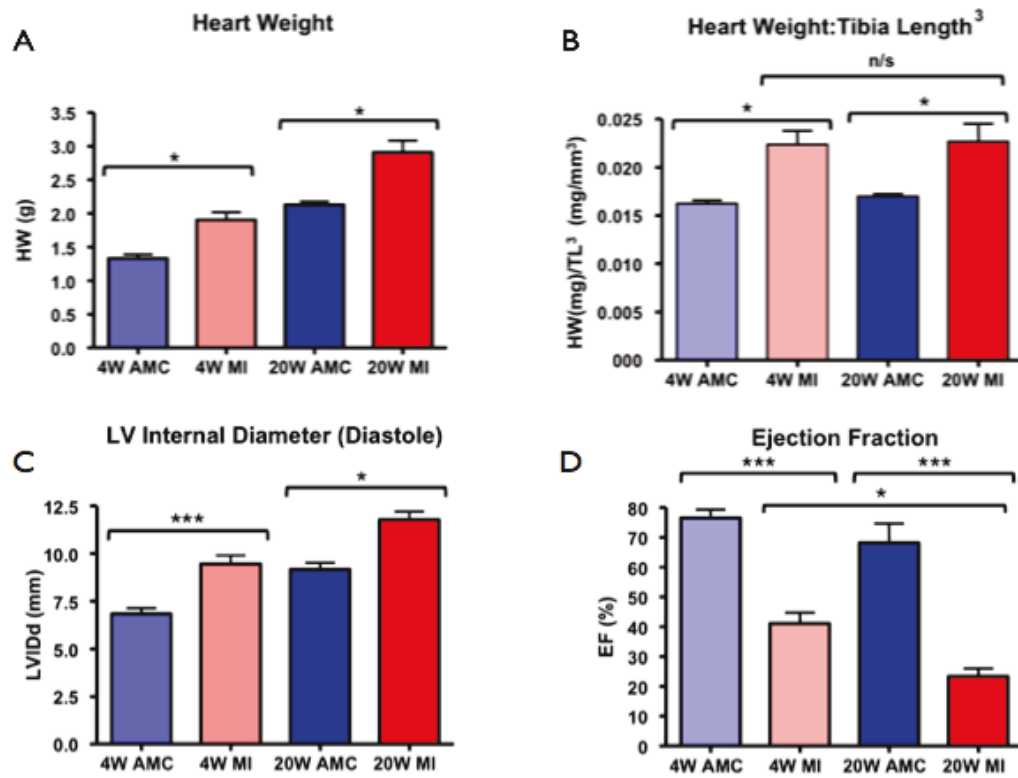


Figure 1: Biometric and echocardiographic measures of cardiac hypertrophy. (A) Heart weight measurements in grams. (B) Heart weight per Tibia length³ (HW:Tibia Length³) Measuring the degree of hypertrophy at both time points. (C) Measurement of left ventricular (LV) internal diameter indicating LV dilatation at both MI4W and MI20W. (D) Ejection fraction (EF) shows decline in MI4W and MI20W. (n=4-6 rats in each group, * p<0.05, *** p<0.001 by one-way ANOVA with Tukey's correction).

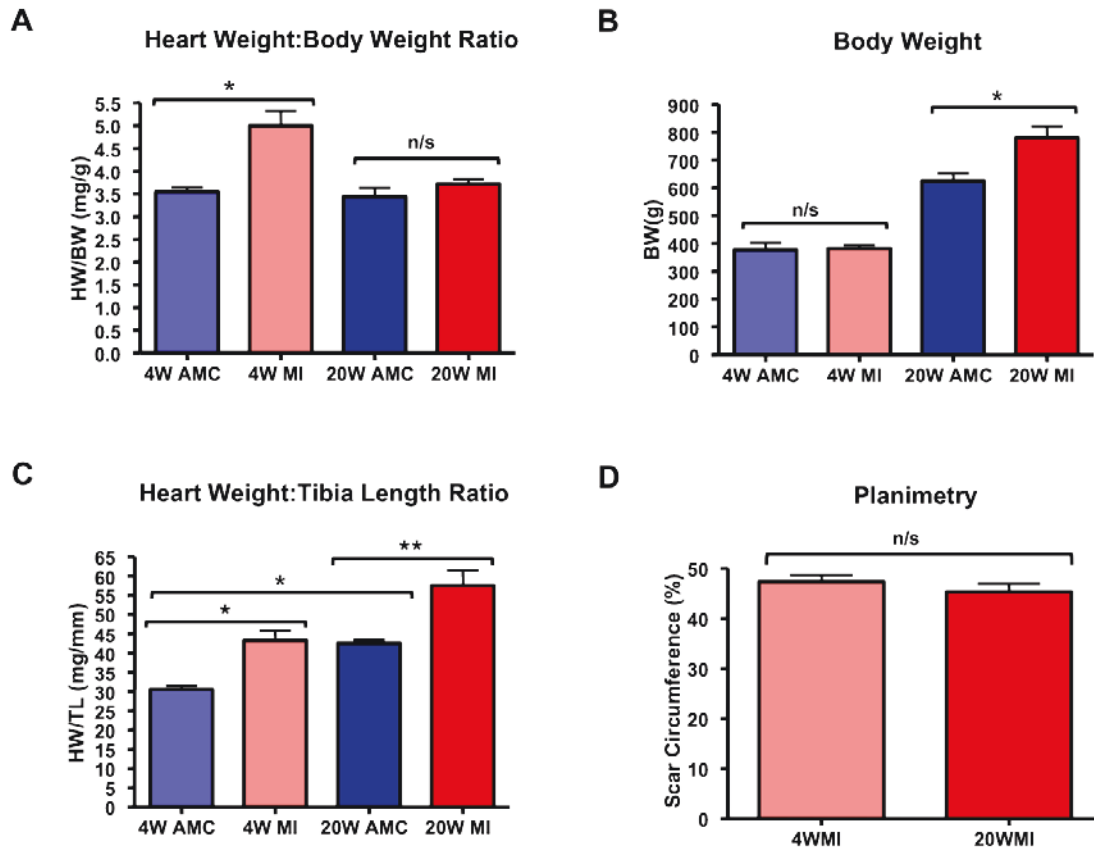


Figure 2: Further measures of hypertrophy and infarct size. (A) Heart weight (HW): Body weight ratio is significantly increased at 4WMI but not 20WMI stage in comparison to their age-matched controls. This may be because of (B) a significant increase in BW at 20WMI stage as compared to AMCs, which could relate to peripheral oedema. (C) HW: Tibia length (TL) ratio is significantly increased at both time-points but given the increase between HW:TL ratio even between 4WAMC and 20WAMC this may relate to division of mass (a measure of muscle volume) by tibia length (a linear measure of size). Since BW varies with the cube of TL. It may be best to correct HW by TL^3 (see main text). (D) Infarct size does not vary between 4WMI and 20WMI as measured by epicardial and endocardial planimetry of a mid-LV transverse section. (n=4-6 rats in each group, * $p<0.05$, ** $p<0.01$, n/s non-significant by one-way ANOVA with significance testing between groups using Tukey's correction (A-C), Student's t-test(D)).

Pressure volume loop analysis indicated the presence of impaired relaxation in diastole by twenty weeks post infarct (Figure 3). End diastolic pressure volume relationship (EDPVR) was raised significantly at twenty weeks indicating increased left ventricular stiffness (Figure 3A). Tau describing the left ventricular relaxation rate is increased at twenty weeks post-infarct (Figure 3B). At four weeks Tau is unaffected by infarction. The left ventricular end diastolic pressure is increased at twenty weeks post-infarct but unaffected at four weeks (Figure 3C). Energetic efficiency is reduced at four weeks in comparison to age-matched controls (Figure 3D). There is a further reduction in energetic efficiency by twenty weeks post-infarct. This data typifies a compensated phenotype at four weeks post-infarct and a phenotype of decompensation by twenty weeks.

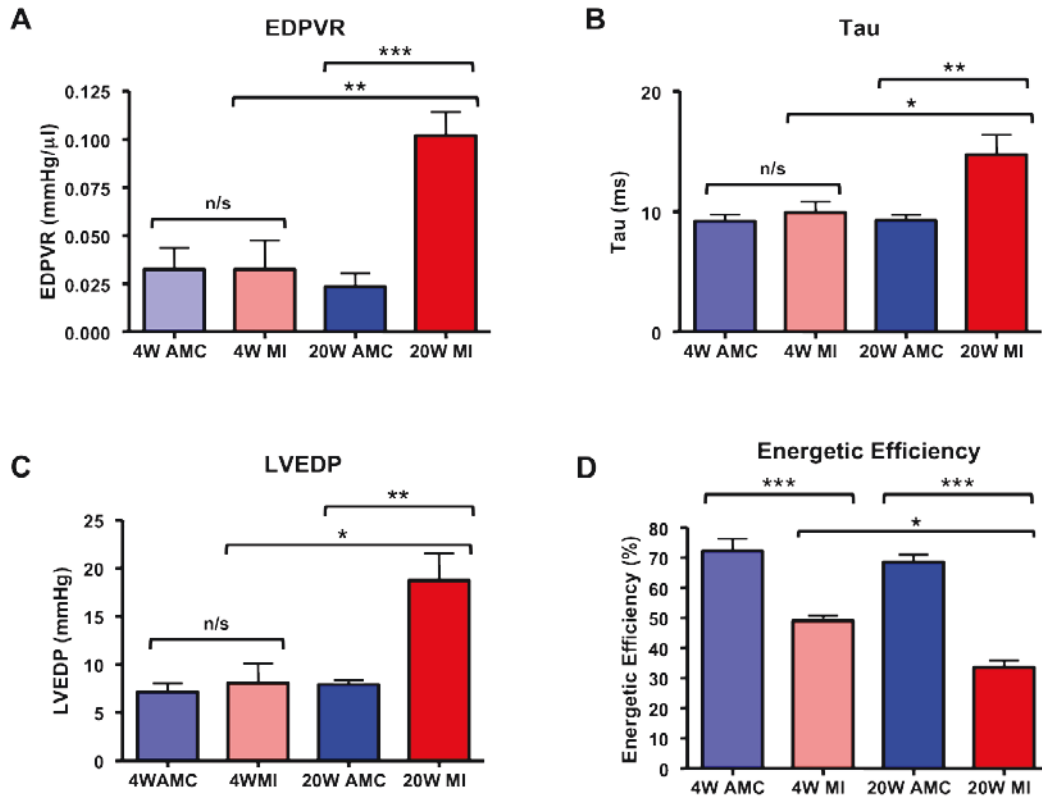


Figure 3: PV loop measurements of cardiac function and energetics. (A) End-diastolic pressure volume relationship (EDPVR) is unchanged between 4WAMC and 4WMI although at 20WMI time point there is a significant increase indicating increased LV stiffness. (B) Tau and (C) Left ventricular end diastolic pressure (LVEDP) are altered in a similar fashion indicating compensated diastolic cardiac function and preload at 4WMI time point but significant decompensation by 20W. (D) Energetic efficiency is reduced in 4WMI compared to 4WAMC. There is significant further decline by 20WMI time point (n=4-8 rats in each group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA with significance testing between groups using Tukey's correction).

Appendix B – Electron microscopy characterisation of the myocardial infarction model

Stereological quantification of myocardium from the rat infarct model was used to establish structural changes to tissue and changes in the volume occupied by different cellular components. Inspection of both four week and twenty week control tissues indicated normal sarcomeric structure, mitochondria and intercalated discs (ICD) (Figure 4A). Myocardium from four weeks post-infarct showed areas of sarcomere disruption, moderate ICD convolution, mitochondrial shrinkage and disruption (Figure 4B). Myocardium at twenty weeks post-infarct showed large-scale tissue disruption and sarcomeric disarray highly convoluted ICDs and elongated enlarged mitochondria (Figure 4C).

Stereological analysis of these tissue indicated that in uninfarcted controls at four weeks there was negligible evidence of cytoplasm and high proportions of healthy mitochondria and myofibrils (Figure 5A & E). Four weeks post-infarct there was a far higher proportion of cytoplasm and a much-reduced volume of healthy mitochondria (Figure 5B & E). Controls at twenty weeks showed a slightly higher proportion of cytoplasm and a small volume of degenerated mitochondria but was otherwise normal (Figure 5C & E). At twenty weeks post-infarct there was an increased volume of degenerated mitochondria and a high proportion of cytoplasm (Figure 5D & E).

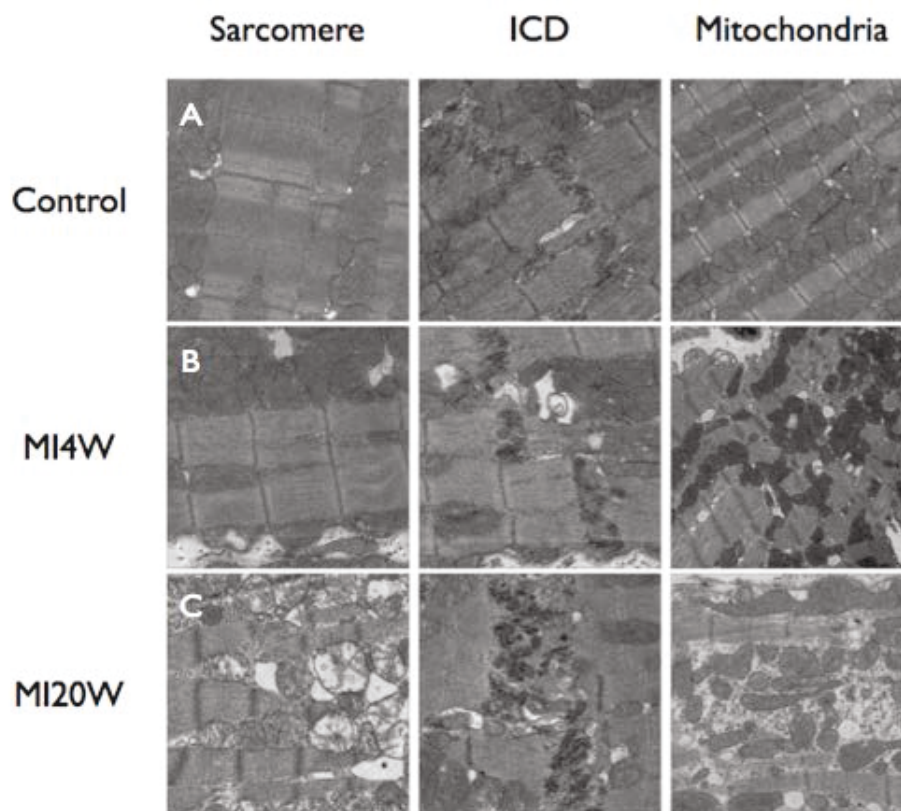


Figure 4: Electron micrographs of age-matched control (Control, A), MI4W (B) and MI20W (C). Columns show typical micrographs of sarcomere order, intercalated disc (ICD) organization and mitochondrial morphology (Mitochondria) of each treatment group.

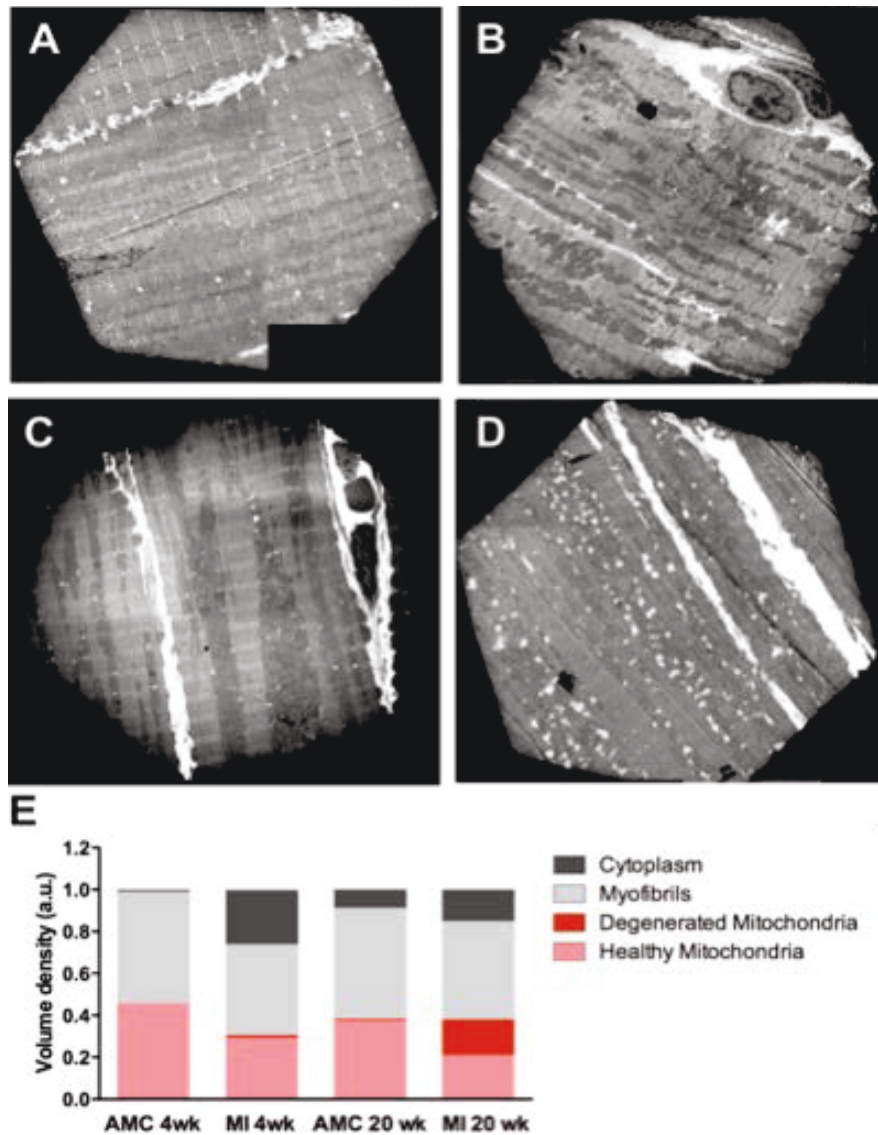


Figure 5: Stereological quantification of tissue organization. Reconstruction of a 34 x 30 μm tissue area using 9 micrographs of cardiac muscle in AMC4W (A), MI4W (B), AMC20W (C) and MI20W (D) rats. Images were acquired at 1000x magnification. Scale bar =250 nm. E, quantification of the volume density occupied by mitochondria, myofibrils and cytoplasm in A-D.