The Actomyosin ATPase Response to Stretch in Cardiac Muscle

By

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A thesis submitted for the degree of

Doctor of Philosophy of Imperial College London

and

Diploma of Imperial College London

March 2012

Molecular Medicine Section,
National Heart and Lung Institute,
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Declaration

The work contained in Chapter 4 has been submitted for publication to the Journal Circulation Research. All work contained in the publication is presented in this thesis. All work presented in this thesis is my own, unless stated otherwise. Contributions of others are mentioned in the acknowledgements section.
Abstract

The aim of this thesis was to examine the molecular mechanism underlying the force response to stretch in the heart. The properties of cardiac muscle were studied in rat trabeculae activated by laser-flash photolysis of NPE-caged ATP at 20°C. The rate of ATP hydrolysis was determined, on a millisecond time scale, using a fluorescently labelled phosphate binding protein to measure the rate of inorganic phosphate release.

The results show that an increase in sarcomere length causes an increase in isometric force with a similar response seen upon increasing thin filament activation. ATPase rate increases with thin filament activation, however, the increase in force with sarcomere length is not accompanied by a corresponding increase in ATPase rate. At a lower activation level a more substantial increase in isometric force is seen with increasing sarcomere length, compared to at a higher activation level. The results show that at a longer sarcomere length less ATP is used per unit of force produced.

Interestingly, stretch of an active muscle causes a substantial and instantaneous reduction in cross-bridge ATPase activity, even though force remains high. Conversely, active shortening resulted in an increase in the ATPase rate, above that of the isometric level. The stretch applied to the active trabeculae was greater than the reach of the cross-bridges therefore cross-bridge detachment must occur, however as force remains high this must be followed by rapid reattachment. As Pᵢ release is low during the stretch this detachment and reattachment cannot occur via the classical view of the cross-bridge cycle where attachment is preceded by ATP hydrolysis. To explain these results a branched cross-bridge cycle is suggested whereby rapid detachment and reattachment can occur without additional Pᵢ release and ATP hydrolysis.
Acknowledgements

I would like to thank my supervisors Professor Mike Ferenczi and Dr Timothy West for their support, guidance and expertise.

I would like to thank the following people at NIMR, London: Martin Webb, Jackie Hunter, John Corrie and Gordon Reid, for developing and perfecting the technique of labelling PBP with MDCC and for generously providing the NPE-caged ATP essential to the experiments described in this thesis. Thanks must also go to Irina Zalivina (NHLI, Imperial College) for her assistance with the production of PBP.

I would also like to thank the members of Professor Ferenczi’s laboratory past and present for their support and assistance and for making it an enjoyable experience: Dale Bickham, Marco Caremani, Weihua Song, Valentina Caorsi, Chris Toepfer, Dmitry Ushakov, So-Jin Holohan and Delisa Ibanez Garcia. Thanks also to Professors Nancy Curtin and Roger Woledge for their expertise. Finally I would like to thank my friends and family who have supported and encouraged me throughout my PhD.

I was supported by a studentship awarded by the National Heart and Lung Institute Foundation.
Contents

Declaration 2
Abstract 3
Acknowledgments 4
Contents 5
List of Figures 9
List of Tables 11
List of Equations and Schemes 12
List of Abbreviations 13

Chapter 1: Introduction 15

1.1 The Cardiac Cycle 16
1.2 The Structure of Cardiac Muscle 17
1.3 Sarcomeric Proteins 20
  1.3.1 Thick Filaments 20
  1.3.2 Thin Filaments 22
    1.3.2.1 Actin 22
    1.3.2.2 Tropomyosin 23
    1.3.2.3 Troponin 23
  1.3.3 Titin 24
1.4 Muscle Contraction 27
  1.4.1 Sliding Filament Model 27
  1.4.2 The Cross-bridge Cycle 27
1.5 Comparison Between Cardiac and Skeletal Muscle 30
  1.5.1 Structure 30
  1.5.2 Length-Force Relationship 31
1.6 The Frank-Starling Relationship 32
1.6.1 Possible Mechanisms of the Frank-Starling Relationship 34
1.7 Current Methods in Muscle Physiology 38
  1.7.1 Chemically Skinned Muscle 38
  1.7.2 Caged Compounds 38
  1.7.3 Phosphate Binding Protein 40
1.8 Context and Objectives of this Study 43

Chapter 2: Materials and Methods 44

2.1 Preparation of Trabeculae 45
  2.1.1 Dissection 45
  2.1.2 Chemical Skinning 46
2.2 Experimental Solutions 48
  2.2.1 Relaxing Solution 48
  2.2.2 Experimental Solutions 48
2.3 Muscle Activation by Laser-flash Photolysis of Caged-ATP 49
  2.3.1 Experimental Apparatus 49
  2.3.2 Laser and Hook Alignment 51
  2.3.3 Force Transducer Calibration 51
  2.3.4 Motor Calibration 51
  2.3.5 Trabecula Dimensions 54
  2.3.6 Sarcomere Length 55
  2.3.7 Experimental Protocol 56
    2.3.7.1 Pre-Activation 56
    2.3.7.2 Activation 57
2.4 Length Changes 58
2.5 Measurement of Actomyosin ATPase Rate 59
  2.5.1 Measurement of Inorganic Phosphate Release 59
  2.5.2 Determination of MDCC-PBP Activity and Fluorescence 61
  2.5.3 Determination of Myosin Active Site Concentration 63
2.6 Muscle Activation by Temperature-Jump 63
  2.6.1 Experimental Setup 64
Chapter 3: Isometric Contractions

3.1. Introduction 68
3.2. Isometric Contractions 68
  3.2.1. Force 68
  3.2.2. Pi Release 70
    3.2.2.1. Initial Rate of Pi Release 71
    3.2.2.2. Steady-state Rate of Pi release 71
    3.2.2.3. Force Economy 72
3.3. Sarcomere Length-Force Relationship and Passive Force 75
3.4. Force-pCa Relationship 77
3.5. Effect of Myosin Heavy Chain Isoform on Force and Pi Release 81
3.6. Discussion 85
  3.6.1. Validity of Preparation 85
  3.6.2. Effect of Sarcomere Length on Force and Pi Release 87
  3.6.3. Effect of Activation Level on Force and Pi Release 88

Chapter 4: Effect of Length Changes

4.1. Introduction 90
4.2. Effect of Length Changes on Active Force and Pi Release 90
  4.2.1. Force Response to Stretch 93
  4.2.2. Force Decay at End of Stretch 93
  4.2.3. Force Enhancement after Stretch 94
  4.2.4. Force during Shortening 97
  4.2.5. Pi Release Rates in Response to Length Changes 97
  4.2.6. Power and Efficiency 99
4.3. Effect of Length Changes in Relaxed Muscle 102
  4.3.1. Force 102
4.3.2. Fluorescence 103
4.4. Shortening Velocity and Power 107
4.4.1. Unloaded Shortening Velocity 107
4.4.2. Force-Velocity Relationship 110
4.4.3. Power-Velocity Relationship 114
4.5. Discussion 116
4.5.1. Response to Stretch 116
4.5.2. Response to Shortening 119

Chapter 5: Effect of ATP Depletion and ADP Accumulation 122

5.1. Introduction 123
5.2. Methods 124
5.3. Length Changes with an ATP Regenerating System 125
5.3.1. Force 125
5.3.2. P_i Release Rate 129
5.4. Discussion 132

Chapter 6: General Discussion 134

6.1. Summary and General Discussion of Results 135
6.1.1. Summary and Discussion of Results 136
6.1.1.1. Effect of Passive Length Changes 136
6.1.1.2. Effect of Length Changes in Contracting Muscle 137
6.1.2. Relevance of the Study 140
6.2. Limitations of the Study 140
6.3. Future Work 142

References 145
Appendix A – Solutions 159
Appendix B – Production of MDCC-PBP 163
| Figure 1.1: | The cardiac cycle | 18 |
| Figure 1.2: | Structure of the sarcomere | 19 |
| Figure 1.3.1: | Structure of the thick filament | 21 |
| Figure 1.3.2: | Structure of the thin filament | 25 |
| Figure 1.3.3: | Passive and restoring force generation by titin | 26 |
| Figure 1.4.2: | The cross-bridge cycle | 28 |
| Figure 1.5.2: | The length-force relationship | 33 |
| Figure 1.6: | The Frank-Starling relationship | 35 |
| Figure 1.6.1: | Compression of myofilament lattice with stretch | 37 |
| Figure 1.7.3: | Structure of MDCC-PBP | 42 |
| Figure 2.1.1: | Trabecula preparation | 47 |
| Figure 2.3.1: | Experimental apparatus for muscle activation by laser flash photolysis of NPE-caged ATP | 50 |
| Figure 2.3.3: | Force transducer calibration | 52 |
| Figure 2.3.4: | Motor calibration | 53 |
| Figure 2.5.2: | Fluorescence change of MDCC-PBP upon titration of phosphate | 62 |
| Figure 2.6.1: | Experimental apparatus for muscle activation by temperature-jump | 65 |
| Figure 3.2: | Effect of sarcomere length and activation level on isometric force and P_i release rate | 73 |
| Figure 3.3: | Sarcomere length-force relationship | 76 |
| Figure 3.4.1: | Typical temperature-jump activation records | 79 |
| Figure 3.4.2: | Force-pCa relationship | 80 |
| Figure 3.5: | Isometric force and P\textsubscript{i} release in rabbit cardiac and skeletal muscles | 83 |
| Figure 4.2: | Effect of length changes on force and P\textsubscript{i} release | 91 |
| Figure 4.2.1: | Force response to stretch | 95 |
| Figure 4.2.2: | Force decay after stretch | 96 |
| Figure 4.2.5.1: | P\textsubscript{i} release rates at each stage of the length change protocol | 100 |
| Figure 4.2.5.2: | P\textsubscript{i} release rate response to stretch | 101 |
| Figure 4.3.1: | Force response to length changes in relaxed muscle | 105 |
| Figure 4.3.2: | Effect of length changes on fluorescence in relaxed muscle | 106 |
| Figure 4.4.1.1: | Typical slack-test recording | 108 |
| Figure 4.4.1.2: | Slack-test determination of unloaded shortening velocity | 109 |
| Figure 4.4.2.1: | Typical recording of force-velocity measurement | 111 |
| Figure 4.4.2.2: | Force-velocity relationship | 112 |
| Figure 4.4.3: | Power-velocity relationship | 115 |
| Figure 5.3: | Effect of length changes on force and P\textsubscript{i} release with an ATP regenerating system | 127 |
| Figure 5.3.1: | Effect of ATP regenerating system on force | 130 |
| Figure 5.3.2: | Effect of ATP regenerating system on P\textsubscript{i} release rate | 131 |
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.3.7.2</td>
<td>Solution incubation times for laser activation</td>
<td>58</td>
</tr>
<tr>
<td>Table 3.2</td>
<td>Summary of results of isometric contractions in rat cardiac trabeculae</td>
<td>74</td>
</tr>
<tr>
<td>Table 3.5</td>
<td>Summary of results of isometric contractions in rabbit cardiac and skeletal muscles</td>
<td>84</td>
</tr>
<tr>
<td>Table 4.2</td>
<td>Summary of results of length change experiments</td>
<td>92</td>
</tr>
<tr>
<td>Table 4.4</td>
<td>Parameters of force-velocity relationship</td>
<td>113</td>
</tr>
<tr>
<td>Table 4.5.2</td>
<td>Ratio of $P_i$ release rates at 1 and 32µM $Ca^{2+}$</td>
<td>121</td>
</tr>
<tr>
<td>Table 5.2</td>
<td>Solution incubation times for laser activation using an ATP regenerating system</td>
<td>124</td>
</tr>
<tr>
<td>Table 5.3</td>
<td>Summary of results of length change experiments with an ATP regenerating system</td>
<td>128</td>
</tr>
<tr>
<td>Scheme</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1.4.2</td>
<td>The cross-bridge cycle</td>
<td>29</td>
</tr>
<tr>
<td>1.8.1</td>
<td>Photolytic release of ATP from NPE-caged ATP</td>
<td>39</td>
</tr>
<tr>
<td>2.3.5</td>
<td>Area of an ellipse</td>
<td>54</td>
</tr>
<tr>
<td>2.3.6</td>
<td>Bragg’s Law</td>
<td>55</td>
</tr>
<tr>
<td>2.5.1</td>
<td>Calculation of P_i release from fluorescence signal</td>
<td>59</td>
</tr>
<tr>
<td>2.5.1</td>
<td>P_i binding to PBP</td>
<td>60</td>
</tr>
<tr>
<td>2.5.2</td>
<td>Determination of MDCC-PBP activity</td>
<td>61</td>
</tr>
<tr>
<td>3.4</td>
<td>Force-pCa relationship: Hill Equation</td>
<td>77</td>
</tr>
<tr>
<td>4.4.2</td>
<td>Force-velocity relationship: Hill’s Hyperbolic Equation</td>
<td>110</td>
</tr>
<tr>
<td>4.5.1</td>
<td>The cross-bridge cycle – Accumulation of P_i containing cross-bridge states</td>
<td>119</td>
</tr>
<tr>
<td>5.1</td>
<td>Phosphorylation of creatine by creatine phosphokinase</td>
<td>123</td>
</tr>
<tr>
<td>5.2</td>
<td>Phosphorylation of free P_i by 7-MEG and PNPase</td>
<td>125</td>
</tr>
</tbody>
</table>
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-band</td>
<td>Anisotropic Band</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>AP</td>
<td>Aortic Pressure</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine Triphosphatase</td>
</tr>
<tr>
<td>BDM</td>
<td>2,3-butanedione monoxime</td>
</tr>
<tr>
<td>[Ca^{2+}]_{i}</td>
<td>Intracellular Calcium Concentration</td>
</tr>
<tr>
<td>CPK</td>
<td>Creatine Phosphokinase</td>
</tr>
<tr>
<td>Cr</td>
<td>Creatine</td>
</tr>
<tr>
<td>CrP</td>
<td>Creatine Phosphate</td>
</tr>
<tr>
<td>CSA</td>
<td>Cross-Sectional Area</td>
</tr>
<tr>
<td>DDIW</td>
<td>Double De-ionised Water</td>
</tr>
<tr>
<td>EGTA</td>
<td>1,2-Di(2-aminoethoxy)ethane-(N,N,N',N'')-tetra-acetic acid</td>
</tr>
<tr>
<td>f-actin</td>
<td>Filamentous Actin</td>
</tr>
<tr>
<td>g-actin</td>
<td>Globular Actin</td>
</tr>
<tr>
<td>HDTA</td>
<td>1,6-Diaminohexane-(N,N,N',N'')-tetraacetic acid</td>
</tr>
<tr>
<td>HMM</td>
<td>Heavy Meromyosin</td>
</tr>
<tr>
<td>I-band</td>
<td>Isotropic Band</td>
</tr>
<tr>
<td>KH</td>
<td>Krebs-Henseleit</td>
</tr>
<tr>
<td>Kprop</td>
<td>Potassium Propionate</td>
</tr>
<tr>
<td>LAP</td>
<td>Left Atrial Pressure</td>
</tr>
<tr>
<td>LDA</td>
<td>Length Dependent Activation</td>
</tr>
<tr>
<td>L_{\text{max}}</td>
<td>Length at which maximum force is produced</td>
</tr>
<tr>
<td>LMM</td>
<td>Light Meromyosin</td>
</tr>
<tr>
<td>LVEDV</td>
<td>Left Ventricular End Diastolic Volume</td>
</tr>
<tr>
<td>LVESV</td>
<td>Left Ventricular End Systolic Volume</td>
</tr>
<tr>
<td>LVP</td>
<td>Left Ventricular Pressure</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>-----------------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>MDCC-PBP</td>
<td>(N-(2[1-maleimidyl]ethyl)-7-diethylamino-coumarin-3-carboxamide phosphate binding protein</td>
</tr>
<tr>
<td>MHC</td>
<td>Myosin Heavy Chain</td>
</tr>
<tr>
<td>ML</td>
<td>Muscle lengths</td>
</tr>
<tr>
<td>MyBP-C</td>
<td>Myosin Binding Protein-C</td>
</tr>
<tr>
<td>NADH</td>
<td>Reduced Nictotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>NPE-caged ATP</td>
<td>$P^3$-[2-(2-nitrophenyl)]ethyl ester of ATP</td>
</tr>
<tr>
<td>PBP</td>
<td>Phosphate Binding Protein</td>
</tr>
<tr>
<td>pCa</td>
<td>$-\log[Ca^{2+}]$</td>
</tr>
<tr>
<td>pCa_{50}</td>
<td>$[Ca^{2+}]$ at which 50% maximum force is produced</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic Phosphate</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenyl Methyl Sulfonyl Fluoride</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions Per Minute</td>
</tr>
<tr>
<td>S1</td>
<td>Myosin Sub-fragment 1</td>
</tr>
<tr>
<td>S2</td>
<td>Myosin Sub-fragment 2</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarcoplasmic/Endoplasmic Reticulum Ca$^{2+}$-ATPase</td>
</tr>
<tr>
<td>SL</td>
<td>Sarcomere Length</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic Reticulum</td>
</tr>
<tr>
<td>$t_{1/2}$</td>
<td>Time at which force is half maximal isometric force</td>
</tr>
<tr>
<td>TES</td>
<td>$N\text{-}tris$(Hydroxymethyl)ethyl-2-aminoethanesulphonic acid</td>
</tr>
<tr>
<td>TnC</td>
<td>Troponin C (Calcium)</td>
</tr>
<tr>
<td>TnI</td>
<td>Troponin I (Inhibitory)</td>
</tr>
<tr>
<td>TnT</td>
<td>Troponin T (Tropomyosin)</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction
1.1. The Cardiac Cycle

The purpose of the heart is to pump blood around the systemic and pulmonary circulatory systems. The heart is therefore vital to sustaining life and thus it is important to understand the mechanisms involved in the normal functioning of the heart. The repeating cycles of contraction and relaxation seen in the heart are referred to as the cardiac cycle (Figure 1.1). The relaxed state is known as diastole, where the ventricles fill with blood, and contraction is known as systole, where blood is pumped out of the ventricles. The cardiac cycle can be further divided up into 4 phases: ventricular filling, isovolumic contraction, ejection and isovolumic relaxation (Figure 1.1.B). Ventricular filling occurs during diastole when the ventricles are relaxed. Isovolumic contraction occurs at the beginning of systole and is characterised by an increase in left ventricular pressure (LVP) with no change in volume. LVP continues to rise until it exceeds aortic pressure, at which point ejection begins and left ventricular volume decreases. At the beginning of diastole, isovolumic relaxation occurs resulting in a decrease in LVP with no change in left ventricular volume. As LVP falls below left atrial pressure, blood flows from the left atrium to the left ventricle causing ventricular filling and completing the cycle (Klabunde, 2011).

The cardiac cycle is initiated by spontaneous depolarisations of the sinoatrial node, a group of pacemaker cells located in the right atrium. Waves of depolarisation spread throughout the heart resulting in contraction. The coupling between depolarisation and contraction is known as excitation-contraction coupling and is reviewed by Bers (2002). Depolarisation causes the opening of voltage-activated Ca\(^{2+}\) channels resulting in the influx of Ca\(^{2+}\) into the sarcoplasm, prolonging the action potential plateau. This influx of Ca\(^{2+}\) activates ryanodine receptors on the sarcoplasmic reticulum (SR) causing a further increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) due to Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores, a process known as calcium-induced calcium release. Calcium influx results in activation of the contractile machinery of the muscle cell (as described in Section 1.4). For relaxation
to occur $[\text{Ca}^{2+}]$, must decrease, requiring transport of $\text{Ca}^{2+}$ out of the sarcoplasm. This can occur via the sarcolemmal $\text{Na}^{\text{+}}/\text{Ca}^{2+}$ exchanger, sarcolemmal $\text{Ca}^{2+}$-ATPase (Adenosine triphosphatase) or the SR $\text{Ca}^{2+}$-ATPase (SERCA).

1.2. The Structure of Cardiac Muscle

Cardiac muscle consists of single elongated cells (cardiomyocytes) connected to each other at intercalated discs, allowing the transmission of force from one cell to another. Gap junctions on the intercalated disc allow ions to pass through and electrical impulses to pass between cardiomyocytes. The sarcomere is the smallest functional unit of a muscle cell, the structure of which is shown in Figure 1.2. Many sarcomeres are arranged in series along the length of the cell producing its characteristic striated appearance.

The sarcomeres of striated muscle (cardiac and skeletal muscles) are highly organised structures consisting of overlapping thick (myosin) and thin (actin) filaments. The sarcomeres are connected to each other by the Z-line which connects actin filaments from adjacent sarcomeres allowing transmission of force along the muscle. Adjacent myosin filaments are cross-linked by the M-line at the centre of the sarcomere. The region of the sarcomere containing myosin filaments is called the A-band (anisotropic) and has a higher protein density than the I-band (isotropic), actin-containing regions where no myofilament overlap occurs.

Another myofilament protein, titin, spans half the sarcomere connecting the Z-line to the M-line and binding to both actin and myosin filaments. Many other smaller proteins are also found in the sarcomere, with most located in the Z-line and M-line, which contribute to maintaining the structure of the sarcomere.
Figure 1.1: The cardiac cycle. A: Plots of left ventricular pressure (LVP), aortic pressure (AP), left atrial pressure (LAP), left ventricular end diastolic volume (LVEDV) and left ventricular end systolic volume (LVESV) changes throughout the cardiac cycle. Numbers across the top represent the different phases of the cardiac cycle: (1) Ventricular filling, (2) Isovolumic contraction, (3) Ejection, (4) Isovolumic relaxation (Klabunde, 2011). B: A pressure-volume loop for the cardiac cycle. Point A represents the end of diastole and point C the end of systole (Lionel, 2005).
Figure 1.2: A: Electron micrograph of the rat cardiac sarcomere (kindly provided by P. Luther, Imperial College London). B: Representation of the arrangement of myofilament proteins in the cardiac sarcomere (adapted from Labeit and Kolmerer, 1995).
1.3. Sarcomeric Proteins

1.3.1. Thick Filaments

Thick filaments are formed from helices of the protein myosin-II. The myosin molecule consists of two myosin heavy chains (MHC), with molecular masses of ~200 kDa each, each of which is associated with an essential light chain and a regulatory light chain, of ~20 kDa each (Figure 1.3.1.A; Sellers et al., 1995).

Proteolytic cleavage of the myosin molecule produces two fragments called heavy meromyosin (HMM) and light meromyosin (LMM). LMM consists of much of the C-terminal tail region of the myosin molecule which is arranged in an extended coiled-coil structure and forms the backbone of the thick filament. Further enzymatic digestion of HMM yields two fragments called sub-fragment 1 (S1) and sub-fragment 2 (S2) (Weeds and Lowey, 1971). S2 is the remainder of the coiled-coil backbone, whereas S1 is the N-terminal head region which forms a globular structure, projecting out from the thick filament backbone and containing actin and nucleotide binding sites. The S1 region forms the motor domain which drives muscle contraction.

The myosin molecules in each thick filament are oriented so that the S1 heads point towards the end of the filament and the tails point towards the centre (Figure 1.3.1.B). This results in a bipolar thick filament with a bare region in the centre, where no myosin heads are present, known as the M-region. This arrangement is important for giving the contractile apparatus directionality, ensuring that thin filaments are pulled towards the centre of the sarcomere from both ends.

Two myosin heavy chain isoforms are found in cardiac muscle, α-MHC and β-MHC, with the two isoforms exhibiting different levels of ATPase activity (described in Section 1.4). α-MHC has a three-fold higher ATPase activity and a higher affinity for actin than β-MHC, thus, hearts expressing α-MHC exhibit increased contractility.
Figure 1.3.1: Structure of the thick filament. A: Structure of a single myosin molecule (Voet and Voet, 1995). B: Arrangement of myosin molecules in the thick filament.
relative to those expressing β-MHC (Pope et al., 1980; McNally et al., 1989). Small mammals, such as mice and rats, express predominantly α-MHC in the ventricles throughout adulthood, whereas larger mammals, such as rabbits, pigs and humans, express predominantly β-MHC (Lompre et al., 1981; Malmqvist et al., 2004). However levels of β-MHC significantly increase in the rat ventricle with ageing (Fitzsimons et al., 1999).

1.3.2. Thin Filaments

The thin filament, extending from the Z-line and ending in the A-band, consists of three components: actin, troponin and tropomyosin (Figure 1.3.2.C). Actin forms the backbone of the thin filament, and the troponin complex and tropomyosin are regulatory proteins associated with the thin filament.

1.3.2.1. Actin

Actin can exist in two forms, globular (g-actin) and filamentous (f-actin). G-actin is a 42kDa protein that exists at low ionic strength and is stabilised by adenosine triphosphate (ATP) binding (Pardee and Spudich, 1982). The g-actin molecule is composed of four subdomains surrounding a nucleotide binding pocket. Increasing the ionic strength to physiological levels causes g-actin to polymerise into f-actin.

The actin filaments themselves are two chains, consisting of 13 monomers, arranged in a double helix which repeats every 36nm (Figure 1.3.2.A). The smaller subdomain (subdomain 1, containing both the N- and C-termini) is located at the periphery of the filament and is available for interaction with myosin (Geeves and Holmes, 1999). In f-actin the monomers of both filaments are arranged with a polarity which is important in determining the manner in which myosin interacts with the filament. Thus, if f-actin is allowed to interact with myosin S1 in vitro the myosin molecules will all be pointing in the same direction (Moore et al., 1970). This
results in the ‘decorated’ f-actin having a so-called arrowhead appearance, with one barbed end and one pointed end. The actin filament is anchored at the barbed end, to the Z-line, by the actin-binding protein α-actinin.

1.3.2.2. Tropomyosin

Tropomyosin is a dimer of two identical α-helical polypeptide chains, with a molecular weight of ~33 kDa each, coiled around each other to form a coiled-coil structure. Each tropomyosin molecule is ~42nm long and lies along the thin filament near the groove in the actin helix (Figure 1.3.2.C). There is one tropomyosin on each side of the thin filament, with each tropomyosin molecule spanning 7 actin monomers. Neighbouring tropomyosin molecules are arranged in an overlapping head-to-tail configuration which repeats every 38.5nm.

Tropomyosin regulates actin-myosin interactions sterically. At low \([\text{Ca}^{2+}]\), the tropomyosin molecules lie outside the groove of the actin helix, blocking the myosin binding site on actin. An increase in \([\text{Ca}^{2+}]\) causes a conformational change resulting in tropomyosin moving into the actin helix groove. This exposes some myosin binding sites on actin allowing myosin heads to bind. Such binding stabilises tropomyosin causing exposure of more myosin binding sites on adjacent actin molecules (McKillop and Geeves, 1993; Gordon et al., 2001).

1.3.2.3 Troponin

The conformation of tropomyosin, and therefore the ability of myosin to interact with actin, is regulated by the troponin complex. Troponin is a complex of three proteins, troponin-C (TnC), troponin-T (TnT) and troponin-I (TnI), that are attached to each tropomyosin molecule and confer the calcium sensitivity (Figure 1.3.2.B).
TnC (~18 kDa) is the calcium-binding subunit of the troponin complex, TnT (31 kDa) binds to tropomyosin to form the troponin-tropomyosin complex and TnI (21 kDa) is an inhibitory subunit, which binds to actin to hold the troponin-tropomyosin complex in place. Binding of Ca^{2+} to TnC causes a conformational change in TnI. This leads to dissociation of TnI from actin allowing tropomyosin to move into the actin helix groove, exposing the myosin binding sites on actin (Farah and Reinach, 1995; Zhang et al., 2011).

1.1.1. Titin

The giant elastic protein titin (3-3.7 MDa) spans half of the sarcomere (~1µm) connecting the Z-line to the M-line (Furst et al., 1988). The A-band region of titin contains sequences of repeating immunoglobulin and fibronectin domains. A-band titin forms an integral part of the thick filament and it is estimated that six titin molecules bind to each half of the thick filament (Cazorla et al. 2000; Liversage et al. 2001) with the main titin binding proteins being myosin and myosin binding protein C (Houmeida et al. 1995; Freiburg & Gautel 1996). The I-band region of titin contains mainly immunoglobulin-like domains with extensible PEVK regions (rich in proline, glutamate, valine and lysine residues). This extensible region is important for maintaining passive stiffness, keeping the thick filaments centred within the sarcomere and providing a restoring force during shortening (Figure 1.3.3).

Two titin isoforms exist in the mammalian heart. Smaller mammals such as the rat and mouse express mainly the shorter and stiffer N2B isoform with larger mammals expressing both the N2B isoform and the longer and more compliant N2BA isoform (Granzier and Labeit, 2004).
Figure 1.3.2: Structure of the thin filament. A: The structure of f-actin (Geeves and Holmes, 1999). B: The crystal structure of human cardiac troponin (Davis and Tikunova, 2008). TnC is shown in grey, TnI in yellow, TnT in green and calcium in black. C: Schematic representation of the arrangement of thin filament proteins (Gordon et al., 2000).
Figure 1.3.3: Passive and restoring force generation by titin. At a slack length (B), titin’s extensible region is in a shortened state and extends with sarcomere stretch (C and D) producing a passive force. Shortening below slack length (A) extends titin’s extensible region in the opposite direction producing a restoring force (Granzier and Labeit, 2004).
1.2. **Muscle Contraction**

1.2.1. *Sliding Filament Model*

The sliding filament theory of muscle contraction was independently developed by A. Huxley and Niedergerke and by H. Huxley and Hanson in 1954. Using light microscopy, it was observed that during stretching and shortening of a muscle fibre the length of the A-band remains constant, whilst the length of the I-band changes. As the length of the myofilaments change very little during contraction it was proposed that shortening of the sarcomere is due the relative sliding of the thick and thin filaments.

1.2.2. *The Cross-bridge Cycle*

The filament sliding, seen during muscle contraction, is caused by the interaction of myosin heads with actin, a process regulated by calcium and fuelled by the hydrolysis of Mg-ATP. This process is known as the cross-bridge cycle and is shown in Figure 1.4.2 and Scheme 1.4.2 (Reviewed by Cooke, 1997). The experiments performed by Lymn and Taylor in 1971, using HMM and actin, relate force production to the biochemical reactions that occur during ATP hydrolysis.

In the absence of ATP the myosin head binds tightly to actin at an angle of 45°, producing a state called rigor. The binding of ATP to the myosin head results in the very rapid dissociation of myosin from actin. This is followed by the hydrolysis of ATP to adenosine diphosphate (ADP) and inorganic phosphate (Pₐ) by the myosin ATPase. The myosin head then re-binds to actin at an angle of 90° with ADP and Pₐ still attached in the nucleotide binding region of the myosin head. Most evidence indicates that force is produced by the strongly bound A.M.ADP.Pₐ state. Subsequently Pₐ is released as the force-bearing A.M.ADP state is formed. The attached cross-bridge then releases ADP and undergoes a conformational change back to an angle of 45°.
Figure 1.4.2: The cross-bridge cycle (Spudich, 2001). Shows the relationship between the mechanical and biochemical steps thought to be involved in force production. The actin filament is shown in red, detached myosin heads in green and attached myosin heads in pink.
The process of myosin attachment and head movement is called the ‘power stroke’. The myosin head is then ready to re-bind ATP and repeat the cycle. The release of reaction products during the power stroke is believed to be the rate-limiting step of contraction (Lionne et al., 2002).

**Scheme 1.4.2:** Schematic representation of the cross-bridge cycle during isometric contraction (West et al., 2009). Italicised states represent post-isomerisation states. A, actin; M, myosin.
1.3. Comparison Between Cardiac and Skeletal Muscle

Much of what we know about striated muscle function has been derived from studies on skeletal muscle as this is easier to study than cardiac muscle. As the ultra-structure of skeletal and cardiac muscle is very similar the knowledge obtained from skeletal preparations is often extrapolated to cardiac muscle, therefore it is important to recognise the similarities and differences between the two muscle types.

1.3.1. Structure

Cardiac myocytes contain one or two nuclei and are relatively small compared to the larger multi-nucleated skeletal muscle cells. Cardiac muscle cells depend entirely on aerobic metabolism to provide them with the energy they need, whereas skeletal muscle utilises both aerobic and anaerobic metabolism. For this reason cardiac muscle contains a far greater number of mitochondria.

X-ray diffraction studies show that the arrangement of the myofilament proteins is very similar between cardiac and skeletal muscle (Matsubara and Millman, 1974) although different isoforms of the proteins are found in different muscle types. The arrangement of cardiac muscle cells and the myofibrils within the cells is less organised than that of skeletal muscle. The larger number of mitochondria and less organised structure of cardiac muscle results in a reduced amount of the contractile machinery being present per given volume, compared to skeletal muscle.
1.3.2. Length-Force Relationship

The force produced by a muscle is dependent on its sarcomere length, due to changes in the degree of myofilament overlap and therefore the number of actin-myosin interactions that can occur (Gordon et al., 1966). In skeletal muscle, force decreases linearly with sarcomere length above 2.2µm, reaching zero at 3.65µm where thick and thin filament overlap ceases. This is known as the descending limb of the sarcomere length-force relationship. Between 2.0 and 2.2µm force is constant at its maximum. This corresponds to the lengths over which the thin filaments cross the bare zone, at the centre of the sarcomere, where no cross-bridges are present. At sarcomere lengths below 2.0µm force decreases and this is known as the ascending limb.

The ascending limb of the skeletal and cardiac sarcomere length-force relationships is shown in Figure 1.5.2. Cardiac muscle possesses greater stiffness than skeletal muscle, probably due to the higher amounts of collagen present in cardiac muscle and the presence of the stiffer titin isoform (Granzier and Irving, 1995). This normally prevents its sarcomeres being stretched beyond 2.2µm, therefore cardiac muscle does not display a descending limb on the sarcomere-length force relationship. In cardiac muscle the slope of the ascending limb varies depending on inotropic state, therefore the shape of the ascending limb is not solely dependent on myofilament lattice geometry, as in skeletal muscle. Within the physiological length ranges, the cardiac force-length curve is much steeper than the skeletal force-length curve, despite the myofilament lengths being similar. This steep ascending limb of the cardiac length-force curve is a feature of the Frank-Starling relationship.
1.4. The Frank-Starling Relationship

In 1895, Otto Frank showed that when he clamped the aorta of an isolated frog heart to render subsequent contractions isovolumic, the systolic pressure increased with diastolic volume (Chapman and Wasserman, 1960). Subsequently in 1918, Ernest Starling demonstrated, in perfused dog hearts, that the stroke volume of the right ventricle increased as soon as the filling pressure was raised. These discoveries were termed the Frank-Starling relationship.

The Frank-Starling relationship is an intrinsic property of cardiac muscle which describes how stretching of the ventricles due to a greater end-diastolic volume results in a greater systolic contraction force (Allen and Kentish, 1985). The effect of cardiac filling on the power output of the heart is important as it contributes to adjusting cardiac output to the body’s needs in an adaptive, beat-to-beat regulation of cross-bridge mechanics and energetics.

The sarcomere length-force relationship (Figure 1.5.2) for skeletal muscle can be explained by the overlap of thick and thin filaments (as described in the previous section). However, the ascending limb of the curve is steeper for cardiac muscle than skeletal muscle, and force can still increase over the sarcomere length range where myofilament overlap remains constant (2.0-2.2 µm). Kentish and Wrzosek (1998) showed that only 40% of the increase in force following stretch is due to myofilament overlap and restoring forces. This means that other mechanisms, not just myofilament overlap, must play an important role in the cardiac length-force relationship.

In 1982, Allen and Kurihara injected the Ca$^{2+}$-sensitive protein aequorin into trabeculae from cat ventricles and made the first observations of force and Ca$^{2+}$-transients in response to length changes (Figure 1.6). Immediately following an increase in muscle length there was a large increase in force with no significant
Figure 1.5.2: Illustration of the ascending limb of the length-force relationship in cardiac and skeletal muscle. Length has been normalised to that at which force is maximal ($L_{\text{max}}$) for each muscle type. Within the physiological length range (80-100% $L_{\text{max}}$) the cardiac force-length curve is much steeper than the skeletal force-length curve, despite myofilament lengths being similar (Fuchs and Smith, 2001).
change in the calcium transient. A further slow increase in force is seen, in the 10 minutes following stretch. This slow increase in force accounts for ~20-30% of the total force increase and is accompanied by an increase in the Ca\(^{2+}\) transient. Thus, the slow force response to stretch involves changes in intracellular Ca\(^{2+}\) homeostasis. The Frank-Starling relationship relates to the initial fast response to stretch.

1.4.1. Possible Mechanisms of the Frank-Starling Relationship

The molecular mechanisms underlying the Frank-Starling relationship are not yet fully understood. The change in myofilament force upon stretch is intrinsic to the sarcomere and is often called myofilament length-dependent activation (LDA). Several factors have been proposed which may contribute to this LDA (Stelzer and Moss, 2006; Shiels and White, 2008).

The experiments of Allen and Kurihara (1982, described previously) show that the rapid increase in force following stretch is not accompanied by an increase in the calcium transient, indicating that an increase in myofilament Ca\(^{2+}\)-sensitivity occurs. Also, in 1982, Hibberd and Jewel demonstrated that an increase in sarcomere length resulted in a leftward shift of the force-pCa curve in skinned rat trabeculae, providing further evidence for an increase in Ca\(^{2+}\)-sensitivity. However, the mechanisms behind this increase in myofilament Ca\(^{2+}\)-sensitivity are yet to be fully determined. Several processes may work together to achieve the total response.

There is evidence to suggest that Ca\(^{2+}\)-sensitivity is partly increased by strong cross-bridge binding (Smith et al., 2009). This process is termed thin filament activation or cooperativity. Formation of strongly-bound cross-bridges results in a further shift of the tropomyosin molecule into the actin groove increasing the probability of more myosin heads binding (Fitzsimons and Moss, 1998). It is also thought that the formation of strongly bound cross-bridges may result in an increased affinity of TnC for Ca\(^{2+}\) (Gordon and Ridgway, 1993).
Figure 1.6: Effect of length changes on force and the amplitude of the calcium transient in intact cat trabeculae. A: Upper panel shows the calcium transients measured by aequorin and lower panel shows force. The muscle was held at $L_{\text{max}}$ (i), released to 82% $L_{\text{max}}$ (ii) for 15 minutes and then restretched back to $L_{\text{max}}$ (iv). Upon stretch a rapid increase in force is seen followed by a further slow increase in force. The rapid increase in force is not accompanied by an increase in the amplitude of the calcium transient. The slow force response, however, is accompanied by a change in the calcium transient. B: Average traces of 32 records taken from A. The muscle was stimulated at 0.33 Hz (Allen and Kurihara, 1982).
Another contributing factor may be that passive muscle stretch during ventricular filling will increase muscle length, resulting in a decrease in the cross-sectional area and compression of the myofilament lattice (Figure 1.6.1). Thus, distance between the thick and thin filaments will decrease, which in turn will increase the probability of strong cross-bridge binding due to the closer proximity of myosin heads and actin filaments. This is suggested from studies investigating changes in the Ca\(^{2+}\)-sensitivity of force production induced by altered sarcomere length using the osmotic agent dextran to preserve a constant cross-sectional area (Fuchs and Wang, 1996).

Another potential mechanism for length dependent activation involves the protein titin. Titin is well positioned in the sarcomere to act as a length sensor as it spans the full half-sarcomere connecting the Z-line to the M-line. Titin is the main determinant of passive tension in the sarcomere and thus is important for offering resistance to ventricular filling. In addition, during stretch titin also produces radial forces, pulling the filaments closer together contributing to the reduction in lattice spacing described earlier (Cazorla et al., 2001). As titin is known to bind both actin and myosin it is possible that titin strain may also influence the number of cross-bridges entering force-generating states (Fukuda et al., 2001; Helmes et al., 2003). Indeed, studies have shown that either selective removal of titin by trypsin digestion or a reduction in titin strain resulted in a reduction in length dependent activation (Cazorla et al., 2001).
Figure 1.6.1: Compression of myofilament lattice with stretch. When muscle is stretched the distance between myosin (grey circles) and actin (black circles) filaments decreases.
1.5. Current Methods in Muscle Physiology

1.5.1. Chemically Skinned Muscle

The study of muscle requires characterisation of the mechanical properties and biochemical states associated with the cross-bridge cycle. The limitation associated with the study of intact muscle fibres is the difficulty in precisely controlling and modifying the chemical and ionic environment of the contractile apparatus. To overcome this skinned muscle preparations were developed. The use of skinned muscle for mechanical experiments has the advantage of having all membranes removed or permeabilised whilst leaving the contractile machinery intact and maintaining the three-dimensional lattice structure (Eastwood et al., 1979). This allows direct control of the chemical and ionic conditions surrounding the contractile apparatus simply by changing the bathing solution. Permeabilisation of the membranes, by chemical skinning, also acts to make all non-myofilament ATPase activity negligible (He et al., 1997).

1.5.2. Caged Compounds

Caged compounds are otherwise active molecules made inert by attachment of a chemical group. This chemical group can be removed by an appropriate wavelength of light, releasing the active molecule. Caged compounds have been used in the study of muscle contraction to probe different steps of the cross-bridge cycle using caged-ATP, caged-ADP, caged-P$_i$, and caged-Ca$^{2+}$. The caged compound must be stable, but with rapid decay upon photolysis, producing a high yield and known quantity of the active molecule. One caged compound used in muscle physiology is $P^3$-1-(2-nitrophenyl)ethyl ester of ATP (NPE-caged ATP; Scheme 1.8.1).

The use of NPE-caged ATP has the advantage that it can be diffused into a skinned muscle allowing rapid activation at a predetermined time. This avoids the delays normally associated with diffusion into the cell. NPE-caged ATP will diffuse into the
cell without causing activation, then at a chosen time point the cage can be removed using a frequency-doubled ruby laser pulse (347nm) and ATP is released throughout the muscle. In addition the muscle can be activated from rigor, ensuring that most of the cross-bridges are synchronised in the attached nucleotide-free state (Goldman et al., 1984). Following photolysis, the NPE-cage forms an aci-nitro intermediate species that absorbs maximally at 406nm, however, this intermediate is short lived (decays at 102s⁻¹ at 20°C) and can be corrected for in fluorescence measurements (He et al., 1998a).

Scheme 1.8.1: Photolytic release of ATP from NPE-caged ATP (Ellis-Davies, 2007).
1.5.3. **Phosphate Binding Protein**

Bacteria use a range of proteins to bind and actively transport essential molecules throughout the periplasm (Hirshberg et al., 1998). In *Escherichia coli* (*E. coli*) a phosphate binding protein (PBP) is produced under low phosphate conditions to scavenge phosphate (P$_i$) with high specificity for translocation into the cytoplasm (Brune et al., 1994; Salins et al., 2004).

PBP is a monomeric 34kDa protein, folded into two globular domains with a phosphate binding cleft in the centre (Figure 1.7.3.B). Using oligonucleotide directed mutagenesis an alanine to cysteine substitution can be made at position 197 (A197C mutant PBP) whilst maintaining the same structure (Hirshberg et al., 1998). This amino acid is subject to large conformational changes upon P$_i$ binding as it is located on the edge of the phosphate binding cleft (Brune et al., 1994).

As PBP contains no other cysteines, site specific labelling can be performed. Labelling with the fluorophore N-2[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide (MDCC; Figure 1.7.3.A) produces a probe capable of rapidly measuring micromolar concentrations of P$_i$. P$_i$ binding to MDCC-PBP is rapid ($\sim 1.4 \times 10^8$ M$^{-1}$s$^{-1}$) and tight ($K_d \sim 0.1 \mu$M) in solution, with a five-fold fluorescence enhancement upon binding (Ferenczi et al., 1995).

Until recently the rate of ATP hydrolysis in muscle was measured using a linked-enzyme assay, linking ADP production to the oxidation of reduced nicotinamide adenine dinucleotide (NADH; Kentish and Stienen, 1994; Wannenburg et al., 1997). However the problem with this technique is that the time-resolution of the measurements is limited to $\sim 1$ second. Therefore this technique is useful for measuring the steady state ATPase rate but it lacks the time resolution and sensitivity required to resolve transient changes in cross-bridge distributions during the approach to steady state and during quick stretches and releases and during fast ramp releases where fibres are producing external work. The development of
MDCC-PBP overcame the time limitations associated with the NADH-linked assay method, enabling real time assays of $P_i$ release with millisecond resolution, including during the first turnover of actomyosin following the hydrolysis of caged ATP.
Figure 1.7.3: A: Structure of MDCC. B: Ribbon representation of MDCC-PBP. MDCC is shown in red and phosphate in blue (Hirschburg et al., 1998).
1.6. **Context and Objectives of this Study**

Understanding the Frank-Starling relationship at the molecular level is required for understanding this complex feature of cardiac function. Modulation of the heart beat is occurring throughout life, but in heart disease, interventions that may allow modulation of cardiac output would have a direct beneficial effect. The design of the best intervention will be based on the correct understanding of the Frank-Starling mechanism. To fully understand the Frank-Starling relationship the molecular mechanisms underlying cardiac LDA must be determined.

This study aims to use the new techniques pioneered in our laboratory, and so far only applied to investigations of skeletal muscle (He et al., 1998a; West et al., 2004; Bickham et al., 2011), to understand the molecular basis of LDA. The hypothesis is that a key component of LDA is brought about by a stretch-dependent shift in the distribution of actomyosin cross-bridge states.

This study utilises NPE-caged ATP and MDCC-PBP to examine the force and rate of $P_i$ release in response to length changes in skinned rat cardiac trabeculae at different activation levels. $P_i$ is released as a result of ATPase activity and progression of cross-bridges through the power stroke during muscle contraction. $P_i$ release is also closely linked with the actomyosin states responsible for force production. Hence, measuring the rate of $P_i$ release, using MDCC-PBP, is a means to evaluate, in real time, ATP usage and energetic efficiency in response to, and during, mechanical events. Length changes in relaxed trabeculae aim to examine the effects of sarcomere length changes that occur during diastolic filling. The Frank-Starling relationship involves stretching during diastole, however, there is also evidence of regional variation within the ventricular wall during systole, providing evidence that some stretch occurs during systole (Stevens and Hunter, 2003; Ashikaga et al., 2009). Therefore, investigation of the effect of length changes in active trabeculae aims to examine the effect of strain on active cross-bridges and may help determine possible cross-bridge based mechanisms of LDA.
Chapter 2: Materials and Methods
2. Methods

The general approach of the experiments described in this thesis is evaluation of the mechanics and energetics of contracting trabeculae and the response to lengthening and shortening protocols. This chapter describes the methods common to the majority of the experiments. Where a different method was used for an individual part of the study the method is described in the relevant chapter.

2.1. Preparation of Trabeculae

2.1.1. Dissection

Trabeculae were dissected from the left ventricle of female Sprague-Dawley rats, weighing 250-350 grams. Animals were killed by cervical dislocation in accordance with Schedule 1 of the UK Home Office Animals (Scientific Procedures) Act 1986. The chest was opened and the heart rapidly explanted and rinsed free of blood in an ice cold 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), containing heparin and 30mM 2,3-butanedione monoxime (BDM), equilibrated to pH 7.4 with 95% O₂-5% CO₂. BDM is a Ca²⁺-desensitizing agent added to reduce spontaneous beating of the heart and to minimise damage to the ends of the trabeculae during dissection (Mulieri et al. 1989). The heart was transferred to a Langendorff apparatus where the aorta was cannulated and the heart retrogradely perfused with oxygenated KH solution for several minutes to remove blood from the coronary vessels.

The heart was then placed on a cooled stage (5°C) of a dissecting microscope in oxygenated KH solution and 30mM BDM. The right ventricular free wall was removed and the inter-ventricular septum bisected to expose the interior of the left ventricle. Thin, unbranched, uniform trabeculae were carefully excised from the left ventricular free wall, typically measuring 1-3mm in length, 80-200µm in width and 50-200µm in depth. T-shaped aluminium foil clips were gently attached to the ends
of the isolated trabeculae (Figure 2.1.1) to allow them to be mounted on the experimental apparatus.

2.1.2. Chemical Skinning

The isolated trabeculae were chemically skinned by immersion in a relaxing solution (see Section 3.2 for details of solutions) containing 2% v/v Triton X-100 for 30 minutes at 5°C. This permeabilisation technique dissolves the cell membrane and intracellular membrane-bound structures such as mitochondria and sarcoplasmic reticulum (SR), allowing direct access to the myofilament space. This skinning procedure also removes all membrane-bound non-myofilament ATPase activity, as well as sources of ATP generation, leaving the contractile myofilaments energetically isolated.

After permeabilisation, trabeculae were stored in a relaxing solution containing 50% v/v glycerol at -20°C for up to 5 days. Protease inhibitors (4mg/l leupeptin, 10mM phenyl methyl sulfonyl fluoride (PMSF) and 50mg/l trypsin inhibitor) are added to the relaxing solution for storage to inhibit proteolysis and maintain structural stability. During skinning and storage the trabeculae are held at a constant resting length.
Figure 2.1.1: Trabecula preparation. A: Aluminium foil T-clip. B: Aluminium T-clips attached to either end of the isolated trabecula.
2.2. Experimental Solutions

All solutions were made from research grade chemicals obtained from either Sigma or BDH, unless stated otherwise. All solutions were made as double strength solutions and diluted down to the final concentration, allowing extra constituents to be added if required. Full details of solution compositions can be found in Appendix A.

2.2.1. Relaxing Solution

An imidazole-buffered relaxing solution was used for skinning, storage and preparation of the trabeculae (See Appendix A, Table 1 for composition). The relaxing solution was stored at 5°C and used within a month of preparation.

2.2.2. Experimental Solutions

Volumes and ionic strength of experimental solutions used for muscle activation were calculated with the aid of a solution mixing programme written by David C.S White and Yale E. Goldman (Revised by M.A. Ferenczi, 1986). Solutions for laser activation were made up to 150 mM ionic strength, adjusted with potassium propionate and pH 7.1, adjusted with potassium hydroxide, at 20°C (See Appendix A, Table 2). Solutions for temperature-jump activation were made up to 200mM ionic strength and adjusted to pH 7.1 at 12 or 20°C (See Appendix A, Table 4). All experimental solutions were stored at -20°C in 1ml aliquots and thawed as required.
2.3. **Muscle Activation by Laser-flash Photolysis of Caged-ATP**

2.3.1. **Experimental Apparatus**

The trabeculae were mounted in a trough built into a stainless-steel stage of a Zeiss ACM upright microscope (Figure 2.3.2). The stage consists of six 30µl troughs, one of which is constructed from quartz allowing illumination of the muscle from either side as well as from below. The stage can be vertically displaced and rotated beneath the muscle allowing quick transfer between different solutions. The stage is connected to a refrigeration system allowing experimental temperature to be maintained throughout the activation protocol. All experiments were performed at 20°C, unless stated otherwise.

The T-shaped aluminium clips on either end of the trabeculae were attached to hooks (made from 0.05mm gauge stainless steel wire) passing through slits at either end of the trough and were glued in place with shellac dissolved in ethanol. One hook was attached to a force transducer (AE801 sensor element, Memscap AS, Norway, frequency response 1.6kHz)) and the other to a servo-motor capable of making longitudinal movements of up to 0.1 mm within 500 µs. The motor consisted of a modified loudspeaker coil (RS Components, Corby, UK, 8 ohms, 40mm diameter) operated in a position feed-back mode (He et al., 1998b).
Figure 2.3.1: Experimental apparatus for muscle activation by laser-flash photolysis of NPE-caged ATP viewed from (A) above and (B) side on. The muscle can be quickly transferred between solutions in the troughs of the rotating stage. One trough is constructed of quartz allowing light from a Helium-Neon laser and a 347nm pulsed laser to reach the muscle (modified from He et al., 1998a).
2.3.2. Laser and Hook Alignment

It is important that the hooks are well aligned with the laser pulse at the start of each experiment. To test the laser and hook alignment the hooks were brought closely together inside the quartz trough and aligned using the dissecting microscope. A small piece of burn paper (Zap-it, Salisbury, NH, USA) was positioned behind the hooks and the laser fired. The laser produces a burn pattern on the paper and if the hooks are correctly aligned they will produce a silhouette on the burn paper. Adjustments can be made to the position of the hooks and the process repeated until the silhouette is in the centre of the burn pattern and the 2 hooks are aligned with each other.

2.3.3. Force Transducer Calibration

Regular calibrations of the force transducer were performed to determine the calibration factor used to convert the voltage output into force. This was done by positioning the transducer vertically and hanging a set of weights of various masses from the hook, thus exerting force in the same direction as a contracting trabecula preparation in an experiment. For each mass, the change in voltage was recorded and plotted against the mass to produce a linear relationship, of which the gradient was the calibration factor (See Figure 2.3.3).

2.3.4. Motor Calibration

The experimental procedure requires motor displacements of ±300µm dependent on muscle length, therefore calibration of the motor signal is necessary to calculate motor displacement at various applied voltages. Motor calibration was performed by placing the motor into the quartz trough and measuring the distance moved by the hook in response to various applied voltages. A linear plot is produced of which the gradient is the calibration factor used to calculate the required voltage for each experiment (See Figure 2.3.4).
Figure 2.3.3: A typical force transducer calibration showing the relationship between transducer output and applied force.

\[ y = 0.0047x \]

\[ R^2 = 0.9995 \]
Figure 2.3.4: A typical calibration of the motor hook displacement at various applied voltages.
2.3.5. *Trabecula Dimensions*

Once mounted in the experimental apparatus the trabecula’s dimensions – length, width, depth - could be measured. Dimensions were measured in the quartz trough loaded with relaxing solution, after the adjustment of the sarcomere length (See Section 3.3.7 below). Length was measured using the platform micrometer scale (graticule calibration: 1 division = 10µm). Width was measured using the Zeiss microscope with a 40x water immersion objective and an eyepiece graticule (calibration: 10 divisions = 28µm), whilst depth was measured by focussing on the top of the muscle followed by the bottom and recording the change in the focussing dial (calibration: 1 division = 10µm).

The cross-sectional area (CSA) was calculated assuming an elliptical cross-section (See Equation 2.3.5) and using the average of three width and three depth measurements along its length. Force measurements were normalised to CSA for each preparation.

**Equation 2.3.5:** Area of an ellipse.

\[
\text{Area (m}^2\text{)} = \text{Height (m) x Width (m) x } \pi
\]

4
2.3.6. **Sarcomere Length**

Experiments were performed at an initial sarcomere length of 1.9 or 2.1µm. To set the initial sarcomere length the beam of a Helium-Neon laser (wavelength 632.8nm, 5mW, 1mm diameter beam, Lambda Photometrics, Herts, UK; LGK 7627) was passed through the muscle whilst in the quartz trough in relaxing solution (Figure 3.3.2). The laser produces a diffraction pattern caused by the regular arrangement of proteins in the sarcomeres. The diffracted light produces a zero order band and a pair of first order diffraction bands which can be viewed by holding a piece of paper in the path of the laser at a fixed distance (k) from the trabecular muscle.

The spacing between the bands can be used to calculate sarcomere length using Bragg’s Law (Equation 2.3.6), where λ is the wavelength of the laser light, θ is the angle of diffraction (Tan θ = (distance between zero order and first order bands)/distance between centre of trabecula and screen) and n is the order of diffraction (1). The muscle length can be adjusted using a micrometer screw gauge to change the distance between the hooks, whilst monitoring the laser diffraction pattern, until the desired sarcomere length is reached.

\[
\text{Sarcomere Length (µm)} = \frac{n \times \lambda (\text{nm})}{1000 \sin \theta}
\]

**Equation 2.3.6:** Bragg’s Law.

A more diffuse diffraction pattern is seen in cardiac muscle compared to skeletal muscle. This is due to scattering of the light by non-contractile elements in the muscle, such as connective tissue and intercalated discs, and the non-uniformity of
the sarcomere lengths (Krueger and Pollack, 1975). The non-uniformity worsens during contraction so that in cardiac muscle it is much more difficult to track sarcomere length than in skeletal muscle. The tracking of the sarcomere length changes throughout the contraction was attempted in the initial experiments of this thesis, however a clear signal was never achieved.

2.3.7. Experimental Protocol

2.3.7.1. Pre-Activation

One major difficulty with using skinned cardiac muscle preparations is the tendency for structural and sarcomeric disorder and instability. Laser activation only allows each muscle to be activated once, therefore it is important that the muscle is in a good state prior to activation. Therefore in these experiments prior to the experimental activation, the muscle was first activated by diffusion of calcium into the muscle. After mounting onto the experimental apparatus in relaxing solution and setting the sarcomere length, the muscle was transferred to a pre-activating solution for 10 seconds followed by activating solution for 20 seconds (see Appendix A, Table 4 for solution compositions), during which the muscle develops force. This pre-activation step stretches any compliant elements, such as the point of attachment between the muscle and the apparatus, and causes a re-ordering and re-registering of the sarcomeres ready for the experimental activation. After this pre-activation the muscle was transferred back to relaxing solution causing the force to return to zero and the sarcomere length checked and adjusted if required.
2.3.7.2. Activation

Prior to laser activation the muscle was incubated for a set period of time in a series of solutions (Table 2.3.7.2).

The muscle was activated from rigor to minimise shortening prior to activation (He 1997). Pre-rigor solution accelerates rigor state development by minimising the amount of ATP that needs to diffuse out of the muscle. ATP was removed rapidly to induce the rigor state uniformly, whilst minimizing the development of rigor force. Once rigor had been established, the trabecula was transferred to a trough containing loading solution to allow 5 mM NPE-caged ATP and 1-1.2 mM MDCC-PBP (see Section 2.5.1) to diffuse into the myofilament lattice accompanied by calcium at a free concentration of 32 or 1 µM. MDCC-PBP has a typical activity of 50-60%, determined by $P_i$ titration (see Section 2.5.2), with the remainder not binding phosphate, therefore the concentration refers to the active concentration.

After loading with NPE-caged ATP and MDCC-PBP the muscle was transferred to the quartz trough containing silicone oil (Dow Corning 200 Fluid 50cS, Coventry, UK) and the microscope objective was lowered over the trabecula to record the fluorescence change. A pulse from a 347nm frequency-doubled ruby laser (30ns light pulses, 80-120mJ; QSR 2, Lumonics, Rugby, UK) was focused to an ellipse of 300x150 µm on the muscle. A single pulse from the laser photolysed NPE-caged ATP liberating approximately 1.3mM ATP (102s$^{-1}$ at 20°C) into the myofilament space, initiating contraction of the muscle (McCray et al., 1980). Silicone oil was used to reduce background fluorescence in the bathing solution as only the muscle contained the fluorophore. For all experiments, the muscle was activated only once to avoid possible fibre deterioration by photo-damage or force generation.
<table>
<thead>
<tr>
<th>Solution</th>
<th>Incubation Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Rigor</td>
<td>5</td>
</tr>
<tr>
<td>Ca(^{2+})-free Rigor</td>
<td>5</td>
</tr>
<tr>
<td>Ca(^{2+}) Rigor</td>
<td>5</td>
</tr>
<tr>
<td>Loading</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 2.3.7.2: Solution incubation times for laser activation. See Appendix A, Table 2 for solution compositions. Ca\(^{2+}\) composition in Ca\(^{2+}\) Rigor was either 1 or 32µM. The ‘Loading’ solution contained Ca\(^{2+}\), caged-ATP (5mM) and MDCC-PBP (to give 1.2mM active PBP).

2.4. Length Changes

One end of the muscle was attached to a servo-motor, allowing the control of longitudinal movements during contraction. In the majority of experiments, a ramp stretch was applied (15% of the preparation length over 300 ms) 400 ms after the laser pulse. At the end of the ramp, the trabecula length was kept constant for 300 ms, following which a ramp release was applied, with the same magnitude and velocity but in the reverse direction as the ramp stretch.
2.5. Measurement of Actomyosin ATPase Rate

2.5.1. Measurement of Inorganic Phosphate Release

The rate of ATP hydrolysis by the cycling actomyosin cross-bridges was studied using an inorganic phosphate (P$_i$) probe allowing high time resolution (ms) assays of P$_i$ release following ATP hydrolysis. Details of the production, labelling and concentration of MDCC-PBP are given in Appendix B.

The rate of P$_i$ release was calculated from the change in fluorescence of the MDCC-PBP. Fluorescence was measured using a 40x water immersion objective (Zeiss, Germany; 40X/0.75NA W) and a photomultiplier tube (Thorn, type 9224 QB). The excitation light (4mW, 440-nm laser diode module (Laser2000, PPM16 (LD1504 G2)) was transmitted through an adjustable aperture and emission recorded through a 460 nm interference filter (Glen Spectra, Stanmore, UK; DF10 0242 DTM, %T = 70, FWHM = 10 nm). A dichroic mirror (reflecting at 350–425 nm, >450 nm %T = 95) separated the excitation and emission beams.

The fluorescence signal was normalized to the signal obtained once the MDCC-PBP was saturated and multiplied by the MDCC-PBP concentration. The amount of P$_i$ released can be calculated using Equation 2.5.1:

\[
[P_i] = \frac{\Delta F \times [\text{MDCC-PBP}]}{\Delta F_{\text{max}}}
\]

where $[P_i]$ is the concentration of P$_i$ released in mM (bound to MDCC-PBP), $\Delta F$ is the change in fluorescence and $\Delta F_{\text{max}}$ is the fluorescence signal when MDCC-PBP is saturated. The ATPase rate can be calculated from the gradient of the rate of release of P$_i$ (assuming a myosin head concentration of 120µM). The active
component of MDCC-PBP is known for each new preparation of the protein. The MDCC-PBP $P_i$ binding is 1:1 (mol:mol), so it is straightforward to calibrate the capacity for $P_i$ to the maximum fluorescence change. Generally, 1-1.2 mM of MDCC-PBP was added to each muscle preparation. The fluorescence record was corrected for saturation of PBP with $P_i$ using the apparent $K_d$ 15.8µM (Curtin et al. 2003). Apparent $K_d$ is the apparent equilibrium constant for the reaction:

\[
\text{Scheme 2.5.1:}
\]

\[P_i,\text{PBP} \rightarrow P_i + \text{PBP}\]

The fluorescence signal shows a transient artefact immediately following the laser flash. This drop in fluorescence is caused by an $aci$-nitro intermediate formed during the photolysis of NPE-caged ATP. This $aci$-nitro intermediate has a high absorbance of light at 420nm (Corrie et al. 1992) thus reducing fluorescence excitation and emission. The rate constant for $aci$-nitro decay at 20°C is 120 s\(^{-1}\) (He et al., 1998a). Fluorescence signals were corrected for $aci$-nitro decay using an exponential with the appropriate rate constant and amplitude to match that of the data. The artifact is short-lived and only affects the ATPase measurements in the first 100ms after the laser flash, therefore it does not affect the response to stretches and releases as these were imposed long after it had subsided.
2.5.2. Determination of MDCC-PBP Activity and Fluorescence

The activity of the MDCC-PBP (the percentage which bound phosphate) and the corresponding increase in fluorescence was determined by titration assay for each new batch of protein. A sample of the MDCC-PBP was diluted down to approximately 6µM using 60mM TES. The diluted sample (670µl) was then decanted into a quartz cuvette and placed in a fluorimeter (Perkin-Elmer, LS 50B, luminescence spectrometer). The fluorimeter chamber was temperature-controlled and all calibrations were performed at the experimental temperature (20°C). The fluorescence of the sample was measured over an emission range of 450-550nm with excitation at 440nm. The fluorescence change was monitored during titration of 10µM phosphate (Fluka, A3-0603) into the cuvette (1µM at a time). The fluorescence read at 464nm was plotted against phosphate concentration (Figure 2.5.2). The saturation point, when fluorescence no longer changes with addition of phosphate indicated the percentage activity of the protein. This was determined more accurately by fitting the curve to Equation 2.5.2.

Equation 2.5.2:

\[
Q = 0.5 \times (1 + M + D - \sqrt{(1 - 2M + M^2 + 2D + 2M^2D + D^2)})
\]

where Q is the proportion of the total MDCC-PBP with phosphate bound to it, M is the ratio of total phosphate present to total MDCC-PBP present and D is the ratio of the apparent dissociation constant for MDCC-PBP \((K_d = 15.8\mu M)\) to the total concentration of MDCC-PBP. The percentage activity was typically 50-60%.
Figure 2.5.2: A typical plot of fluorescence change of MDCC-PBP upon titration of phosphate. The data fitted to Equation 2.5.2 is shown in red.
2.5.3. **Determination of Myosin Active Site Concentration**

In fast mammalian skeletal muscle fibers the active site concentration is considered to be 150 μM (He et al., 1997). In rat trabeculae the active site concentration is expected to be lower because of the additional space taken by mitochondria and sarcoplasmic reticulum in the heart. In the myofibril lattice, the myosin concentration is calculated from the lattice geometry (Millman, 1998), with 294 myosin molecules per filament with a length of 1.6 μm. At a sarcomere length of 1.9 μm, the myosin head concentration is 306 μM. Microscopical reconstruction of the cross-section of trabeculae (performed by V. Caorsi, Imperial College London) suggest that 50-60% of the trabecula cross-section is occupied by myofilament lattice, giving an estimate for the myosin head concentration of 150-180 μM. However, in skinned muscle preparations there is evidence of swelling (Elliott, 1973; Godt and Maughan, 1977) and increased lattice spacing (Matsubara and Elliott, 1972). To account for lattice swelling a value of 120μM was used in the calculation of the ATPase rate described in Section 2.5.1.

2.6. **Muscle Activation by Temperature-Jump**

A subset of experiments was performed by activating the trabecula by calcium diffusion, whereby a muscle already loaded with ATP was transferred to a calcium-containing solution. Activation time using this technique is limited by diffusion of calcium into the muscle also resulting in a non-uniform initiation of contraction. However at temperatures <5°C, force level attained in calcium-containing solution is low so in spite of slow activation, little distortion of the sarcomeric structure is seen. Force generation develops markedly and rapidly when the calcium-activated trabecula is then switched to a trough containing activating solution at 20°C. This technique of temperature-jump activation was used in the experiments, thereby removing the time-limiting diffusion step and producing a quicker activation with better preservation of sarcomeric structure.
2.6.1. Experimental Setup

The muscle was mounted between a force transducer and a motor on a rapid solution exchange system driven by a stepper motor. Force transducer and motor calibrations were performed in the same way as for laser activation (Sections 2.3.3 and 2.3.4). The solution exchange system consists of a movable platform carrying two aluminium plates that can be maintained at two different temperatures (Figure 2.6.1), with each plate consisting of two pedestals to support the drops of solution (Linari et al., 2007). One platform also contains a glass trough into which the muscle can be vertically displaced allowing high magnification visualisation (10-60x) of the muscle for dimension measurements. The glass trough also allows measurement of sarcomere length by laser diffraction as described previously (Section 2.3.6.).

2.6.2. Experimental Protocol

The first plate was used to transfer the muscle between pre-activating solution and activating solution at ~0°C (see Appendix A, Table 4 for composition of solutions). The second plate was used to transfer the muscle between activating solution at the test temperature and relaxing solution, with the transition between the two plates producing a rapid change of temperature (Linari et al., 2004). Most of the force develops after the temperature jump, thereby reducing activation time which would otherwise be limited by diffusion of calcium into the muscle. The rapid transfer of the active muscle back to relaxing solution allows multiple contractions to be performed on the same muscle without a reduction in force production.
Figure 2.6.1: Experimental apparatus for muscle activation by temperature-jump viewed from (A) side on and (B) above. The muscle is mounted between two hooks bathed in a drop of solution supported on top of a platform on one of two pedestals. The pedestals are temperature-controlled, one maintained at a low temperature and one at high temperature. The pedestals are connected to two stepper motors allowing vertical and horizontal movements to provide quick transfer of the muscle between different solutions.
2.7. Data Collection and Analysis

Data were collected using a personal computer and a LabView programme designed by M. Caremani (University of Florence). Four channels were recorded (force, fluorescence, motor input and motor output) at 10 kHz for 3 seconds for laser activation or 2 kHz for 14 seconds for temperature-jump activation. Data were analysed in Microsoft Excel 2007 and Origin 8.5. All data are expressed as mean ± standard error of the mean (S.E.M). Data was compared for statistical significance using either a Student’s t-test or two-way ANOVA where p < 0.05 is considered significant. The statistical test used for each data set is described in the relevant sections.
Chapter 3: Isometric Contractions
3.1. Introduction

The Frank-Starling relationship describes how an increase in end-diastolic volume leads to increased force being produced during the next contraction. The molecular mechanism behind this relationship is currently unclear. This chapter aims to examine the mechanics and aspects of the cross-bridge molecular mechanism (i.e., the phosphate, $P_i$, release step) of cardiac trabeculae during isometric contractions at different sarcomere lengths. Increasing the sarcomere length of the trabeculae aims to replicate the stretch seen in the myocardium during diastolic filling. In the heart, calcium concentrations within cardiac muscle cells are constantly fluctuating therefore experiments were performed at two different levels of calcium-dependent activation (32$\mu$M and 1$\mu$M $Ca^{2+}$) in order to quantify actomyosin $P_i$ release, and ATPase activity, during maximal and approximately half-maximal isometric force responses.

3.2. Isometric Contractions

Isometric contractions examining the $P_i$ release rate were performed by photolytic release of ATP from NPE-caged ATP using a laser pulse, at sarcomere lengths of 1.9 and 2.1$\mu$m, and calcium concentrations of 32 and 1$\mu$M. Records of force and $P_i$ release during isometric contractions are shown in Figure 3.2 with the data summarised in Table 3.2. Records were averaged from single experiments on separate trabeculae. Following the photolytic release of ATP (at time 0s), isometric force developed and ATP hydrolysis led to $P_i$ release, which bound to MDCC-PBP and was detected as an increase in the fluorescence signal.

3.2.1. Force

An increase in sarcomere length caused an increase in passive force (see Section 3.3). To account for this during activation, the passive force measured at each
sarcomere length (0.8 kN.m\(^{-2}\) at 1.9µm and 3.8 kN.m\(^{-2}\) at 2.1µm) was subtracted from the total force measured in these experiments to give the active force. The passive forces were 1.6% and 5.5% of the maximal force response at sarcomere lengths 1.9 µm and 2.1 µm, respectively. All forces described are the active forces.

The laser flash produced a transient artefact, lasting ~4ms, in both the force and fluorescence recordings. Thereafter, force rose steadily until an isometric plateau was reached approximately, 0.4s after the laser flash. In maximally activated trabeculae (32µM Ca\(^{2+}\)) the isometric force produced was 47.7 ± 5.2 kN.m\(^{-2}\) and 64.6 ± 5.6 kN.m\(^{-2}\) at sarcomere lengths 1.9 (n = 9) and 2.1µm (n = 18), respectively (P < 0.001).

Prior to activation the muscle is taken through a series of solutions to remove ATP (described in Section 2.3.7.2). During these steps a ‘rigor force’ develops. Most of this rigor force develops in Ca\(^{2+}\)-free rigor solution and usually decreases upon the addition of Ca\(^{2+}\). Rigor force varies between muscles, amounting to between 10 and 20% of the isometric force produced in maximally activated muscle. As the trabeculae are activated from rigor all cross-bridges should be strongly attached at the time of activation. Upon photolytic release of ATP the cross-bridges detach causing a transient relaxation before force development. This is seen as a brief drop in force of approximately 5 to 10 kN.m\(^{-2}\) immediately following the laser flash.

The rate of force development (T\(_{1/2}\)) was calculated as the time until half maximal force is reached. T\(_{1/2}\) in maximally activated trabeculae was 64.7 ± 4.7 ms at sarcomere length 1.9µm and 70.2 ± 4.1 ms at 2.1µm (P > 0.05).

Activation in the presence of 1µM Ca\(^{2+}\) produced an isometric force of 24.7 ± 4.7 kN.m\(^{-2}\) at sarcomere length 1.9µm (n = 5) and 48.2 ± 7.2 kN.m\(^{-2}\) at sarcomere length 2.1µm (n = 4; P < 0.001). This represents 51.7% and 74.7% of maximal force at 1.9 and 2.1µm, respectively. This is consistent with the increased Ca\(^{2+}\)-sensitivity of cross-bridges in the stretched trabeculae. T\(_{1/2}\) was 77.8 ± 7.7 ms at sarcomere length
1.9µm and 79.2 ± 4.2 ms at 2.1µm (P > 0.05). Decreasing the Ca²⁺ concentration from 32 to 1µM caused the rate of force development to slow by 20% and 13% at sarcomere lengths 1.9 and 2.1µm, respectively.

The effects of pre-activation sarcomere length and [Ca²⁺] on average peak force generation and on average T₁/₂ (see Table 3.2) were analysed using two-way ANOVA, and the Bonferroni post-hoc comparison of the main factors and interactions. Neither starting sarcomere length nor [Ca²⁺] affected T₁/₂ (P > 0.05). As is clearly evident from Figure 3.2.A, there were significant effects of both starting sarcomere length (P < 0.001) and [Ca²⁺] (P < 0.001) on peak isometric force. However, the interaction between starting sarcomere length and [Ca²⁺] was not significant (P = 0.08), indicating that pre-activation stretch of the fibres neither enhanced nor diminished the overall effect of [Ca²⁺] on peak isometric force. The average time course of force generation in maximally activated (32 µM Ca²⁺) fibres at sarcomere length 1.9 µm was the same as for the lower level of activation (1 µM Ca²⁺) at the longer sarcomere length (2.1 µm).

3.2.2. Pᵢ Release

The Pᵢ release records were derived from the fluorescence signals, as described in Section 2.5.1. The rate of Pᵢ release was determined in each activation at different points in the contraction time course by linear fits to sections of the Pᵢ release record. The rate of Pᵢ release was determined during the initial rise in force and again during the period where once isometric force had reached a steady state. A fast initial rate of Pᵢ release is seen during force development which then decreases to a pseudo-steady state rate during the isometric force plateau.
3.2.2.1. Initial Rate of $P_i$ Release

The initial rate of $P_i$ release immediately following activation was measured for the first turnover, defined as the first 120µM of $P_i$ released (myosin head concentration is assumed to be 120µM, see Section 2.5.3). Measurements were actually made from 20 to 120µM $P_i$ to avoid the brief laser flash artefact. In maximally activated trabeculae the initial rate of $P_i$ release was $19.7 \pm 2.4$ and $16.3 \pm 1.1 \text{ s}^{-1}$ at sarcomere lengths 2.1 and 1.9µm, respectively. At 1µM $\text{Ca}^{2+}$ the initial rates of $P_i$ release were $11.1 \pm 2.0$ and $14.2 \pm 3.1 \text{ s}^{-1}$ at sarcomere lengths 2.1 and 1.9µm, respectively. Neither sarcomere length nor $[\text{Ca}^{2+}]$ had significant effects on $P_i$ release rate during the period of the first myosin turnover (two-way ANOVA; $P > 0.05$). Therefore the initial fast rate of $P_i$ release is independent of sarcomere length and degree of calcium activation.

3.2.2.2. Steady-State Rate of $P_i$ Release

The steady-state rate of $P_i$ release was calculated by linear least-square fits made between 0.35s and 0.4s of the time-course of $P_i$ release. The steady state $P_i$ release rate was shown to be independent of sarcomere length ($8.1 \pm 0.7 \text{ s}^{-1}$ at 2.1µm and $8.0 \pm 0.4 \text{ s}^{-1}$ at 1.9µm when maximally activated). There is not a significant effect of sarcomere length on $P_i$ release rate (Two-way ANOVA; $P > 0.05$). However a reduction in the degree of activation, induced by lowering free $[\text{Ca}^{2+}]$ from 32µM to 1µM, resulted in a 39.6% and 39.75% reduction in the steady-state $P_i$ release rate at sarcomere lengths 2.1 ($4.9 \pm 0.9 \text{ s}^{-1}$) and 1.9µm ($4.6 \pm 0.9 \text{ s}^{-1}$), respectively. The effect of $[\text{Ca}^{2+}]$ on steady state $P_i$ release is significant (Two-way ANOVA; $P < 0.001$). There was not a significant interaction between sarcomere length and $[\text{Ca}^{2+}]$ on $P_i$ release rate (Two-way ANOVA; $P > 0.05$).
3.2.2.3. Force Economy

Force economy, defined as the amount of ATP used per unit of force generated and calculated by dividing steady state ATPase rate by isometric force, improved with sarcomere length, changing from $0.18 \pm 0.017 \text{ s}^{-1}/\text{kN.m}^{-2}$ at 1.9µm to $0.13 \pm 0.012 \text{ s}^{-1}/\text{kN.m}^{-2}$ at 2.1µm at 32µM Ca$^{2+}$ and from $0.24 \pm 0.052 \text{ s}^{-1}/\text{kN.m}^{-2}$ at 1.9µm to $0.10 \pm 0.017 \text{ s}^{-1}/\text{kN.m}^{-2}$ at 2.1µm at 1µM Ca$^{2+}$. The effect of sarcomere length on force economy was significant (Two-way ANOVA; $P < 0.05$) but the effect of [Ca$^{2+}$] was not significant (Two-way ANOVA; $P > 0.05$). There was not a significant interaction between sarcomere length and [Ca$^{2+}$] on force economy (Two-way ANOVA; $P > 0.05$).
Figure 3.2: Effect of sarcomere length and activation level on isometric force and P<sub>i</sub> release rate. A: force response to photolytic release of ATP at longer sarcomere length (2.1µm; solid symbols) and shorter sarcomere length (1.9µm; open symbols) and maximal (32µM Ca<sup>2+</sup>; circles) and half-maximal (1µM Ca<sup>2+</sup>; squares) activation. B: P<sub>i</sub> release derived from the change in fluorescence of MDCC-PBP. For clarity, SEM’s are not shown – See Table 3.2 for SEM’s and n’s. Data was collected at 10 kHz, but for clarity only one in eighty values are shown.
Table 3.2: Summary of results of isometric contractions in rat cardiac trabeculae. ATPase rates (rate of P<sub>i</sub> release) are calculated assuming a myosin head concentration of 120µM. The initial ATPase rate was measured for the first turnover, defined as the first 120µM of phosphate released. The steady-state rate of P<sub>i</sub> release was calculated by linear least-square fits made between 0.35s and 0.4s of the time-course of P<sub>i</sub> release. Data expressed as mean ± SEM. SL, sarcomere length; T<sub>½</sub>, time to half maximal force. Force economy is calculated as the ratio of the steady state ATPase rate to the isometric force. \( \dagger P < 0.001 \) difference between 1 and 32µM Ca<sup>2+</sup>, \( \ast P < 0.05 \), \( \ast\ast P < 0.001 \) difference between SL 2.1 and 1.9µm.
3.3. **Sarcomere Length-Force Relationship and Passive Force**

According to established theories of muscle contraction the isometric force produced by a muscle should be proportional to the degree of myofilament overlap (Section 1.5.2). Therefore the dependence of isometric force on sarcomere length is essential for proving the validity of the model. Figure 3.3 shows the sarcomere-length force relationship measured in my preparations. Trabeculae were activated, by temperature-jump activation, at either 32\(\mu\)M (n=5) or 1\(\mu\)M Ca\(^{2+}\) (n=4) at various sarcomere lengths between 1.8\(\mu\)m and 2.3\(\mu\)m and the isometric force recorded. Passive force was measured by stretching the muscle to various sarcomere lengths, within the same range, whilst in relaxing solution. The passive force recorded was subtracted from the total force produced by the active muscle at each sarcomere length to give the active force.

The results show that between 1.8\(\mu\)m and 2.3\(\mu\)m an increase in sarcomere length causes a corresponding increase in isometric force. This is in accordance with results shown by Gordon et al. (1966), ter Keurs et al. (1980) and Allen and Kentish (1985). Measurements were not taken below 1.8\(\mu\)m as the muscle became slack.
Figure 3.3: Sarcomere length-force relationship. Passive force (dashed line, n = 8) has been subtracted from total force to give active force at 32µM Ca$^{2+}$ (diamonds, n = 5) and 1µM Ca$^{2+}$ (circles, n = 4). Data expressed as mean ± SEM. Passive force was fitted by a single exponential decay. The sarcomere-length force relationship was fitted to a power function (F = a.[SL + b]^c, de Tombe and ter Keurs, 1991) where F is force, SL is sarcomere length and c is the constant relating to the curvature. The fit parameters were as follows: 32µM Ca$^{2+}$, a = 90.0 kN.m$^{-2}$, b = -1.79 µm, c = 0.29; 1µM Ca$^{2+}$, a = 88.2 kN.m$^{-2}$, b = -1.72 µm, c = 0.68.
3.4.  Force-pCa Relationship

Temperature-jump activation was used to examine the relationship between force and calcium concentration at 20°C. Isometric force was measured at various calcium concentrations (pCa, -log([Ca^{2+}]), ranging from 4.5 to 7) at sarcomere lengths of 1.9 or 2.1µm. The muscle was maximally activated followed by multiple activations at submaximal [Ca^{2+}], interspersed with maximal activations to check for deterioration in maximal force. Maximal force begins to deteriorate after 10-15 contractions at which point the experiment was terminated. Solutions containing submaximal [Ca^{2+}] were produced by mixing relevant amounts of relaxing and activating solutions (see Appendix A, Table 4) calculated by a solution mixing programme. Submaximal force measurements were normalised to the maximal force values. Data were fit with the Hill equation (Hill, 1913):

**Equation 3.4:**

\[
\frac{P}{P_0} = \frac{[Ca^{2+}]^n}{k^n + [Ca^{2+}]^n}
\]

where \(P\) is the steady-state force produced at each pCa, \(P_0\) is the maximal force at pCa 4.5, \(k\) is the pCa\(_{50}\) ([Ca\(^{2+}\)] at which 50% maximum force is produced) and \(n\) is the Hill coefficient indicating the steepness of the relation and thus the cooperativity.

Figure 3.4.1 shows typical force recordings of rat trabeculae activated by temperature-jump at pCa 4.5 and 5.5. Very little force (0-3 kN.m\(^{-2}\)) develops when the muscle is transferred to activating solution at low temperature (0°C) with most of the force developing after the temperature-jump to 20°C. The force-pCa relationships are shown in Figure 3.4.2. At a sarcomere length of 2.1µm (n = 4) the pCa\(_{50}\) was 5.8 ± 0.1 with a slope (Hill coefficient) of 1.76 ± 0.11. Whereas at sarcomere length 1.9µm (n = 4), pCa\(_{50}\) was 5.6 ± 0.1 (t-test P < 0.05) and the slope was 1.98 ± 0.10 (t-test P > 0.05).
The results are consistent with existing data showing that an increase in sarcomere length causes an increase in the sensitivity of the contractile apparatus without a significant effect on the slope (ter Keurs et al., 1988; Wolff et al., 1995 and McDonald and Moss, 1995).
Figure 3.4.1: Typical recordings of rat cardiac muscle activation by temperature-jump at sarcomere length 2.1μm. Transition from pre-activating solution (0°C) to activating solution (0°C) begins at 0.3s. Transition from activating solution (0°C) to activating solution (20°C) begins at 2.7s. The noise seen in the force records during the transition between solutions is due to the surface tension of the drops of solution pulling on the muscle as it leaves and enters each drop. Activation at A, maximal [Ca$^{2+}$] (pCa 4.5) and B, submaximal [Ca$^{2+}$] (pCa 5.5).
Figure 3.4.2: The force-pCa relationship at sarcomere length 2.1µm (circles; n = 4) and 1.9µm (squares; n = 4). Forces are expressed relative to maximum force at pCa 4.5. Data are fitted to the Hill equation. Data expressed as mean ± SEM.
3.5. Effect of Myosin Heavy Chain Isoform on Force and $P_i$ Release

The speed and efficiency of muscle shortening is dependent on the intrinsic ATPase activity of the myosin heavy chain (MHC) isoform (Barany, 1967). In skeletal muscle there are several isoforms of MHC; however cardiac muscle expresses only two isoforms, α-MHC and β-MHC (see Section 1.4). The ventricles of smaller mammals such as the rat express predominately α-MHC whereas larger mammals such as the rabbit express predominantly β-MHC.

Experiments were performed to compare isometric force and rate of $P_i$ release in rabbit cardiac trabeculae and fast (psoas) and slow (soleus) skeletal muscles. Records are shown in Figure 3.5 with the data summarised in Table 3.5. All muscles were maximally activated with 32µM Ca$^{2+}$. Cardiac trabeculae were activated from a sarcomere length of 2.1µm and skeletal muscles were activated from a sarcomere length of 2.4µm. ATPase rate was calculated assuming a myosin head concentration of 120µM in cardiac muscle and 150µM in skeletal muscle (He et al., 1997).

The isometric force produced by rabbit cardiac trabeculae ($64.31 \pm 18.8$ kN.m$^{-2}$) was not significantly different to that produced by rat trabeculae ($64.56 \pm 5.60$ kN.m$^{-2}$, see Section 3.2.1) activated from the same sarcomere length. The steady state ATPase rate was not significantly different between rabbit trabeculae ($6.47 \pm 0.99$ s$^{-1}$) and rat trabeculae ($8.07 \pm 0.67$ s$^{-1}$; one-way ANOVA P > 0.05). Rabbit soleus muscle, a slow-twitch skeletal muscle fibre type, produced significantly more force than the rabbit cardiac trabeculae ($64.32 \pm 18.8$ vs. $146.91 \pm 11.96$ kN.m$^{-2}$; one-way ANOVA P < 0.05; see table 3.5). The force produced in rabbit psoas ($270.4 \pm 12.18$ kN.m$^{-2}$), a fast-twitch skeletal muscle fibre type, is clearly significantly (one-way ANOVA P < 0.01) greater than that of the rabbit trabeculae. However, the rate of force development was the same in soleus muscle and in cardiac muscle ($T_{1/2} = 76.4 \pm 13.04$ ms in soleus and $86.4 \pm 8.66$ ms in cardiac; see table 3.5). The rates of $P_i$ release, both during the first cross-bridge turnover and steady state, were not
different for the rabbit trabecula and rabbit soleus muscles, however a significant increase in $P_i$ release rate is seen in rabbit psoas (one-way ANOVA $P < 0.05$).

Rabbit cardiac muscle displays significantly lower force economy (ATP used per unit force) than soleus (one-way ANOVA $P < 0.05$). Rabbit cardiac and soleus muscles show similar steady state rates of $P_i$ release but soleus muscle produces 2.3-fold more force. This difference may be related to the relative importance of the isometric properties of cardiac and slow skeletal muscles. For example, the isometric phase of the cardiac cycle is short compared to soleus (a postural muscle) which may be required to generate force for relatively long periods of time, thus benefiting from more economical force generation.
Figure 3.5: Comparison of isometric force (A) and \( P_i \) release (B) in rabbit cardiac (open circles), soleus (closed circles) and psoas (closed squares) muscles.
Chapter 3: Isometric Contractions

Table 3.5: Summary of results of isometric contractions in rabbit cardiac, fast (psoas) and slow (soleus) skeletal muscles. The ATPase rate is calculated assuming a myosin head concentration of 120µM in cardiac muscle and 150µM in skeletal muscle. The first turnover is defined as the first 120µM $P_i$ released in cardiac muscle and the first 150µM $P_i$ released in skeletal muscle. Force economy is calculated as the ratio of the steady state ATPase rate to isometric force. Data expressed as mean ± SEM. Data were analysed using one-way ANOVA. Pairwise multiple comparison of groups was achieved using the Holm-Sidak method, with significance level $P = 0.05$. * indicates significantly different from cardiac and soleus muscle; † indicates significantly different from cardiac muscle.

<table>
<thead>
<tr>
<th></th>
<th>Rabbit Cardiac</th>
<th>Rabbit Psoas</th>
<th>Rabbit Soleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Rigor Force (kN.m⁻²)</td>
<td>12.2 ± 2.9</td>
<td>27.4 ± 19.6</td>
<td>30.9 ± 6.3</td>
</tr>
<tr>
<td>Isometric Force (kN.m⁻²)</td>
<td>64.3 ± 18.8</td>
<td>270.4 ± 12.8</td>
<td>146.9 ± 13.1</td>
</tr>
<tr>
<td>$T_{1/2}$ (ms)</td>
<td>86.4 ± 9.5</td>
<td>39.0 ± 2.3</td>
<td>76.4 ± 14.3</td>
</tr>
<tr>
<td>ATPase Rate (s⁻¹):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial (First Turnover)</td>
<td>17.4 ± 2.8</td>
<td>41.2 ± 4.2</td>
<td>11.8 ± 1.8</td>
</tr>
<tr>
<td>Steady State (Isometric)</td>
<td>6.5 ± 1.0</td>
<td>10.8 ± 0.5</td>
<td>4.85 ± 0.7</td>
</tr>
<tr>
<td>Force Economy (s⁻¹ / kN.m⁻²)</td>
<td>0.10 ± 0.017</td>
<td>0.04 ± 0.025</td>
<td>0.03 ± 0.032</td>
</tr>
</tbody>
</table>
3.6. Discussion

3.6.1. Validity of Preparation

The isometric force measured at 20 °C in maximally activated trabeculae was similar to that measured previously by Ebus and Stienen (1996) and Dobesh et al. (2002) at the same temperature. The isometric force was consistent between preparations under the same experimental conditions and was similar between the two activation techniques used in this study; laser-flash release of ATP from NPE caged-ATP at 20°C, and temperature-jump activation (from zero to 20°C).

The rate of $P_i$ release measured during the first turnover of the total cross-bridges (assuming a myosin head concentration of 120 µM) was 19.7s$^{-1}$. The rate decreased to a steady state of 8s$^{-1}$ after the eighth turnover (0.5-0.6s after activation). This is within the range of previous steady state measurements, using an NADH-linked enzyme assay for the rate of ADP appearance (see Section 6.2), of 10s$^{-1}$ (Wannenburg et al., 1997; 23°C), but higher than values in other studies, 3s$^{-1}$ (Ebus and Stienen, 1996; 20°C; assuming a myosin head concentration of 160 µM, 4s$^{-1}$ if recalculated as 120 µM as is used in this thesis) and 3.3s$^{-1}$ (Kentish and Stienen, 1994).

The sarcomere length-force relationship shows an increase in isometric force with sarcomere length. This is in accordance with existing data (Gordon et al., 1966; ter Keurs et al., 1980; Allen and Kentish 1985) and is partially due to the degree of myofilament overlap increasing with sarcomere length (described in Section 1.6), however this cannot explain the steepness and continual increase in force above optimal overlap so other processes must be involved. Isometric force also increases with the degree of calcium activation as seen in the force-pCa relationship, with an increase in calcium sensitivity seen as sarcomere length increases (ter Keurs et al., 1988; Wolff et al., 1995 and McDonald and Moss, 1995).
These results show preservation of LDA in my preparation at the activation levels and sarcomere lengths used in these studies. The sarcomere lengths used are representative of 50% of the physiological working length range of cardiac muscle (Rodriguez et al., 1992). This confirms the validity of my preparation for investigation of possible mechanisms of LDA.

Experiments performed on different muscle types (rat cardiac, rabbit cardiac, rabbit soleus and rabbit psoas) show that the technique is sensitive enough to detect differences in force and the rate of P\(_i\) release caused by differential expression of MHC isoforms, as a significant difference in force and P\(_i\) release is seen between cardiac muscle and fast skeletal muscle. No difference was seen between rat and rabbit cardiac muscle, even though β-MHC expression is known to be higher in larger mammals. The body mass differences between rats and rabbits may not be great enough to detect effects of different expression levels of β-MHC.

Isometric force per cross-sectional area in maximally activated cardiac muscle is considerably less (~43%) than that observed for slow skeletal muscle at the same temperature, despite similar MHC isoform expression. The organisation of the myocytes in the trabeculae in which sarcomere alignment is not as well organised as in skeletal muscle fibres may account for part of the difference. Myocytes also contain a higher fraction of mitochondria and sarcoplasmic reticulum resulting in a lower myosin head concentration than skeletal muscle. The recent demonstration of the existence of a population of super-relaxed cross-bridges that do not contribute to force under the experimental conditions used here (Hooijman et al., 2011) may explain the lower force of fully-activated cardiac muscle compared to that of slow skeletal muscle.
3.6.2. Effect of Sarcomere Length on Force and $P_I$ Release

The results show that an increase in sarcomere length from 1.9 to 2.1µm causes an increase in isometric force corresponding to the sarcomere length-force relationship. However the steady state rate of $P_I$ release is independent of sarcomere length.

As force increases significantly with sarcomere length, it may be expected that $P_I$ release would also increase. The sarcomere length-force relationship in cardiac muscle can only partially be explained by the degree of myofilament overlap, the remainder must be explained by other unknown processes. The results shown here indicate that these processes do not result in an increase in ATP hydrolysis. By assuming that the rate of $P_I$ release corresponds to the ATPase rate, the force economy (amount of ATP hydrolysed per unit of force produced) can be calculated. An increase in sarcomere length from 1.9 to 2.1 µm leads to a significant increase in force economy at both activation levels. The results described in Chapter 4 show that stretch of active muscle causes a reduction in $P_I$ release, whilst high force is maintained, thus similar processes may be involved here. Stretch-induced recruitment of cross-bridges (as suggested in Chapter 4) does not result in an increase in ATP hydrolysis, thus the turnover of stretched cross-bridges is slower than that of un-stretched cross-bridges.

Alternative possibilities are that the increase in force is due to stretching of the parallel elastic components, such as titin, rather than due to cross-bridge recruitment, or that the force per cross-bridge increases with stretch, with no change in the number of attached cross-bridges and therefore no increase in the rate of $P_I$ release.
3.6.3. Effect of Activation Level on Force and $P_i$ Release

*In vivo* the heart operates over a range of calcium concentrations that does not reach maximal activation levels. Diastolic calcium concentration is thought to be in the region of 0.1µM, with systolic calcium concentrations in the range of 1-2.5µM (Kirschenlohr et al., 2000). Thus, the submaximal calcium concentration used in this study (1µM Ca$^{2+}$) is a good representation of the physiological calcium levels found in the heart during systole, whereas 32 µM is above the intracellular calcium range, but does ensure that calcium effects are fully saturated.

Sarcomere stretch, from 1.9 to 2.1 µm, increased isometric force significantly in both the high (32µM Ca$^{2+}$) and low (1µM Ca$^{2+}$) activation states. Clearly there were also significant effects of Ca$^{2+}$ alone on peak force generated (Figure 3.2). While there was not a significant interaction of sarcomere length and [Ca$^{2+}$] (two-way ANOVA), there was nevertheless a trend towards sarcomere stretch promoting greater cross-bridge attachment at submaximal activation – force was increased by a factor of 1.35 at 32µM Ca$^{2+}$ and 1.95 at 1µM Ca$^{2+}$. This effect is likely to be an important contributing factor to the overall effect of cardiac muscle stretch on isometric force development. Stretching of sarcomeres and parallel elastic elements may cause further activation of thin filaments, thus increasing the availability of cross-bridge binding sites.

The steady-state rate of $P_i$ release increases by a factor of 1.65 upon increasing [Ca$^{2+}$] from 1 to 32µM Ca$^{2+}$. This increase in ATPase rate is independent of sarcomere length. $P_i$ release rate increases with activation level due to increased activation of thin filaments, allowing more cross-bridges to bind, resulting in a higher rate of ATP hydrolysis.
Chapter 4: Effect of Length Changes
4.1. Introduction

In the previous chapter the effects of sarcomere length and activation level on actomyosin kinetics in isometric trabeculae were explored. The experiments described in this chapter aim to examine how length changes imposed upon active trabeculae affect the force and energy turnover. During systole the volume of the ventricle decreases as working myocytes shorten, but there is regional variation within the ventricle wall providing evidence that some myocytes are stretched during systole (Stevens and Hunter, 2003; Ashikaga et al., 2009). A stretch and release protocol was applied with the aim of investigating the properties of cross-bridge mechanical and energetic properties during lengthening and shortening that the myocardium would experience during the cardiac cycle.

4.2. Effect of Length Changes on Active Force and P\textsubscript{i} Release

Experiments examining the effect of length changes on P\textsubscript{i} release were done using laser activation from a starting sarcomere length of 1.9\,\mu m. Upon activation, force rises rapidly until an isometric plateau is reached. 400ms after activation, once force has reached an isometric steady state the muscle was stretched by 15% of its length over 300ms. Length was held constant at this longer length for 300ms and then released back to the initial starting length at the same velocity as the stretch. Experiments were performed at two activation levels (1 and 32\,\mu M Ca\textsuperscript{2+}; n = 5 for both groups). Records of force and P\textsubscript{i} release in response to length changes are shown in Figure 4.2 and the results are summarised in Table 4.2. The records presented here are an extension of some of the isometric data presented in Section 3.2. The force and P\textsubscript{i} release rate seen during the initial isometric phase is described previously in Section 3.2. The P\textsubscript{i} release records become noisy after 1.5s because of the correction for the K\textsubscript{d} of MDCC-PBP when the protein approaches saturation with P\textsubscript{i}.
Figure 4.2: Length change protocol applied to trabeculae at an initial sarcomere length of 1.9µm. Trabeculae were activated with either 32µM Ca$^{2+}$ (Black) or 1µM Ca$^{2+}$ (Grey). n = 5 for both groups. Top panel, force response; Middle panel, P$_i$ release derived from the change in fluorescence of MDCC-PBP; Bottom panel, motor movement corresponding to a length change of 15% of total muscle length. Vertical lines across the panels indicate the timing of changes in motor movement.
### Chapter 4: Effect of Length Changes

#### Table 4.2: Summary of results of length-change experiments.

Starting sarcomere length is 1.9µm. $T_{1/2}$, Time to half maximal force. Efficiency calculated assuming a $\Delta G_{\text{ATP}}$ of 50 kJ.mol$^{-1}$. ATPase rates are calculated assuming a myosin head concentration of 120µM. The initial ATPase rate was measured for the first turnover, defined as the first 120µM of phosphate released. Data expressed as mean ± SEM. ML, muscle lengths. * $P < 0.05$; ** $P < 0.005$, analysed by t-test.

<table>
<thead>
<tr>
<th></th>
<th>32µM Ca$^{2+}$</th>
<th>1µM Ca$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong> Length (mm)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Cross-sectional area (mm$^2$)</td>
<td>0.0135 ± 0.003</td>
<td>0.0118 ± 0.002</td>
</tr>
<tr>
<td>Rigor force (kN.m$^{-2}$)</td>
<td>9.6 ± 2.3</td>
<td>10.5 ± 2.3</td>
</tr>
<tr>
<td>Isometric force (kN.m$^{-2}$)</td>
<td>49.8 ± 6.4</td>
<td>24.7 ± 4.7*</td>
</tr>
<tr>
<td>$T_{1/2}$ (ms)</td>
<td>58.2 ± 2.2</td>
<td>77.5 ± 11.7</td>
</tr>
<tr>
<td>Stiffness (kN.m$^{-2}$.µm$^{-1}$)</td>
<td>364 ± 98</td>
<td>412 ± 105</td>
</tr>
<tr>
<td>Peak force at end of stretch (kN.m$^{-2}$)</td>
<td>84.3 ± 18.0</td>
<td>61.6 ± 12.9</td>
</tr>
<tr>
<td>Force enhancement after stretch (kN.m$^{-2}$)</td>
<td>71.7 ± 14.0</td>
<td>49.1 ± 9.1</td>
</tr>
<tr>
<td>Force at end of release (kN.m$^{-2}$)</td>
<td>10.9 ± 1.7</td>
<td>6.1 ± 3.4</td>
</tr>
<tr>
<td>Isometric force after release(kN.m$^{-2}$)</td>
<td>36.3 ± 3.0</td>
<td>16.0 ± 4.8**</td>
</tr>
<tr>
<td>$T_{1/2}$ - Force redevelopment (ms)</td>
<td>86.1 ± 9.5</td>
<td>127.3 ± 11.8*</td>
</tr>
<tr>
<td>Power output (W.L$^{-1}$)</td>
<td>5.4 ± 0.9</td>
<td>3.1 ± 1.7</td>
</tr>
<tr>
<td>Thermodynamic efficiency</td>
<td>0.11 ± 0.02</td>
<td>0.04 ± 0.03</td>
</tr>
</tbody>
</table>

#### ATPase Rate (s$^{-1}$):

<table>
<thead>
<tr>
<th></th>
<th>32µM Ca$^{2+}$</th>
<th>1µM Ca$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial (First turnover)</td>
<td>15.2 ± 1.3</td>
<td>14.4 ± 1.1</td>
</tr>
<tr>
<td>Steady state (Isometric)</td>
<td>8.2 ± 0.5</td>
<td>4.8 ± 0.9*</td>
</tr>
<tr>
<td>During stretch</td>
<td>1.0 ± 0.9</td>
<td>1.5 ± 0.7</td>
</tr>
<tr>
<td>After stretch</td>
<td>4.7 ± 0.3</td>
<td>4.4 ± 0.4</td>
</tr>
<tr>
<td>During release</td>
<td>10.9 ± 1.5</td>
<td>7.8 ± 1.2</td>
</tr>
<tr>
<td>End isometric</td>
<td>7.8 ± 1.3</td>
<td>3.7 ± 1.1*</td>
</tr>
</tbody>
</table>
4.2.1. Force Response to Stretch

During the stretch force rapidly increases. A short-lasting dip is observed approximately 30ms after the start of the stretch with 32µM Ca\(^{2+}\), and is followed by a linear rise in force. At 1µM Ca\(^{2+}\), a similar response is seen, but the dip is not observed (Figure 4.2.1.A).

Stiffness per half-sarcomere was derived from the gradient of linear fits of force plotted against half-sarcomere length (Figure 4.2.1.B). Half-sarcomere length was determined from the motor movement assuming the initial sarcomere length at the start of the stretch was 1.9µm and that a total length change of 7.5% was transmitted to the sarcomeres (see Section 4.3.2). Stiffness of the trabeculae was 364 ± 98 kN.m\(^{-2}\).µm\(^{-1}\) at 32µM Ca\(^{2+}\) and 412 ± 105 kN.m\(^{-2}\).µm\(^{-1}\) at 1µM Ca\(^{2+}\) (\(P = 0.83\) calculated by Student’s t-test), indicating that stiffness is not affected by calcium concentration.

4.2.2. Force Decay at End of Stretch

The peak force at the end of the stretch is higher than the steady state force after stretch due to the viscoelastic properties of the cross-bridges, titin and other parallel elastic components (see Section 4.2.3). The peak force at the end of stretch was 84.3 ± 17.9 kN.m\(^{-2}\) at 32µM Ca\(^{2+}\) which decreased to a steady state of 71.7 ± 14.0 kN.m\(^{-2}\). No significant difference (\(P > 0.05\)) was seen at 1µM Ca\(^{2+}\) where the peak force at the end of the stretch was 61.6 ± 12.9 kN.m\(^{-2}\) which decreased to 49.1 ± 9.1 kN.m\(^{-2}\).

The force decay from the peak after the stretch at 32µM Ca\(^{2+}\) was fit by a single exponential decay (Figure 4.2.2) characterized by a rate constant of 59.8 ± 0.2 s\(^{-1}\) and amplitude of 12.6 ± 0.03 kN.m\(^{-2}\) (\(\chi^2/DoF =0.0029; R^2=0.992\)). At 1µM Ca\(^{2+}\), the force decay was fit by a double exponential, with one rate constant set at the same value as that seen at 32µM Ca\(^{2+}\), namely \(k_1 = 59.8\) s\(^{-1}\) with the fit parameters being \(A_1=7.8\) ± 0.03 kN.m\(^{-2}\), \(k_2=10.0\) ± 0.1 s\(^{-1}\), \(A_2=4.8\) ± 0.0 kN.m\(^{-2}\) (\(\chi^2/DoF =0.016; R^2=0.997\)).
fast and slow decays represent 62.0% and 38.0% of the total force decay, respectively.

4.2.3. **Force Enhancement After Stretch**

After the end of the stretch the force dropped to a new isometric plateau that was significantly higher than the initial isometric force before stretch. Stretch increased the isometric force by 44% at 32µM Ca\(^{2+}\) and by 99% at 1µM Ca\(^{2+}\).

This force enhancement after stretch has previously been described in skeletal muscle (Herzog et al., 2003; Pinniger et al., 2006; Leonard et al., 2010) and can only partially be explained by stretch causing an increase in sarcomere length resulting in a higher force being produced. In these experiments the trabeculae were stretched by 15% of their total length. Initial sarcomere length was 1.9µm, therefore if the full stretch is transmitted to the sarcomeres the stretched sarcomere length will be 2.18µm. However experiments examining the change in cross-sectional area (Section 4.3.2) suggest only a stretch of 7.5% of total muscle length is applied to the sarcomeres in the area of focus, indicating the stretched sarcomere length is 2.04µm. According to the sarcomere length-force relationship described in Section 3.3 a stretch from sarcomere length from 1.9µm to 2.0µm causes an increase in force of 30% and 37.5% at 32µM and 1µM Ca\(^{2+}\) respectively. This leaves a residual force enhancement of 14% at 32µM Ca\(^{2+}\) and 61.5% at 1µM Ca\(^{2+}\) which cannot be explained by moving up the ascending limb of the sarcomere length-force relationship. Residual force enhancement after stretch has been well documented in skeletal muscle (Herzog et al., 2006) and possible mechanisms are described in Section 4.5.1.
Figure 4.2.1: Expanded time course of force response to stretch (A) at 32μM Ca$^{2+}$ (Black) and 1μM Ca$^{2+}$ (Grey). Dotted line represents motor length change. Stiffness per half-sarcomere was calculated by fitting a straight line (red) through the force-half-sarcomere length relationship (B). Data are the same as those shown in Figure 4.2.
Figure 4.2.2: Analysis of force decay immediately following the end of the stretch, at 32µM (A) and 1µM Ca²⁺ (B). Data are shown in black and are the same as those shown in Figure 4.2. The time axis was reset so that zero marks the time at which the stretch ends. Force decay at 32µM Ca²⁺ was fit by a single exponential and at 1µM Ca²⁺ was fit by a double exponential. Exponential fits are shown in red.
4.2.4. Force During Shortening

After the isometric phase at long length the trabeculae are shortened by a ramp release of 15% over 300ms back to the initial starting sarcomere length of 1.9µm. The release causes the force to decrease rapidly to 10.87 ± 1.7 kN.m⁻² and 6.1 ± 3.4 kN.m⁻² at 32µM and 1µM Ca²⁺ ($P > 0.05$), respectively. This decrease in force corresponds to the force-velocity relationship for cardiac muscle (see Section 4.4.5).

Following the release, force rises again, but more slowly than after photolytic release of ATP ($t_{1/2}$ of 86.0 and 127.3 ms) to 36.3 ± 3.0 and 16.0 ± 4.8 kN.m⁻² at 32 and 1 μM Ca²⁺ ($P < 0.005$), respectively. These force levels are about half the force attained during the initial force rise to an isometric plateau.

4.2.5. $P_i$ Release Rates in Response to Length Changes

The initial rates of $P_i$ release during force development and the isometric steady state are described in detail in Section 3.2.2. Length changes were timed to occur after development of isometric force but before saturation of the $P_i$ probe. $P_i$ release rates were calculated by linear fits to the $P_i$ release record for the periods of the first turnover, 50ms before the stretch, the duration of the stretch, the duration of the period after the stretch at longer length, the duration of release and 50ms after the release. The rates of $P_i$ release at each stage of the length change protocol are shown in Figure 4.2.5.1. Stretch causes a significant reduction in $P_i$ release to 1.0 ± 0.9 s⁻¹ and 1.5 ± 0.7 s⁻¹ ($P > 0.05$), corresponding to 12.4% and 30.3% of the rate in the preceding isometric period, at 32 and 1µM Ca²⁺, respectively. The time from the start of the stretch until a change in $P_i$ release is seen was calculated by fitting straight lines through the data immediately before and after the start of stretch and determining the position of the intercept relative to that of the motor movement (Figure 4.2.5.2). A reduction in the rate of $P_i$ release is seen 6ms and 13ms after the start of the motor movement at 32 and 1µM Ca²⁺, respectively. This reduction in $P_i$ release lasts for the duration of the stretch.
During the isometric period following the stretch the $P_i$ release rate partially recovers to $4.7 \pm 0.3 \text{ s}^{-1}$ and $4.4 \pm 0.4 \text{ s}^{-1} (P > 0.05)$ at 32 and 1µM Ca$^{2+}$ respectively. This corresponds to a recovery to 56.6% of the $P_i$ release rate seen during the isometric phase prior to stretch at 32µM Ca$^{2+}$, compared to 90.6% at 1µM Ca$^{2+}$. The difference in the isometric $P_i$ release rates before and after stretch are not due to the change in sarcomere length (see Section 3.2.2 for the effect of sarcomere length on $P_i$ release).

During the ramp release the $P_i$ release rate increased to $10.9 \pm 1.5 \text{ s}^{-1}$ and $7.8 \pm 1.1 \text{ s}^{-1} (P > 0.05)$ at 32µM and 1µM Ca$^{2+}$, respectively. This corresponds to a 2.3-fold and 1.8-fold increase in $P_i$ release compared to the preceding isometric phase. This acceleration of $P_i$ release during shortening has been demonstrated previously in skeletal muscle (He et al., 1998).

Following the ramp release another isometric phase develops at the same sarcomere length as at the start of the protocol (1.9µm). The rates of $P_i$ release during the re-establishment of the isometric steady state are $7.8 \pm 1.3 \text{ s}^{-1}$ and $3.7 \pm 1.1 \text{ s}^{-1} (P < 0.05)$ at 32µM and 1µM Ca$^{2+}$, respectively. These rates are close to, but slightly lower than, the rates seen during the initial isometric steady state that followed photolytic release of ATP.

The $P_i$ release records shown become noisy after 1.5s because of the correction for the apparent $K_d$ of MDCC-PBP when the protein approaches saturation with $P_i$ (see Section 2.5.1).
4.2.6. **Power and Efficiency**

Power output was calculated by multiplying the velocity of the release (0.5 ML.s\(^{-1}\)) by the force at the end of the release. At this velocity the power output and thermodynamic efficiency is expected to be suboptimal (i.e. \(V_{\text{max}}\) at 20°C is > 8 ML.s\(^{-1}\); see section 4.4.2). A power of 5.4 ± 0.9 W.L\(^{-1}\) was produced at 32µM Ca\(^{2+}\) and 3.0 ± 1.7 W.L\(^{-1}\) at 1µM Ca\(^{2+}\) \((P > 0.05)\).

Thermodynamic efficiency was calculated by dividing power by the rate of energy release. Energy release is the product of the ATPase rate (rate of Pi release in mol.L\(^{-1}\).s\(^{-1}\)) and the free energy of ATP hydrolysis (\(\Delta G_{\text{ATP}}\), assumed to be 50 kJ.mol\(^{-1}\), He et al., 2000). At 32µM Ca\(^{2+}\) the efficiency was 0.11 ± 0.03, compared to 0.04 ± 0.03 at 1µM Ca\(^{2+}\) \((P > 0.05)\).
Figure 4.2.5.1: $P_i$ release rates at each stage of the length change protocol shown in Figure 4.2. First turnover: during the time taken for the first 120µM $P_i$ to be released. Isometric phase: at 0.4s of isometric contraction at SL 1.9µm. During stretch: average rate during stretch from SL 1.9 to 2µm. After stretch: average rate during isometric phase at SL 2µm. During release: average rate during shortening from SL 2 to 1.9µm. End isometric: average rate during isometric phase at SL 1.9µm. Data expressed as mean ± SEM. * $P < 0.05$. n = 5 for both groups.
Figure 4.2.5.2: Expanded time course of $P_i$ release and motor movement at beginning of stretch. Data are the same as those shown in Figure 4.2. A reduction in the rate of $P_i$ release is seen 6ms and 13ms after the start of the motor movement at 32µM (Black line) and 1µM $Ca^{2+}$ (Grey line), respectively.
4.3. Effect of Length Changes in Relaxed Muscle

4.3.1. Force

The same stretch-release protocol was also performed on relaxed trabeculae. The experiments were performed both in the presence (n=5) and absence (n=4) of 30mM BDM to determine the degree of relaxation of the muscle and how much of the force enhancement after stretch, seen in active muscle (described in Section 4.2.3), is due to stretching of parallel elastic elements such as titin.

In the presence of BDM no cross-bridges should be attached (or they will be only weakly attached) and therefore the muscle will be fully relaxed. Stretching of the muscle in the presence of BDM caused a rapid increase in force (Figure 4.3.1) to a peak of $10 \pm 1.10$ kN.m$^{-2}$ which then decayed to a steady state of $8 \pm 0.94$ kN.m$^{-2}$ as the muscle length was held constant at the longer length. The peak force at the end of the stretch is higher than the steady state force due to the viscoelastic properties of titin and other parallel elastic components. During the release force decreased back to the initial starting value. In the absence of BDM force increased to a peak of $10.5 \pm 0.88$ kN.m$^{-2}$ and decreased to a steady state of $8.5 \pm 0.97$ kN.m$^{-2}$. Comparison of the two groups with an unpaired $t$-test showed no significant difference (peak $p=0.96$, steady state $p=0.98$) indicating the muscle is fully relaxed whilst in the relaxing solution. Stiffness was calculated as described in Section 4.2.1. Stiffness was $124 \pm 7.4$ kN.m$^{-2}$.µm$^{-1}$ in the relaxed trabeculae with BDM, significantly lower than the stiffness observed in active trabeculae at 32 $(P < 0.05)$ and 1 µM Ca$^{2+}$ $(P < 0.05)$.

The force produced during these experiments is a passive force and was therefore subtracted from the force produced by the active muscle (as described in Section 4.2).
4.3.2. **Fluorescence**

Isolated trabeculae are thought to act as constant volume systems, therefore stretching of the muscle will cause a corresponding decrease in cross-sectional area, with the opposite applying to a release. Relaxed trabeculae in the presence of MDCC-PBP were subjected to the same stretch-release protocol as described in Section 4.2. Figure 4.3.2.A shows that a small decrease in fluorescence is seen during the stretch and a corresponding increase is seen during the release. This change in fluorescence is due to the muscle obeying the constant volume hypothesis described above. Stretching of the muscle causes a decrease in cross-sectional area resulting in fluorescence being collected from a smaller volume of muscle and therefore less fluorescence is collected. This can be corrected for by applying a correction factor.

To accurately correct the fluorescence measurements for the change in cross-sectional area it is necessary to know the extent of the cross-sectional area change. Attempts were made to measure the difference in cross-sectional area under the microscope objective before and after stretch had been imposed, however this is relatively inaccurate as the change is so small.

A more efficient way is to use the fluorescence change observed in relaxed muscle and determine the correction factor required so that no change in fluorescence is seen. Figure 4.3.2.B shows the change in fluorescence after application of a correction factor assuming a 15% decrease in cross-sectional area, as would be expected if the muscle was of a constant volume and the full 15% length change was applied to the full length of the muscle. Application of a 15% correction factor causes a change in fluorescence of almost equal magnitude but opposite to that seen with no correction factor. Application of a correction factor of 7.5% shows no change in fluorescence (Figure 4.3.2.C) therefore it can be assumed that the imposed length changes cause a 7.5% change in cross-sectional area. A correction
factor of 7.5% was applied to the experimental fluorescence records described in Sections 4.2 and 5.2.

Figure 4.3.2 also shows a representative fluorescence record from an active muscle with the relative correction factors applied. With a correction factor of 7.5% a clear decrease in fluorescence is seen during the stretch and an increase in fluorescence during the release (as described in Section 4.2). As no fluorescence change is seen in the relaxed muscle it confirms that the observed fluorescence change in the active muscle is a physiological response to length changes and not a movement artefact caused by the imposed length changes.
Figure 4.3.1: Effect of length changes on relaxed muscle in the presence (blue, n = 5) and absence (red, n = 4) of 30mM BDM. Motor movement is shown in black.
Figure 4.3.2: Effect of length changes on fluorescence in relaxed muscle (red). A representative fluorescence trace from active muscle is shown in blue. Motor movement is shown in black. A: Fluorescence change observed; B: After correction for 15% volume change; C: After correction for 7.5% volume change.
4.4. **Shortening Velocity and Power**

4.4.1. **Unloaded Shortening Velocity**

To determine the speed of unloaded muscle shortening ($V_0$) the slack-test was used (Edman, 1979). Maximally activated trabeculae, at sarcomere length 1.9µm, were quickly released by different lengths (7 – 14 % of muscle length) that reduce force to zero so the muscle is slack. The time interval ($\Delta t$) is then measured from the release until force starts to re-develop. $\Delta t$ is calculated as the difference between the start of the release and the intercept of straight lines fitted through the slack force and the initial period of force redevelopment. A typical force record of a slack-test is shown in Figure 4.4.1.1.

The linear regression between the amount of shortening and the time taken to take up the slack gives a slope corresponding to $V_0$ and is shown in Figure 4.4.1.2. $V_0$ was calculated as $5.8 \pm 0.45$ ML.s$^{-1}$ (n=3). This is comparable to values reported previously by de Tombe and ter Keurs (1990). The slack-test is thought to give a more accurate determination of the maximum shortening velocity ($V_{max}$), than the force-velocity curve (see Section 4.4.2), as it excludes any contribution of the recoil of the series elastic elements.
Figure 4.4.1.1: Example of typical slack-test recording. A large amplitude quick release (12% of muscle length at time 50ms) is applied so that force is reduced to zero. Δt is the time taken until the start of force redevelopment after the release. Δt is calculated as the difference between the start of the release and the intercept of straight lines fitted through the steady-state slack force and the initial period of force redevelopment (shown in red).
Figure 4.4.1.2: Slack-test determination of unloaded shortening velocity, $V_0$. $n = 3$. 
4.4.2. Force-Velocity Relationship

A force-velocity curve was produced, using temperature-jump activation of maximally activated trabeculae, to study shortening velocity and power output. After isometric force had developed, ramp releases (5% of muscle length) were performed at various different velocities, ranging from $0.2 - 5 \text{ ML.s}^{-1}$ (ML, muscle lengths). A small step release was applied at the beginning of the ramp to accommodate the series elasticity. The step size ranged from 10-20% of the total length change and was adjusted as required until a steady state force is reached where no further decrease in force is seen during the ramp release. Isometric force was measured and the force during shortening was calculated as the average force obtained once the initial force decrease had ended. A typical record showing measurement of force during shortening is shown in Figure 4.4.2.1. Force is expressed as a fraction of the maximum isometric force produced during each contraction and plotted against shortening velocity to produce the force-velocity curves shown in Figure 4.4.2.2. The force-velocity relationship was fitted with Hill’s hyperbolic equation (Hill, 1938):

\[
P/P_o = a \cdot \frac{(V_{\text{max}} - V)/(V + b)}
\]

where $P_o$ is the isometric force, $P$ is the force during shortening at velocity $V$ and $a$ and $b$ are constants with dimensions of force and velocity, respectively. The value for unloaded shortening velocity ($V_0$) as measured by the slack—test ($5.8 \text{ ML.s}^{-1}$, see Section 4.4.1) is included in the force-velocity curve and is included in the fit. $V_{\text{max}}$ was measured to be $8.6 \text{ ML.s}^{-1}$. Results are summarised in Table 4.4.
Figure 4.4.2.1: Example of typical force-velocity measurement. Muscle is released by 5% of muscle length at a velocity of 0.2ML.s\(^{-1}\). \(P_0\), isometric force; \(P\), force during shortening.
Figure 4.4.2.2: The force-velocity relationship of maximally activated rat cardiac trabeculae at sarcomere length 1.9µm and 20°C. Data fitted by Hill’s hyperbolic equation (Equation 4.4.2). Open circle represents the unloaded shortening velocity, \( V_0 \), as determined by the slack-test (see Section 4.4.1). The abscissa intercept represents the maximum shortening velocity, \( V_{\text{max}} \).
## Chapter 4: Effect of Length Changes

### Table 4.4: Parameters of the force-velocity relationship in rat trabeculae maximally activated at sarcomere length 1.9µm and at 20°C. $P_0$, isometric force; $a$ and $b$, constants of Hill’s force-velocity equation; $V_{max}$, maximum shortening velocity extrapolated from force-velocity curve; $V_0$, unloaded shortening velocity determined by slack test (n=5).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_0$</td>
<td>58.2 kN.m$^{-2}$</td>
</tr>
<tr>
<td>$a/P_0$</td>
<td>0.058</td>
</tr>
<tr>
<td>$b$</td>
<td>0.53 ML.s$^{-1}$</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>8.6 ML.s$^{-1}$</td>
</tr>
<tr>
<td>$V_0$</td>
<td>5.8 ± 0.45 ML.s$^{-1}$</td>
</tr>
</tbody>
</table>
4.4.3. Power-Velocity Relationship

Power-velocity curves were obtained by multiplying force by velocity for each point on the force-velocity curve. The power-velocity relationship is shown in Figure 4.4.3. A maximum power output of 18.0 W.L$^{-1}$ is produced at a shortening velocity of 1.7 ML.s$^{-1}$. The velocity of shortening used in the length change experiments examining $P_i$ release was 0.5 ML.s$^{-1}$. The power output calculated during $P_i$ release experiments (5.43 W.L$^{-1}$; see Table 4.2) when maximally activated is approximately half the power interpolated from the force-velocity curves at the same velocity of shortening (10.2 W.L$^{-1}$). This is most likely due to ATP depletion by the time of the release phase in $P_i$ release experiments, whereas force-velocity curves were performed by temperature-jump activation where ATP concentration will be high (see Chapter 5).
Figure 4.4.3: Power-velocity curve constructed from force-velocity data.
4.5. Discussion

4.5.1. Response to Stretch

The results show that stretch causes a rapid rise in force. Stiffness remains constant during the stretch and is not affected by calcium concentration. The stiffness is very similar between the 2 groups, suggesting that the elasticity is linear and largely caused by passive components such as titin. The amplitude of the stretch is far greater than the reach of the cross-bridges (~75 vs. ~10 nm, respectively), indicating cross-bridges must be forcibly detached, however as stiffness remains high this indicates rapid reattachment.

Force is not constant during stretch, but continues to rise for the whole duration of the stretch, a situation clearly different from that seen in skeletal muscle (Bickham et al., 2011) where force reaches a plateau during stretch. The difference is probably due to the higher stiffness of parallel elastic components, such as titin, in the heart compared to skeletal muscle (Cazorla et al., 2000; LeWinter and Granzier, 2010). Thus in the heart force is enhanced by the stretch of this elasticity which provides a recoil force to enhance ejection.

At 32μM Ca$^{2+}$ the rate of force decay after stretch is well described by a single exponential decay of 59 s$^{-1}$. However at 1μM Ca$^{2+}$ the rate of force decay after stretch consists of a fast component (59 s$^{-1}$) and a slow component (10 s$^{-1}$). The fast component of force decay indicates detachment of additional cross-bridges that have attached during the stretch at both 32μM Ca$^{2+}$ and 1μM Ca$^{2+}$. The slow component of force decay seen at 1μM Ca$^{2+}$ could be due stretch-induced activation of thin filaments beginning to disappear at the end of the stretch. No slow component of force decay is seen at 32μM Ca$^{2+}$ as the thin filaments are fully activated and remain so during the force decay. The amplitude of the slow component of force decay, seen at 1μM Ca$^{2+}$ (4.8 kN.m$^{-2}$, 38% of stretch-induced cross-bridge binding) may correspond to the force generated by cross-bridges.
recruited during stretch, which is not seen at 32µM Ca$^{2+}$ as most cross-bridge binding sites are already occupied, and therefore stretch cannot cause further thin filament activation.

After the end of the stretch, force dropped to a steady-state that was significantly higher than that prior to the stretch, a process known as force enhancement after stretch (Herzog et al., 2003, Pinniger et al., 2006, Leonard et al., 2010). Experiments performed in skeletal muscle have shown that enhanced force after stretch can exceed isometric forces at the plateau of the sarcomere-length force relationship (Peterson et al., 2004; Schachar et al., 2004) suggesting that there is a passive component that contributes to residual force enhancement (Herzog and Leonard, 2002; Raaier et al., 2003) as well as stretch-induced cross-bridge recruitment. The residual force enhancement seen in these experiments is greater than previously reported in skeletal muscle (Hilsey et al., 2009; Lee and Herzog, 2008). This is most likely due to the greater stiffness of the cardiac titin isoform compared to that of the skeletal titin isoform (Cazorla et al., 2000; LeWinter et al., 2010). Residual force enhancement is greater at 1µM (61.5%) than at 32µM Ca$^{2+}$ (14%). This may be due to fewer additional cross-bridge binding sites being available for cross-bridge recruitment at 32µM Ca$^{2+}$, as most are already occupied, resulting in a smaller force enhancement.

During stretch the rate of P$_i$ release (and therefore the ATPase rate) decreases to a value lower than the isometric rate at both maximal and submaximal activation. The decrease in P$_i$ release lasts for the duration of the stretch. A similar reduction in ATPase activity has previously been reported in fully activated intact skeletal muscle (Curtin and Davies, 1973 and 1975). This effect of stretch is not caused by the change in sarcomere length or force as the rate of P$_i$ release during stretch remains constant for the duration of the stretch, although sarcomere length and force vary during this time.
Within the time resolution of the experiment, the change in P$_i$ release occurs almost simultaneously with the application of the stretch. As the trabeculae are permeabilised, membrane structures will have been destroyed and soluble proteins will have diffused away, suggesting that the stretch is having a direct effect on the cross-bridge ATPase site itself.

The results described in Chapter 3 show that the isometric rate of P$_i$ release is higher at 32µM than 1µM Ca$^{2+}$. In contrast, the results described in this chapter show that during a stretch, and the period immediately following a stretch, the rate of P$_i$ release at 32µM Ca$^{2+}$ is almost the same as at 1µM Ca$^{2+}$. Thus, during and immediately after stretch, less ATP is hydrolyzed although force remains high, indicating stretch leads to thin filament activation through cross-bridge recruitment. This stretch-induced activation is proportionately greater at lower activation. This may be due to more cross-bridge binding sites being available for cross-bridge recruitment at lower calcium compared to at higher calcium where most are already occupied.

The low rate of P$_i$ release seen during stretch indicates that P$_i$ remains bound to myosin, resulting in an accumulation of the A.M.ADP.P$_i$ state (see Scheme 4.5.3.). This means that there is a higher population of cross-bridges waiting to undergo their power stroke, resulting in a higher force of contraction. Further work needs to be done to model the distribution of cross-bridge states and how they change in response to length changes. Accumulation of this P$_i$ containing state this cannot be due to a simple reversal of the power stroke as this would involve generation of ATP which is not thought to be possible (Bickham et al., 2011). A possible reaction scheme to explain this is described in Chapter 6.
Scheme 4.5.1: During stretch of active cardiac muscle, a low rate of $P_i$ release is seen. This suggests an accumulation of the force-generating $P_i$ containing state A.M.AD.P_i.

The majority of ventricular stretch occurs during diastolic filling when the muscle is not active. However modelling by Stevens and Hunter (2003) and experiments by Ashikaga et al. (2009) have shown that stretch does occur in the endocardial apical and basal regions of the left ventricular free wall during systole. These are the regions where trabeculae for the present studies were dissected from. Therefore the experiments in this study, involving the stretching of active muscle, are relevant to exploring the Frank-Starling mechanism and LDA.
4.5.2. Response to Shortening

After being held at the longer sarcomere length the trabeculae were allowed to shorten. Following shortening force redeveloped to an isometric plateau, however the rate of force redevelopment and the new isometric force were both lower than during the initial isometric phase at the start of the protocol. Throughout the course of the experiment ATP is being hydrolysed to ADP and P$_i$, resulting in a progressive depletion of ATP and accumulation ADP, explaining the lower isometric force and rate of force development. Free P$_i$ remains low as it binds to MDCC-PBP, until saturation of MDCC-PBP after approximately 1.5 seconds. The effects of ATP depletion and ADP accumulation are studied more in Chapter 5.

During shortening the rate of P$_i$ release increases to a value higher than the isometric rate, indicating that extra ATP hydrolysis occurs during the power generating shortening phase. The acceleration of P$_i$ release during shortening is seen even though ADP will have accumulated and ATP will have been largely depleted. This increase in ATP hydrolysis is representative of the Fenn effect (Fenn, 1924). Using heat measurements in skeletal muscle, Fenn showed that energy output during shortening is in excess of that of an isometric muscle.

After shortening the rate of P$_i$ release partially recovers towards the rate during the initial isometric period. Full recovery does not occur due to ATP depletion and ADP accumulation towards the end of the experiment. The recovery is more complete at 1µM than at 32µM Ca$^{2+}$ due to lower ATP utilisation at lower activation.

The ratios of the rate of P$_i$ release at 1 and 32µM Ca$^{2+}$ are shown in Table 4.5.2. The stretch-induced activation described previously is seen as an increase in the ratio towards 1 during stretch and the period after stretch, compared to 0.58 during the isometric phase. During the release the ratio (0.71) shifts back towards the isometric value, suggesting the stretch-induced activation is beginning to wear off during the release.
Table 4.5.2: Ratio of P$_i$ release rates at 1 and 32µM Ca$^{2+}$ calculated from the means in Table 4.2.

<table>
<thead>
<tr>
<th></th>
<th>Ratio of P$_i$ Release Rates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1µM : 32µM Ca$^{2+}$</td>
</tr>
<tr>
<td>Initial (First Turnover)</td>
<td>0.95</td>
</tr>
<tr>
<td>Isometric (Steady State)</td>
<td>0.58</td>
</tr>
<tr>
<td>During Stretch</td>
<td>1.42</td>
</tr>
<tr>
<td>After Stretch</td>
<td>0.93</td>
</tr>
<tr>
<td>During Release</td>
<td>0.71</td>
</tr>
<tr>
<td>End Isometric</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Force-velocity curves and slack-tests were performed to determine where on the power-velocity curve the P$_i$ release experiments lie. 32µM Ca$^{2+}$ has a higher power output and thermodynamic efficiency than 1µM Ca$^{2+}$ at a shortening velocity of 0.5 ML.s$^{-1}$. At maximal activation, maximal power occurs at a velocity of 1.7 ML.s$^{-1}$. A velocity of 0.5 ML.s$^{-1}$ produces less than maximal power, but was chosen for these experiments as this mimics the velocity of physiological shortening seen in the heart. Force-velocity curves and slack-tests were only performed on trabeculae at sarcomere length 1.9µm. In my length change experiments the initial starting sarcomere length was 1.9µm, however prior to the release the muscle was stretched to a sarcomere length of 2.04µm (see Sections 4.2.3 and 4.3.2). However these force-velocity curves and slack tests are still valid for comparison to the P$_i$ release rate data as several previous studies (Daniels et al., 1984 and de Tombe and ter Keurs 1990) have shown that there is no difference in $V_0$ within the sarcomere length range 1.85-2.3µm in cardiac muscle.
Chapter 5: Effect of ATP Depletion and ADP Accumulation
**5.1. Introduction**

The experiments studying $P_i$ release rate described in Chapters 3 and 4 were performed in the absence of an ATP regenerating system. Therefore ATP hydrolysis by the muscle results in a gradual depletion of ATP and an accumulation of ADP. $P_i$ will also begin to accumulate more as the saturation of MDCC-PBP is approached. These processes possibly explain the reduction in isometric force, rate of force development and reduced ATP hydrolysis towards the latter part of the $P_i$ release time courses.

The aim of this study was to investigate the effects of ATP depletion and ADP accumulation using an ATP regenerating system (He et al., 1998b; Thirlwell et al., 1994) consisting of 10mM creatine phosphate (CrP) and 4mg/ml creatine phosphokinase (CPK) from rabbit skeletal muscle. Creatine phosphokinase is a near equilibrium enzyme found in high activities in vertebrate muscles. During conditions of elevated ATP turnover in muscle tissue the sarcomeric isoform of CPK catalyses the conversion of CrP to creatine (Cr) whilst phosphorylating ADP to ATP (Scheme 5.1); thereby maintaining a relatively low [ADP]:[ATP] ratio in the vicinity of the active cross-bridges.

**Scheme 5.1:**

![Scheme 5.1](image_url)
5.2. Methods

The experimental protocol described in Section 4.2 was repeated but in the presence of a CrP–CPK ATP regenerating system. The solution compositions are described in Appendix A, Table 3. The time that the trabeculae are incubated in each solution is shown in Table 5.2.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Incubation Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Rigor</td>
<td>5</td>
</tr>
<tr>
<td>Ca(^{2+})-free Rigor</td>
<td>5</td>
</tr>
<tr>
<td>Ca(^{2+}) Rigor (+ 10 U.ml(^{-1}) Apyrase)</td>
<td>5</td>
</tr>
<tr>
<td>Ca(^{2+}) Rigor</td>
<td>5</td>
</tr>
<tr>
<td>Loading</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 5.2: Solution incubation times for laser activation using a creatine phosphate – creatine phosphokinase ATP regenerating system.

The main differences between the solutions used in this study and those used previously are that the ADP scavenger, apyrase (10 U.ml\(^{-1}\), Sigma A-6535), is added to the Ca\(^{2+}\)-free rigor solution and the first incubation in Ca\(^{2+}\)-rigor, to remove ADP. The NPE-caged ATP present in the loading solution is also pre-treated with apyrase (20 U.ml\(^{-1}\)) prior to the experiment.

A so-called phosphate mop is also added to the Ca\(^{2+}\)-rigor solution to remove free Pi. Phosphate mop consists of 16.5 U.ml\(^{-1}\) PNPase (bacterial purine nucleoside phosphorylase, Sigma, N-8264) and 1 mM 7-MEG (7-methylguanosine, Sigma M-
0627). This results in any free $\text{P}_i$ being phosphorylated to 7-MEG (Scheme 5.2). 10mM CrP is included in the $\text{Ca}^{2+}$-rigor and loading solutions, with 370 U.ml$^{-1}$ CPK also added to the loading solution.

**Scheme 5.2:**

$$\begin{align*}
\text{PNPase} \\
\text{P}_i + \text{7-MEG} & \rightarrow \text{7-MEG-P}_i
\end{align*}$$

5.3. Length Changes with an ATP regenerating system

Records of force and $\text{P}_i$ release from trabeculae activated in the presence of an ATP regenerating system are shown in Figure 5.3. The data are summarised in Table 5.3. Data are compared to those described in Chapter 4, where no ATP regenerating system was used, to determine the effects of ATP depletion and ADP accumulation on the force and $\text{P}_i$ release rate response to length changes.

5.3.1. Force

The force response to length changes in the presence of an ATP regenerating system has similar features to those described in Chapter 4 where no ATP regenerating system was used. An increase in $[\text{Ca}^{2+}]$ from 1 to 32$\mu$M causes a 1.5 fold increase in isometric force, with a slower rate of force development seen with 1$\mu$M $\text{Ca}^{2+}$. The peak isometric force is similar to that seen when no ATP regenerating system is used (Figure 5.3.1 A; $P > 0.05$).
Stretch causes a rapid rise in force which falls to a new steady state at the end of the stretch. At 32\(\mu\)M Ca\(^{2+}\) the force decay at the end of the stretch can be fit with a single exponential decay with a rate constant of 59.0 ± 0.3 s\(^{-1}\) and amplitude of 10.5 ± 0.04 kN.m\(^{-2}\) \((\chi^2/\text{DoF} = 0.0029; R^2 = 0.970)\). This is similar to that seen with no ATP regenerating system (see Section 4.2.2; Rate constant, 59.8 ± 0.2 s\(^{-1}\); Amplitude, 12.6 ± 0.03 kN.m\(^{-2}\)). However at 1\(\mu\)M Ca\(^{2+}\) the rate of force decay at the end of the stretch cannot be fit to an exponential decay as a slow rise in force is seen whilst being held at the longer length after the stretch. This gradual rise in force may be due to a phenomenon called ‘stretch-activation’ which has previously been seen in cardiac muscle (Campbell and Chandra, 2006; Steiger et al., 1978; Stelzer and Moss, 2006; Stelzer et al., 2007). Stretch-activation is thought to be due to an increase in the number of force generating cross-bridges by cooperative recruitment (Stelzer et al., 2006). Stretch-activation is not seen at 32\(\mu\)M Ca\(^{2+}\), which may be due to the thin filaments already being maximally activated and therefore no further activation can take place in response to stretch. As stretch-activation is not seen without an ATP regenerating system (see Chapter 4) it is possible that a high concentration of ATP or low concentration of ADP is required to observe the effect.

Stretch-induced force enhancement was seen with force increasing by ~30% and ~58% at 32 and 1\(\mu\)M Ca\(^{2+}\), respectively. This phenomenon of force enhancement after stretch was also seen without an ATP regenerating system being present (described in Section 4.2.3). Isometric experiments with the ATP regenerating system were not performed at a longer sarcomere length, therefore it cannot be determined how much of this force enhancement is due to moving up the ascending limb of the sarcomere length-force curve.
Figure 5.3: Records of force (top panel) and $P_i$ release (middle panel) from trabeculae activated in the presence of an ATP regenerating system. Trabeculae were activated from an initial sarcomere length of 1.9µm with either 32µM Ca$^{2+}$ (Black; force, n = 10; $P_i$ release, n = 4) or 1µM Ca$^{2+}$ (Grey; force, n = 5; $P_i$ release, n = 3). Bottom panel shows motor movement. Vertical lines across the panels indicate the timing of changes in motor movement.
### Chapter 5: Effect of ATP Depletion and ADP Accumulation

<table>
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<tr>
<th></th>
<th>32µM Ca\textsuperscript{2+}</th>
<th>1µM Ca\textsuperscript{2+}</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (Force)</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Length (mm)</td>
<td>1.7 ± 0.1</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>Cross-sectional area (mm(^2))</td>
<td>0.0127 ± 0.002</td>
<td>0.0132 ± 0.002</td>
</tr>
<tr>
<td>Rigor force (kN.m(^{-2}))</td>
<td>7.4 ± 1.7</td>
<td>8.9 ± 1.9</td>
</tr>
<tr>
<td>Isometric force (kN.m(^{-2}))</td>
<td>54.0 ± 6.1</td>
<td>36.0 ± 8.4**</td>
</tr>
<tr>
<td>(T_{\frac{1}{2}}) (ms)</td>
<td>65.3 ± 8.3</td>
<td>86.6 ± 12.5</td>
</tr>
<tr>
<td>Stiffness (kN.m(^{-2}).µm(^{-1}))</td>
<td>413 ± 90</td>
<td>387 ± 107</td>
</tr>
<tr>
<td>Peak force at end of stretch (kN.m(^{-2}))</td>
<td>80.0 ± 7.3</td>
<td>61.9 ± 15.2</td>
</tr>
<tr>
<td>Force enhancement after stretch (kN.m(^{-2}))</td>
<td>69.4 ± 6.1</td>
<td>57.1 ± 11.9</td>
</tr>
<tr>
<td>Force at end of release (kN.m(^{-2}))</td>
<td>14.5 ± 2.4</td>
<td>7.7 ± 2.5*</td>
</tr>
<tr>
<td>Isometric force after release(kN.m(^{-2}))</td>
<td>38.7 ± 3.9</td>
<td>27.2 ± 5.9*</td>
</tr>
<tr>
<td>(T_{\frac{1}{2}}) - Force redevelopment (ms)</td>
<td>83.8 ± 9.2</td>
<td>80.2 ± 15.8</td>
</tr>
<tr>
<td>Power output (W.L(^{-1}))</td>
<td>7.3 ± 1.2</td>
<td>3.8 ± 1.2*</td>
</tr>
<tr>
<td>Thermodynamic efficiency</td>
<td>0.16 ± 0.03</td>
<td>0.06 ± 0.02*</td>
</tr>
</tbody>
</table>

**ATPase Rate (s\(^{-1}\)):**

<table>
<thead>
<tr>
<th></th>
<th>4</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (ATPase rate)</td>
<td>14.7 ± 1.4</td>
<td>14.8 ± 1.4</td>
</tr>
<tr>
<td>Initial (First turnover)</td>
<td>7.5 ± 0.4</td>
<td>5.5 ± 0.4*</td>
</tr>
<tr>
<td>Steady state (Isometric)</td>
<td>1.4 ± 1.2</td>
<td>0.3 ± 0.6</td>
</tr>
<tr>
<td>During stretch</td>
<td>4.5 ± 0.17</td>
<td>4.4 ± 0.5</td>
</tr>
<tr>
<td>After stretch</td>
<td>7.6 ± 0.8</td>
<td>11.0 ± 0.6</td>
</tr>
<tr>
<td>During release</td>
<td>6.8 ± 1.0</td>
<td>4.8 ± 0.8*</td>
</tr>
<tr>
<td>End isometric</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 5.3:** Summary of results from length change experiments in the presence of a creatine phosphate – creatine phosphokinase ATP regenerating system as shown in Figure 5.3. * \(P < 0.05\); ** \(P < 0.01\), analysed by t-test.
After the release force redevelops back to an isometric plateau. As described in Chapter 3, ATP depletion and ADP accumulation towards the end of the experiment may cause a slower rate of force development after the release than during the initial force rise, with isometric force not fully recovering to the initial isometric force seen at the start of the experiment. The ATP regenerating system had no effect on the rate of force redevelopment after the release or on the percentage recovery towards initial isometric force with 32µM Ca\(^{2+}\). However with 1µM Ca\(^{2+}\) force redeveloped significantly faster (\(P < 0.05\)) with the addition of an ATP regenerating system, and a significant increase in the percentage recovery towards initial isometric force was seen (\(P < 0.05\); see Figure 5.3.1 B and C).

### 5.3.2. \(P_i\) Release Rate

The rate of \(P_i\) release shows a similar response to length changes with an ATP regenerating system to that described in Chapter 4, where no ATP regenerating system was used. An initial fast rate of \(P_i\) release is seen which then slows down to a pseudo-steady state once force has reached the isometric plateau. Stretch causes a reduction in \(P_i\) release to almost zero and an increase in \(P_i\) release above the isometric rate is seen during the release. The use of the ATP regenerating system itself produced no significant differences in the rates of \(P_i\) release at any stage of the length change protocol, at either activation level, compared to when ATP regenerating system was not used. However, the ATP regenerating system did cause the \(P_i\) release rate, after the release, to recover by an additional 10% towards the initial steady state rate (Figure 5.3.2). This additional recovery was not seen at 32µM Ca\(^{2+}\).
Figure 5.3.1: Comparison of force measurements at 32 and 1\mu M \text{Ca}^{2+} in the presence (Grey) and absence (Black) of an ATP regenerating system. * $P < 0.05$, difference between experiments with and without ATP regenerating system at same [Ca$^{2+}$].
Figure 5.3.2: Comparison of $P_i$ release rates at 32 and 1μM $Ca^{2+}$ in the presence (Grey) and absence (Black) of an ATP regenerating system. * $P < 0.05$, difference between experiments with and without ATP regenerating system at same $[Ca^{2+}]$. 
5.4. Discussion

The aim of this study was to determine the effects of ATP depletion and ADP accumulation on the response of force and $P_i$ release rate to the stretching of active myocardial sarcomeres. This was done using a CrP – CPK ATP regenerating system resulting in re-phosphorylation of ADP (released by the actomyosin ATPase during cross-bridge cycling) to ATP.

The use of the ATP regenerating system had no significant effect on the isometric force and steady state $P_i$ release rate (compared to the data presented in Chapter 4 where no ATP regenerating system was used). This suggests that the ADP that would have accumulated in the absence of an ATP regenerating system (~0.3-0.5 mM after 0.35 – 0.4 s of activation was insufficient to significantly affect isometric force and steady state $P_i$ release. At 0.35 – 0.4 s into the time courses, the [ATP] would still have been greater than 0.5 mM.

The ATP regenerating system does, however, improve the rate of force redevelopment after the release, the percentage recovery of force after the release (towards initial isometric force) and the percentage recovery of steady state $P_i$ release (towards the initial steady state rate) at 1µM Ca$^{2+}$. This is perhaps because relatively more ATP was available, and less ADP accumulated, towards the end of the activation time course; ADP would have been recycled into the ATP pool by the action of CPK, and kept the ADP:ATP ratio very low in the vicinity of the active cross-bridges.

The ATP regenerating system used in this study does not prevent accumulation of $P_i$ as MDCC-PBP becomes saturated late in the contraction time course. Increased [P$_i$] is known to cause a reduction in isometric force and an increase in the rate constant of force development (Caremani et al., 2008). This may explain why there was no improvement in force recovery and $P_i$ release rate in the presence of 32µM Ca$^{2+}$. At 32µM Ca$^{2+}$ more $P_i$ is likely to have been released throughout the activation time.
course due the higher rate of ATP hydrolysis; therefore, MDCC-PBP will have saturated earlier and resulted in greater build-up of $P_i$ towards the end of the experiment, than with 1µM Ca$^{2+}$. Further experiments are required to determine the effect of an accumulation of $P_i$ on force and rate of $P_i$ release.

At maximal activation, power output (7.3 W.L$^{-1}$) was closer to that measured with temperature-jump activation (10.2 W.L$^{-1}$) than it was without the ATP regenerating system (5.43 W.L$^{-1}$; see Section 4.4.3). This improvement in power output is due to more ATP being available and less ADP having accumulated at the time of the release.
Chapter 6: General Discussion
6.1 Summary and General Discussion of Results

The aims of this thesis were to examine the effects of sarcomere length changes and activation level on the mechanoenergetic properties of force production, at specific stages of the cardiac cycle, using a novel technique for measuring the rate of $P_i$ release – the fluorescent $P_i$ probe MDCC-PBP. This is thought to be the first time that MDCC-PBP has been used to study biochemical and mechanical events in permeabilised cardiac muscle. Contraction protocols were designed to simulate the different phases of the heart beat: passive stretch (due to ventricular filling during diastole), isometric force development (during isovolumic contraction) and cardiac work (ejection phase during systole).

The main advantage of using MDCC-PBP to study the rate of ATP hydrolysis is that the rate of $P_i$ release can be monitored with a very high time resolution (on a sub-millisecond time scale). Methods previously used to measure ATPase rate, such as the NADH-linked enzyme assay for ADP appearance, limits the time resolution of the measurements to tens of milliseconds (Ebus and Stienen, 1996; Griffiths et al., 2009). The NADH assay is useful for measuring the steady state ATPase rate during isometric contractions and during low speed length changes, however it cannot be used to resolve the ATPase rate during the approach to steady state and instantaneous changes in response to length changes. MDCC-PBP was used to resolve changes in $P_i$ release in real time which is important for detecting the early time course during force development as there may only be time for a single or few cross-bridge turnovers during the isometric phase of the cardiac cycle in vivo.
6.1.1. Summary and Discussion of Results

6.1.1.1. Effect of Passive Length Changes

Passive stretch was performed by increasing the sarcomere length of relaxed trabeculae. An increase in sarcomere length from 1.9 to 2.1µm caused a 1.35-fold increase in maximal isometric force. At submaximal activation (1µm Ca\(^{2+}\)) a more substantial increment in isometric force with sarcomere length is seen (1.95-fold) although absolute force is lower due to less thin filament activation.

Although an increase in force is seen with increasing sarcomere length, this is not accompanied by an increase in the rate of Pi release, measured by MDCC-PBP. However, the steady-state rate of Pi release does increase with activation level. Increasing the activation level from 1 to 32µM Ca\(^{2+}\) causes a 1.65-fold increase in the rate of Pi release at both sarcomere lengths. The results show that at the longer sarcomere length less ATP is used per unit of force produced at both activation levels. Thus, cardiac muscle is more economical at longer sarcomere lengths. This suggests that either stretch causes an increase in the force produced per cross-bridge, resulting in more force being produced without an increase in Pi release, or the increase in force is due to stretching of parallel elastic components, such as titin. Note however that as resting tension due to titin has already been subtracted, its further involvement in these processes would infer a calcium-sensitivity of titin, which has been suggested previously (Labeit et al., 2003; Joumaa et al., 2008).

The increase in force is due to moving up the ascending limb of the sarcomere length-tension curve, which is known to be steep in cardiac muscle. The steepness of the sarcomere length-tension curve is a feature of the Frank-Starling relationship and cannot be explained by changes in myofilament overlap (as it can in skeletal muscle). The steep ascending limb is thought to be due to an increase in myofilament calcium sensitivity although it is unknown how this occurs. As the heart is not normally maximally activated, but operates over submaximal levels of
activation, the experiments performed at 1μM calcium are closer to physiological conditions. Thus, the ascending limb of the sarcomere length-tension relationship appears to be steeper at submaximal activation.

Data analysis by two-way ANOVA shows a significant effect of calcium (P < 0.001) and sarcomere length (P < 0.001) on force, but no interaction between the two on either force or P_i release. Interaction between Ca^{2+} and sarcomere length would be expected, at least with respect to force, as a leftward shift in the force-pCa curve is seen with increasing sarcomere length. Currently interaction is close to being significant (P = 0.08) therefore it is possible an increase in the number of experiments in the low activation state may determine if there is indeed significant interaction.

6.1.1.2. Effect of Length Changes in Contracting Muscle

The effect of length changes were also examined in active trabeculae by applying a stretch-release protocol 400ms after activation, once isometric force had developed. Stretch causes a rapid rise in force accompanied by a substantial and instantaneous decrease in the rate of P_i release and therefore the ATPase rate. At the end of the stretch the trabecula was held at the longer length for 300ms. During this period force is higher than the initial isometric force, termed force enhancement after stretch, and the rate of P_i release is lower. During shortening force rapidly decreases in accordance with the force-velocity relationship and an increase in the rate of P_i release is seen.

These results agree with numerous earlier studies in skeletal muscle which show that during stretch force is maintained higher than isometric value (Herzog et al., 2006), ATPase rate is lower during stretch and after a stretch than it is during isometric contraction (Curtin and Davies, 1975; Linari et al., 2003) and energy turnover increases during shortening (He et al., 1999).
The decrease in ATPase rate lasts for the duration of the stretch. The ATPase rate is constant throughout the duration of the stretch. As sarcomere length and force are constantly changing during the stretch, this indicates that the change in ATPase rate is independent of sarcomere length and force. Less ATP is hydrolysed although force remains high during the stretch, suggesting stretch-induced thin filament activation.

The rate of $P_i$ release is similar during the stretch at 1 and 32µM Ca$^{2+}$, in contrast to isometric contractions where $P_i$ release increases with activation level. The low rate of $P_i$ release during stretch indicates an accumulation of $P_i$-containing pre-power stroke force bearing cross-bridge states (A.M.ADP.$P_i$; Bickham et al., 2011). The effect of stretch on $P_i$ release appears to be instantaneous, suggesting that stretch is having a direct effect on the cross-bridge ATPase site (e.g., stretch may cause distortion of the active site, thus reducing $P_i$ release). Force enhancement after stretch was relatively greater at low Ca$^{2+}$ yet the rate of $P_i$ release was similar at high and low Ca$^{2+}$, suggesting that stretched cross-bridges at low Ca$^{2+}$ are more economical. The results suggest that stretch causes cross-bridge attachment and/or cooperative activation of the thin filaments, thus reducing the calcium concentration needed to activate thin filaments.

The amplitude of the stretch is greater than the reach of the cross-bridges, thus cross-bridges must be forcibly detached, however as force remains high this must be followed by a rapid reattachment. The classical view of the cross-bridge cycle, as developed by Huxley and Simmons (1971), involves an unbranched cross-bridge cycle that consists of sequential attached and detached states (described in Section 1.4.2). This model is not sufficient for explaining the detachment and reattachment which must occur during stretch (as described above) with no additional ATP hydrolysis. Therefore, stretch-induced detachment and reattachment must be occurring via a different pathway to that of isometric contraction where cross-bridge attachment is preceded by ATP hydrolysis. It has recently been suggested that in skeletal muscle the cross-bridge cycle may include a detachment and reattachment step which does not involve ATP hydrolysis (Bickham et al., 2011; Lombardi and Piazzesi, 1990; Piazzesi et al., 1992). The results presented in this
thesis suggest that a similar branched cross-bridge cycle may be involved in the response of cardiac muscle to stretch (Scheme 6.1.1.2).

During shortening an increase in the rate of P\textsubscript{i} release is seen. This corresponds to the ejection phase of the cardiac cycle. An increase in the rate of P\textsubscript{i} release is seen during shortening due to the Fenn effect (Fenn, 1924). Using heat measurements in skeletal muscle, Fenn showed that energy output increases during muscle shortening, however there is still controversy over the significance of a Fenn effect in intact cardiac muscle.

By using the ATP regenerating enzyme creatine kinase, it can be seen that the key observation of a stretch-induced decrease in the rate of P\textsubscript{i} release is not attributable to depletion of ATP and accumulation of ADP, although these processes contribute to the gradual slowing down of the response from the time of photolytic release of ATP.

\textbf{Scheme 6.1.1.2}: A branched cross-bridge reaction scheme allowing cross-bridge detachment followed by reattachment without release of P\textsubscript{i}. A, actin; M, myosin.
6.1.2. Relevance of the Study

The classical view of the Frank-Starling relationship, describes the effect of end-diastolic volume on myocardial force production, thus, the Frank-Starling relationship occurs via stretch of the muscle at rest. However, it is also well known that transmural patterns of stretch occur throughout the ventricular wall during systole (Stevens and Hunter, 2003; Ashikaga et al., 2009). The experiments and results presented in Chapter 3 examine the effect of stretch at rest whereas the experiments in Chapters 4 and 5 involve stretching of an active muscle. Both types of experimental protocols simulate processes that occur physiologically within the heart and are thus relevant to studying cardiac function.

There is also evidence that some degree of active cross-bridge formation occurs during diastole in intact cardiac muscle (Bai et al., 2011; King et al., 2011). Thus, the study of the effect of stretch on active muscle can also be applied to the Frank-Starling relationship. Similar studies have also been performed to investigate the effect of length changes in active skeletal muscle, therefore we can compare and contrast the cardiac and skeletal muscle data to further evaluate Frank-Starling mechanisms of cross-bridge stretch.

6.2 Limitations of the Study

Due to the importance of the heart and the current prevalence of heart disease it is very important to study cardiac muscle, however it is not very well ordered (compared to skeletal muscle) invariably making experiments more difficult to perform. For example, sarcomere length could not be tracked throughout the course of the contraction, as it can in skeletal muscle. This is a common problem with studies of cardiac muscle (Kentish and Stienen 1984) and is due to non-uniformity and scattering of light by non-contractile elements. The lack of a sarcomere length signal means the exact sarcomere length at each stage of the
contraction protocol cannot be known, it can only be assumed from muscle length changes and cross-sectional area changes.

Compliance at the muscle ends due to damage during dissection and attachment to the experimental set-up can result in internal shortening of the sarcomeres during force development and therefore an increase in ATP hydrolysis and further uncertainty of sarcomere length. In the experiments described in this thesis efforts were made to reduce end compliance by careful dissection in the presence of BDM to minimise damage to the muscle ends (Mulieri et al. 1989) and the use of shellac to hold the aluminium clips in place on the experimental set-up. Initial sarcomere shortening due to compliance may partly contribute to the initial fast rate of $P_i$ release seen in these experiments.

Ideally experiments studying physiological processes should be performed under physiological conditions, however due to the nature of the experiments used in this study this was not entirely possible:

- The majority of studies on both skeletal and cardiac muscle are usually performed at sub-physiological temperatures. This is because at 37°C (physiological temperature) muscles produce significantly more force and as they are not securely attached at each end (as they are in vivo) they tend to pull themselves apart. Also at higher temperatures biochemical reactions occur much faster, which in this case would cause a much faster saturation of MDCC-PBP therefore less time to detect any changes in $P_i$ release rate. For these reasons experiments were performed at 20°C in this study, with the added advantage of allowing comparisons with previous studies on cardiac muscle that were also performed at this temperature.

- During the cardiac cycle only very short isometric periods are seen, during isovolumic contraction and isovolumic relaxation, and sustained activation does not occur. In these experiments, trabeculae were continuously activated and allowed to develop isometric force for periods of time.
significantly greater than would occur physiologically. However, this was necessary as fluorescence recordings need to be made over a suitable time frame to determine changes in $P_i$ release rate.

- In these experiments it is necessary to activate the trabeculae from rigor although muscles never go into rigor in vivo. The activation protocol requires all ATP to be removed to prevent premature contraction before laser-flash photolysis of NPE-caged ATP. As soon as the laser-flash occurs ATP is released from NPE-caged ATP causing a transient relaxation before force development. Therefore, although the trabeculae are activated from rigor, force develops from a relaxed state, as occurs physiologically. Prior to activation the time that the trabeculae are kept in the rigor state was kept to a minimum.

- The use of skinned muscle preparations is also not physiological and is thought to cause some degree of lattice swelling. The skinning process may cause loss of some soluble proteins and of some parallel elastic components such as the sarcolemma. Skinned trabeculae have also been shown to be more compliant than intact trabeculae (Kentish et al., 1986). However, skinned trabeculae must be used in these experiments as the activation process and measurement of $P_i$ release requires direct control of the biochemical environment of the myofilament lattice.

### 6.4. Future Work

The results of the experiments described in this thesis show that an increase in sarcomere length results in more force being produced per unit of ATP hydrolysed. However, further work is required to determine what causes this increase in force economy.
Current theories of the Frank-Starling relationship suggest the increase in Ca\(^{2+}\)-sensitivity may be due to a reduction in myofilament lattice spacing in response to stretch (described in Section 1.4.1). Experiments performed by Fuchs and Wang (1996) used varying concentrations of the osmotic agent dextran to preserve lattice spacing whilst changing sarcomere length. They observed that Ca\(^{2+}\)-sensitivity correlated more closely with interfilament spacing than with sarcomere length. This is thought to be due to myosin heads being in closer proximity to the actin filament, thus increasing the probability of cross-bridge formation. Experiments measuring ATPase rate using MDCC-PBP could be performed in the presence of varying concentrations of dextran to change lattice spacing (without a change in sarcomere length) to see if there is a similar effect on force economy as is seen with sarcomere length changes. If there is then this would suggest that the improved force economy with increased sarcomere length is due to an effect on the cross-bridges themselves, rather than stretching of parallel elastic elements.

One possible way that cross-bridges could sense sarcomere length is through myosin binding protein-C (MyBP-C), a protein known to bind to and stabilise thick filaments (Fukuda and Granzier, 2005). MyBP-C can also bind to titin which, due to spanning the whole sarcomere, is able to sense sarcomere length (Palmer et al., 2004). MyBP-C has been shown to restrain actin-myosin interaction during diastole (Pohlmann et al., 2007). Thus, myocyte stretch, transmitted through titin, may influence MyBP-C’s control over actin-myosin interaction in active muscle. Therefore, studying the energetics of cardiac muscle contraction and the response to stretch in cardiac MyBP-C knock-out mice will help determine if this protein does indeed influence the cardiac stretch response at a cross-bridge level.

Heart disease is one of leading causes of death in developed countries. The generation of mice expressing mutations leading to hypertrophic and dilated cardiomyopathies or subjected to experimental myocardial infarctions (e.g. by ligation of the left anterior descending coronary artery) are powerful tools in cardiac research. In order to successfully treat and prevent such conditions a good
understanding of the genetics, molecular mechanisms and pathophysiology is required. Investigating the mechanics and energetics of cardiac function in such animal models using experimental methods similar to those described in this thesis could provide valuable information about the molecular changes that occur in such diseases.
References


References


References


Appendices
Appendix A: Solutions

All solutions were made up as double strength stock solutions and diluted with dH₂O. Concentrations listed are final concentration after dilution. Solutions were stored at -20°C in 1ml aliquots and thawed as required.

Relaxing Solution:

<table>
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<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>K⁺ Propionate</td>
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</tr>
<tr>
<td>Mg Acetate</td>
<td>8mM</td>
</tr>
<tr>
<td>K₂EGTA</td>
<td>5mM</td>
</tr>
<tr>
<td>Na₂ATP (Sigma A3377)</td>
<td>7mM</td>
</tr>
<tr>
<td>Imidazole</td>
<td>6mM</td>
</tr>
<tr>
<td>PMSF *</td>
<td>10µM</td>
</tr>
<tr>
<td>Trypsin Inhibitor (Sigma T-9003)</td>
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</tr>
<tr>
<td>Leupeptin (Sigma L-2884) *</td>
<td>4mg.L⁻¹</td>
</tr>
</tbody>
</table>

Table 1: Composition of relaxing solution. Solutions were made up to 150mM ionic strength and pH 7.1 at 20°C. For storage of the muscle at -20°C a 50% v/v glycerol/relaxing solution was made. Protease inhibitors were added if the solution was to be used for storage of the muscle.

Abbreviations: EGTA, 1,2-Di(2 aminoethoxy)ethane- N,N,N',N'-tetra-acetic acid; ATP, Adenosine 5'-Triphosphate (Sigma A3377); PMSF, Phenyl methyl sulfonyl fluoride.
Solutions for Laser Activation:

<table>
<thead>
<tr>
<th></th>
<th>Pre-Rigor</th>
<th>Ca(^{2+})-free Rigor</th>
<th>Ca(^{2+}) Rigor (1µM Ca(^{2+}))</th>
<th>Ca(^{2+}) Rigor (32µM Ca(^{2+}))</th>
<th>Loading (1µM Ca(^{2+}))</th>
<th>Loading (32µM Ca(^{2+}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>TES</td>
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<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
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<tr>
<td>MgCl(_2) (^{(1)})</td>
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<td>2.38</td>
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<td>CaEGTA (^{(2)})</td>
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<td>-</td>
<td>15.40</td>
<td>19.85</td>
<td>15.40</td>
<td>19.88</td>
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<tr>
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<td>0.15</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glutathione</td>
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<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>K(^+) Propionate</td>
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<td>63.94</td>
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<td>NPE-caged ATP (^{(3)})</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>1.2</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**Table 2:** Composition of solutions for activation by laser-flash photolysis of NPE-caged ATP. Concentration given in mM. Solutions were made up to 150mM ionic strength, adjusted with K\(^+\) Propionate, and pH 7.1 at 20°C. (1) Concentration corresponding to free [Mg\(^{2+}\)] 2.0mM; (2) Concentration corresponding to free [Ca\(^{2+}\)] 1µM or 32µM; (3) The loading solution contains 5mM NPE-caged ATP. Upon photolysis 1.5mM ATP is released. In the case of solutions containing NPE-caged ATP the contribution of NPE-caged ATP to the ionic strength was assumed to be equal to that contributed by an equal concentration of ADP.

### ATP Regenerating System:

<table>
<thead>
<tr>
<th></th>
<th>Pre-Rigor</th>
<th>Ca\textsuperscript{2+}-free Rigor</th>
<th>Ca\textsuperscript{2+} Rigor (1µM Ca\textsuperscript{2+})</th>
<th>Ca\textsuperscript{2+} Rigor (32µM Ca\textsuperscript{2+})</th>
<th>Loading (1µM Ca\textsuperscript{2+})</th>
<th>Loading (32µM Ca\textsuperscript{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>TES</td>
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<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>MgCl\textsubscript{2}</td>
<td>3.76</td>
<td>3.66</td>
<td>2.62</td>
<td>2.25</td>
<td>6.16</td>
<td>5.78</td>
</tr>
<tr>
<td>CaEGTA</td>
<td>-</td>
<td>-</td>
<td>15.40</td>
<td>19.85</td>
<td>15.40</td>
<td>19.88</td>
</tr>
<tr>
<td>EGTA</td>
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<td>20</td>
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<td>0.15</td>
<td>4.60</td>
<td>0.12</td>
</tr>
<tr>
<td>Na\textsubscript{2}ATP</td>
<td>0.11</td>
<td>-</td>
<td>-</td>
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<tr>
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<tr>
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**Table 3:** Composition of solutions for laser activation using a creatine phosphate – creatine phosphokinase ATP regenerating system. Concentrations are given in mM unless stated otherwise. Ionic strength 150mM, pH 7.1. The muscle is incubated in the Ca\textsuperscript{2+} rigor solutions twice, the first time being in the presence of 10 U.ml\textsuperscript{-1} apyrase. * NPE-caged ATP is pre-treated with apyrase (20 U.ml\textsuperscript{-1}) prior to these experiments.

**Abbreviations:** CP, Creatine phosphate (N-[imino(phosphonoamino)methyl]-N-methylglycine); PNPase, Purine nucleoside phosphorylase; MEG, Methylguanisine; CPK, Creatine phosphokinase.
Solutions for Temperature-Jump Activation:

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<th>Activating Solution</th>
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<td>Glutathione</td>
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</tr>
</tbody>
</table>

**Table 4:** Composition of solutions for activation by temperature-jump. Concentrations given in mM. Ionic strength 200mM, pH 7.1.

Abbreviations: HDTA, 1,6-Diaminohexane-$N,N,N',N'$-tetraacetic acid.
Appendix B: Production and Labelling of MDCC-PBP

The probe used to assay inorganic phosphate ($P_i$) release in this thesis was the A197C mutant phosphate binding protein (PBP) from *Escherichia coli*. The mutant protein has a cysteine residue introduced at position 197 by oligonucleotide-directed mutagenesis (Brune *et al.*, 1994), allowing labelling at this site with the fluorophore N-(2-[1-maleimidyl]ethyl)-7-diethylaminocoumarin-3-carboxamide (MDCC). The mutant *E. Coli* glycerol stock was provided by M. Webb and J. Hunter (NIMR, London) and the production, purification and labelling of PBP was carried out in our lab at Imperial College, London.

Production and purification of PBP

The method described below was developed by M. Webb and J. Hunter (NIMR, London). The production and purification of PBP was initially carried out by D. Ushakov (Imperial College, London). Following his departure from Imperial College, I carried out the production and purification of PBP following the protocol detailed here.

**Pre-preparation:**

Sterile techniques are used until the cells are harvested.

Prepare 10cm L. Agar + tetracycline (0.0125mg.ml$^{-1}$) plates and store at +4°C. Streak dried and warmed to 37°C L.Agar + tetracycline plates with glycerol stock of PBP. The glycerol stock should be kept in dry ice, not allowed to thaw, and returned as soon as possible to -80°C. Incubate inverted plates overnight at 37°C. There should be several colonies the following morning. Seal the plates with Parafilm and store at 4°C inverted.
**Day 1:**

Take 100ml Lysogeny Broth (L. Broth). To 10ml L.Broth add 10µl 12.5mg.ml⁻¹ tetracycline in a 50ml flask and warm to 37°C. Inoculate with 3-4 colonies off the plate. Grow for 6-7 hours with shaking at 220 revolutions per minute (rpm). Check it is thick with cell growth after 2.5 hours.

Add 90µl 12.5mg.ml⁻¹ tetracycline to the remaining 90ml L.Broth, transfer to a 250ml flask and warm to 37°C. Add the 10ml incubation mixture and incubate at 37°C overnight with shaking at 220rpm.

Prepare 8 x 490ml TG+ and allow to come to room temperature overnight. TG+ (filter membrane sterilised and adjusted to pH 7.2): 120mM Tris, 80mM NaCl, 20mM KCl, 20mM NH₄Cl, 3mM Na₂SO₄, 50mg.L⁻¹ L-leucine, 10mg.L⁻¹ L-tryptophan, 40mg.L⁻¹ L-histidine HCl, 100mg.L⁻¹ L-arginine HCl, 20mg.L⁻¹ L-methionine, 20mg.L⁻¹ L-adenosine.

**Day 2:**

Check the overnight culture is thick with cells.

Weigh 120mg L-tryptophan and 60mg thiamine. Dissolve together in 60ml double distilled deionised water (DDIW, 18MΩ.cm⁻¹). Filter sterilise through a 0.2µm membrane into a sterile 100ml flask.

Prepare high phosphate (HiPi) supplement mixture. To 18ml sterile DDIW add 50ml 64mM KH₂PO₄, 20ml 50% glucose, 5ml 10mM FeSO₄, 1ml 1M CaCl₂, 1ml 1M MgSO₄. Mix well.

Add 5ml of the tryptophan/thiamine solution to 4 of the 490ml TG+ aliquots at room temperature. Add 5ml 12.5mg tetracycline to the HiPi supplements, mix and IMMEDIATELY add 10ml HiPi supplement to each TG+ (The tetracycline and FeSO₄ precipitate out if allowed to stand together).

Transfer the TG+ with supplements to 4 x 2L baffled flasks and warm in an incubator to 37°C. Inoculate each flask with 10ml of the 100 ml overnight culture. Incubate at 37°C with shaking at 220rpm for 6 hours.

Using a spectrophotometer measure the absorbance at 600nm (A600) of the overnight culture diluted 1:10 in 10% formaldehyde against L.Broth diluted in the same way. A reading of 2 or more should be expected.
Read A600 of one of the 500ml cultures, at inoculation and each hour throughout the day, to monitor cell growth. Dilute culture 1:2 with 10% formaldehyde against TG+ with HiPi supplements diluted in the same way. Plot time against A600 values on a logarithmic scale (Figure 1).

![Figure 1: Logarithmic plot of E.coli cell growth.](image)

There should be an initial lag phase of approximately 1.5 hours followed by a doubling of cell concentration over the next 3-4 hours when the cell growth reaches a plateau. This is the time to transfer the cells to a low phosphate medium to induce protein production. The cells usually take 6-7 hours to reach this point.
**Appendix B: Production and Labelling of MDCC-PBP**

**Induction:**

Warm 4 x 490ml TG+ to 37°C in a water bath.

Prepare a tryptophan/thiamine solution and sterilise as before.

Prepare low phosphate (LoPi) supplements. To 63ml sterile DDIW add 5ml 64mM KH$_2$PO$_4$, 20ml 50% glucose, 5ml 10mM FeSO$_4$, 1ml 1M CaCl$_2$, 1ml 1M MgSO$_4$.

Transfer the cells from the 2L flasks into 4 x 1L centrifuge bottles. Centrifuge at 4000rpm for 25 minutes at room temperature.

Complete the LoPi TG+ by adding 5ml tryptophan/thiamine solution and 10ml LoPi supplements to each of the 4 490ml TG+ solutions, as near as possible to the centrifuge finishing.

After centrifuging, discard the HiPi medium and resuspend the cell pellets in LoPi TG+. Ensure the cells are well suspended and distribute evenly between 4 x 2L flasks. Incubate at 37°C with shaking at 220rpm for 16 hours.

**Day 3:**

At 16 hours incubation read A600 of the cells, diluted 1:10 in 10% formaldehyde against LoPi TG+ diluted in the same way.

Transfer cells to 4 x 1L centrifuge bottles and centrifuge at 4000rpm for 25 minutes at room temperature.

Make 1L 30mM NaCl in 10mM Tris HCl, pH7.6 at room temperature.

Weigh one marked centrifuge bottle. Discard the LoPi TG+ and resuspend the pellets in 650ml NaCl/Tris buffer in the weighed centrifuge bottle. Centrifuge at 4000rpm for 25 minutes at room temperature.

Discard the buffer and resuspend the cells in the remaining 350ml of the NaCl/Tris buffer. Centrifuge as before.

Discard the buffer and weigh the bottle to calculate the weight of the pellet. The pellet should weigh approximately 15g.

Resuspend the pellet into 100ml 33mM Tris HCl (pH 7.6 at 25°C). Transfer to a 250ml centrifuge bottle containing a stirring bar. While stirring briskly add 100ml
40% sucrose, 33mM Tris HCl, 0.1mM EDTA. Continue to stir briskly for 10 minutes. Remove the stir bar and centrifuge at 10,000rpm for 30 minutes at 4°C.

Discard the supernatant and resuspend the pellet in 200ml ice cold 0.5mM MgCl₂. Stir briskly on ice for 15 minutes. Centrifuge at 10,000rpm for 30 minutes at 4°C.

Measure volume of the shockate and make to 10mM with respect to Tris HCl. Dilute 1:10 with 10mM Tris HCl and scan on a spectrophotometer against 10mM Tris HCl. There should be evidence of a shoulder at 290.5nm (Figure 2). This is typical of PBP, however the peak will probably be nearer 260nm which is most likely adenosine.

Figure 2: Absorbance spectrum of shockate. The shoulder at 290.5nm is typical of PBP.
Column Chromatography:

Equilibrate a Q-Sepharose Fast Flow column (120-130ml, 2.6 x 25 cm) with 10mM Tris HCl + 1mM MgCl₂ buffer (pH 7.6) at 4°C. Check the speed of the column is 2ml.min⁻¹ and that the conductivity and pH of the buffer post column is the same as that going in.

Check the pH and conductivity of the shockate. Add cold water to adjust the conductivity to that of the column equilibration buffer.

Load the shockate onto the column. Monitor A₂₈₀ and collect 20ml fractions whilst loading, reduced to 6ml fractions during the wash and gradient.

Wash the column with at least one column volume of 10mM Tris HCl + 1mM MgCl₂.

Apply a gradient 1L total volume, 0-200mM NaCl in 10mM Tris HCl + 1mM MgCl₂.

PBP is eluted at approximately 220ml after the start of the gradient. Scan fractions at 280nm on the spectrophotometer to find the start of the peak and where it ends. Pool together tubes within the peak for subsequent concentration. It is best to cut short the tail end of the peak to avoid wild-type PBP.

When finished wash the column with at least one column volume of a high salt solution (10mM Tris HCl + 500mM NaCl) to clean the column of any remaining protein.

Concentration:

Concentrate the pooled fractions using Centricon centrifugal filter devices (Millipore, YM-10). Centrifuge at 4000rpm for 60 minutes at 4°C. Scan the final preparation on the spectrophotometer diluted 1:100 with 10mM Tris against 10mM Tris HCl.

Protein concentration is calculated based on absorbance at 280nm assuming an extinction coefficient of 17.8cm⁻¹.

Thus: \[
[PBP] = \frac{A_{280} \times \text{dilution factor}}{1.78} \text{ mg.ml}^{-1}
\]
Division of the concentration by the molecular weight of PBP (\(M_r = 34.5\text{kJD}\)) will further generate the molarity in mM. Aim for a final concentration of approximately 1 - 1.5mM (35-60mg.ml\(^{-1}\)). Split into ~28 mg aliquots quick frozen in dry ice and store at -80°C for subsequent labelling with MDCC.
Labelling PBP with MDCC

The method described below was written by J. L. Donnelly (Imperial College, London). The PBP labelling was initially carried out by D. Bickham (Imperial College, London). Following his departure from Imperial College, I carried out the protein labelling following the protocol detailed here.

Briefly, preparation involves conjugation of the raw protein with MDCC fluorophore by simple mixing under phosphate-free conditions, followed by separation of bound and unbound protein by ion exchange column chromatography. The optical density at 280nm of fractions of column eluent is recorded and individual fractions assessed spectrophotometrically to identify bound protein. Selected fractions are then pooled and concentrated centrifugally. MDCC-PBP activity can be assessed by titration against \( P_i \) standards, and the finished product is aliquoted and stored at \(-80°C\).

Conjugation of protein with fluorophore

Thaw several aliquots of unlabelled PBP as required. Measure volume and concentration of PBP. Concentration is measured as before, based on absorbance at 280nm zeroed against 10mM Tris HCl buffer with the protein diluted 1:100 with 10mM Tris HCl.

Use the volume and concentration of PBP to calculate the required volumes of the components of the labelling solution (based on a final concentration of 100µM PBP). In a tube mix together the components to give the required final concentration – 20mM Tris HCl, 100µM PBP, 150µM MDCC and \( P_i \) Mop components 0.2U.ml\(^{-1}\) PNPase and 200µM MeG. NB: Let the \( P_i \) Mop do its work for about 10 minutes before adding the MDCC last.

Ensure the lid is tight and cover with foil. Place on an end-over-end rotator for 30 minutes at 20°C.
**Q-Sepharose Fractionation**

The underlying principle of Q-Sepharose fractionation is that the protein-coumarin complex will initially adhere to the gel, and subsequently be released by a narrow threshold polarity as the salt gradient is introduced. The more gradual an increase in salt concentration, the greater the spread of the peaks of fluorescence within the eluent over a larger number of fractions, facilitating identification and purification (but also increasing volume that needs to be concentrated). Small ‘breakout’ peaks are normally followed by two peaks representing MDCC-PBP and subsequently eluted free MDCC.

Set up a C16/20 (20ml loading volume) column at 4°C containing Q-Sepharose Fast Flow beads and supplied with freshly made up 10mM Tris buffer (pH 8.0 at room temperature, pH 8.2 at 4°C; ζ (= conductivity) ≤ 400 S.cm\(^{-1}\)) via a downstream peristaltic pump calibrated to 2ml.min\(^{-1}\). The system also consists of a combined control and optical unit (which should be set to read absorbance levels at 280nm (OD\(_{280}\))) with a chart recorder to record absorbance and conductivity, and a fraction collector. Switch on machine and allow UV lamp 15 minutes to warm up. Fill fraction collector with tubes.

Wash the column with at least one column volume of 10mM Tris HCl + 1mM MgCl\(_2\). Check the conductivity of the PBP/MDCC mixture is equal to that of the 10mM Tris HCl buffer, dilute with DDIW as required. Load the column with the diluted PBP/MDCC mixture and wash with at least one column volume of 10mM Tris HCl + 1mM MgCl\(_2\). Run a 100ml gradient of 0-50mM NaCl collecting 6ml fractions. When the MDCC-PBP has eluted, clean the column by running through 200-250ml 500mM NaCl in 10mM Tris.

Use the graph generated by the chart recorder (Figure 3) as an indicator of which fractions can be pooled to provide a source of pure MDCC-PBP, and which a source of dissociated MDCC.

Confirm this indication by measuring maximum wavelength values of the second (unbound MDCC) peak and calculating the ratio of absorbance levels at 280nm and at the maximum wavelength in aliquots of fractions from the ascending limb, descending limb and subsidiary peaks. Use a dedicated phosphate-free cuvette and a plastic pipette.
Figure 3: $A_{280}$ ($OD_{280}$) of fractions of MDCC-PBP eluted from Q-Sepharose column.

Figure 4: A, Absorption spectrum of fractions from the ascending limb of Figure 3. B, Absorption spectrum of fractions from the descending limb of Figure 3. C, Absorption spectrum of fractions from the subsidiary peaks of Figure 3.
Pool fractions for further concentration of MDCC-PBP (or recycling of disassociated MDCC) on the basis of the ratio (between 1.57 and 1.67) and maximum absorbance (<434nm). In the above example (Figures 3 and 4) fractions 76-98 were pooled for further concentration, and fractions 99-133 were taken for recovery of MDCC.

**Concentration of MDCC-PBP**

Concentration of MDCC-PBP is achieved using a Millipore Amicon Centriprep. Each Centriprep holds 15ml. Take two Centripreps and divide the protein solution equally between them (filling the outer, larger compartment) and weigh each Centriprep to check that they are balanced. Spin for 50mins at 3,665 rpm (20°C).

Once spinning is completed, decant the liquid from the centre of the Centriprep (i.e. the clear liquid that has passed through the filter – the protein is too large to pass through the filter, so will remain in the outside chamber). Spin again as before.

Decant the filtered protein into the smaller Centricons (each holds 2ml) – make sure that the centrifuge is balanced and spin at 5,000 rpm for 99mins. Repeat spinning until the volume looks small enough to be fully concentrated (should have ~150µl protein when fully concentrated).

Measure the final concentration on a spectrophotometer, as before. Ideally the concentration should be no less than 4mM. Continue spinning if not concentrated enough.

The MDCC-PBP is then ready for the fluorescence and activity assay described in Section 2.5.2. It can then be split into aliquots of the required volume and frozen at -80°C until needed.
References

