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The role of extracellular matrix in modulation of myocyte Ca^{2+} signalling and contraction in the isolated rat heart

Sarbjot Kaur

A thesis submitted in complete fulfillment of the requirements for the degree of Doctor of Philosophy, The University of Auckland, 2015.
Abstract
Traditionally the extracellular matrix (ECM) in the heart has been thought to provide mechanical scaffolding for the contracting myocytes. However, it was observed that the amplitude of the intracellular Ca\(^{2+}\) transients that underlie contraction was reduced in isolated myocytes in comparison to Ca\(^{2+}\) transients from multicellular trabeculae. The role of ECM in myocyte Ca\(^{2+}\) modulation is poorly understood. We therefore hypothesised that Ca\(^{2+}\) handling might be modulated by (bidirectional) signalling between myocytes and the ECM, and that this important link could be lost during the standard cell isolation by enzymatic process.

The aim of this thesis was to investigate the contribution of ECM in myocyte Ca\(^{2+}\) handling in cardiac trabeculae. To achieve this, measurements of isometric force and intracellular Ca\(^{2+}\) (340/380 ratio) in electrically stimulated rat cardiac trabeculae before and after enzyme exposure that dismantle the linkages between the ECM and the myocytes was continuously measured at room temperature. Immuno-histochemical techniques were used to determine the extent of loss of key proteins linking the ECM and the myocytes during this dismantling.

Experiments revealed that partial enzymatic digestion increased the resting Ca\(^{2+}\) and stress in trabeculae. Furthermore, this study showed that there is decreased RECA-1 labelling in partial digested trabeculae and its complete absence in single myocytes but without any loss in laminin, Cxn-43, and vinculin labelling. In addition, the evaluation of ET-1 effect show neither increase in stress nor in [Ca\(^{2+}\)], in partial dismantled trabeculae. Additionally, the ET-1 application in isolated single myocytes significantly increase the amplitude of [Ca\(^{2+}\)]. Together, these results suggest the contribution of ECM components especially endothelial cells to myocyte Ca\(^{2+}\) modulation. Next, this study also looked at the evidence of presence of primary cilia in adult rat hearts and found no evidence of primary cilia in either ventricular tissue sections or isolated myocytes from the adult rat hearts.

The effect of novel EPAC signalling pathway which contributes to the EC-coupling and Ca\(^{2+}\) homeostasis was also investigated by activating with cpTOME (an EPAC agonist) in intact trabeculae. It was depicted that cpTOME do not affect trabeculae until the experimental conditions of trabeculae are matched with isolated myocytes. EPAC activation increase the myofilament Ca\(^{2+}\) sensitivity when Ca\(^{2+}\) currents were lowered by reducing the [Ca\(^{2+}\)] to 0.5 mM which was removed by CamKII inhibition. This work indicates that EPAC contributes to Ca\(^{2+}\) signalling in a CamKII-dependent fashion in the cardiac tissue preparations.
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I would like to express my cordial and sincere thanks to my supervisor Dr Marie Ward, for her valuable guidance, constructive suggestions, patience and for all the time she devoted to bring this work in the present form. The completion of my PhD would not have been possible without her continued support.

I would also like to thank Professor Mark Cannel for his constructive criticism to refine this work. I am grateful to Dr Sue McGlashan for the kind gifts of antibodies and her suggestions for the primary cilia study. I am thankful to Dr David Crossman for being my advisor and for his guidance at time to time. I am thankful to Dr Cherrie Kong for carrying out calcium spark experiments and for her valuable contribution in my manuscript. I am thankful to Dr Xin Shen for his time and patience during the discussions.

I would like to thank Dr Kimberely Mellor for providing her laptop during my thesis writing. I acknowledge the support of my fellow graduate students, and colleagues in the Department of Physiology, who have encouraged me along the way. I, especially thank Ming, Amelia, YuFeng, Rani, Bindu, Lucy, and Michelle for their company who made this study more enjoyable. I sincerely thank Sam, Ravi, and Rani for helping me in my thesis writing. I would like to thank everyone in the Cardiac Muscle Function lab for their weekly inputs to my project. I acknowledge the financial support of the University of Auckland Doctoral Scholarship contributed to my PhD stipend. Part of the financial support to my stipend was provided by the Marsden grant.

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Abbreviations – General

Abs  antibodies
AC  adenyl cyclase
Ang-II  angiotensin-II
AP  action potential
ARL13B  ADP-ribosylation factor-like 13B
AV ring  atrio-ventricular ring
BDM  2, 3-butanedione monoxime
BSA  bovine serum albumin
Ca\(^{2+}\)  calcium
CaM  calmodulin
CamKII  calmodulin kinase II
cAMP  cyclic adenosine 3’, 5’-monophosphate
CICR  calcium-induced calcium release
cMyBP-C  cardiac myofilament binding protein-C
CNG cation channel  cyclic nucleotide gated cation channel
cpTOME  8-(4-chlorophenylthio)-2’-O-methyladenosine-3’,5’-cyclic monophosphate
Cx43  connexin-43
Cx45  connexin-45
DMSO  dimethyl sulfoxide
EC coupling  excitation-contraction coupling
ECM  extracellular matrix
EE  endocardial endothelium
eNOS  endothelial nitric oxide synthase
EPAC  exchange protein-directly activated by cAMP
ET-1  endothelin-1
FFR  force-frequency relationship
GEF  guanine nucleotide exchange factors
GPCRs  G-protein-coupled receptors
Hz  hertz
IL-6  interlukin-6
KH  Krebs-Henseleit
L-NAME  N – Nitro – L – arginine methyl ester hydrochloride
LTCC  L-type Ca\(^{2+}\) channels
NCX  Na\(^{+}\) -Ca\(^{2+}\) exchanger
NGS  normal goat serum
NO  nitric oxide
OCT  Optical Coherence Tomography
OCT  Optimal Cutting Temperature
PBS  phosphate-buffered saline
PDEs  phosphodiesterases
PFA  paraformaldehyde
PGF  prostaglandin
PKA  protein kinase A
PLB  phosphalamban
PLC  phospholipase C
RECA-1  rat endothelial cell antigen-1
RV  right ventricle
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarcoplasmic-reticulum Ca(^{2+})-ATPase</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>TnC</td>
<td>troponin C</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-α</td>
</tr>
<tr>
<td>TnI</td>
<td>troponin I</td>
</tr>
<tr>
<td>T(_{room})</td>
<td>Room temperature (20-22 °C)</td>
</tr>
<tr>
<td>T-tubules</td>
<td>transverse tubules</td>
</tr>
<tr>
<td>VSMC</td>
<td>vascular smooth muscle cells</td>
</tr>
<tr>
<td>Wt/vol</td>
<td>weight/volume</td>
</tr>
<tr>
<td>β-AR</td>
<td>β-adrenergic receptor</td>
</tr>
<tr>
<td>[Ca(^{2+})](_i)</td>
<td>intracellular calcium</td>
</tr>
<tr>
<td>[Ca(^{2+})](_o)</td>
<td>extracellular calcium</td>
</tr>
</tbody>
</table>
**Table of contents**

Abstract .................................................................................................................................................. i

Acknowledgements ................................................................................................................................. ii

Abbreviations – General .......................................................................................................................... iii

Table of contents ................................................................................................................................... v

List of Figures ....................................................................................................................................... ix

List of Tables ....................................................................................................................................... xi

Chapter 1. General Introduction ........................................................................................................... 1

1.1. Thesis Overview ............................................................................................................................. 1

1.2. The Functions of the Heart ............................................................................................................ 2
    1.2.1. Composition of myocardium ................................................................................................. 2

1.3. Cardiac muscle structure ............................................................................................................... 4
    1.3.1. Structure of the cardiac myocyte ........................................................................................... 4
    1.3.2. Intercalated discs ..................................................................................................................... 4
    1.3.3. Transverse Tubules (T-tubules) .............................................................................................. 5

1.4. A cardiac muscle ............................................................................................................................ 6
    1.4.1. The Extracellular Matrix (ECM) ........................................................................................... 8
    1.4.1.1. The structural proteins ........................................................................................................ 9
    1.4.1.2. The adhesive biomolecules ................................................................................................ 10
    1.4.1.3. The cytokines and growth factors ....................................................................................... 12
    1.4.1.4. The Endothelial cells .......................................................................................................... 13
    1.4.1.5. The Cardiac fibroblasts ....................................................................................................... 15

1.5. Excitation-Contraction (EC) Coupling ........................................................................................... 18
    1.5.1. Ventricular action potential .................................................................................................... 19
    1.5.2. Contractile Proteins and Cross-Bridge Cycle ........................................................................ 21
    1.5.3. Regulation of contraction ........................................................................................................ 23
    1.5.3.1. β-adrenergic receptor (β-AR) signalling .............................................................................. 23
    1.5.3.2. The PKA pathway ................................................................................................................. 25
    1.5.3.3. The EPAC pathway and its proteins .................................................................................... 26
    1.5.3.4. The role of EPAC in excitation-contraction (EC) coupling ................................................... 27
1.6. Thesis Objectives

Chapter 2. General Methodology

2.1. Chapter Overview

2.2. Ethical Approval

2.3. The Cardiac Trabecula

2.3.1. Isolation, Dissection and Mounting of Trabeculae

2.3.2. Force Measurement

2.3.2. Measurement of [Ca$^{2+}$], using Fura-2/AM

2.3.4. Autofluorescence

2.3.5. Loading of Fura-2/AM

2.4. The cardiac myocyte

2.4.1. Ventricular myocytes

2.4.2. Measurement of [Ca$^{2+}$], in myocytes

2.4.3. Measurement of Ca$^{2+}$ sparks in myocytes

2.4.4. Elimination of SR Ca$^{2+}$ storage using caffeine

2.5. Chemicals and solutions

2.5.1. K-H buffer

2.5.2. Tyrode’s buffer

2.5.3. Dissection

2.6. Experimental protocol for sequential dismantling of the ECM in isolated trabeculae

2.7. Data Acquisition and Statistical Analysis of trabeculae experiments

2.7.1. Data acquisition

2.7.2. Statistical analysis of data

2.8. Immunohistochemistry

2.8.1. Tissue section preparation, reagents and antibodies

2.8.2. Protocol for fixing isolated tissue

2.8.3. Protocol for cardiomyocytes fixation

2.8.4. Embedding and Cryosectioning of fixed trabeculae

2.8.5. Immunohistochemistry for ECM proteins in trabeculae

2.8.6. Immunohistochemistry for ECM proteins in single cells

2.9. Image acquisition and analysis
Chapter 3.  Role of ECM in myocyte intracellular Ca\(^{2+}\) signalling ------------------------------------------ 49

3.1.  Chapter Overview ----------------------------------------------- 49

3.2.  Modulation of myocyte Ca\(^{2+}\) handling by intact ECM  -------------------------------- 51

3.3.  Material and Methods ------------------------------------------------------------- 53
   3.3.1. Experimental Protocols ----------------------------------------------------------- 53
   3.3.2. Reagents, enzymes and antibodies ------------------------------------------------- 53
   3.3.3. Immunolabelling of cardiac trabeculae and single myocytes ------------------------ 54
   3.4.4. Optical Coherence Tomography imaging and analysis ----------------------------------- 54

3.4.  Measurement of [Ca\(^{2+}\)]\(_i\) transients in single cardiomyocytes and trabeculae -------- 56
   3.4.1. Measurement of [Ca\(^{2+}\)]\(_i\) transients in single cardiomyocytes and trabeculae -------- 56
   3.4.2. Measurement of stress and Ca\(^{2+}\) transients in pre- and post- enzyme treatment in trabeculae -------- 57
   3.4.3. Optical Coherence Tomography scanning and analysis ------------------------------- 66

3.5.  The Loss of ECM proteins during digestion of trabeculae ------------------------------- 68
   3.5.1 Reduced Collagen-I labelling--------------------------------------------------------- 68
   3.5.2 Endothelial cell Marker (RECA-1) and Laminin-------------------------------------- 71
   3.5.3. Connexin-43 and Laminin------------------------------------------------------------- 75

3.6.  Contribution of Endothelial cells to myocyte intracellular Ca\(^{2+}\) signalling ----------- 79
   3.6.1 Effect of bradykinin and L-NAME in intact trabeculae -------------------------------- 79
   3.6.2. Effect of ET-1 in intact trabeculae ----------------------------------------------- 81
   3.6.3. Effect of ET-1 in presence of ET\(_A\) receptor blocker BQ123 in intact trabeculae------- 82
   3.6.4. Effect of ET-1 after ECM dismantling in digested trabeculae------------------------ 83
   3.6.5. ET-1 direct application elevated Ca\(^{2+}\) transients in isolated cells -------------- 84

3.7.  Contribution of Autocrine and Paracrine Factors to myocytes intracellular Ca\(^{2+}\) signalling - 85
   3.7.1. Inotropic increase in force by PGF\(_{2\alpha}\) in isolated trabeculae ------------------ 86

3.7.  Chapter Summary and Discussion ------------------------------------------------------- 88

Chapter 4.  Evidence of Primary cilia in ventricular tissue --------------------------------------- 91

4.1.  Chapter Overview ------------------------------------------------------------- 91

4.2.  Material and Methods ------------------------------------------------------------- 92
   4.2.1. Primary antibodies and normal serum ----------------------------------------------- 92
   4.2.2. Immunolabelling of Primary cilia in rat cardiac tissue sections and isolated myocytes --- 92
   4.2.3. Immunolabelling in adult single cardiomyocyte and cardiac tissue ------------------ 93
4.2.4. Immunolabelling in embryonic, neonatal, young and adult rat cardiac tissue .......................... 95

4.2.5. Primary cilia association to a particular cell type in neonatal rat cardiac tissue .................. 98

4.3. Chapter Summary and Discussion ......................................................................................... 99

Chapter 5. Identification of novel Ca\(^{2+}\) signalling pathways ............................................. 101

5.1. Chapter Overview .................................................................................................................. 101

5.2. EPAC-selective activators .................................................................................................... 103

5.3. EPAC activation of isolated rat RV trabeculae and single myocytes ................................. 104

5.3.1. Reagents and solutions ..................................................................................................... 104

5.3.2. Data and statistical analysis ............................................................................................... 104

5.4. EPAC activation of isolated trabeculae ................................................................................ 106

5.4.1. EPAC activation of isolated adult rat ventricular myocytes ........................................... 109

5.5. EPAC activation of depotentiated trabeculae ..................................................................... 111

5.6. Myofilament Ca\(^{2+}\) sensitivity ............................................................................................ 112

5.6.1. Measurement of myofilament calcium sensitivity in trabeculae .................................... 112

5.7. Myofilament Ca\(^{2+}\) sensitivity in presence of CamKII inhibition ........................................ 115

5.8. Caffeine evoked Ca\(^{2+}\) transients in isolated trabeculae ..................................................... 116

5.8.1. Measurement of SR Ca\(^{2+}\) by caffeine ........................................................................ 116

5.8.2. Estimation of SR Ca\(^{2+}\) load before and after EPAC activation ...................................... 116

5.9. Chapter Summary and Discussion ....................................................................................... 118

Chapter 6. Thesis Summary ........................................................................................................ 122

6.1. Summary ............................................................................................................................. 122

Bibliography ........................................................................................................................... 126
# List of Figures

## Chapter 1

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Scanning electron microscope (SEM) images</td>
<td>3</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>A. Illustrative diagram of the transverse</td>
<td>6</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>A longitudinal section of feline cardiac muscle</td>
<td>7</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>Schematic diagram of costamere adhesion complex</td>
<td>9</td>
</tr>
<tr>
<td>Figure 1.5</td>
<td>Schematic diagram showing linkages between</td>
<td>11</td>
</tr>
<tr>
<td>Figure 1.6</td>
<td>Schematic diagram showing the link between</td>
<td>14</td>
</tr>
<tr>
<td>Figure 1.7</td>
<td>Transversal cross-section of rabbit ventricular</td>
<td>15</td>
</tr>
<tr>
<td>Figure 1.8</td>
<td>Schematic diagram showing proposed signaling</td>
<td>16</td>
</tr>
<tr>
<td>Figure 1.9</td>
<td>Schematic representation of excitation-contraction</td>
<td>19</td>
</tr>
<tr>
<td>Figure 1.10</td>
<td>A trace showing typical ventricular action</td>
<td>20</td>
</tr>
<tr>
<td>Figure 1.11</td>
<td>Schematic organization of the sarcomere</td>
<td>22</td>
</tr>
<tr>
<td>Figure 1.12</td>
<td>Schematic diagram showing signal</td>
<td>25</td>
</tr>
<tr>
<td>Figure 1.13</td>
<td>Schematic diagram showing the proposed EPAC</td>
<td>27</td>
</tr>
</tbody>
</table>

## Chapter 2

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2.1</td>
<td>Schematic diagram of the dissected out right ventricle</td>
<td>32</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>Schematic diagram of the Perspex muscle bath showing</td>
<td>33</td>
</tr>
<tr>
<td>Figure 2.3</td>
<td>Schematic diagram of a standard force transducer showing</td>
<td>34</td>
</tr>
<tr>
<td>Figure 2.4</td>
<td>The freshly isolated rod shaped cardiomyocytes</td>
<td>36</td>
</tr>
<tr>
<td>Figure 2.5</td>
<td>Summary of the experimental protocol used for</td>
<td>40</td>
</tr>
<tr>
<td>Figure 2.6</td>
<td>Example of data analysed using IDL software</td>
<td>42</td>
</tr>
<tr>
<td>Figure 2.7</td>
<td>The 2% PFA fixed myocytes showing striated</td>
<td>47</td>
</tr>
<tr>
<td>Figure 2.8</td>
<td>Schematic diagram of a cryomold base lined with</td>
<td>47</td>
</tr>
</tbody>
</table>

## Chapter 3

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 3.1</td>
<td>A comparison of Ca(^{2+}) transients between isolated</td>
<td>58</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Enzyme exposure protocols for a representative RV</td>
<td>59</td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>Mean data from trabeculae (n = 7)</td>
<td>60</td>
</tr>
<tr>
<td>Figure 3.4</td>
<td>Mean ± SEM data from trabeculae</td>
<td>61</td>
</tr>
</tbody>
</table>
Figure 3.5 Estimation of SR Ca\textsuperscript{2+} load-----------------------------63
Figure 3.6 A representative trabecula showing OCT scans before --------------68
Figure 3.7 The confocal images showing Collagen-I ---------------------------70
Figure 3.8 RECA-1, laminin and actin labelling in fixed trabeculae------------73
Figure 3.9 RECA-1, laminin and actin labelling in rat ventricular------------74
Figure 3.10 Representative data showing confocal images of 2% --------------76
Figure 3.11 Vinculin, laminin and actin labelling in rat ventricular---------78
Figure 3.12 Vinculin and F-actin labelling in cardiac trabeculae------------79
Figure 3.13 The effect of ET-1 in intact trabeculae--------------------------82
Figure 3.14 The effects of ET-1 in presence of ET\textsubscript{A}-receptor --------83
Figure 3.15 The effects of ET-1 after ECM dismantling in -------------------84
Figure 3.16 Effect of ET-1 on Ca\textsuperscript{2+} transients in isolated ---------85
Figure 3.17 Effects of PGF\textsubscript{2\alpha} on contractile force in rat trabecula-88

Chapter 4

Figure 4.1 Representative data showing confocal images ---------------------95
Figure 4.2 Representative data showing confocal images ---------------------97
Figure 4.3 Representative data showing confocal -----------------------------99

Chapter 5

Figure 5.1 Structure of EPAC-selective agonist -----------------------------104
Figure 5.2 EPAC activation of RV trabeculae -------------------------------108
Figure 5.3 EPAC activation of RV trabeculae during PKA ---------------------109
Figure 5.4 EPAC activation of isolated myocytes----------------------------111
Figure 5.5 EPAC activation of trabeculae in low (0.5 mM [Ca\textsuperscript{2+}]) ------113
Figure 5.6 Myofilament Ca\textsuperscript{2+} sensitivity--------------------------115
Figure 5.7 CaMK-II inhibition and myofilament Ca\textsuperscript{2+} sensitivity ----116
Figure 5.8 Estimation of Sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} load------118
Figure 5.9 Mechanism of EPAC activation proposed --------------------------119
Figure 5.10 Proposed schematic outline of Ca\textsuperscript{2+}signalling pathway --121
List of Tables

Chapter 2
Table 2.1 A list of primary and secondary antibodies-------------------------------------45

Chapter 3
Table 3.1 The time constant of decay in fluorescence------------------------------------64
Table 3.2 List of parameters obtained for Ca$^{2+}$----------------------------------------66
Table 3.3 Relative fractional area of collagen I------------------------------------------72
Table 3.4 Relative fractional area of RECA-1---------------------------------------------76
Table 3.5 Relative fractional area of Cxn-43---------------------------------------------78
Table 3.6 Percentage change of amplitude of [Ca$^{2+}$]----------------------------------81
Table 3.7 Percentage change of amplitude of [Ca$^{2+}$]----------------------------------82
Chapter 1. General Introduction

1.1. Thesis Overview

The contraction and relaxation of the heart critically depends upon the changes in calcium concentration within the cardiomyocytes (Bers, 2008). Most of our knowledge of Ca\textsuperscript{2+} handling in myocytes comes from the studies done in single isolated ventricular myocytes which are devoid of extracellular matrix (ECM) and its components. In the cellular environment within the myocardium, each myocyte is in close communication with ECM and its components which is disrupted during the enzymatic isolation procedures.

The ECM acts as a dynamic structure by transmitting the force generated by individual cardiomyocytes into an organized ventricular contraction. The ECM is an important component of the heart and it is essential to understand its role in cell communication and calcium signalling in the normal heart. Usually, ECM is considered as an inert, supportive, scaffold for cardiomyocytes, but we hypothesized that the cardiac ECM could also influence cardiomyocyte Ca\textsuperscript{2+} modulation. Therefore, we asked the question whether dismantling of ECM and its components disturbs myocyte Ca\textsuperscript{2+} handling. The role of ECM in Ca\textsuperscript{2+} homeostasis in the normal heart is the focus of my thesis.

This chapter provides background information on cardiac muscle and its function, followed by a review of what is known about the ECM and its components.
1.2. The Functions of the Heart

The heart is a specialized muscle that contracts and relaxes continuously, pumping blood to the body and the lungs. It consists of four chambers - two atria at the top (left & right) and two ventricles at the bottom (left & right). The deoxygenated blood enters into the heart through the veins and pulmonary artery takes blood from the heart to the lungs for oxygenation. From the lungs, the blood returns again to the left side of the heart and is pumped to the systemic circulation. Thus, the primary function of the heart is to provide oxygen and nutrients to all peripheral tissues to meet their local metabolic demands. The pumping action is initiated by a spread of electric signal from the sino-atrial (SA) node of the right atrium to the atrio-ventricular (AV) node of the ventricles. This causes the heart to contract and pump blood from top chambers (atria) to the bottom chambers (ventricles). The right ventricle pumps blood into the lungs and the blood from left ventricle supply the systemic circulation. The left ventricle provides the cardiac output by contracting myocytes to generate sufficient pressure to pump the blood into the aorta. The synchronous contraction of the myocytes, regulated by spatially arranged ion channels and exchangers in the cell membranes, results in the efficient pumping of blood.

1.2.1. Composition of Myocardium

The term “myocardium” (from Greek, *myos* = muscle + *kardio* = heart) means a mass of muscle. The myocardium forms the thick middle layer between epicardium (outer layer) and endocardium (inner layer) and is composed of contracting myofibrils. The layers of muscle fibers are arranged in planes, separated by connective tissue, and are supplied by numerous blood vessels. The myocardium is composed of myocytes and ECM. The arrangement of myocytes in form of sheets, surrounded by adjacent layers of connective tissue (collagen) and capillaries is shown in figure 1.1.
Figure 1.1. Scanning electron microscope (SEM) images of fixed tissue from canine left ventricle (A) tangential surface showing layered organization of myocytes, arrow pointing branching of layers, and collagen fibres between adjacent sheets (scale bar, 100 µm) and (B) transverse surface perimysial connective tissue surrounding myocardial sheets and covering surface capillaries from the specimens (Scale bar, 25 µm) (modified from LeGrice et al., 1995).
1.3. Cardiac muscle structure

1.3.1. Structure of the cardiac myocyte

The myocytes are the fundamental units of the heart and are responsible for generating force. In a healthy heart, cardiomyocytes account for up to 25 - 35% of the cell number, ~90% of myocardial mass and 67 - 75% of myocardial volume (Vliegen et al., 1991; Cleutjens, 1996). A typical ‘rod-shaped’ myocyte measures approximately 10 - 25 µm (width) and from 80 - 130 µm (in length) after enzymatic cell isolation (Egorova et al., 2005), figure 1.2). An important aspect of cardiac muscle is that each individual myocyte is activated with each and every heartbeat. This property of cardiac muscle that allows the heart to function as a syncytium is a result of the connections between cells. Myocytes are connected to each other through intercalated discs made up of gap junctions (with connexins forming channels for ion transfer), adherens (with N-cadherin stabilizing gap junctions), and desmosomes (structural organization of the intercalated disc).

1.3.2. Intercalated discs

Classically, the intercalated discs contain three functional zones: the fascia adherens, the desmosomes, and the gap junctions. Recently, another region called “transitional junction’ has been reported at the perimeter of fascia adherens (Bennett et al., 2006).

Fascia Adherens and Desmosomes

The fascia adherens and desmosomes are responsible for mechanical coupling of myocytes at the intercalated discs. The adherens junctions, consisting of cadherins (Ca²⁺-dependent adhesion), link the intercalated disc to the actin cytoskeleton inside the myocytes whilst desmosomes in which desmoplakin (protein associated with desmosome junctions) is the major component, attach to intermediate filaments (cytoskeletal components) (Ian Gallicano et al., 1998).

Gap junctions

The gap junctions are intracellular junctions containing membrane channels that mediate the movement of ions and small molecules (secondary messengers below 1.2 kDa) between the myocytes (Li et al., 2002). The gap junctions are mainly responsible for electric coupling
between cardiomyocytes (Burt et al., 1982; Gutstein et al., 2003; Chilton et al., 2007). They are narrow tunnels that consist of proteins called connexins. Connexin-43 is expressed predominantly in the heart although other isoforms are also found (connexin 40, connexin 45 and connexin 37) in mammalian hearts (Blackburn et al., 1995). Recently, ‘tunneling nanotubes’ have been discovered in vitro and in vivo which are responsible for making the direct cytosolic links between cardiomyocytes and fibroblasts in neonatal rat ventricular myocyte cultures (He et al., 2011). Membrane nanotubes have been reported in other types of cells, including endothelial progenitor cells with cardiomyocytes and mesenchymal stem cells with cardiomyocytes (Koyanagi et al., 2005; Plotnikov et al., 2008). The connections between fibroblasts and myocytes are further explained in detail in section 1.4.1.5.

1.3.3. Transverse Tubules (T-tubules)

The transverse-tubules (t-tubules) found in mammalian cardiomyocytes are invaginations of the sarcolemma and glycocalyx that occur at the Z lines, illustrated in figure 1.2, roughly at intervals of ≈2 µm along the longitudinal axis of the myocytes (Brette & Orchard, 2003). Within mammalian cardiac tissue, t-tubules are predominantly present in ventricular myocytes but are absent or far less developed in atrial, pacemaking, and conducting tissue (Ayetey & Navaratnam, 1978). Immuno-histochemical studies have revealed a number of key excitation contraction (EC)-coupling proteins either within the t-tubular membrane or very close to it. These key proteins are the L-type Ca^{2+} channels (LTCC), ryanodine receptors (RyR), sarcoplasmic-reticulum Ca^{2+}-ATPase SERCA, and Na^{2+}–Ca^{2+} exchanger (NCX).
A

Illustrative diagram of the transverse tubules (t-tubules), the sarcoplasmic reticulum (SR) and the abundant mitochondria that surrounds the sarcomeres of the mammalian cardiac muscle (Katz, 2001).

B

Electron micrograph of a longitudinal section of rabbit ventricular myocyte, showing T-tubules (T), junctional SR (jSR) and free SR (fSR). Arrowheads show connections between jSR and fSR (Brochet et al., 2005)

Figure 1.2.  A. Illustrative diagram of the transverse tubules (t-tubules), the sarcoplasmic reticulum (SR) and the abundant mitochondria that surrounds the sarcomeres of the mammalian cardiac muscle (Katz, 2001). B. Electron micrograph of a longitudinal section of rabbit ventricular myocyte, showing T-tubules (T), junctional SR (jSR) and free SR (fSR). Arrowheads show connections between jSR and fSR (Brochet et al., 2005)

1.4. A cardiac muscle

The characteristic striated appearance of cardiac muscle is due to the organization of the distinct myofibrils. Each sarcomere lies between two Z lines and is the measure of contractile unit. Figure 1.3 shows a section of feline cardiac muscle displaying the sarcomere arrangement that gives myocytes their striated appearance as well as the intercalated discs that connect the individual myocytes.
Figure 1.3. A longitudinal section of feline cardiac muscle fibers viewed by electron microscopy. The photomicrograph show each sarcomere lies between two Z discs, and M line is present in the center of the A-band. (Mt) represents rows of mitochondria, (Lp) are lipid droplets, (Cap) represents the capillary, while (In D) represents intercalated discs. x 15,000.
1.4.1. The Extracellular Matrix (ECM)

Pioneering studies on the ECM in the late 1970s and early 1980s identified a complex fibrillary collagen network in the heart. The cardiac ECM is a complex structure containing many cell types: endothelial cells, neurons, blood vessels, fibrous proteins, proteoglycans, and others such as fibroblasts, and macrophages. Some growth factors, extracellular proteases, and cytokines are also produced by ECM (Brown et al., 2005). The fibrous proteins are categorized into structural proteins (collagens and elastins) and adhesive biomolecules (fibronectin, vitronectin and laminin), that provide scaffolding to the heart. Many of these proteins found in the ECM are secreted by the fibroblasts (Miner & Miller, 2006). Although the extracellular matrix is important in transmitting the force generated by individual cardiomyocytes into an organized ventricular contraction, it is also a dynamic structure. There are multiple Ca\(^{2+}\) signalling pathways which regulate the intracellular Ca\(^{2+}\) activity in cardiac fibroblasts (Chen et al., 2010).

**Linkage between ECM and cardiomyocytes**

In the normal heart, myocytes are anchored to the ECM with vinculin rich rib-like bands distributed between Z-lines and sarcolemma, called costameres that encircle the cardiomyocytes perpendicular to its long axis (Pardo et al., 1983b). These costamere adhesion complexes are composed of vinculin, talin, integrin α6β1, laminin and other cytoskeletal and extracellular molecules (Danowski et al., 1992; Imanaka-Yoshida et al., 1996; Samarel, 2005). In addition to the attachment of myocytes to the ECM, costameres help in maintaining the spatial organization of the contractile apparatus and contribute to force transmission to myofibrils. The costameres, intermediate filaments, and membrane skeleton are linked to the glycocalyx and ECM by integrins and dystrophin-glycoprotein complex as shown in figure 1.4. Intercalated disks help in joining the cardiomyocytes together and form a functional syncytium. Costamere adhesions are well known for their role in transmitting forces between the cells, and acting as a mechanotransducer (Danowski et al., 1992; Ross & Borg, 2001).
Figure 1.4. Schematic diagram of costamere adhesion complex showing the linkage between the cardiomyocytes and ECM during contraction. The myofibril Z-lines ($\alpha$-actinin) are anchored to connective tissue by costamere adhesion complexes including vinculin, talin, integrin $\alpha$6$\beta$1 and laminin. The other molecules such as dystrophin, dystroglycan and desmin might be also involved in forming the complexes (Imanaka-Yoshida et al., 2004).

1.4.1.1. The structural proteins

Fibrillar collagens: Collagen types I and III are the major fibrillar collagen synthesized by cardiac fibroblasts. The dominant fibrillar collagen, Collagen type I, constitutes approximately 85% of the total myocardial collagen whilst 11% is collagen type III (Graham et al., 2008). In combination with sarcomeric protein, titin (which connects the Z line to the M line in the sarcomere), type I collagen fibrils determine the stiffness of the myocardium, whereas type III collagen helps in maintaining the structural integrity of the cardiomyocytes (Weber, 1989). The circulation of blood in the vessels of myocardium, also known as coronary circulation, is very important and is supported by the structural proteins of ECM. The precise distribution of collagen types was visualized in arterial wall by immuno-histochemical techniques in 1970s (Gay et al., 1975; Duance et al., 1977). Collagen types I and III predominate in human arterial wall (Chung & Miller, 1974; McCullagh & Balian, 1975) and in bovine aorta in ratio 3:2 (Rauterberg, 1974).
1.4.1.2. The adhesive biomolecules

**Fibronectins:** This is an adhesive glycoprotein which is secreted by cardiac fibroblasts, and binds to the membrane-spanning receptor proteins called integrins. It has binding sites for ECM components such as collagen, fibrin, and heparan sulphate proteoglycans. Cellular elements (T-tubules) and collagen are embedded in the fibronectin and are homogenously localized in the extracellular space. Therefore, fibronectins serve as a bridge from cardiomyocytes to the extracellular collagen. Fibronectin has essential roles in development, cell adhesion, growth and migration and its altered expression, by mechanical and chemical factors is associated with a number of cardiac pathologies (Corda *et al.*, 2000; Bowers *et al.*, 2010).

**Laminins:** Laminin is localized in the basement membrane of both cardiomyocytes and fibroblasts. It consist of three α, β,γ chains and at least eight genetically distinct chains with different assembly forms which helps in binding to various ECM components including collagens and cell membrane receptors. It is largely produced by myocytes, endothelial cells and vascular smooth muscle cells (VSMCs) (Timpl & Brown, 1994). Laminins play three essential roles: (i) they act as structural elements of the basement membrane by forming self-assembling networks (Yurchenco & O’Rear, 1994; Timpl, 1996); (ii) they interact with cell surface components such as dystroglycan to attach cells to ECM (Henry & Campbell, 1996); (iii) they act as signalling molecules by interacting with cellular receptors such as the integrins (Clark & Brugge, 1995; Yamada & Miyamoto, 1995). See figure 1.5 for details.

**Integrins:** Integrins are membrane receptors which are located on ECM proteins such as collagen, fibronectin, and laminin and act as mechano-transducers. These receptors anchor the intracellular cytoskeletal proteins of myocytes to the ECM across the plasma membrane and trigger intracellular signalling (See figure 1.5 for details). Studies have reported that cardiac myocyte hypertrophy is promoted through β1-integrin receptors (Ieda *et al.*, 2009). Integrins are known to activate multiple signalling pathways such as control of cell cycle, proliferation as well as apoptosis (Hynes, 1992; Giancotti & Ruoslahti, 1999). The expression of several cytokines such as TGF-β and angiotensin-II (Ang-II) is associated with integrin regulation (Sun *et al.*, 2003; Bowers *et al.*, 2010). There is an important link between cytokines and integrin-mediated signalling pathways. Integrins and dystrophin are the two major complexes involved in trans-sarcolemmal mechanical connection to the
ECM. This includes the cytoskeletal proteins such as actin, alpha-actinin, vinculin, talin, paxillin, tensin, laminin, and collagen.

**Vinculin:** Vinculin is a 117-kDa protein which is ubiquitously expressed in cardiac tissues. The function of this protein in cardiomyocytes appears to serve as a lateral Z-line attachment of the myofibre to the plasma membranes and T-tubules via cell-surface integrins (Danowski et al., 1992). Vinculin links the ECM to the actin-based cytoskeleton of the myocyte (see figure 1.5 for details). It also binds to talin, α-actinin, and actin. In addition to its position within cell-matrix linkages, vinculin is also localized in cell-cell adheren junctions that link one cell to another cell (Geiger et al., 1980). It is also localized and distributed throughout the Z-lines (Pardo et al., 1983a). During cardiac myofibrillogenesis (formation of myofibrils), vinculin is also reportedly involved in proper alignment of the developing myofibrils.

![Diagram showing linkages between ECM and cytoskeletal proteins](image)

**Figure 1.5.** Schematic diagram showing linkages between ECM and cytoskeletal proteins. The connections between various ECM (fibronectin and laminin) and cytoskeletal proteins (integrins, talin, vinculin, dystrophin, and actin) are responsible for triggering intracellular signalling. (Redrawn from (Jaalouk & Lammerding, 2009))
1.4.1.3. The cytokines and growth factors

Many cytokines such as TNF-α, IL-1β, IL-6, and TGF-β have signalling role at cellular and physiological levels of cardiac function. These growth factors and cytokines, specifically TNF-α, IL-1β, and IL-6 have further role in stimulating cardiac remodelling process by activating matrix metalloproteinases (MMPs) (Corda et al., 2000; Brown et al., 2005). It is also seen that the expression of cytokines and growth factors is upregulated in response to mechanical stretch (Ruwhof et al., 2000; van Wamel et al., 2001). Studies have reported that loss of IL-6 results into many abnormalities including those related to an increased number of fibroblasts and interstitial collagen deposition (Banerjee et al., 2009). Cardiac fibroblasts are the major source of TNF-α, IL-1β, and IL-6 in the myocardium (Yue et al., 1998; Ancey et al., 2002; Bujak & Frangogiannis, 2007). Cytokines, such as TNF-α, are consistently reported as increased in patients with angina and myocardial infarction (Neumann et al., 1995; Mulvihill & Foley, 2002). Several TNF-α mediated mechanisms may cause endothelial dysfunction (Locksley et al., 2001; Madge & Pober, 2001). Cardiac fibroblasts are the main source of IL-1β in the heart following injury (Long, 2001). IL-1β expression can also be induced by β-AR stimulation, hypoxia, or serotonin (Long, 2001; Jaffré et al., 2004). IL-1β is also known to induce IL-6 expression and Angiotensin type 1 receptor expression in neonatal rat cardiac fibroblasts (Cowling et al., 2005). IL-6 is responsible for regulation of cell growth, apoptosis, and differentiation of heart. It is a critical component in cell-cell interaction that occur between myocytes and cardiac fibroblasts (Banerjee et al., 2009). Data from several studies suggests that IL-6 and the gp 130-JAK-STAT signalling pathway are important in cardiac function and cardioprotection (Hirota et al., 1995, 1999; Hubner et al., 2006).
1.4.1.4. **The Endothelial cells**

In 1980, Furchgott and Zawadski discovered the role of the vascular endothelium in vasomotor tone (Furchgott & Zawadzki, 1980). Endothelial cells produce a special peptide called endothelin which is an extremely potent vasoconstrictor, as well as having a positive inotropic effect on the myocardium (Yanagisawa et al., 1988a, 1988b). There are three isoforms- endothelin-1, endothelin-2, and endothelin-3. Endothelin-1 (ET-1) is the most well characterised isoform, and its actions are mediated through two receptor subtypes: ET\(_A\) and ET\(_B\) (Arai et al., n.d.; Sakurai et al., 1990). Mostly, ET\(_A\) receptor activation has been reported to influence the myocardial inotropic effects. ET-1 is synthesised by the myocytes (Nunez et al., 1990), fibroblasts (Fujisaki et al., 1995) and endothelial cells (Kedzierski & Yanagisawa, 2001) of the heart. ET-1 is involved in controlling aortic arch formation during development (Kurihara et al., 1995), is necessary for myocyte survival (Zhao et al., 2006) and prevents myocyte loss during ageing (Zhao et al., 2006). In the adult, ET-1 modulates coronary blood flow and cardiac muscle by acting on atrial and ventricular myocyte receptors (Hirata et al., 1989). The role of ET-1 is considered to both the aetiology and pathology of the heart. Acutely (within minutes of application), ET-1 is known to induce arrhythmias by modulating muscle contractility (Baltogiannis et al., 2005), whereas over longer time periods (days to weeks), the peptide induces growth associated with maladaptive hypertropic remodelling of the heart (Yorikane et al., 1993) and its progression to heart failure (Francis et al., 1990). The myocyte secreted ET-1 is reported to contribute to stretch induced increased contractility during periods of increased workload (Pikkarainen et al., 2006). During stretch, ET-1 production is directly stimulated or induced in response to AngII, which is also secreted by the stretched myocytes (Sadoshima et al., 1993; Pikkarainen et al., 2006; Cingolani et al., 2011).

It has been reported that the contractile performance of isolated cardiac muscle is intensely modified by the presence of endocardial endothelial cells (Brutsaert et al., 1988; Brutsaert, 1989). The cardiac trabeculae are encased in a layer of endocardial endothelial cells as in vascular endothelial cells (Hanley et al., 1999). Under normal conditions, endothelin is present in very low concentrations but there is significant increase during myocardial infarction and congestive heart failure (Stewart et al., 1991). *In vivo* and *in vitro* observations of the effects of cardiac endothelium and of related autocrine and paracrine factors on cardiac function were made by many investigators (Smith et al., 1991; Ramaciotti et al., 1992; Balligand et al., 1993; Bourreau et al., 1993; Calaghan & White, 2001). It has been reported that endothelin stimulates endothelium-dependent relaxation factor and
prostaglandin I₂ release (Yanagisawa et al., 1988a, 1988b). It is also reported that the effects of endothelin are independent of β-adrenergic, α-adrenergic, H₁-histaminergic, serotonergic, or cyclooxygenase mechanisms. See figure 1.6 for details on paracrine communication between endothelial cells and myocytes.

**Figure 1.6.** Schematic diagram show the link between endothelial cells and myocytes via paracrine communication. ET-1(endothelin-1), eNOS (endothelial nitric oxide synthase), NO (nitric oxide). Figure modified from (Brutsaert, 2003).
1.4.1.5. The Cardiac fibroblasts

In a healthy heart, cardiac fibroblasts account for more than 50% of all cells, and are interspersed within collagen that forms the bulk of ECM (Vliegen et al., 1991). Figure 1.7 shows the cardiac structure overview (Camelliti et al., 2005). The fibroblasts are mesenchymal in origin and produce interstitial collagen (along with I, III and VI). They contribute to myocardial structure, biochemical and electro-mechanical function (MacKenna et al., 2000; Camelliti et al., 2004a; Camelliti et al., 2004b; Long & Brown, 2002. The number of fibroblasts increase with normal development and aging (Adler et al., 1981). In cardiovascular diseases, fibroblasts play an important role in the myocardial remodelling process (changes in size, shape, structure, and physiology of the heart after injury to the myocardium). This includes hypertrophy of cardiomyocytes, migration and proliferation of fibroblasts, and changes in the ECM composition and content (Sun & Weber, 2000; Sun et al., 2002).

Figure 1.7. (A) Transversal cross-section of rabbit ventricular myocardium (stained with Hematoxylin and Eosin) shows layered sheets of cardiac muscle cells separated by ‘voids’ (un-labelled, non-myocytes) and (B) Confocal image of ventricular myocardium cross-section immunolabelled with anti-myomesin (red) to mark myocytes, anti-vimentin (blue) to mark fibroblasts, anti-connexin-43 (green dots) and DAPI (4’-6-Diamidino-2-phenylindole) to label nuclei (pale yellow-green) show dense network of fibroblasts that surrounds myocyte clusters (2-4 cells). Image taken from (Camelliti et al., 2005).
**Fibroblast-myocyte interactions**

The fibroblasts communicate with myocytes in three different ways: (i) directly through fibroblast-myocyte interactions by connexins and cadherins; (ii) indirectly by influencing surrounding ECM composition by secreting collagen, fibronectin and activating matrix metalloproteinases and (iii) in a paracrine manner by secreting various growth factors and cytokines (see figure 1.8).

![Schematic diagram showing proposed signalling pathways between cardiac fibroblasts and myocytes](image)

**Figure 1.8.** Schematic diagram showing proposed signalling pathways between cardiac fibroblasts and myocytes either (i) directly via ECM through collagen synthesis, (ii) indirectly via gap junctions or (iii) or in a paracrine fashion via cytokines and growth factors. Diagram modified from (Brutsaert, 2003).

**Fibroblast gap-junctional coupling**

The gap junctions that form between cardiac fibroblasts and myocytes express connexin 43 and have been investigated mainly in cell cultures (Rook et al., 1989, 1992; Doble & Kardami, 1995). Fibroblasts are able to conduct electrical signals *in vitro* over extensive distances (up to 300 µm) and express both Cx43 and Cx45 in neonatal rat heart cultures (Gaudesius et al., 2003). But the fibroblast junctions are much smaller than those in cardiomyocytes (De Maziere et al., 1992). The fibroblast-fibroblast structural and functional
Coupling in rabbit SA node revealed its contribution in several processes, including electrical, chemical and mechanical signaling (Camelliti et al., 2004b). Several *in vitro* evidence suggest that myocyte-fibroblast coupling is able to form functional gap junctions (Kohl et al., 1994; Gaudesius et al., 2003; Camelliti et al., 2004b).
1.5. **Excitation-Contraction (EC) Coupling**

The term excitation-contraction (EC) coupling was first introduced by Alexander Sandow in 1952, and is the process where an action potential triggers a myocyte to contract (Sandow, 1952). Contraction in cardiomyocytes is initiated in response to depolarization of the sarcolemma during the phase 1 and 2 of the cardiac action potential. This wave of depolarization opens voltage gated LTCC predominantly in the sarcolemma of T-tubules, resulting in a small influx of Ca\(^{2+}\) into the cell. This triggers a large Ca\(^{2+}\) release from RyR of sarcoplasmic reticulum (SR) in the form of ‘Calcium transients’. This process is known as calcium-induced calcium release (CICR) (Fabiato, 1983) and the binding of cytoplasmic Ca\(^{2+}\) to troponin C (Fabiato, 1985). The Ca\(^{2+}\) entry plus the amount released from the SR via CICR raises cytosolic free Ca\(^{2+}\), causing Ca\(^{2+}\) binding to multiple cytosolic Ca\(^{2+}\) buffers. Troponin C is one of the most important Ca\(^{2+}\) buffers. When Ca\(^{2+}\) binds to troponin C (TnC), it induces conformational changes in the regulatory complex such as troponin I (TnI) and movement of tropomyosin exposed the myosin binding site on the actin molecule. This binding results in ATP hydrolysis which provides energy in conformational change to occur in the actin-myosin complex. These changes result in the movement between the myosin heads and actin, causing force development and/or shortening sarcomere length. The removal of Ca\(^{2+}\) from the cytosol at the end of phase 2 is primarily via SERCA, with a small amount of Ca\(^{2+}\) extruded out of cell via NCX. This returns the cytoplasmic-Ca\(^{2+}\) back to resting levels, removing Ca\(^{2+}\) from TnC, leading to relaxation of myofilaments. Figure 1.9 provides a diagrammatic overview of EC coupling in cardiomyocytes.
Figure 1.9. Schematic representation of excitation-contraction (EC) coupling in a ventricular myocyte. The propagation of AP across the sarcolemma (1) triggers an influx of Ca\(^{2+}\) through LTCC (2). This small influx of Ca\(^{2+}\) leads to a much larger release of Ca\(^{2+}\) from the SR via CICR (3). Ca\(^{2+}\) then binds to the myofilament complex, resulting in contraction (4). Relaxation occurs when cytosolic Ca\(^{2+}\) is removed, either by SERCA activity (5), or through NCX (6). (Redrawn from (Bers, 2002).)

1.5.1. Ventricular action potential

The action potential (AP) is responsible for the propagation of electrical signals from cell to cell through gap junctions which allow the heart to function as an electrical and mechanical syncytium. The propagation of AP across the sarcolemma marks the onset of EC-coupling. The cardiac impulse originates in the pacemaker cells of sino-atrial (SA) node and propagates through atria to atrio-ventricular (AV) node. The electrical activity then rapidly passes through His-Purkinje system of the ventricles, triggering EC-coupling (Katz, 2001). Figure 1.10 shows a typical ventricular action potential. In ventricular myocytes, each AP has distinct five phases.
Figure 1.10. A trace showing typical ventricular action potential with five different phases. Phase 0-depolarization due to opening of sodium channels, Phase 1-partial repolarization due to decrease in sodium ions, Phase 2- plateau phase due to extrusion of calcium ions and maintains depolarization, Phase 3-repolarization, caused by delayed inward K\(^+\) (\(I_k\)) currents, closure of Na\(^+\) and Ca\(^{2+}\) channels and return of membrane potential back to baseline, and Phase 4-resting membrane potential (-90mV), a negative intracellular potential due to the activity of Na\(^+\)/K\(^+\) ATPase pump (Katz, 2001).

The first dynamic model of the ventricular action potential was given by (Luo & Rudy, 1994) based on the major ionic currents which determine the shape of the action potential. The resting membrane potential is primarily by the inward K\(^+\) rectifier and secondarily by the Na\(^+\)/K\(^+\) ATPase, and the sarcolemmal Ca\(^{2+}\) ATPase. During the resting phase, sarcolemma is permeable only to K\(^+\), thus it is the K\(^+\) equilibrium potential that primarily determines the resting membrane potential of the myocyte. During phase 0, the Na\(^+\) channels rapidly activate (<1 ms) and remain activated for a duration of 2 to 10 ms. The activation of these channels allow Na\(^+\) to flow into the cell and produces rapid depolarization from -90mV to +10mV. During phase 1, due to decrease in Na\(^+\) permeability, there is partial repolarization. Phase 2 is the plateau phase and is determined by the influx of Ca\(^{2+}\) through LTCC which maintains the depolarization and prolong the action potential (Balke & Shorofsky, 1998). Towards the end of phase 2, the membrane permeability to Ca\(^{2+}\) decreases, and the plateau is maintained by influx of Na\(^+\) through Na\(^+\)/Ca\(^{2+}\) exchanger. Phase 3 represents repolarization carried by delayed inward K\(^+\) currents (\(I_k\)) which eventually brings the cell back to the resting membrane potential (Katz, 2001). In phase 4, resting membrane potential becomes -90mV and is maintained by the activity of Na\(^{2+}\)/K\(^+\) ATPase pump which exchanges three Na\(^+\) ions for only two K\(^+\) ions (Gadsby & Nakao, 1989; Nakao & Gadsby, 1989; Fozzard & Lipkind, 1995).
1.5.2. Contractile Proteins and Cross-Bridge Cycle

The cardiac sarcomere serves as the fundamental contractile unit of the myocardium. The characteristic arrangement of striations seen under the light microscope is due to the arrangement of myofilaments, comprising of thick and thin filaments (Hanson & Huxley, 1953). The thin filaments are composed of double stranded helical polymer complex containing actin, tropomyosin and the regulatory complex of troponin (Bailey K., 1946; Hanson J and Lowy J., 1963; Katz, 1966). The thick filaments are largely made up of a heavy protein myosin (MW ~ 480,000 Da) and extends from the center of the sarcomere (M-line) and spans the length of A-band (Holtzer A & Lowey S., 1959). Each myosin contains two heads having ATPase activity. There are three subunits of troponin complex: TnC, TnI and TnT and have different roles. TnC serves as a binding site for Ca$^{2+}$ during EC-coupling (four Ca$^{2+}$ can bind per TnC); TnI inhibits the binding site (actomyosin ATPase) via its linkage with actin, while TnT acts as structural scaffold attaches to the troponin-tropomyosin-actin complex together. Figure 1.11 illustrates the spatial arrangement between thick and thin myofilaments.
**Figure 1.1**: Schematic organization of the sarcomere contractile proteins during relaxed and contracted phase of the muscle. N, NH2-terminal domains of TnC and TnI; C, COOH-terminal domains of TnC and TnI; I, inhibitory region of TnI. (Farah & Reinach, 1995).
The cross-bridge cycle describes the sliding filament theory of muscle contraction (Huxley & Niedergerke, 1954). In a relaxed phase, the myofilaments are tightly bound, where the troponin (Tn)-tropomyosin complex prevents the binding of myosin heads to the actin. Following the depolarisation and release of Ca\(^{2+}\) from the SR, Ca\(^{2+}\) binds to TnC and allosterically modulates the tropomyosin. This in turn sterically obstructs the binding sites for myosin. The dissociation of myosin from actin takes place when ATP binds to the myosin head. The myosin then hydrolyses the ATP and uses the energy for muscle contraction. The formation of cross-bridges as a result leads to the production of force.

1.5.3. Regulation of contraction

Every myocyte contributes to each heart beat but the heart can regulate cardiac output by the varying forces generated by the contraction of each myocyte. The amplitude of the Ca\(^{2+}\) transient produced by the SR Ca\(^{2+}\) release determines the contractile force developed by cardiomyocytes. Most of the signal transduction pathways that stimulate contractility involve Ca\(^{2+}\), either by increasing Ca\(^{2+}\) influx (via LTCC) during the AP (primarily during phase 2), by increasing the release of Ca\(^{2+}\) by the SR, or by sensitizing TnC to Ca\(^{2+}\). The arrangement that regulates SR Ca\(^{2+}\) release include: (1) triggers through sarcolemmal Ca\(^{2+}\) influx through L-type calcium channels (2) SR calcium release via RyR channels (3) SR Ca\(^{2+}\)-reuptake pump (SERCA) and its regulation PLB. This process of trigger, release and reuptake is modulated by signalling pathways such as \(\beta\)-adrenergic receptor (\(\beta\)-AR) signalling and cAMP (PKA and EPAC) signalling pathways (Sperelakis & Schneider, 1976; Reuter & Scholz, 1977; Kamp & Hell, 2000).

1.5.3.1. \(\beta\)-adrenergic receptor (\(\beta\)-AR) signalling

The \(\beta\)-adrenergic receptor (\(\beta\)-AR) belongs to the superfamily of membrane proteins known as G-protein-coupled receptors (GPCRs). The trans-membrane signalling by GPCRs is initiated by the binding of agonists such as neurotransmitters or hormones. There are four subtype's known- \(\beta_1\)-AR, \(\beta_2\)-AR, \(\beta_3\)-AR and \(\beta_4\)-AR (Singh et al., 2001). The \(\beta_1\)-AR is predominantly present in heart and comprises 75-80% of \(\beta\)-ARs found in the heart. The G protein consists of \(\alpha\), \(\beta\) and \(\gamma\) subunits. The \(\beta\)-AR binds to norepinephrine and is released from sympathetic adrenergic nerves, and to epinephrine which circulates in the blood. Overall, the effect of \(\beta\)-agonists on the heart is (positive chronotropy (increased heart rate),
positive dromotropy (increased conduction of electrical signals through AV node), positive inotropy (increased force of contraction), and positive lusitropy (increased myocardial relaxation after contraction). The agonist binding induces conformational change in GPCRs that cause G-protein coupling and dissociation of the G-protein in active $G_\alpha$ and $G_\beta$ subunits. These subunits modulate the activity of various molecules such as adenylyl cyclase (AC) or phospholipase C (PLC) which further regulate downstream signalling molecules of the pathway.
1.5.3.2. The PKA pathway

The mechanisms underlying the response of the heart to β-AR stimulation is complex and involves cAMP/PKA-dependent phosphorylation of several proteins including LTCC and PLB. The stimulation of β-AR activates AC which increases the cytosolic cAMP production and binds to regulatory subunits of PKA, liberating the catalytic subunits to phosphorylate their substrates on specific serine and threonine positions (Kamp & Hell, 2000). An A kinase adapter protein (AKAP) adjacent to the LTCC and RyR facilitates its phosphorylation. The other proteins phosphorylated by PKA include PLB and TnI (Bers, 2008). Figure 1.12 shows a schematic diagram of PKA pathway involving cAMP-dependent phosphorylation of several proteins. In the β-AR cardiac studies, most work is focused on β1-AR stimulation which is the most predominant form in the heart.

![Schematic diagram showing signal cascades regulating L-type calcium channels. Stimulation of β1- or β2-AR activates AC which increases the cAMP production and further stimulates PKA (as shown). PKA can phosphorylate the LTCC and nearby RyR at multiple potential sites making it more sensitive to phosphatases PP1 and PP2A. Abbreviations: PKA: protein kinase A; AC: Adenylyl cyclise; AKAP: A kinase adapter protein PP1: phosphatise 1 and PP2A: phosphatise 2A (modified from (Kamp & Hell, 2000)).]
1.5.3.3. The EPAC pathway and its proteins

Cyclic adenosine 3′, 5′-monophosphate (cAMP) is an important second messenger in the heart that regulates many physiological processes such as contractility, relaxation and automaticity (Métrich et al., 2010). Protein kinase A (PKA) is the usual known and recognised primary effector of cAMP signalling but other effectors such as cyclic nucleotide gated (CNG) cation channels and phosphodiesterases (PDEs) are known to transduce cyclic nucleotide encoded information. A recently identified protein, activated by cAMP, named as exchange protein-directly activated by cAMP (EPAC) adds complexity to classical PKA-dependent signalling mechanism (de Rooij et al., 1998). EPAC proteins function independently of PKA, and are guanine nucleotide exchange factors (GEF) for the Ras family of GTPases, Rap1 and Rap2. Upon binding cAMP, the EPAC proteins activate the Rap GTPases that subsequently give rise to the various biological effects. Out of two isoforms of EPAC, named EPAC1 and EPAC2, EPAC1 is highly expressed in the heart (Métrich et al., 2008).

Upon cardiac β-AR stimulation, cAMP via PKA influences the activity of many key proteins such as LTCC, PLB, RyR and cardiac TnI which are involved in EC coupling (Cheng et al., 2008). The schematic diagram showing a proposed mechanism of EPAC pathway in figure 1.13.
Figure 1.13. Schematic diagram showing the proposed EPAC (red) and classical PKA pathways (black) regulated by AMP. Important effectors in the pathway are shown in red.

1.5.3.4. The role of EPAC in excitation-contraction (EC) coupling

Holz (2004) reported that activation of the EPAC1 isoform stimulated CICR in pancreatic β-cells and confirmed cAMP was a part of the signalling pathway (Holz et al., 2008a). The first evidence of involvement of EPAC in CICR in adult cardiomyocytes were reported (Morel et al., 2005; Oestreich et al., 2007; Pereira et al., 2007). An increase in spontaneous spark activity in isolated rat myocytes following activation of EPAC, with a reduction in the evoked Ca\(^{2+}\) transient amplitude but with no change in cell shortening was reported (Pereira et al., 2007). Oestreich et al. (Oestreich et al., 2007) reported increased Ca\(^{2+}\) transient amplitudes in isolated cardiomyocytes from mouse heart during EPAC activation, with no change to Ca\(^{2+}\) transients in myocytes from phospholipase C\(_{\varepsilon}\) knockout mice (PLC\(_{\varepsilon}^{-/-}\)) (Oestreich et al., 2007). However, Cazorla et al. (Cazorla et al., 2009) reported reduced Ca\(^{2+}\) transient amplitude, and increased cell shortening with EPAC activation in rat myocytes. Further studies by the Oestreich group identified the sequential activation of EPAC, PLC\(_{\varepsilon}\), PKC\(_{\varepsilon}\) and CaMKII, downstream of β-adrenergic stimulation. They also reported increased myofilament Ca\(^{2+}\) sensitivity in myocytes infected in vivo with a constitutively active form of EPAC (Oestreich et al., 2007). In addition, EPAC induces the
expression of calmodulin which maintains the inotropic effect in cardiomyocytes (Ruiz-Hurtado et al., 2012). More recently isolated cells from EPAC1 knock out mice have been found to have decreased cardiac contractility, in combination with decreased SR Ca^{2+} storage and smaller Ca^{2+} transients (Okumura et al., 2014).
1.6. Thesis Objectives

In the cellular environment within the myocardium, each myocyte communicates with ECM and its components structurally and through signalling pathways. The microenvironment around each cell is disrupted when myocytes are separated from the ECM during the enzymatic isolation procedure. Isolated myocytes are frequently thought of as the basic physiological model of cardiac function in many studies, yet they are devoid of the ECM and its components. The structural importance of ECM in the heart is very clear, but there is evidence that the ECM could also play an important role in myocyte Ca\(^{2+}\) metabolism. A number of observations reported in the literature suggest its role in Ca\(^{2+}\) metabolism (Valencik et al., 2006), (Wang et al., 2009a), (Browe & Baumgarten, 2004), (Iribe et al., 2009). A brief comparison was made on Ca\(^{2+}\) transients obtained from single myocytes and intact trabeculae. Our observation that Ca\(^{2+}\) signals in single myocytes were smaller than those recorded from intact trabeculae led us to believe that there were linkages between cardiac ECM and cardiomyocytes which might alter Ca\(^{2+}\) metabolism. Therefore, my study will examine the role of the ECM in relation to myocyte Ca\(^{2+}\) signalling.

The overall aim of this thesis is to better understand the interaction between the ECM and the contracting myocytes. In particular, I aim to investigate the direct and indirect links between the ECM and myocyte and the role of ECM in Ca\(^{2+}\) signalling pathways with the following specific objectives:

Objective 1: To measure Ca\(^{2+}\) transients in trabeculae and single myocytes at the same sarcomere length (Chapter 3).

Objective 2: To study changes in myocyte [Ca\(^{2+}\)], as the ECM is systematically dismantled using a protocol based on standard cell isolation procedures (Chapter 3).

Objective 3: To measure the loss of ECM components during dismantling by fixing the trabeculae and labelling key ECM proteins (Chapter 3).

Objective 4: To look for evidence of direct signalling between endothelial cells and cardiomyocytes via ET-1 and prostaglandins (Chapter 3).

Objective 5: To use immunohistochemistry to look for the evidence of important surface structures (e.g. primary cilium) which might contribute to myocyte Ca\(^{2+}\) signalling (Chapter 4).
Objective 6: To study the novel EPAC pathway in trabeculae and its contribution to Ca^{2+} signalling and EC-coupling (Chapter 5).
Chapter 2. General Methodology

2.1. Chapter Overview

Investigating the role of ECM in myocyte Ca\(^{2+}\) modulation required a number of techniques. This includes the live cardiac muscle preparation at maximal sarcomere length (Lmax) to measure force changes (mN scale) and to observe intracellular [Ca\(^{2+}\)]\(_i\), simultaneously. For this study, intracellular [Ca\(^{2+}\)]\(_i\) was monitored whilst subjecting the trabeculae to a procedure that sequentially dismantled the linkages between the ECM and the myocytes, mimicking the standard cell isolation process (Farmer et al., 1983). Furthermore, to look for the extent to which key surface proteins and structures are lost during the cell isolation process by using immunohistochemistry. My study investigates autocrine/paracrine signalling and its contribution to myocyte Ca\(^{2+}\) homeostasis in RV trabeculae from rat hearts. The following chapter outlines the general equipment and techniques used during this study.

2.2. Ethical Approval

The experiments in this study were carried out in accordance with the recommendations in the Guide for the Care and use of Laboratory Animals of the National Institutes of Health. Experimental protocols were approved by the Animal Ethics Committee, The University of Auckland, approval number AEC R857.
2.3. The Cardiac Trabecula

Cardiac trabeculae are free running, multicellular ventricular muscles located in the RV just beneath the atrio-ventricular (AV) ring with one end attached to AV ring and the other to the ventricular wall. Trabeculae were obtained from adult wistar rat hearts (figure 2.1). The longitudinal alignment of myocytes within each trabecula makes them ideal cardiac preparations for studying force and \([\text{Ca}^{2+}]_i\) as they have a uniform distribution of strain across the entire preparation (Greenbaum et al., 1981; LeGrice et al., 1995).

![Image 2.1](Image)

**Figure 2.1.** Schematic diagram of the dissected out right ventricle (RV) showing the relative position of cardiac trabeculae near the AV valve within ventricular wall. Image from (Hanley & Loiselle, 1998).

2.3.1. Isolation, Dissection and Mounting of Trabeculae

Un-branched trabeculae (length 2.2 ± 0.1 mm, cross-sectional area 0.03 ± 0.01 mm\(^2\)) were micro-dissected from the RV of rat hearts (Wistar, ~300g) of either sex. The rats were anaesthetized with isoflurane using 100% O\(_2\) as a carrier gas prior to decapitation. Hearts were quickly excised and submerged in cold physiological buffer (see section 2.5). The hearts were then transferred to a dissection chamber where the aorta was cannulated and perfusion of coronary vasculature was started. In the dissection chamber, the heart was oriented with the right ventricular free wall in the uppermost position. An initial cut across the right atrium was made followed by a second cut perpendicular to the apex along the septal wall. The right ventricular free wall was then peeled back, as shown in figure 2.1. When present, a free running trabecula was micro-dissected with a block of tissue at one end and a piece of the AV ring at the other end. The trabecula was then transferred to a chamber on the stage of an inverted light microscope (Nikon Diaphot 300, Japan) and mounted between a wire cradle extending from the beam of a force transducer (KX801
Micro Force Sensor, Kronex Technologies) and a monofilament snare at the other end (figure 2.2). Both the force transducer and the snare were attached to 3-D micromanipulators that enabled the trabecula to be positioned in the middle, and close to the base, of the chamber, as well as SL to be adjusted. Once mounted, the trabecula was superfused continuously with oxygenated buffer using a non-pulsatile roller pump (Minipuls 2, Gilson, France) with a flow rate set at 10mL/min. The muscle chamber was designed (as shown in figure 2.2) in such a way to reduce any baseline noise to the force measurements due to the flow of buffer solution. Thin platinum electrode plates extended along the sides of the chamber, and were used for electrical stimulation of the trabeculae.

![Diagram of muscle bath](image)

**Figure 2.2.** Schematic diagram of the Perspex muscle bath showing mounted trabecula. There is inflow and outflow for the superfusate into the chamber. The tissue block at one end of the trabecula was placed into a wire cradle extending from the beam of a force transducer, while the small AV ring block at the other end was held within a nylon loop of snare attached to the micromanipulator.

### 2.3.2. Force Measurement

The RV wall tissue block of the trabecula was placed in the wire cradle (hook) extending from the silicon beam of a force transducer (figure 2.3), while the small tissue block at the other end was held within a nylon loop of snare attached to micromanipulator (see figure 2.2). The force transducer is equipped with silicon strain-gauge and is sensitive to as low as 1 µN range of force. The three-axis micromanipulator allowed the trabeculae length changes to obtain the optimal length. The electrodes of muscle chamber were coupled to a stimulator (model D100, Digitimer™, UK) as shown in figure 2.2. It was field stimulated with pulses of 5 ms duration (~20% above threshold voltage) at 0.2 Hz frequency at $T_{room}$. The central portion of the trabecula was viewed, using x40 objective and a charge-coupled device (CCD) camera connected to a video monitor. The muscle striations were clearly visible at
this magnification. The twitch amplitude and duration are sarcomere-length dependent in cardiac muscle, trabeculae were extended to give a sarcomere length of 2.1 – 2.2 µm (Allen & Kentish, 1985). Therefore, at this sarcomere length, twitch force is at the peak of the length-tension relationship for cardiac muscle (ter Keurs et al., 1980). This length was then maintained throughout the experiment.

**Figure 2.3.** Schematic diagram of a standard force transducer showing the silicon cantilever beam. The silicon beam is attached to the wire cradle.

The force transducer was calibrated by converting the output signals from mass of 0.1 mg to mN using a linear interpolation between the output signal and the actual force applied.

### 2.3.2. Measurement of [Ca$^{2+}$]$_i$ using Fura-2/AM

The ratiometric fluorescent indicator, Fura-2 in its acetoxymethyl (AM) ester, a membrane permeable, form was used to record intracellular [Ca$^{2+}$]$_i$. A spectrophotometric system (Cairn Research, Faversham, Kent, UK) coupled to the inverted light microscope (Nikon Diaphot 300, Japan) was used to record Fura-2 signals from trabeculae illuminated using a 75 W xenon arc lamp. A six-filter wheel (3 × 340 nm, 1 × 360 nm, 2 × 380 nm) attached to the system was run at 47 Hz to provide rapidly changing excitation wavelengths. A combiner module provided a continuous average of the emitted fluorescence for each of the excitation wavelengths. A window size of approximately 100 µm × 200 µm (depending upon the individual preparations) was used to record the fluorescence from the central portion of the trabecula. Ratiometric measurements for the 340/380 excitation wavelengths were then obtained; i.e. the ratio of fura-2 fluorescence for Ca$^{2+}$$_{bound}$/Ca$^{2+}$$_{free}$. The emitted fluorescence was collected by the objective piece and directed via a 600 nm dichroic mirror.
and a 480 nm long pass filter to a photomultiplier tube (EMI9124B, Thorn EMI Electron tubes, UK).

2.3.4. Autofluorescence

It has previously been reported that a significant portion of cardiac muscle autofluorescence on UV light excitation comes from reduced nicotinamide adenine nucleotides (NADH and NADPH) and oxidized flavoproteins (Vuorinen et al., 1995; Hanley & Loiselle, 1998). Like fura-2, NADP is also excited by UV light at ~340 nm and has a broad emission spectrum at ~460 nm. Depending upon the measured tissue autofluorescence for both wavelengths (340 and 380 nm), subtraction was made for these wavelengths.

2.3.5. Loading of Fura-2/AM

Intracellular Ca$^{2+}$ measurements were made as described previously (Ward et al., 2003). The trabeculae were loaded at T$_{room}$ with 2 x 50 µg of Fura-2/AM (Teflabs, TX, USA) dissolved in 20 µL of anhydrous dimethyl sulfoxide (DMSO) with 5% wt/vol of Pluronic F-127 (Teflabs, TX, USA). The mixture of Pluronic F-127/DMSO facilitates the loading by making the AM form more soluble (Takahashi et al., 1999). The 40 µL of Fura-2/AM loading solution was added to 10 ml of physiological buffer to make up a final concentration of 10 µM. The formation of excess foam due to continuous oxygenation of the loading solution, was prevented by adding 10 µL antifoam A (Sigma Chemical Co., St Louis, USA). The loading solution was continuously recirculated through the muscle chamber for 2 hours (0.1 Hz, 1mM [Ca$^{2+}$]o and at T$_{room}$ (20-22 ºC)).
2.4. The cardiac myocyte

2.4.1. Ventricular myocytes

Ventricular myocytes were enzymatically-isolated from adult Wistar rat (200–300 g) hearts as previously described (Cooper et al., 2010). The rats were anaesthetized with isoflurane using 100% O₂ as a carrier gas prior to decapitation. The heart was quickly dissected out and mounted by the aorta onto a Langendorff perfusion system. Initially, heart was perfused with oxygenated Ca²⁺-free Tyrode’s buffer (see section 2.5 for details) for 5 min at 37 °C. This was then followed by perfusion with Tyrode’s buffer containing 200 µM [Ca²⁺]₀, 1.0 mg/ml Type II Collagenase and 0.1 mg/ml Type I Protease. The end of digestion was detected as a fast flow rate of enzyme solution in the form of a string coming out from the coronary circulation. Ventricles were then dissected, diced into smaller blocks of ~ 1mm wide and triturated within Tyrode’s buffer containing 0.15 mM [Ca²⁺]₀ to release single myocytes. The resulting suspension was collected in 50 ml falcon tubes and samples of the myocytes obtained were examined under the light microscope. Only-rod shaped cells that were not spontaneously contracting, and had visible clear striations were used for experiments. According to the experimental requirements, cells were either kept at T_room for experimentation (1 mM [Ca²⁺]₀) or fixed (see section 2.8.3) for immunolabelling. The Ca²⁺ was raised to 1 mM for cell storage, and experiments were carried out at T_room. See figure 2.4 for structure of freshly isolated myocytes.

![Figure 2.4](image1.jpg)

**Figure 2.4.** Freshly isolated rod shaped cardiomyocytes showing a clear striated pattern under transmitted light.
2.4.2. Measurement of \([Ca^{2+}]_i\) in myocytes

Intracellular Ca\(^{2+}\) measurements in myocytes were made with Fura-2/AM. Cells were incubated with 5 \(\mu\)M Fura-2/AM (Teflabs, TX, USA) dissolved in anhydrous DMSO with 5\% wt/vol of Pluronic F-127 (Teflabs, TX, USA) for 20 min at \(T_{room}\). The cells were then washed with 1 mM Ca\(^{2+}\)-Tyrode’s solution, without Fura-2/AM. Ca\(^{2+}\) transients were recorded only from rod-shaped myocytes, which responded to electrical stimulation with a rapid twitch. Cells at \(T_{room}\) were stimulated at 0.2 Hz using a supramaximal voltage pulse of 4ms duration which was delivered by platinum electrodes placed 2 mm apart along the length of the cell chamber.

2.4.3. Measurement of Ca\(^{2+}\) sparks in myocytes

Cells were aliquoted and incubated with 5 \(\mu\)M Fluo-4/AM (2 mM stock in 2.5 \% Pluronic in DMSO; Invitrogen, California, U.S.A) for 25 min at \(T_{room}\). Cells were then washed with 1 mM Ca\(^{2+}\)-Tyrode’s solution. Line-scan images of Ca\(^{2+}\) sparks and transients were obtained with an inverted line-scanning confocal microscope (LSM 410; Zeiss, Germany). Ca\(^{2+}\) sparks were measured in quiescent cells with well-defined striations within ~8 hours of their isolation.

Ca\(^{2+}\) spark frequency was visually quantified. Cells were stimulated at 0.2 Hz for ~20 s before Ca\(^{2+}\) sparks were measured in quiescent cells. F/F\(_0\) was converted to \([Ca^{2+}]_i\) using the pseudo-ratio method (Cheng et al., 1993), where \([Ca^{2+}]_i\), rest and KD of Fluo-4 were set to 100 and 800 nM. All analyses were performed in IDL. Calibration to [Ca\(^{2+}\)] was performed using \(K_d\), Fluo-4 = 800 nM and \([Ca^{2+}]_{rest} = 100 \text{ nM}\) (Woodruff et al., 2002).

2.4.4. Elimination of SR Ca\(^{2+}\) storage using caffeine

Caffeine is a pharmacological agent used experimentally to release the SR Ca\(^{2+}\) stores in cardiac muscle (Bers, 1989). The contractile effects of the action of caffeine are dependent on concentration, rate, length of exposure, and the species (Chapman & Léoty, 1976; Kitazawa, 1988). The caffeine application to cardiac preparations causes a transient increase in both \([Ca^{2+}]_i\) and force (Kitazawa, 1988; Smith et al., 1988). The peak amplitude of caffeine contractures in multicellular preparations depend critically on the diffusion of
caffeine to the innermost cells and is limited by the diameter of trabeculae (Bers, 1989). But this is not the case when caffeine is applied to isolated myocyte preparations, the release of SR Ca\(^{2+}\) is rapid, and the peak of the resulting transient increase in [Ca\(^{2+}\)], is used as the measure of amount of Ca\(^{2+}\) stored in the SR. The sustained application of caffeine to isolated muscle preparations completely eliminates the contribution of the SR as a Ca\(^{2+}\) storage site. There is no net Ca\(^{2+}\) storage as the RyRs remain in an open state, though SR Ca\(^{2+}\)-ATPase still transports Ca\(^{2+}\) into the SR.

### 2.5. Chemicals and solutions

#### 2.5.1. K-H buffer

The trabeculae were superfused with Krebs-Henseleit (K-H) solution containing (mM): 118 NaCl; 4.75 KCl, 1.18 MgSO\(_4\); 1.18 KH\(_2\)PO\(_4\); 24.8 NaHCO\(_3\); and 10 D-glucose. The superfusate was continuously bubbled with 95% O\(_2\) and 5% CO\(_2\) to maintain pH at 7.4.

#### 2.5.2. Tyrode’s buffer

For some experiments, a Tyrode’s buffer bubbled with 100% O\(_2\) was used containing (mM): 141.8 NaCl, 6 KCl, 1.2 MgSO\(_4\), 1.2 Na2HPO\(_4\), 10 HEPES and 10 D-glucose. [Ca\(^{2+}\)]\(_o\) was adjusted, as required, by addition of CaCl\(_2\) from a 1 M stock solution. For isolated cells, a Ca\(^{2+}\)-Tyrode’s solution was used containing (mM): 140 NaCl; 10 HEPES; 10 D-Glucose; 4 KCl; MgCl\(_2\); pH = 7.4 with NaOH. All experiments were performed at T\(_{\text{room}}\). Collagenase Type 2 (Worthington, NJ, USA) and Protease Type I: Crude from Bovine Pancreas (Sigma Aldrich, MO, USA) were the enzymes used for trabeculae and single cell isolation experiments.

#### 2.5.3. Dissection

The dissection solution contained 0.25 mM Ca\(^{2+}\) plus 20 mM 2, 3-butanedione monoxime (BDM) to protect the myocardium from reperfusion injuries. BDM is a non-competitive ATPase inhibitor of myosin-II and acts as cardioprotectant from reoxygenation injuries (Mulieri et al., 1989). 2% paraformaldehyde (PFA) is used to fix the trabeculae and single myocytes.
2.6. Experimental protocol for sequential dismantling of the ECM in isolated trabeculae

Trabecula loaded with Fura-2/AM were subjected to partial digestion using a protocol that mimicked the enzymatic single cell isolation procedure after first measuring force and [Ca\(^{2+}\)]\(_i\) (see figure 2.5 for summary). The trabecula was continuously perfused with 1.0 mg/mL collagenase, type 2 (Worthington, NJ, USA) and 0.1 mg/mL Protease, type I (Sigma Aldrich, MO, USA) dissolved in 10 mL of Tyrode's buffer with 0.2mM [Ca\(^{2+}\)]\(_o\) until a change in the amplitude of [Ca\(^{2+}\)]\(_i\) transients was detected. During enzyme wash out, the [Ca\(^{2+}\)]\(_o\) was gradually increased back to 1mM. The force and [Ca\(^{2+}\)]\(_i\) transients were recorded again in 1mM [Ca\(^{2+}\)]\(_o\) at T\(_{room}\).

![Diagram of experimental protocol](image)

**Figure 2.5.** Summary of the experimental protocol used to examine Ca\(^{2+}\) handling during the dismantling of the ECM in cardiac trabeculae.* Enzyme solution (see section 2.5.2.)
2.7. Data Acquisition and Statistical Analysis of trabeculae experiments

2.7.1. Data acquisition

Raw data were acquired using Lab View Signal Express (National Instruments, Austin, USA). An analogue form of the data was also stored using a conventional chart recorder (Graphtek Linearorder Mark VII, Leatham Electronic Limited, Wellington). The recorded force and fluorescence data were sampled at 400 Hz and sections of interest were exported as text files for off-line analysis using a custom-written program (IDL Research Systems Inc., Boulder, CO, USA). The data were averaged across a number of cardiac cycles, and a five-parameter Boltzmann-exponential function shown below (eq. 1) was used to describe the Ca$^{2+}$ transient (Ward et al., 2003):

\[
y = A_0 \exp(-t/A_1)/(1 + \exp(-(t + A_2)/A_3)) + A_4 \text{ (eq. 1)}
\]

where $A_0$ represents the maximum change in fluorescence 340/380 ratio, $A_1$ is the time constant of exponential decay during the relaxation phase of the Ca$^{2+}$ transient, $A_2$ is the time difference between the rising and the decaying phases of the Ca$^{2+}$ transient, $A_3$ is the time constant of rise of the transient and $A_4$ is the diastolic or minimum fluorescence before stimulation. Peak systolic fluorescence, diastolic fluorescence, time-to-peak fluorescence, the time course of fluorescence decay, and the maximum rate-of-rise of fluorescence were obtained from both the averaged and fitted-data (see Figure 2.6). Force was also averaged for the same number of cardiac cycles, and measurements were made for the peak systolic force, the resting force, the time-to-peak force, the maximum rate-of-rise of force, and the times to 50% and 90% relaxation.
Figure 2.6. Example of data analysed using IDL software for 12 consecutive Ca$^{2+}$ transients obtained from a representative trabecula (0.2 Hz, 1.5 mM [Ca$^{2+}$]$_o$, T$_{room}$). A shows the averaged Ca$^{2+}$ transient (top), with the averaged twitch (bottom). B shows the average of the Ca$^{2+}$ transients (solid line) in E. C shows the differential of the averaged transient, from which the maximum rate-of-rise of fluorescence was obtained. D shows the differential of fitted transient (dotted line in B). E shows 12 consecutive Ca$^{2+}$ transients, vertically aligned for comparison, and F shows phase plots of the averaged force, plotted as a function of averaged fluorescence.
2.7.2. Statistical analysis of data

Statistical analysis was performed using the Excel Data Analysis Toolpak (Microsoft Corporation, USA). All results were expressed as mean ± standard error of means (SEM) for n number of experiments and statistical significance as $P \leq 0.05$. In this study, not more than one trabecula per heart was utilised. Two-tailed Paired or unpaired t-tests, as well as One-way Analysis-of-variance for repeated measures (ANOVA) was applied where appropriate using the SPSS statistics software (IBM) and statistical significance was determined at the 95% level of confidence limit with Bonferroni post-test as confidence interval adjustment. However, for the results under section 5.4 figure 5.4, LSD post-test was used because of the small n value.
2.8. Immunohistochemistry

2.8.1 Tissue section preparation, reagents and antibodies

Neonatal and adult rats were anaesthetized with isoflurane prior to decapitation. Hearts from neonatal (full term), postnatal day 2 (P2), postnatal day 21 (P21), and postnatal day 28 (P28) were quickly excised, immediately washed with PBS, and suspended in 2% PFA for 30 min at TRoom. Adult rat hearts were cut into approximately 0.5 x 0.5 cm chunks before fixation. All tissues were then cryoprotected in sucrose solutions and stored at -80 °C until cryosectioning (described in detail in Section 2.8.2).

Rabbit polyclonal to ARL13b (Proteintech, USA) and Mouse monoclonal to acetylated alpha-tubulin (clone 6-11 B-1, Sigma-Aldrich) antibodies were kind gifts from Dr. Sue McGlashan. During the development of immunohistochemical protocols, all primary antisera were titrated to give maximal immunohistochemical reactivity. Normal goat serum (Invitrogen, Life Technologies) and phosphate-buffered saline (PBS) (Gibco, Life Technologies, NZ), pH 7.5 was already available in this laboratory. Paraformaldehyde (PFA, 8% aqueous solution, Electron Microscopy Sciences, Hatfield, PA) was diluted to 2% with PBS.
<table>
<thead>
<tr>
<th>Primary/Secondary antibodies</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit polyclonal to collagen I</td>
<td>1:100</td>
<td>Abcam Cambridge, UK</td>
</tr>
<tr>
<td>Mouse monoclonal to endothelial cells (RECA-1)</td>
<td>1:50</td>
<td>Abcam Cambridge, UK</td>
</tr>
<tr>
<td>Mouse monoclonal to Vinculin</td>
<td>1:200</td>
<td>Abcam Cambridge, UK</td>
</tr>
<tr>
<td>Rabbit monoclonal to Integrin beta 1</td>
<td>1:100</td>
<td>Abcam Cambridge, UK</td>
</tr>
<tr>
<td>Rabbit polyclonal to Laminin</td>
<td>1:100</td>
<td>Abcam Cambridge, UK</td>
</tr>
<tr>
<td>Rabbit polyclonal to ARL13B</td>
<td>1:500</td>
<td>Proteintech, USA</td>
</tr>
<tr>
<td>Mouse monoclonal to Acetylated Tubulin (clone 6-11 B-1)</td>
<td>1:500</td>
<td>Sigma Aldrich, MO, USA</td>
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<tr>
<td>Mouse monoclonal to Ryanodine receptor</td>
<td>1:100</td>
<td>Thermo Scientific, Rockford, USA</td>
</tr>
<tr>
<td>Mouse monoclonal to Vimentin</td>
<td>1:100</td>
<td>Sigma Aldrich, MO, USA</td>
</tr>
<tr>
<td>Alexa fluor 488 conjugated with Goat anti Mouse</td>
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<tr>
<td>Alexa fluor 694 conjugated with Phalloidin</td>
<td>1:5</td>
<td>Invitrogen, Life Technologies, USA</td>
</tr>
</tbody>
</table>

**Table 2.1:** A list of primary and secondary antibodies with their dilution and source
2.8.2. **Protocol for fixing isolated tissue**

After recording force and $[\text{Ca}^{2+}]_i$, the trabeculae were fixed in 2% PFA for 10 min at $T_{\text{room}}$ for immunolabelling purposes from a method modified from Ward et al., 2008. The trabeculae were fixed at Lmax and cryoprotected in 10%, 20%, and 30% sucrose solution in PBS with 0.1% sodium azide (Sigma Aldrich, USA) for a minimum time of 2 hours, 2 hours, and overnight, respectively. On the following day, trabeculae were embedded in Optimal Cutting Temperature (OCT) (Tissue-Tek; Sakura Finetek, USA) in cryomolds (Tissue-Tek; Sakura Finetek, USA) and stored at -80°C until cryosectioning. The OCT embedded trabeculae were cut into 10 to 20 µm thin sections using a cryostat (LEICA CM1900) at -18°C, and sections were placed on superfrost glass slides. The temperature of the cryostat was adjusted if necessary. Slides were kept at -20°C in slide folders for later use.

2.8.3. **Protocol for cardiomyocytes fixation**

Isolated myocytes suspended in Tyrode’s buffer (0.15mM $[\text{Ca}^{2+}]_i$) were first gently spun at 800 rpm for 2 min at $T_{\text{room}}$ and the supernatant was discarded without disturbing the cell pellet. Cells were then re-suspended in 2% PFA (w/v; pH~7.4) at RT for 10 min. The cells were again centrifuged at 1200 rpm (Ultracentrifuge) for 2 min and the supernatant was discarded before repeating the centrifugation-suspension step once again to remove residual PFA. After the final centrifugation, the fixed cells were re-suspended in ~2-4 ml of storage buffer (0.5% BSA, 0.1% Sodium azide in PBS) (Jayasinghe *et al.*, 2009). The image showing fixed cells under transmitted light in figure 2.6.
Figure 2.7. The 2 % PFA fixed myocytes showing striated pattern even after fixation under transmitted light. Scale bar is given.

2.8.4. Embedding and Cryosectioning of fixed trabeculae

Fixed trabeculae were embedded in biopsy cryomolds (10 (l) x 10 (w) x 5 (d) mm) (Tissue-Tek, Sakura Finetek, USA) with OCT and kept at -80 °C. The trabeculae were placed horizontally on 4-fold strip of parafilm (Pechiney Plastic Packaging, Menasha, WI) of same size as cryomold on the surface of cryomold, immersed in OCT and pinned in position whilst frozen at -20 °C for 2 hours (see figure 2.7). The pins were taken out once the OCT was set.

Figure 2.8. Schematic diagram of a cryomold base lined with parafilm showing a trabecula held by pins during OCT embedding.

The OCT embedded tissue blocks were oriented during cryosectioning in such a way to obtain horizontal and transverse sections (10 µm) of the trabeculae, as required. The slides were kept at -20°C until used further for immunolabelling.
2.8.5. **Immunohistochemistry for ECM proteins in trabeculae**

The 2% PFA fixed control and partially digested trabeculae kept at -20°C, were sectioned at 10 µm thickness. Sections were rehydrated with cold PBS (10 min at T\text{room}) followed by rinsing with 1% BSA (bovine serum albumin) in PBS (10 min). Sections were then blocked with 5% NGS in 1% BSA in PBS (1h at T\text{room}) and incubated (4°C, overnight) with primary antibodies (Abs) (see Table 2.1 for more details) as appropriate (diluted in PBS/ 5% NGS in PBS). After washing, slides were stained for 2h with secondary Abs (1:200) (diluted in PBS/ 5% NGS in PBS) at T\text{room}. Slides were washed (PBS, 3 x 5 min each), sections were mounted in Prolong gold antifade reagent (Invitrogen, Life Technologies, NZ) and kept in a slide folder at 4 °C until viewed under the fluorescence microscope.

2.8.6. **Immunohistochemistry for ECM proteins in single cells**

The 2 % PFA fixed myocytes in a storage buffer at 4°C were re-suspended into Eppendorf tubes (1.5 ml). The tubes with the cell-suspension were spun in the centrifuge (IEC MicroCL 17 centrifuge, Thermo Electron Corporation) at 3000 rpm for 3min and supernatant was discarded. The pellet was incubated with PBS/ 1% BSA/ 0.1% triton X-100 on rocker (10 min) before re-spinning (3000 rpm, 3 min). Next, the cells were blocked with blocking solution (PBS with 10% NGS and 0.05% triton X-100) (1 h at T\text{room}). At the end of each step, the cells were re-spun at 3000 rpm, 3 min to obtain the cell pellet. The primary Abs were incubated diluted in incubation buffer (5% NGS in PBS with 0.01% triton X-100) (4 °C, overnight) followed by secondary Abs (1:200) for 2h at T\text{room}. The cells suspension was mixed with 30-40 µl of Prolong gold antifade reagent and mounted on glass slides/coverslips and kept in a slide folder at 4 °C until ready to view.
2.9. Image acquisition and analysis

Fluorescent images (1024 x 1024 pixels) were recorded in xy/xyz scan mode either with a Zeiss LSM410 laser scanning (63x, NA 1.25, oil-immersion objective) or with a Olympus FV1000 (60x, NA, 1.35 oil-immersion objective, located in BIRU facility of Faculty of Medical and Health Sciences, The University of Auckland) confocal microscope. Alexa 488, 594 and 694 fluorochromes were excited with 473, 559, and 635 nm lasers on Olympus FV1000 confocal microscope. Some of the sections were selected for Z-scanning and Z-stacks (0.3-1.0 µm spacing between adjacent sections) were acquired with a sampling speed of 8.0 µs / pixel for offline data processing. Z-projected images were generated offline from the stacks of confocal sections wherever required. The images were processed using Image J (http://imagej.nih.gov/ij) and FV10-ASW 3.0 Viewer software (Olympus Fluoview Ver.3.0 Viewer) for those acquired by confocal microscope. Images for RECA-1, Cxn-43, Laminin, and Vinculin were quantified by thresholding and measuring the intensity using Image J. The results were expressed as mean ± SEM percentage of fractional area of labelled tissue viewed, and Students’s t-test was used to test for statistical significance.
Chapter 3. Role of ECM in myocyte intracellular Ca\textsuperscript{2+} signalling

3.1. Chapter Overview

Contraction and relaxation of the heart critically depends upon changes in cytosolic Ca\textsuperscript{2+} concentration in myocytes (Moulin & Wilbrandt, 1955). Our knowledge of EC-coupling derives mainly from isolated cardiac myocyte studies (Bers, 2002, 2008). However, isolated myocytes lack the ECM components of multicellular tissue and may not provide the true complexity of exact tissue function. The ECM is a key component of heart which provides three-dimensional network for contracting myocytes and other cell types to perform their function correctly. Though, myocytes make up the largest volume of the heart, there are other cell types such as fibroblasts and vascular endothelial cells that are the major components of ECM. These cell types can possibly contribute to the myocyte Ca\textsuperscript{2+} signalling, and speculated that this was lost in single myocytes during the cell isolation process. Previously, we have observed differences in the intracellular Ca\textsuperscript{2+} transients between isolated myocytes and multicellular trabeculae under the same experimental conditions (unpublished data). This finding provides the background to this current study which investigates the possible contribution of ECM in myocyte Ca\textsuperscript{2+} signalling.

Evidence from previous studies has suggested linkages between the ECM and cardiomyocytes that might act as Ca\textsuperscript{2+} regulatory pathways. For example: (i) Cardiac endothelial cells release many autocrine and paracrine factors such as NO, endothelin, prostaglandin 2\textalpha{}, and angiotensin II, which directly influence cardiac metabolism, growth, contractile performance, and rhythmicity (Li et al., 1993; Brutsaert et al., 1998; Brutsaert, 2003); (ii) Endocardial endothelium (EE) reportedly affects the force-frequency relationship (FFR) in rat myocardium, with dysfunctional EE-myocyte coupling contributing to a negative FFR in heart failure (Shen et al., 2013b); (iii) Sarcolemmal integrins regulate both mechanical and electrical coupling in the adult heart (Valencik et al., 2006); (iv) Axial stretch of rat cardiomyocytes acutely increases the SR Ca\textsuperscript{2+} spark rate via a mechanism that links the cell surface proteins with the cytoskeletal microtubules (Iribe et al., 2009).

The aim of this study was therefore to investigate the possible contribution of ECM (non-muscle cells) to contracting myocyte Ca\textsuperscript{2+} signalling in isolated rat ventricular muscle preparations. To carry out my aim, I compared Ca\textsuperscript{2+} transients and isometric stress from
cardiac trabeculae before and after an enzyme exposure protocol that mimicked the standard cell isolation procedure. Immuno-histochemical techniques were then used to determine the “loss” of key proteins linking the ECM to the myocyte cytoskeleton during the enzyme exposure.
3.2. Modulation of myocyte Ca\(^{2+}\) handling by intact ECM

Cardiomyocytes account for only 30-40% of the cells by number in the heart (Vliegen et al., 1991). Also present in large numbers are fibroblasts, endothelial cells and vascular smooth muscle cells that contribute to the non-myocyte cell population that forms the ECM (Adler et al., 1981). In healthy cardiac tissue, every myocyte is closely associated with fibroblast and vascular endothelial cells. Traditionally the ECM has been thought to provide mechanical scaffolding for the contracting myocytes. Whilst the structural importance of the ECM in the heart is clear, we believe it also plays an important role in modulation of myocyte Ca\(^{2+}\) metabolism based on our observation that isolated myocytes have smaller Ca\(^{2+}\) transients than isolated cardiac tissue (unpublished data).

The extracellular matrix is directly linked to the myocyte cytoskeleton by costameres that are composed of vinculin, talin, integrin α6β1, laminin and other cytoskeletal and extracellular molecules (Hynes, 1992; Henry & Campbell, 1996; Imanaka-Yoshida et al., 2004) (see section 1.4.). Also, costamere proteins are known to form the linkage between myofibrils and the ECM via the Z-lines to the sarcolemma (Danowski et al., 1992). It was found by many groups that cardiac Ca\(^{2+}\) handling is affected by the mechanical environment (Allen & Kurihara, 1982; Kentish & Wrzosek, 1998; Calaghan & White, 2001; Shen et al., 2013a). Also, the capillaries supply blood to every myocyte, and therefore endothelial cells are associated with smooth muscle cells. The endothelial cells are situated in close vicinity to myocytes modulate the contractile activity of smooth muscle (Farrer-Brown, 1968). The release of auto- and paracrine factors such as NO, ET-1 bradykinin and prostaglandins (PGF2α) from endothelial cells can affect intracellular signalling cascades (Yew et al., 1998; Dallas & Khalil, 2003; Zeng et al., 2009). The release of angiotensin II in response to stretch has been shown to regulate ion channels (Browe & Baumgarten, 2004). The 21 amino acid peptide ET-1, originally characterized as an endothelium derived vasoconstrictor regulates many physiological processes, including vasoconstriction, cell proliferation and angiogenesis (Mencarelli et al., 2009). The vascular endothelial cells are the predominant sources of ET-1 (Yanagisawa et al., 1988a, 1988c). Most of the effects of ET-1 are associated with increase in intracellular calcium concentration and in calcium mobilization (Jouneaux et al., 1994; Dallas & Khalil, 2003). Li et al. revealed that endothelin increases contractility and modifies twitch configuration in rabbit papillary muscles (Li et al., 1993). It is clearly evident from the literature that there are number of different ECM interactions that directly or indirectly contribute to the myocyte Ca\(^{2+}\) signalling in intact cardiac muscle via number of different pathways. Taken together, it is important to look further for ECM
and myocyte interactions that might can act as $\text{Ca}^{2+}$ entry pathways and contribute to intracellular $\text{Ca}^{2+}$ homeostasis in the heart.
3.3. Material and Methods

3.3.1. Experimental Protocols

Trabeculae micro-dissected from rat hearts and mounted in the muscle chamber were electrically stimulated at 0.2 Hz in 1 mM [Ca$^{2+}$]$_o$ at T$_{room}$. Fura-2/AM loaded trabeculae were then subjected to an experimental protocol mimicking the standard cell isolation procedure whilst continuously measuring force and [Ca$^{2+}$], (see section 2.6 for details). Once steady-state had been reached and recordings made, trabeculae were fixed whilst held at fixed length in the muscle chamber with 2% PFA (10 min, T$_{room}$) for immunolabelling purposes (Ward et al., 2008).

For the measurement of SR Ca$^{2+}$, the trabeculae were exposed to 20 mM caffeine solution. After recording [Ca$^{2+}$] and force (0.2 Hz, 1 mM [Ca$^{2+}$]$_o$ at T$_{room}$), the stimulation frequency was turned off and 0 mM [Ca$^{2+}$]$_o$ Tyrode’s buffer was introduced for 30 s and was followed by 20 mM caffeine solution (Ward et al., 2011; Zhang et al., 2013). The trabeculae exposed to caffeine solution were followed by washout with caffeine-free 1 mM [Ca$^{2+}$]$_o$ Tyrode’s buffer, and were stimulated at 0.2 Hz (to restore the SR Ca$^{2+}$). This protocol was repeated in same trabecula before and after enzyme treatment.

Data analysis

Labview Signal express text files before the onset of the caffeine transient were created for analysis using a custom-written IDL programme (see section 2.7.1, Chapter 2). The resting Ca$^{2+}$ and the amplitude of the peak of the caffeine-transient were recorded before and after the enzyme treatment. The kinetics of the decay phase of the caffeine-induced fluorescence were determined by fitting a three-parameter exponential functional from 90% of the peak fluorescence (see Chapter 2).

3.3.2. Reagents, enzymes and antibodies

Collagenase Type 2 (Worthington, NJ, USA) and Protease Type I: Crude from Bovine Pancreas (Sigma Aldrich, MO, USA) were the enzymes used for trabeculae and single cell isolation experiments. A Tyrode solution was used for trabeculae and isolated myocytes
experiments, continuously bubbled with 100% O₂. [Ca^{2+}]₀ was adjusted, as required, by addition of CaCl₂ from a 1 M stock solution. All experiments were performed at T_{room}. Caffeine and Enothelin-1 (ET-1) were purchased from Sigma-Aldrich, MO, USA. BQ-123 sodium salt was purchased from Enzo Life Sciences Inc., NJ, USA. Mouse monoclonal (RECA-1) to endothelial cells, rabbit polyclonal to laminin, rabbit polyclonal to collagen I, mouse monoclonal to integrin beta 1 and mouse monoclonal to vinculin antibody were purchased from Abcam Cambridge, UK and mouse anti-connexin-43, alexafluor 488/594 conjugated with goat anti mouse and Prolong gold antifade reagent from Invitrogen Company (see Table 2.1 for details).

3.3.3. Immunolabelling of cardiac trabeculae and single myocytes

The 2% PFA fixed control and partially digested trabeculae kept at -80 ºC, were sectioned at 10 µm thickness (see section 2.8.4.). Sections were rehydrated with cold PBS (10 min at T_{room}) followed by rinsing with 1% bovine serum albumin (BSA) in PBS (10 min). Sections were then blocked with 5% NGS in 1% BSA in PBS (1h at T_{room}) and incubated (4 ºC, overnight) with anti-endothelial cell Ab (RECA-1) (1:50) or anti vinculin (1:200) or anti connexin-43 (1:1000) or anti-laminin (1:100) diluted in 1% BSA in PBS. After washing, slides were stained for 2h with secondary Abs (1:200) (Alexa Fluor 488 goat anti mouse Ab) at T_{room}. Slides were washed (PBS, 3 x 5 min each), mounted (Prolong gold antifade reagent), and imaged with a confocal microscope (Olympus FV1000). The images were processed using Image J. The 2% PFA fixed single myocytes were stored in cell storage buffer and were immunolabelled for RECA-1.

3.4.4. Optical Coherence Tomography imaging and analysis

Optical Coherence Tomography is an imaging technique, based on low-coherence interferometry. It enables non-invasive dynamic 1D, 2D, and 3D imaging of biological structures. To obtain the geometry of trabeculae in pre- and post-enzymatic conditions, the trabeculae (n = 2) were scanned using Optical Coherence Tomography on a different microscope setup in the Associate Professor Andrew Taberner’s laboratory located at Auckland Bioengineering Institute, The University of Auckland. The Optical Coherence Tomography system used was a Spectral Domain Optical Coherence Tomography, capable of imaging about 1 mm deep with a lateral resolution of 23 µm and axial resolution of 3.6
μm (Cheuk ML, Lippok N, Dixon AW, Ruddy BP, Vanholsbeeck F, Nielsen PM, 2014). A total envelope volume of 3 mm x 1.5 mm x 1.5 mm was imaged in 1.8 s. This was repeated twice and the average was taken. The density was calculated by means of drop in contrast and the contrast was calculated by the sum of intensity of pixels in the region of interest.
3.4. Measurement of $[\text{Ca}^{2+}]_i$ transients in single cardiomyocytes and trabeculae

Previously, our group had observed that the $\text{Ca}^{2+}$ transients from the isolated cardiomyocytes were reduced in amplitude in comparison to those from multicellular trabeculae under the similar experimental conditions. One possible explanation was that it could be due to differences in sarcomere length between the two preparations. Isolated myocytes in control solution with $[\text{Ca}^{2+}]_o$ of 1 mM are at a short length of $\sim 1.8 \, \mu\text{m}$, whereas experiments were usually carried out on multicellular trabeculae at a sarcomere length of $\sim 2.1 - 2.2 \, \mu\text{m}$ where force was maximal ($L_{\text{max}}$). Therefore, we shortened trabeculae to 1.8 $\mu\text{m}$ and recorded the $\text{Ca}^{2+}$ transients to compare them with transients from isolated myocytes.

3.4.1. Measurement of $[\text{Ca}^{2+}]_i$ transients in single cardiomyocytes and trabeculae

The $[\text{Ca}^{2+}]_i$ from the single myocytes was recorded ($\sim 1.8 \, \mu\text{m}$) after electrically stimulating at 0.2 Hz, $T_{\text{room}}$, and in 1mM $[\text{Ca}^{2+}]_o$ Tyrode’s buffer. Figure 3.1 shows the mean ± SEM 340/380 ratio of $\text{Ca}^{2+}$ transients recorded from single cells ($n = 11$, from $n = 4$ rats) and trabeculae ($n = 6$) obtained using the same ratiometric indicator and spectrofluorometric equipment. It was found that the $\text{Ca}^{2+}$ transients from isolated myocytes remained smaller in amplitude than those from multicellular trabeculae, even though both were at a sarcomere length of 1.8 $\mu\text{m}$ (Figure 3.1). The asterisks in the figure depict statistical difference for resting (lower) and peak (upper) $\text{Ca}^{2+}$ between isolated myocytes and trabeculae at short length (1.8 $\mu\text{m}$).
Figure 3.1. A comparison of Ca$^{2+}$ transients between isolated cardiomyocytes (open bars, n = 11, 4 hearts) and multicellular trabeculae (solid bars, n = 6, 6 hearts). Each bar shows resting (lower) and peak (upper) Ca$^{2+}$ (as fura-2 340/380 ratio). *$P \leq 0.05$, two-tailed unpaired t-test for the means.

3.4.2. Measurement of stress and Ca$^{2+}$ transients in pre- and post- enzyme treatment in trabeculae

The aim was to investigate the myocyte Ca$^{2+}$ changes during the ECM dismantling by mimicking the cell isolation procedure in multicellular trabeculae whilst monitoring intracellular Ca$^{2+}$. Exemplar recording of [Ca$^{2+}$]$_i$ and force from a trabecula before, during and after enzymatic digestion are illustrated in Figure 3.2 A & B. Panel A (i) shows Ca$^{2+}$ transients and force before enzyme treatment, and (ii) shows that the changes in the transients and force after initial exposure to enzymes. With increased time in enzyme solution the myocytes showed signs of Ca$^{2+}$ overload, indicated by the "undershoot" immediately after the Ca$^{2+}$ transient (iii). Continued enzyme exposure caused a sudden increase in resting Ca$^{2+}$ and a drop in the overall amplitude of Ca$^{2+}$ transients (iv). At this point the enzyme solution was replaced with enzyme-free control solution, and [Ca$^{2+}$]$_o$ gradually increased from 0.5 to 1 mM (v). However, the trabeculae did not fully recover from the enzyme treatment, with signs of Ca$^{2+}$ overload remaining even after ~10 min in control solution (vi). These data suggest that either the enzyme treatment increased the RyR opening probability during diastole, resulting in increased cytosolic Ca$^{2+}$, or that the cell membrane has become leaky with enzyme treatment.
Figure 3.2. Enzyme exposure protocols for a representative RV trabecula. A, records of 340/380 ratio (top) and force (bottom), with the solution changes for [Ca\textsuperscript{2+}]\textsubscript{o} and enzymes are schematically shown underneath. B, individual Ca\textsuperscript{2+} transients (black) and force twitches (green) from the continuous record shown in A above, as indicated by the arrows and labels. Note: Label (iv) shows the crucial point when Ca\textsuperscript{2+} transient amplitude was reduced in response to enzyme exposure.
Averaged data from 7 trabeculae experiments before and after enzyme treatment showed that developed stress was decreased from $39.35 \pm 5.28 \text{ mN mm}^{-2}$ to $12.62 \pm 2.21 \text{ mN mm}^{-2}$, and resting (or diastolic) stress was increased from $1.92 \pm 0.79 \text{ mN mm}^{-2}$ to $2.10 \pm 0.51 \text{ mN mm}^{-2}$. Likewise, the $\text{Ca}^{2+}$ transients increased from $1.60 \pm 0.07$ to $1.85 \pm 0.11$ ($n = 7$, $P \leq 0.05$) after partial enzymatic digestion. Figure 3.3 shows mean ± SEM results for stress (i) and (ii), $[\text{Ca}^{2+}]_i$ (iii) and the time constant of decay (iv) from $n = 7$ trabeculae. These experiments show no alteration in the $\text{Ca}^{2+}$ transients on enzyme treatment that mimicked the standard cell isolation protocol. Table 3.2 shows the list of other parameters analysed before and after enzyme exposure in trabeculae.

![Figure 3.3](image)

**Figure 3.3.** Mean ± SEM data from trabeculae ($n = 7$) showing (i) developed stress (mN mm$^{-2}$), (ii) resting stress (mN mm$^{-2}$), (iii) 340/380 ratio of $\text{Ca}^{2+}$ transients, and (iv) the time constant $[\text{Ca}^{2+}]_i$ of decay (s) before (pre-enzyme) and after (post-enzyme) digestion (0.2 Hz; 1mM $[\text{Ca}^{2+}]_o$; at $T_{room}$). $* P \leq 0.05$, Two-tailed paired t-test for the means.
There was also a change in the maximum-rate-of-rise of [Ca\(^{2+}\)]\(_i\), transient fluorescence in post-enzyme trabeculae. Figure 3.4 shows a significant decrease in the maximum-rate-of-rise of fluorescence from 0.20 ± 0.02 to 0.12 ± 0.03 (ms\(^{-1}\)) (n = 4, P ≤ 0.05) after partial enzymatic digestion of trabeculae. No significant change was observed in the time-to-peak fluorescence between the groups (see table 3.1 for details).

**Figure 3.4.** Mean ± SEM data from trabeculae (n = 4) showing maximum-rate-of-rise of fluorescence (ms\(^{-1}\)) of [Ca\(^{2+}\)]\(_i\) transient before and after enzyme digestion (0.2 Hz; 1 mM [Ca\(^{2+}\)]\(_o\); at T\(_{room}\)). *P ≤ 0.05, Two-tailed paired t-test for the means.
To further investigate the enzyme related changes in intracellular \([\text{Ca}^{2+}]_i\), the SR \(\text{Ca}^{2+}\) load before and after enzyme exposure was probed by application of caffeine (20mM) in the absence of external \([\text{Ca}^{2+}]_o\) in two trabeculae (a preliminary data). Caffeine causes a transient rise in \([\text{Ca}^{2+}]_i\) within the preparation as \(\text{Ca}^{2+}\) is released from the SR, and then slowly decays as \(\text{Ca}^{2+}\) is extruded from the myocytes, primarily by the SL NCX. The size, and time course, of the caffeine induced \(\text{Ca}^{2+}\) transients are representative of the amount of \(\text{Ca}^{2+}\) accumulated in the SR prior to addition of caffeine (Smith et al., 1988). Its addition to cardiac muscle leads to a site-specific interaction on the Ryr2, which in turn increases the open probability of channel (Rousseau & Meissner, 1989). Figure 3.5 shows an example of \(\text{Ca}^{2+}\) transients (ratio 340/380) during caffeine application for a representative trabecula (a preliminary data only). An important observation of the caffeine-evoked \(\text{Ca}^{2+}\) transients was that the decay of the relaxation phase appeared prolonged for the post-enzyme condition (table 3.1 for details).
Figure 3.5. Estimation of SR Ca\textsuperscript{2+} load in a representative trabecula, before and after enzyme exposure. (A), representative caffeine-evoked [Ca\textsuperscript{2+}]\textsubscript{i} transients (340/380 ratio) recorded in 1 mM [Ca\textsuperscript{2+}]\textsubscript{o}, and in the absence of stimulation before enzyme exposure. (B) caffeine-evoked [Ca\textsuperscript{2+}]\textsubscript{i} transients (340/380 ratio) recorded in 1 mM [Ca\textsuperscript{2+}]\textsubscript{o}, and in the absence of stimulation in the same trabecula as (A), post-enzyme exposure. The amplitude of the caffeine transient provides an estimation of SR Ca\textsuperscript{2+} load.
<table>
<thead>
<tr>
<th>Condition (trabecula)</th>
<th>Tau Ca(^{2+}) decay (s)</th>
<th>Area* under caffeine transient</th>
<th>Amplitude of caffeine transient (AU)</th>
<th>Resting 340/380 ratio before caffeine-transient (AU)</th>
<th>Resting 340/380 ratio after caffeine-transient (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-enzyme (trabecula 1)</td>
<td>9.17</td>
<td>4.95</td>
<td>0.35</td>
<td>2.0</td>
<td>1.98</td>
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<tr>
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<td>0.2</td>
<td>1.8</td>
<td>1.8</td>
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<tr>
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<td>11.02</td>
<td>0.3</td>
<td>2.1</td>
<td>2.35</td>
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<tr>
<td>Post-enzyme (trabecula 2)</td>
<td>18.25</td>
<td>4.8</td>
<td>0.15</td>
<td>1.9</td>
<td>2.05</td>
</tr>
</tbody>
</table>

**Table 3.1:** The time constant of decay in fluorescence (Tau) of caffeine-induced Ca\(^{2+}\) transient, amplitude of caffeine-induced Ca\(^{2+}\) transient, and resting Ca\(^{2+}\) before and after enzyme treatment in (n = 2) trabeculae (preliminary data).

*Area of the caffeine transient is calculated with the formula:

\[ A = \frac{h \times b}{2} \]

where ‘h’ is the height and ‘b’ is the base of triangle (time measured in seconds from the onset of the caffeine-transient till the decay reaches base line).
Preliminary results from the experiments described in table 3.1 showed the time constant of the decay of the caffeine-induced Ca\(^{2+}\) transients was longer, suggesting that SL Ca\(^{2+}\) extrusion was slower after the enzyme treatment. An exponential fitted to the decay phase of the caffeine transient (from 90% of the peak [Ca\(^{2+}\)]\(_i\)) was used as a measure of SL Ca\(^{2+}\) transport in the absence of SR Ca\(^{2+}\) storage. This shows slower NCX activity for Ca\(^{2+}\) extrusion in the enzyme treated trabeculae (n = 2). The SR Ca\(^{2+}\) content was also calculated by measuring the area under each caffeine transient. However, the preliminary data showed the increased SR Ca\(^{2+}\) content under post-enzyme conditions. A possible reason for the slower NCX Ca\(^{2+}\) extrusion from the cell could be due to increased Ca\(^{2+}\) in the SR following enzyme treatment. This needs to be examined further. The low “n” number is due to the difficulties in making these measurements in trabeculae post-enzyme treatment. We chose only trabeculae that recovered from the caffeine treatment and responded again to stimulation.
<table>
<thead>
<tr>
<th>Trabeculae (N)</th>
<th>Condition</th>
<th>Time-to peak fluor* (s)</th>
<th>Tau ± SE of Boltz fit decay (s)</th>
<th>Diastolic fluor (AU)</th>
<th>Systolic fluor (AU)</th>
<th>Max-rate-of rise-of fluor (ms⁻¹)</th>
<th>Peak developed force (mN)</th>
<th>Diastolic force (mN)</th>
<th>Time of peak force (s)</th>
<th>Time to half relaxation (s)</th>
<th>Time to 90% relaxation (s)</th>
<th>Max-rate-of rise-of force (mN ms⁻¹)</th>
<th>Time delay, fit F &amp; T</th>
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<tbody>
<tr>
<td>1</td>
<td>Pre-enz*</td>
<td>0.10</td>
<td>0.42</td>
<td>1.37</td>
<td>3.17</td>
<td>0.25</td>
<td>0.39</td>
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<td>0.21</td>
<td>0.13</td>
<td>0.25</td>
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<td>0.05</td>
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<td>3.39</td>
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<td>0.19</td>
<td>0.34</td>
<td>0.03</td>
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<td>0.06</td>
<td>0.12</td>
<td>0.18</td>
<td>0.02</td>
<td>0.51</td>
<td>0.05</td>
<td>0.02</td>
<td>0.03</td>
<td>0.04</td>
<td>0.01</td>
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<td>1.35</td>
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<td>0.20</td>
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<td>0.01</td>
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<td>0.14</td>
<td>0.15</td>
<td>0.03</td>
<td>0.19</td>
<td>0.21</td>
<td>0.39</td>
<td>0.00</td>
<td>0.09</td>
</tr>
<tr>
<td>3</td>
<td>Post-enz.</td>
<td>0.12</td>
<td>0.14</td>
<td>2.19</td>
<td>3.12</td>
<td>0.06</td>
<td>0.39</td>
<td>0.19</td>
<td>0.19</td>
<td>0.20</td>
<td>0.37</td>
<td>0.01</td>
<td>0.08</td>
</tr>
<tr>
<td>4</td>
<td>Post-enz.</td>
<td>0.08</td>
<td>0.12</td>
<td>2.07</td>
<td>2.94</td>
<td>0.07</td>
<td>0.78</td>
<td>0.05</td>
<td>0.23</td>
<td>0.13</td>
<td>0.24</td>
<td>0.01</td>
<td>0.13</td>
</tr>
<tr>
<td>Mean</td>
<td>Post-enz.</td>
<td>0.09</td>
<td>0.20</td>
<td>1.84</td>
<td>3.16</td>
<td>0.12</td>
<td>0.38</td>
<td>0.08</td>
<td>0.20</td>
<td>0.17</td>
<td>0.31</td>
<td>0.01</td>
<td>0.11</td>
</tr>
<tr>
<td>SEM</td>
<td>Post-enz.</td>
<td>0.01</td>
<td>0.05</td>
<td>0.19</td>
<td>0.09</td>
<td>0.03</td>
<td>0.14</td>
<td>0.04</td>
<td>0.01</td>
<td>0.02</td>
<td>0.04</td>
<td>0.00</td>
<td>0.01</td>
</tr>
</tbody>
</table>

**Table 3.2:** List of parameters obtained for Ca²⁺ transients with IDL program before and after enzyme exposure in (N = 4) trabeculae.

*Fluor denotes 340/380 fluorescence and enz. denotes enzyme.
3.4.3. **Optical Coherence Tomography scanning and analysis**

Ventricular trabeculae differ considerably between hearts in their dimensions, with bigger trabeculae producing comparatively more force. For this reason when comparing the results between experiments, we normalise force to cross sectional area (stress, as mN mm$^{-2}$). However, in our experimental set up, at best we can only record the wider dimension of any single trabecula, which potentially might result in errors in measurements of stress. We therefore utilised a newly developed technique “Optical Coherence Tomography” by researchers at the Auckland Bioengineering Institute to gain insight into the actual shape of one of our trabeculae.

The side view and cross-sectional images of a representative trabecula were captured before and after the enzyme treatment by using Optical Coherence Tomography (Figure 3.6). The cross-sectional area of the trabecula shown was 109,400 µm$^2$, and was approximately 300 µm by 470 µm in minor and major transverse axis, respectively. It was revealed that enzyme treatment decreased the density by ~34% in two trabeculae examined using Optical Coherence Tomography. This qualitative analysis showed that the trabeculae tissue became more disorganised after enzyme treatment with a drop in overall intensity after digestion (Figure 3.6, side view). An increase in cross sectional area was also observed on enzyme treatment that accompanied the reduction in tissue density (Figure 3.6, cross-section). Unfortunately, it could not be determined from these trabeculae whether the change in density was due to sarcomere length shortening, or to ECM disruption.
Figure 3.6. A representative trabecula showing Optical Coherence Tomography scans before and after enzyme treatment in side and cross-section views. Note: red dotted line depicts the position from where the cross-sectional scan of trabecula is taken. Scale bar is given for each scan.
3.5. The Loss of ECM proteins during digestion of trabeculae

Optical Coherence Tomography study confirmed that enzyme treatment made large structural changes to the partially digested trabeculae in comparison to control. Therefore, my next step was to investigate which proteins were lost from partially digested trabeculae by using immunohistochemistry.

3.5.1 Reduced Collagen-I labelling

Because ECM is composed of mainly structural proteins such as collagens (collagen type I) and elastins, we examined the extracellular collagen labelling and the organization of sarcomeric thin filaments (F-actin) in control and partially enzyme treated trabeculae (Weber, 1989). The 2% fixed sections from control and partially digested trabeculae (n = 3) were immunolabelled with Collagen I antibody. Collagen I labelling showed abundance of collagen in between the myocytes compared to partially digested sections which showed weak and occasional labelling. Quantitative analysis of images revealed no significant difference between Collagen I labelling in enzyme treated and control trabeculae sections. Analysis of images from Collagen I and F-actin (labelled with phalloidin) dual-immunolabelled trabeculae sections are summarized in Table 3.2. Confocal images in figure 3.7 show the collagen-I labelling (green) and F-actin (red) in control and digested trabeculae sections.
Figure 3.7. The confocal images showing Collagen I (green) and F-actin (red) in control and partially digested trabeculae sections (Confocal at 63x/1.5NA). Long arrows point collagen I labelling in between myocytes in control trabecula section and short arrows indicate reduced collagen I labelling in digested trabecula section.
<table>
<thead>
<tr>
<th>Fractional area (%)</th>
<th>Control (n = 3)</th>
<th>Enzyme treated (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen I</td>
<td>14.3 ± 2.07</td>
<td>12.9 ± 1.17</td>
</tr>
<tr>
<td>F-actin</td>
<td>61.2 ± 5.76</td>
<td>51.4 ± 1.13</td>
</tr>
</tbody>
</table>

**Table 3.3:** Relative fractional area of collagen I and F-actin in control and enzyme treated trabeculae sections. Data are mean ± SEM. Number of animals is shown in parentheses.
3.5.2  Endothelial cell Marker (RECA-1) and Laminin

The 2% PFA fixed sections from control and partially digested trabeculae (n = 3) were immunolabelled for endothelial cell marker (RECA-1) (mouse monoclonal to RECA-1 antibody) and laminin proteins. Figure 3.8 shows images of RECA-1 and laminin labelling in control and enzyme treated trabeculae sections. The enzyme treated trabeculae showed decreased RECA-1 labelling (Control trabeculae: 6.5 ± 1.29%; enzyme treated trabeculae: 11.7 ± 1.28%, P ≤ 0.05). RECA-1 labelling was reduced at the edges of enzyme treated trabeculae in comparison to control, as shown in longitudinal and transverse trabeculae sections in figure 3.8. Quantitative analysis of these images revealed that laminin was not affected by enzyme treatment, and was present along the sarcolemma and T-tubules of myocytes in a punctate pattern. Laminin was absent at intercalated disks. Analysis of images from RECA-1 and laminin immunolabelled trabeculae sections are summarized in Table 3.3.
Figure 3.8. RECA-1, laminin and actin labelling in fixed trabeculae

Upper panels show confocal images of 2% PFA fixed control trabeculae sections (longitudinal section (LS) & transverse section (TS)) with triple immunofluorescence staining for RECA-1 (green), laminin (red), actin (blue), and the merged image (long arrows show clear RECA-1 staining, rectangle shows the part of TS). Lower panels show digested trabeculae sections (LS and TS) (short arrows show absence of RECA-1 staining).
As expected, RECA-1 labelling for the presence of endothelial cells was absent in enzymatically isolated single myocytes ($n = 3$ hearts, with 20-100 cells per heart) (Figure 3.9).

**Figure 3.9.** RECA-1, laminin and actin labelling of rat ventricular myocytes. Representative confocal images from 2% PFA fixed ventricular myocyte triple-immunolabelled for RECA-1 (green), laminin (red), actin (blue), and the merged image. Note: RECA-1 was completely lost in isolated myocytes without affecting laminin.
<table>
<thead>
<tr>
<th>Fractional area (%)</th>
<th>Control (n = 3)</th>
<th>Enzyme treated (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RECA-1</td>
<td>11.7 ± 1.28</td>
<td>6.5 ± 1.29*</td>
</tr>
<tr>
<td>Laminin</td>
<td>16.7 ± 0.59</td>
<td>17.1 ± 0.60</td>
</tr>
</tbody>
</table>

**Table 3.4:** Relative fractional area of RECA-1 and laminin in control and enzyme treated trabeculae sections. Data are mean ± SEM. Number of animals is shown in parentheses. *P ≤ 0.05, Student’s unpaired t-test.
3.5.3. **Connexin-43 and Laminin**

Fixed sections from control and partially enzyme digested trabeculae (n = 3) were also immunolabelled for connexin-43 (Cxn-43) and laminin proteins. Figure 3.10 shows images from control and enzyme treated trabeculae sections. No significant difference was seen in either Cxn-43 or laminin labelling (Control trabeculae: 2.4 ± 0.10%; enzyme treated trabeculae: 1.5 ± 0.29%). Analyses of images from Cxn-43 and laminin dual-immunolabelled trabeculae sections are summarized in Table 3.4.

![Representative data showing confocal images of section from 2% PFA fixed trabeculae (control and digested) immunolabelled for Connexin-43 (green) and laminin (red).](image)

**Figure 3.10.** Representative data showing confocal images of section from 2% PFA fixed trabeculae (control and digested) immunolabelled for Connexin-43 (green) and laminin (red).
<table>
<thead>
<tr>
<th>Fractional area (%)</th>
<th>Control (n = 3)</th>
<th>Enzyme treated (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cxn-43</td>
<td>2.4 ± 0.10</td>
<td>1.5 ± 0.29</td>
</tr>
<tr>
<td>Laminin</td>
<td>16.5 ± 0.41</td>
<td>16.5 ± 0.48</td>
</tr>
</tbody>
</table>

**Table 3.5:** Relative fractional area of Cxn-43 and laminin in control and enzyme treated trabeculae sections. Data are mean ± SEM. Number of animals is shown in parentheses.
Vinculin showed strong labelling at the intercalated disks, and at T-tubules, in both trabeculae and single myocytes, as shown in figure 3.11. The single myocyte vinculin labelling revealed a sarcolemmal distribution in the form of a costameric pattern of regularly repeating rib like structures, perpendicular to the long axis of the cell. No difference was observed in vinculin labelling between control trabeculae, enzyme treated trabeculae, and single myocytes.

**Figure 3.11.** Vinculin, laminin and actin labelling in rat ventricular myocytes. Representative confocal images from 2% PFA fixed ventricular myocyte triple-immunolabelled for vinculin (green), laminin (red), actin (blue), and the merged image. Note: Clear sarcolemmal surface labelling in the form of punctate pattern for both vinculin and laminin, T-tubules also labelled for both proteins; intercalated disks are strongly labelled for vinculin alone.
Figure 3.12. Vinculin and F-actin labelling in cardiac trabeculae. 
Representative confocal images from control and digested trabeculae sections immunolabelled for vinculin (green) and F-actin (red).
3.6. Contribution of Endothelial cells to myocyte intracellular Ca\(^{2+}\) signalling

The next question to address was whether the decrease in the Ca\(^{2+}\) transients was due to the loss of endothelial cells during the ECM dismantling process in intact trabeculae. I therefore investigated the direct signalling between endothelial cells and cardiomyocytes by examining the effect of either bradykinin, N–Nitro–L–arginine methyl ester hydrochloride (L-NAME), or ET-1 and their appropriate blockers.

3.6.1. Effect of bradykinin and L-NAME in intact trabeculae

In intact trabeculae, force and [Ca\(^{2+}\)]\(_i\) was recorded before and the application of 1 µM bradykinin (Sigma Aldrich, USA). However, the results obtained were inconsistent, in some experiments bradykinin increased force and [Ca\(^{2+}\)]\(_i\), but in others force and [Ca\(^{2+}\)]\(_i\) were decreased. A summary of these data is shown in Table 3.5.

<table>
<thead>
<tr>
<th>Trabecula (n)</th>
<th>Amplitude of [Ca(^{2+})](_i) (% change)</th>
<th>Stress (% change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.02</td>
<td>-50.57</td>
</tr>
<tr>
<td>2</td>
<td>0.35</td>
<td>2.00</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>3.00</td>
</tr>
<tr>
<td>4</td>
<td>0.1</td>
<td>4.00</td>
</tr>
<tr>
<td>Mean</td>
<td>0.12</td>
<td>-10.39</td>
</tr>
<tr>
<td>SEM</td>
<td>0.08</td>
<td>13.40</td>
</tr>
</tbody>
</table>

**Table 3.6:** Percentage change of amplitude of [Ca\(^{2+}\)]\(_i\) transients and stress for n = 4 trabeculae after bradykinin application.

The changes with bradykinin are given for each experiment with the mean ± SEM for all experiments included.
The effect of 1 mM L-NAME on force and \([\text{Ca}^{2+}]_i\) transients was also recorded in intact trabeculae within ~20 min of application. But, again, the trabeculae did not respond consistently. A summary of these data is shown in Table 3.6:

<table>
<thead>
<tr>
<th>Trabecula (n)</th>
<th>Amplitude of ([\text{Ca}^{2+}]_i) (%)</th>
<th>Stress (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-8.8</td>
<td>0.05</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>0.17</td>
</tr>
<tr>
<td>3</td>
<td>8.8</td>
<td>0.24</td>
</tr>
<tr>
<td>Mean</td>
<td>2</td>
<td>0.15</td>
</tr>
<tr>
<td>SEM</td>
<td>5.46</td>
<td>0.06</td>
</tr>
</tbody>
</table>

**Table 3.7:** Percentage change of amplitude of \([\text{Ca}^{2+}]_i\) transients and stress for \(n = 3\) trabeculae after L-NAME application.

The changes with L-NAME are given for each experiment separately, and with the mean ± SEM for \(n = 3\) trabeculae included.

Hence, I excluded the bradykinin and L-NAME experiments from this study. I therefore looked only at the effects of ET-1 in intact trabeculae and isolated cells with and without the ET\(_A\)-receptor blocker BQ123. It is known that endothelial cells are responsible for the release of endothelin and is further taken up by myocytes via ET\(_A\) receptors. Therefore, with the ET-1 application, the affects on force and intracellular \(\text{Ca}^{2+}\) were also observed in partially digested trabeculae.
3.6.2. Effect of ET-1 in intact trabeculae

The effect of ET-1 on stress, Ca\(^{2+}\) transients, and time constant of decay was recorded in intact trabeculae. Within 5 minutes of the ET-1 application, there was a significant increase in Ca\(^{2+}\) transients from 1.60 ± 0.18 to 3.16 ± 0.23 (AU) without affecting force in intact trabeculae (n = 6, p ≤ 0.05). The resting calcium was also significantly increased from 1.54 ± 0.08 to 1.66 ± 0.07 (AU), p ≤ 0.05 in early ET-1 stage (Figure 3.12 (iv)). The initial increase in Ca\(^{2+}\) transients was followed by a return back to the pre-endothelin Ca\(^{2+}\) transients, with an increase in stress from 35.97 ± 3.97 to 44.35 ± 5.4 mN mm\(^{-2}\) (p ≤ 0.05). There was no change observed in time constant of decay as shown in Figure 3.12 (iii).

Figure 3.13. The effect of ET-1 in intact trabeculae (n = 6) on (i) stress, (ii) Ca\(^{2+}\) transients, (iii) time constant of decay, and (iv) resting calcium at early (+ 5 mins) and later (+ 15 mins) stages of application. *p ≤ 0.05, Two-tailed paired t-test for the means.
To investigate if the effect of ET-1 is via ET$_A$-receptor in intact trabeculae, the trabeculae were incubated with 1 µM ET$_A$-receptor blocker BQ123 before adding ET-1. It was observed that the early increase in Ca$^{2+}$ transients and later increase in stress in prolonged application was completely abolished by ET$_A$-receptor blocker as shown in figure 3.13. There was no change in amplitude of Ca$^{2+}$ transients from 1.31 ± 0.14 to 1.28 ± 0.14 ($p \geq 0.05$) with and without ET-1 in presence of BQ123 as shown in figure 3.11.

Figure 3.14. The effects of ET-1 in presence of ET$_A$-receptor blocker BQ123 intact trabeculae (n = 3) on (i) stress, (ii) Ca$^{2+}$ transients, and (iii) time constant of decay at early and late stages. *$p \leq 0.05$, Two-tailed paired t-test for the means.
3.6.4. Effect of ET-1 after ECM dismantling in digested trabeculae

To further address that endothelial cells were lost during the enzyme treatment process, the ET-1 was added after the ECM digestion while the trabeculae were in post-enzyme solutions. Interestingly, the results obtained showed that there was no change in stress, Ca$^{2+}$ transients, and time constant of decay of Ca$^{2+}$ transients after the addition of ET-1 as shown in figure 3.14.

![Graphs showing the effects of ET-1 on (i) stress, (ii) Ca$^{2+}$ transients, and (iii) time constant of decay at early and late stages.](image)

**Figure 3.15.** The effects of ET-1 after ECM dismantling in post-enzyme condition in partially digested trabeculae (n = 3) on (i) stress, (ii) Ca$^{2+}$ transients, and (iii) time constant of decay at early and late stages.
3.6.5. **ET-1 direct application elevated Ca\(^{2+}\) transients in isolated cells**

To measure the Ca\(^{2+}\) transient response of myocytes to ET-1, the Fura-2/AM loaded cells (as described in Methods section) were incubated with 0.1 \(\mu\)M ET-1. It was found that ET-1 caused an increase in Ca\(^{2+}\) transients (340/380 ratio) at \(T_{room}\). Figure 3.15 shows that treatment of isolated cells with ET-1 caused a significant increase in \([\text{Ca}^{2+}]_i\) levels. This elevated effect of ET-1 was abolished in cells incubated with ET\(_A\)-receptor blocker BQ123 for 30 min.

![Graph showing Ca\(^{2+}\) transients in isolated cardiomyocytes](image)

**Figure 3.16.** Effect of ET-1 on Ca\(^{2+}\) transients in isolated cardiomyocytes. The cells treated with 0.1 \(\mu\)M ET-1 showed elevated \([\text{Ca}^{2+}]_i\) (Mean \(\pm\) SEM) which was abolished by 1 \(\mu\)M of ET\(_A\) receptor blocker BQ123 (\(n = 7\) cells from 1 heart). \(*p \leq 0.05\), Two-tailed paired t-test for the means.
3.7. Contribution of Autocrine and Paracrine Factors to myocytes intracellular Ca\textsuperscript{2+} signalling

Autocrine/paracrine release of factors such as NO, ET-1, bradykinin and prostaglandins (PGF\textsubscript{2\alpha}) from endothelial cells can affect intracellular signalling cascades (D’Souza \textit{et al.}, 2004; Iribe \textit{et al.}, 2009). The divergent effects of these factors on intracellular signalling cascades effect ECM turnover. In this study, we have examined the effects of PGF\textsubscript{2\alpha} on the cardiac trabeculae at stretched length (L\textsubscript{max}). It is reported to increase cardiac contractility (Karmazyn \textit{et al.}, 1981), coronary vessels constriction (Balwierczak, 1991) and release atrial natriuretic factors (Gardner & Schultz, 1990). Prostaglandin F\textsubscript{2\alpha} is known to have a positive ionotropic effect in isolated ventricular myocytes (Yew \textit{et al.}, 1998). The role of PGF\textsubscript{2\alpha} in intracellular Ca\textsuperscript{2+} signalling in multicellular preparations such as the cardiac trabeculae needs to explore further.

I, therefore examined the effects of PGF\textsubscript{2\alpha} on contractile stress and intracellular Ca\textsuperscript{2+} in electrically stimulated (0.2 Hz, 1 mM [Ca\textsuperscript{2+}]\textsubscript{o}) cardiac trabeculae at T\textsubscript{room}.
3.7.1. Inotropic increase in force by PGF$_{2\alpha}$ in isolated trabeculae

It was observed that the direct application of PGF$_{2\alpha}$ to isolated trabeculae at Lmax at room temperature produces increase in force and Ca$^{2+}$ transients. A representative trabecula showing increase in stress after PGF$_{2\alpha}$ application as shown in figure 3.16 (A). Also, panel B shows mean ± SE of stress and Ca$^{2+}$ transients from n = 5 (p ≤ 0.05) trabeculae. The data revealed that there is significant increase in stress from 28.63 ± 1.61 to 38.06 ± 3.98 mN mm$^{-2}$ with PGF$_{2\alpha}$ application but the increase in Ca$^{2+}$ transients is not significant. There was no change in time constant of decay with PGF$_{2\alpha}$ application was observed.
Figure 3.17. Effects of PGF$_{2\alpha}$ on contractile force in rat trabecula. Panel A, representative force recording showing ionotropic response following the application of 1 µM PGF$_{2\alpha}$. Panel B, shows the mean ± SE increase in (i) stress and (ii) Ca$^{2+}$ transient amplitude from $n = 5$ (*$p \leq 0.05$, Two-tailed paired t-test for the means) trabeculae in response to PGF$_{2\alpha}$.
3.7. Chapter Summary and Discussion

Our data show that difference in sarcomere lengths in isolated cells and trabeculae did not explain the smaller amplitude of Ca\(^{2+}\) transients in isolated cells compared to intact trabeculae. The Ca\(^{2+}\) transients recorded from trabeculae at short sarcomere length of 1.8 µm still remain bigger in amplitude (Figure 3.1). The most obvious difference between isolated myocytes and intact trabeculae preparations is the removal of the ECM during the cell isolation process. The data obtained from intact and partially digested trabeculae showed that resting and peak Ca\(^{2+}\) was altered by enzyme treatment (Figure 3.3). There was significant increase in resting Ca\(^{2+}\) and stress after digestion. However, the preliminary data showed the increased SR Ca\(^{2+}\) content under post-enzyme conditions. Since, the relaxation phase of caffeine-induced Ca\(^{2+}\) transient appeared prolonged for the post-enzyme condition (Figure 3.5); one might expect the slow Ca\(^{2+}\) extrusion via the NCX here. This is due to the longer time taken for Ca\(^{2+}\) extrusion by the activity of NCX. Other Ca\(^{2+}\) transporters, such as the sarcolemmal Ca\(^{2+}\)-ATPase and the mitochondrial uniporter, might also be inactivated during the enzyme exposure. However, these transporters only extrude very small amounts of Ca\(^{2+}\) from the cytosol under normal circumstances (Bassani et al., 1994; Eisner et al., 1998). Therefore, combined together these observations directed this study towards the involvement of ECM in myocyte Ca\(^{2+}\) signalling.

In addition, the structural changes in trabeculae before and after enzyme treatment were also recorded in terms of optical coherence tomography scans. The optical scans revealed increased cross-sectional area and reduced tissue density in enzyme treated trabeculae and confirmed some structural loss after enzyme treatment (Figure 3.6). Our, results were the first to provide any structural changes in trabeculae subjected to enzyme treatment by using this technique. Though, quantitative analysis of collagen I labelling does not show any significant difference between control and enzyme treated trabeculae sections. But we have found with our visual observations in the intermyocyte regions that collagen I was reduced at some places in enzyme treated sections. One of the possible explanations is that enzyme treatment had removed some of the links between collagen and myocytes without affecting the total collagen in the tissue. In addition, immuno-histological examination of fixed enzyme treated trabeculae revealed the decreased number of endothelial cells, and their absence in enzymatically isolated single myocytes (Figure 3.8 & 3.9). However, any contractile dysfunction arising from reduced RECA-1 labelling would be compounded by
the reduction of force and Ca\textsuperscript{2+} transient amplitude. It is known that endothelium-derived signals modulate the contractile activity of smooth muscle (Hirata et al., 1989). Hence, the affect of ET-1 on active force and Ca\textsuperscript{2+} transient amplitude was measured later in study. Laminin, an abundant ECM protein that connects to the myocytes by membrane spanning β-integrins was also investigated. The results report no change in laminin between control and enzyme treated trabeculae. The protein was not lost from partially digested trabeculae and from single cells during the enzymatic isolation process (Figure 3.8 & 3.9). Data from isolated atrial myocytes shows that laminin, an abundant ECM protein that connects to the myocytes by β-integrins, down-regulates β-adrenergic signalling by inhibiting adenylate cyclase activity (Wang et al., 2009b). For the ECM-myocyte linkage, see section 1.4.1., Figure 1.5. The data from immuno-histochemical analysis showed no change in gap junctional protein, Cxn-43 after enzyme treatment. This is in accordance with the data from one group that Cxn-43 associated gap junctions has role in regulation of resting Ca\textsuperscript{2+} signalling in normal rat ventricular myocytes (Li et al., 2012). Our study showed the costameric distribution of vinculin in both control and enzyme treated trabeculae as evidenced in single cardiomyocytes (Figures 3.11 and 3.12). The contractile deficit might therefore arise, in part, from an overall reduction in the endothelial cells available for endothelin production. Additionally, removal of the links between ECM and myocytes might also explain the reduced stress and time constant of decay in enzyme treated trabeculae.

Several lines of evidence support the hypothesis that ET-1 could alter Ca\textsuperscript{2+} transients. Our, results showed that ET-1 application in intact trabeculae caused the early Ca\textsuperscript{2+} transient elevation and followed by a return in Ca\textsuperscript{2+} transients and increase in stress. This is supported by studies from (Ko et al., 2005) showed that in freshly isolated rabbit pulmonary artery smooth muscle cells, ET-1 induced a transient increase in Ca\textsuperscript{2+} transients followed by a return to the initial Ca\textsuperscript{2+} transients. Later, found that ET-1 induced increase in Ca\textsuperscript{2+} transients was attenuated by ET\textsubscript{A} receptor blocker, BQ123 in intact trabeculae and isolated myocytes (Figure 3.14 & 3.16). It was proposed in the hypothesis and from the results of immuno-histological analysis that endothelial cells were lost during the enzyme treatment. The results from the enzyme treated trabeculae experiments showed no change in force and Ca\textsuperscript{2+} transients with ET-1 application (Figure 3.15). One possible explanation here is because of the loss of endothelial cells there is no release of endothelin from ET-1, and was not available to myocytes either. The second explanation could be as the myocytes were already overloaded with Ca\textsuperscript{2+} right after the enzyme treatment (Figure 3.2) which was either
due to the increased activity of SERCA/PLB and prolonged opening of Ryr channels and reduced activity of NCX exchanger. Hence, post-enzyme treated trabecula did not respond to ET-1. (Zeng et al., 2009) reported that ET-1 regulates calcium overload through LTCC activation and CICR mechanism, AT1 receptors and via PKC and PKA pathway. The direct application of PGF$_2$α to isolated trabeculae at fixed length showed significant increase in active force with small increase in Ca$^{2+}$ transients. This demonstrated that the response to PGF$_2$α is reminiscent of the response to β-adrenergic stimulation in isolated cardiac trabeculae but without any change in Ca$^{2+}$ transient decay. This suggests the cellular transduction mechanism is via a different intracellular signalling pathway.

All these results onfirmed that ECM components do play role in myocyte Ca$^{2+}$ modulation either directly or indirectly. Also, the auto- and paracrine factors show inotropic affect but Ca$^{2+}$ transients were only slightly affected, which might be due to the reason that these experiments being carried out at T$_{room}$. 
Chapter 4. Evidence of Primary cilia in ventricular tissue

4.1. Chapter Overview

The primary cilium is a solitary, non-motile extension of centriole-based basal body and bears a microtubule-based cytoskeleton called axoneme. The axoneme of primary cilia typically has a ring of nine outer microtubule triplets (called 9 + 0 axoneme). The primary cilium is only ~200-300 nm in diameter and 1 to 3 µm long (Davis et al., 2006). Microtubules are the major constituent of the cells cytoskeleton. They consist of two subunits, α- and β-tubulin. The function of the primary cilium has remained elusive until the studies by (Praetorius & Spring, 2001) showed that primary cilium of kidney epithelial cells acts as a ‘flow sensor’ and its bending activates intra-cellular calcium signalling. Primary cilia are important in the development of a number of organs, including heart (Tasouri & Tucker, 2011). They have sensory functions and are reported as being ubiquitously expressed in many cell types that are also found in cardiac tissue (Praetorius & Spring, 2005) & (Wheatley et al., 1996). They coordinate multiple signalling pathways, such as receptor tyrosine kinase (RTK), Hedgehog (Hh), Wnt, and TGF-β (Goetz & Anderson, 2010; Satir et al., 2010; Christensen et al., 2012; Lienkamp et al., 2012; Clement et al., 2013). They are the sites for co-ordination of TGF-β signalling reported in fibroblasts and stem cells during cardiomyogenesis.

Cardiac primary cilia were first discovered in 1969 when Rash and colleagues identified primary cilia in embryonic and adult hearts in chickens, rabbits, mice, and lizards (Rash et al., 1969). Later, they were also observed in the embryonic and adult human heart (Myklebust et al., 1977) as well as other parts of the cardiovascular system, including endothelial cells of the aorta (Bystrevskaya et al., 1992). Evidence from previous studies has suggested primary cilia might provide a Ca\(^{2+}\) entry pathway between the ECM and cardiomyocyes (Myklebust et al., 1977; Clement et al., 2009; Willaredt et al., 2012). These mechano-sensitive organelles have a role in transducing external forces via intracellular Ca\(^{2+}\) signals (Praetorius & Leipziger, 2009).

The aim of this study was therefore to look for evidence of primary cilia in ventricular tissue and isolated myocytes from rat hearts using immuno-histochemical techniques. The primary cilia specific antibodies were selected to target the primary cilia in rat ventricle tissues.
4.2. Material and Methods

4.2.1. Primary antibodies and normal serum

Affinity-purified polyclonal anti-rabbit ARL13B (ADP-ribosylation factor-like 13B) is a specific marker of primary cilia. It is marker of ARL13B protein, a small ciliary G protein of the Ras superfamily localized in cilia and is required for cilium biogenesis and maintenance. The immunospecificity of this antibody for the ARL13B was confirmed by the suppliers in immunofluorescense studies. The immuno-histochemical specificity of anti-ARL13B antibody employed in this study has been demonstrated recently by others who have shown specific primary cilia immuno-labelling in developing cultured cardiomyocytes, sections of kidney, neurons from cerebral cortex (Clement et al., 2009; Higginbotham et al., 2012; Deane et al., 2013).

4.2.2. Immunolabelling of Primary cilia in rat cardiac tissue sections and isolated myocytes

Rat hearts from different developmental stages - neonatal, embryonic (P2), young (P21 & P28) and adult (~ 2 months) were used in this study. Hearts were excised carefully from anaesthetized rats after decapitation and were washed with PBS. Hearts were then fixed in 2% PFA for 30 min at T\textsubscript{room}, with adult hearts first cut into ~ 0.5 × 0.5 cm small pieces before fixation. The tissues were cryoprotected with sequential 10%, 20%, and 30% sucrose solutions and stored at -80 °C. PFA fixed tissue was OCT embedded and blocks from neonatal (n = 3 hearts), P2 (n = 2 hearts), P21 (n = 3 hearts), P28 (n = 3 hearts) and adult rat hearts were stored (-20 °C) until sectioning (10 µm). Sections were rehydrated with cold PBS (10 min at T\textsubscript{room}) followed by blocking with 10 % NGS in PBS (1h at T\textsubscript{room}). After washing with PBS (2 x 10 min at RT), sections were incubated (overnight at 4 °C) with either acetylated alpha-tubulin (1:500 diluted in 5% NGS with 0.1% Triton X-100-X 100 in PBS) or anti-ARL13B Ab (1:500 diluted in 5% NGS with 0.1% Triton X-100-X 100 in PBS. The following day, slides were washed with PBS (2 x 10 min at T\textsubscript{room}) and stained with secondary Ab (1:500) (Alexa Fluar 594 goat anti mouse) and (1:200) (Alexa Fluar 488 goat anti rabbit), respectively, 2h at RT. Slides were washed with PBS and counterstained with Hoechst (1:200, 10 min) before a final wash with PBS, and were mounted in Prolong Gold antifade reagent. Selected tissue sections were also triple-labelled to differentiate the
cell types in ventricle tissue with either mouse monoclonal to RECA-1 (endothelial cell marker) or Vimentin (fibroblast marker) along with Phalloidin conjugated with Alexa Fluor 694 and Hoechst. A similar protocol was used to label single myocytes isolated from adult rat hearts.

4.2.3. **Immunolabelling in adult single cardiomyocyte and cardiac tissue**

Initially, the primary cilia labelling in rat ventricular tissue was targeted by acetylated-α-tubulin antibody. But staining by this antibody did not show clear results as this antibody targets the tubulin which is the building block of microtubules. Microtubules are required for many well characterised functions in eukaryotic cells such as movement of chromosomes in mitosis and meiosis, intracellular transport, establishment and maintenance of cellular morphology, cell growth, cell migration, and morphogenesis (Vale, 2003; Howard & Hyman, 2007). The tubulin subunit is a heterodimer of alpha- and beta-tubulin, and both these monomers are found in eukaryotes. The acetylated alpha-tubulin has a role in stabilizing intracellular structure by binding to mitotic spindles, centrioles, and subsets of cytoplasmic microtubules, including the primary cilia (Vale, 2003).

A representative confocal image taken at the surface of an isolated myocyte labelled for acetylated-α-tubulin is shown in figure 4.1 (A), with the transmitted light shown in green. A very bright region of labelling near the nucleus was observed in all cells (20-50 cells, from n = 2 hearts). A fine network of positively stained filaments were also seen within all of the the myocytes, revealing this antibody also labels the cytoskeletal tubulin (microtubule) in single cells, and not just primary cilia if they were present. In rat ventricular tissue sections, intense staining in the extracellular spaces in between the myocytes was observed using this antibody, as shown in figure 4.1 (B). The size of the staining patterns, and its distribution in tissue sections, suggested neuronal labelling which has both α- and β-tubulin. Therefore, the acetylated α-tubulin results showed it was not a reliable primary antibody for primary cilia in adult rat tissue. Therefore, a more specific primary antibody, such as ARL13B, was used in further experiments.
Figure 4.1. Representative data showing confocal images immunolabelled for acetylated α-tubulin in red (A) and green (B) in adult rat single cardiomyocyte and ventricular tissue, respectively. Note: ‘N’ denotes the nucleus in image A. The image (B) immunolabelled with anti-RyR2 (red) to myocytes (t-tubules).
4.2.4. Immunolabelling in embryonic, neonatal, young and adult rat cardiac tissue

The cardiac tissue sections from embryonic, neonatal, P2, young (P21 & P28) and adult hearts were immunolabelled for primary cilia and nuclei with anti ARL13B antibody shown in figure 4.2 (C - G). The adult rat kidney sections were also labelled with and without primary antibody for the positive and negative controls (Figure 4.2 (A & B). The tissue sections from embryonic (n = 3 hearts), neonatal (n = 2 hearts) and young (n = 3 hearts) showed abundance of primary cilia and each of them is present in the close vicinity of nucleus in most cells. The appearance of primary cilia was more like small dots in embryonic tissues compared to their comma like appearance in neonatal and young ones (Figure 4.2 C, D, E & C). Immuno-staining revealed that primary cilia were present exclusively in embryonic and young cardiac tissue associated with ECM. Confocal images showed that primary cilia were absent in adult rat cardiac tissue sections (n = 4 hearts) and single myocytes (n = 4 hearts, 20-50 cells per heart) (Figure 4.2 (G & H). There were only n = 2 neonatal rat hearts used in this study. The low “n” was associated due to the less availability of neonatal rats as our laboratory had approval to isolate only adult rat hearts (section 2.2). The neonatal rat hearts were mostly collected from other laboratories in the faculty on availability.
Figure 4.2. Representative data showing confocal images immunolabelled for ARL 13B in red and nuclei in blue in (A) rat kidney section (positive control), (B) rat kidney section (negative control), (C) embryo, (D) P2, (E) P21, and (F) P28 neonatal rat cardiac tissue. Vimentin (Vim, green) and phalloidin (magenta) is also shown in (G) adult cardiac tissue.
and in (H) in single rat myocyte. Scale bar is shown for each individual image. **Note:** ARL 13B labelling was completely absent in single myocytes and adult rat heart tissue.
4.2.5. Primary cilia association to a particular cell type in neonatal rat cardiac tissue

To further investigate which particular cell type was associated with primary cilia, the triple immunofluorescence labelling for primary cilia, endothelial cells and nucleus or primary cilia, fibroblasts and nucleus or primary cilia, f-actin and nucleus was carried out in selected tissue sections (cardiac tissue sections from P2 and P21 rats where primary cilia were evidenced). For such experiments only neonatal (P2, \(n = 2\) hearts) and young (P21, \(n = 3\) hearts) cardiac tissue sections were triple immunolabelled and analysed under the confocal microscope. Preliminary results revealed that primary cilia labelling did not display any noticeable association with endothelial cells (RECA-1 labelling) as shown in confocal image in figure 4.3 (A & B). It looks from the confocal images that primary cilia are associated with myocyte sarcomeric protein F-actin as shown in figure 4.3 (C).

![Figure 4.3](image)

**Figure 4.3.** Representative data showing confocal images of ventricular tissue from P2 rats immunolabelled with (A) anti - ARL 13B (red) to mark primary cilia, Hoechst (blue) to label nuclei, and anti - RECA-1 (green) to mark endothelial cells, and (B) anti - ARL 13B (red) to mark primary cilia, Hoechst (blue) to label nuclei, and anti - Vimentin (green) to mark fibroblasts. (C) The confocal image of ventricular tissue from P21 rat immunolabelled for ARL 13B (red), nuclei (blue), and Phalloidin to mark F-actin (green). Arrows point to primary cilia labelling in red.
4.3. Chapter Summary and Discussion

The analysis of images labelled with acetylated-α-tubulin antibody revealed cytoskeletal tubulin (microtubule) labelling in single cells and intense extracellular spaces labelling in between myocytes in cardiac tissue sections. Unfortunately, the acetylated-α-tubulin antibody was not specific for primary cilia, also labelling the neurons (tubulin) present in cardiac tissue. Such neuronal labelling was already reported by Siddiqui et al., in C. elegans model by using this antibody (Siddiqui et al., 1989). Therefore, we introduced a more specific antibody (ARL13B) to primary cilia. It is marker of ARL13B protein, a small ciliary G protein of the Ras superfamily localized in cilia and is required for cilium biogenesis and maintenance. The immunospecificity of this antibody for the ARL13B was confirmed by the suppliers in immunofluorescence studies. The immuno-histochemical specificity of anti-ARL13B antibody employed in this study has been demonstrated recently by others who have shown specific primary cilia immuno-labelling in developing cultured cardiomyocytes, sections of kidney, neurons from cerebral cortex (Clement et al., 2009; Higginbotham et al., 2012; Deane et al., 2013).

Using the more specific ARL13B antibody, no evidence of primary cilia in either ventricular tissue sections or isolated myocytes from adult rat hearts using an immunofluorescent approach (Figure 4.2 G & H). This was surprising, since primary cilia had previously been reported in embryonic and adult human hearts, identified by electron microscopy (Myklebust et al., 1977). The absence of primary cilia in adult hearts showed they could not account for any trans-sarcolemmal Ca^{2+} entry which would explain our previously observed Ca^{2+} transient differences between isolated myocytes and intact trabeculae.

In contrast to adult hearts, evidence of cardiac primary cilia was observed in tissue sections from embryonic, neonatal (P2) and young (P21 & P28) rat hearts (Figure 4.2 C, D, E & F). It was also observed that primary cilia had a small dot-like appearance in embryonic hearts compared to their comma-like appearance in young (P21 & P28) rat hearts. Our results are first to show presence of primary cilia by using ARL13B antibody in embryonic and neonatal (P21 & P28) rat cardiac tissue sections using confocal microscopy. The confocal results from this study are in accord with others where they have found presence of primary cilia in embryonic day 9.5 mouse embryos by using other antibodies (Slough et al., 2008). Other studies has also reported the presence of primary cilia at intermediate stages of heart development in accord with our observations (McGrath & Brueckner, 2003; Norris, 2012; Koefoed et al., 2014). Many studies documented the role of cardiac primary cilia in
regulating cellular signalling pathways, important for the progressive differentiation, morphogenesis and maturation of the heart (Clement et al., 2009, 2013; Gerhardt et al., 2013).

Because my data investigated primary cilia labelling in cardiac tissue, it was necessary to investigate whether the cilia were associated with cardiomyocytes or other vascular cell types. I therefore carried out triple-immunolabelling of tissue samples. It appeared from confocal imaging that primary cilia were associated with the myocytes and not endothelial cells, or fibroblasts in rat cardiac tissue (Figure 4.3 (C)). However, it has been reported that primary cilia are present in epicardial, myocardial, and endocardial cells in human embryonic and adult cardiac tissues using electron microscopy (Myklebust et al., 1977). The presence of primary cilia in mouse embryonic day 11.5 ventricular cells is also reported by another group (Gerhardt et al., 2013). The cardiac primary cilia were also observed in the parts of cardiovascular system, including endothelial cells of the aorta (Bystrevskaya et al., 1992), cardiomyocytes differentiating from mice stem cells (Clement et al., 2009, 2013), and in cultured human umbilical endothelial cells (Iomini et al., 2004).

I, therefore, conclude that primary cilia are present in the neonatal and early stages of development of rat hearts but are absent in adult hearts. Thus, primary cilia has no such role in myocyte calcium modulation required during the early development of the heart, and their absence during this early developmental stages has been linked to disorders and diseases known as 'ciliopathies'.
Chapter 5. Identification of novel Ca$^{2+}$ signalling pathways

5.1. Chapter Overview

Protein kinase A (PKA) is the usual primary effector associated with cAMP signalling. Cyclic adenosine 3′, 5′-monophosphate (cAMP) is one of the most important second messengers of the heart. The discovery of the EPAC protein, activated directly by cAMP, raises the question of its potential role in Ca$^{2+}$ signalling in cardiac cells. Although the exact role of EPAC in heart is still unclear, results from a number of different studies have suggested it is important. These include some conflicting results as to the role of EPAC, particularly as a regulator of EC-coupling. A number of studies have shown that activation of EPAC in cardiomyocytes modulates intracellular Ca$^{2+}$ homeostasis (Morel et al., 2005; Oestreich et al., 2007, 2009; Pereira et al., 2007; Cazorla et al., 2009), but the acute effects of EPAC activation are still debated (Oestreich et al., 2007; Pereira et al., 2007). Pereira et al. (2007) reported an increase in spontaneous spark activity in isolated rat myocytes following activation of EPAC, with a reduction in the evoked Ca$^{2+}$ transient amplitude, a slower decay of the transient but with no change in cell shortening (Pereira et al., 2007). Similarly, Cazorla et al. (2009) also reported reduced Ca$^{2+}$ transient amplitude with EPAC activation of rat myocytes, but in their study cell shortening was increased (Cazorla et al., 2009). In contrast, Oestreich et al. (2007) reported increased Ca$^{2+}$ transient amplitudes in isolated myocytes from mouse hearts during a brief exposure to EPAC activation, with no change to Ca$^{2+}$ transients in myocytes from phospholipase Cε knockout mice (PLCε$^{-/-}$) (Oestreich et al., 2007). Their data suggested that EPAC acts upstream of PLC in regulating myocyte SR Ca$^{2+}$ handling. It is also reported that EPAC activation increased myofilament Ca$^{2+}$ sensitivity in myocytes infected in vivo with a constitutively active form of EPAC (Oestreich et al. 2009). These observations combined with EPAC expression in the heart prompted us to focus on its role in Ca$^{2+}$ signalling in the heart. Overall, our understanding of the role of the EPAC pathway in cardiac muscle remains unclear. Since the majority of studies have been carried out in isolated myocytes, my plan was to activate the EPAC pathway in an intact cardiac preparation and examine EC coupling.

Since most of the studies have been carried out in isolated myocytes and the isolated myocytes are in absence of any paracrine/hormonal input such as β-adrenergic stimulation unlikely the multicellular cardiac preparations. The regulation of the heart in vivo is
modulated by autonomic nervous system. Therefore, we speculate there might be
differences in signalling pathways in isolated myocytes and multicellular cardiac
preparations such as cardiac trabeculae which are influenced by β-adrenergic stimulation.

The aim of this chapter is to explore novel EPAC signalling pathway which contribute to the
EC-coupling and Ca\(^{2+}\) homeostasis. This was achieved by activating the EPAC pathway in
cardiac trabeculae whilst measuring intracellular Ca\(^{2+}\) and isometric force. To enable
comparison between my study and others, I also examined the effects of EPAC activation in
isolated myocytes. Experiments described in this chapter were all performed in the rat RV
trabeculae at T\(_{room}\).
5.2. EPAC-selective activators

The synthesis of cell permeant and EPAC specific cAMP analogs is an important progress in the functional characterisation of EPAC. These EPAC-selective cAMP analogs incorporate a 2’-O-methyl substitution on the ribose ring of cAMP, a modification that impairs their ability to activate PKA (Enserink et al., 2002; Holz et al., 2008b). The most commonly used EPAC selective agonist is 8-pCPT-2’-O-Me-cAMP (8-cpTOME) which exhibits high affinity for both EPAC1 and EPAC2 (Enserink et al., 2002; Rehmann et al., 2003, 2008). The structure of 8-cpTOME is shown in figure 5.1. The cAMP analog 8-cpTOME activates EPAC with a \( K_d \) 2.9 \( \mu \)M compared with 45 \( \mu \)M for cAMP as endogenous ligand (Rehmann et al., 2003).

![8-pCPT-2'-O-Me-cAMP](image)

**Figure 5.1.** Structure of EPAC-selective agonist (8-pCPT-2’-O-Me-cAMP) for EPAC1 and EPAC2 (structure reproduced from (Schmidt et al., 2013)).
5.3. **EPAC activation of isolated rat RV trabeculae and single myocytes**

5.3.1. **Reagents and solutions**

A modified K-H solution containing (mM): 142 KCl, 1.2 KH$_2$PO$_4$, 20 NaHCO$_3$, 1.2 MgSO$_4$.7H$_2$O and 10 D-Glucose; was used for a number of trabeculae in which K$^+$ contractures were obtained for myofilament Ca$^{2+}$ sensitivity experiments. All experiments were performed at room temperature. For both trabeculae and single myocyte experiments, an EPAC-selective cAMP analog, 8-(4-chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (cpTOME, Biolog. Life Science Institute, Germany) was used to activate the EPAC pathway. A 5 mM stock solution of cpTOME was prepared and stored at -20 °C in small aliquots. Final drug concentrations of 10 µM were obtained by dilution with physiological buffer solution. Trabeculae were exposed to 10 µM cpTOME for 20 min before measurements were made. At this concentration, cpTOME is highly specific for EPAC over PKA (Enserink et al., 2002). Inhibition of PKA was carried out by pre-treating trabeculae with 1 µM H-89 (CalBiochem.), and activation of β-adrenergic signalling was carried out using 0.1 µM isoproterenol hydrochloride (Sigma-Aldrich) from a 10 mM stock solution. CaMKII activity was inhibited by addition of 1 µM KN93 (Sigma-Aldrich) to the superfusate (Sumi et al., 1991).

5.3.2. **Data and statistical analysis**

For steady-state conditions, measurements of Ca$^{2+}$ transient and isometric force parameters were obtained from data averaged over a number of cardiac cycles. Data are presented as mean± standard error of the mean (SEM) from trabeculae and isolated myocytes. Statistical analysis was carried out using repeated measures ANOVA (IBM SPSS software), and a $P$ value ≤ 0.05 was considered significant. The force-[Ca$^{2+}$], curves were fitted with a modified Hill equation and half-maximal (EC$_{50}$) was calculated using the following relationship:
\[
\text{Force} = F_{\text{min}} + F_{\text{max}} \left( \frac{[340/380 \text{ ratio}]^{nH}}{K_a^{nH} + [340/380 \text{ ratio}]^{nH}} \right)
\]

where \( nH \) is the cooperativity, or slope; \( K_a \) is the dissociation constant; and \( F_{\text{min}} \) and \( F_{\text{max}} \) are the minimal and maximal forces.
5.4. EPAC activation of isolated trabeculae

Figure 5.2 (A) shows examples of \([\text{Ca}^{2+}]_i\) (ratio 340/380) transient amplitude and force in control and during cpTOME application (after 20 min). Figure 5.2 (B) shows normalised \(\text{Ca}^{2+}\) transient amplitude (%), (C) normalised active force (%), and (D) normalised time constant of decay (%) from trabeculae (n = 7) at 1.5 mM \([\text{Ca}^{2+}]_o\) and 0.2 Hz stimulation frequency. There was no effect after 20 min of application of cpTOME on either the normalised \(\text{Ca}^{2+}\) transient amplitude, normalised active force, and normalised time constant of fluorescence decay for trabeculae at 1.5 mM \([\text{Ca}^{2+}]_o\). Since EPAC activation acts in parallel with PKA, the lack of effect seen above might be due to overlap between these signalling pathways.
Figure 5.2.  
EPAC activation of RV trabeculae. Mean ± SEM data from n = 7 trabeculae before (control), and during cpTOME exposure ([Ca\(^{2+}\)]\(_{o}\) 1.5 mM, 0.2 Hz). A, Ca\(^{2+}\) transients (340/380 fluorescence ratio) and stress (mN mm\(^{-2}\)) from a representative trabecula before and during EPAC activation with cpTOME; B, normalised Ca\(^{2+}\) transient amplitude (%); C, normalised active force (%); D, normalised time constant of Ca\(^{2+}\) transient decay (%).

The effects of cpTOME were therefore investigated in trabeculae in which PKA was inhibited by addition of H-89, a competitive inhibitor of PKA. H-89 was added to partially block the PKA response to addition of 0.1µM isoproterenol to the superfusate. Addition of cpTOME in the presence of isoproterenol and H-89 still had no effect on either the [Ca\(^{2+}\)]\(_{i}\) transients, or twitch force (Figure 5.3). These experiments were carried out in n = 4 trabeculae. Further experiments were not thought to be necessary since H-89 did not reveal any effect of cpTOME.
Figure 5.3. EPAC activation of RV trabeculae during PKA inhibition and β-adrenergic activation. Mean ± SEM data recorded from n = 4 trabeculae sequentially superfused with control solution (control), solution containing 1 µM H-89 (an inhibitor of PKA), solution containing H-89 and 0.1 µM isoproterenol (a β-agonist), and solution containing 10 µM cpTOME (to activate EPAC) with H-89 and isoproterenol ([Ca^{2+}]_o 1.5 mM, 0.2 Hz). A, Ca^{2+} transient amplitude (340/380 ratio); B, stress (mN mm^{-2}); C, the time constant of Ca^{2+} transient decay (s). * p ≤ 0.05 (One way ANOVA for repeated measures).
5.4.1. EPAC activation of isolated adult rat ventricular myocytes

As there were some negative results obtained from trabeculae experiments, therefore it was important to re-examine the effect of EPAC activation on isolated rat cardiomyocytes. The spontaneous calcium sparks in quiescent myocytes before (control) and during cpTOME application was recorded. Figure 5.4 (A) shows representative examples of line scan images taken from a single cardiac myocyte. EPAC activation significantly increased the spark rate in isolated myocytes. Averaged data from \( n = 10 \) myocytes are shown in figure 5.4 (B). EPAC activation significantly increased the \( \text{Ca}^{2+} \) spark frequency (from 6.62 per 100 \( \mu \text{m}^3 \) s \( \pm \) 2.49 to 32.26 per 100 \( \mu \text{m}^3 \) s \( \pm \) 7.34, \( p \leq 0.05 \)). Results obtained from the isolated myocytes showed that EPAC activation initially increased spark frequency (black and red bars). With prolonged exposure to cpTOME spark frequency decreased again towards control conditions, but resting \([\text{Ca}^{2+}]_i\) was increased (Fig. 5.4 (B) and (C)). The blue bars in figure 5.4 (C) indicate the cells that underwent the same imaging and perfusion protocol, but without the addition of cpTOME (\( n = 3 \)).
Figure 5.4. EPAC activation of isolated myocytes. A, shows line-scan images of Ca\(^{2+}\) sparks from a representative myocyte before (i) and during early (ii) and late (iii) cpTOME perfusion. B, shows the measured Ca\(^{2+}\) spark frequency for the myocyte shown in A, averaged with a box car window of three images. The solid red line indicates the period of cpTOME perfusion. The inset shows pseudo-calibrated resting [Ca\(^{2+}\)]\(_i\) from F/F\(_0\) (see Methods, Chapter 2), which increased over time. C, shows pooled data from cells treated with cpTOME (black and red bars, n = 10) and cells that underwent the same imaging and perfusion protocol, but without the addition of cpTOME (blue bars, n = 3). The inset shows resting [Ca\(^{2+}\)]\(_i\), which had a mean of 100 nM at the beginning of the experiment. (Ca\(^{2+}\) spark data and analysis was carried out by Dr. CH Kong)
5.5. **EPAC activation of depotentiated trabeculae**

The lack of effect of cpTOME on intact trabeculae shown earlier seems at variance with the clear effect of EPAC activation in isolated myocytes. However, the possible reasons of this explanation could be unlike isolated myocytes (unstretched), multicellular trabeculae have intact ECM, and are held at fixed length (~2.1 µm). In this regard, it can be considered to be in depotentiated state since it is well known that the amplitude of the Ca\(^{2+}\) transient depends on the state of mechanical loading (Marban *et al.*, 1986; Solaro & Van Eyk, 1996; McClellan *et al.*, 2001). Therefore, experiments were done at reduced \([\text{Ca}^{2+}]_o = 0.5 \text{ mM}\), \([\text{Ca}^{2+}]_i\) and force were compared before and during superfusion with cpTOME. Under these experimental conditions, EPAC activation showed no change in the amplitude of the \([\text{Ca}^{2+}]_i\) transients, or in the time constant of decay, whereas stress was increased from 8.35 ± 2.07 mN mm\(^{-2}\) in control to 11.42 ± 2.46 mN mm\(^{-2}\) during cpTOME exposure (n = 7, p ≤ 0.05) in Figure 5.5 A). This possibility was further examined with an experimental protocol for determining myofilament Ca\(^{2+}\) sensitivity in intact cardiac trabeculae.
Figure 5.5. EPAC activation of trabeculae in low (0.5 mM [Ca$^{2+}$]$_o$). Mean ± SEM data for control and cpTOME from n = 7 trabeculae ([Ca$^{2+}$]$_o$ 0.5 mM, 0.2 Hz). A, representative raw data of force (mN) and Ca$^{2+}$ transients (340/380 ratio) from a single trabecula. B, normalised Ca$^{2+}$ transient amplitude (%). C, normalised active force (%). D, normalised time constant of Ca$^{2+}$ transient decay (%) in control and during cpTOME application, * $p \leq 0.05$ (One way ANOVA for repeated measures).

5.6. Myofilament Ca$^{2+}$ sensitivity

5.6.1. Measurement of myofilament calcium sensitivity in trabeculae

After recording [Ca$^{2+}$]$_i$ and force at 0.2 Hz and 0.5 mM [Ca$^{2+}$]$_o$, the stimulation frequency was turned off and the force-[Ca$^{2+}$]$_i$ relationship determined using a modified K-H solution containing high [K$^+$]$_o$. On introduction of the high [K$^+$]$_o$ superfusate, both [Ca$^{2+}$] and force increased. Once force had reached a new steady-state level, the high [K$^+$]$_o$ solution was washed out and superfusion with control K-H buffer re-commenced. The force-[Ca$^{2+}$]$_i$ relationship was then determined from the relaxation phase of the contracture when the myofilaments and the [Ca$^{2+}$]$_i$ were assumed to be in equilibrium (Varian et al., 2006). Data
were obtained from consecutive high $[K^+]_o$ contractures in trabeculae ($n = 4$) before (control) and during EPAC activation (to eliminate the inter-muscle variation). The force-$[Ca^{2+}]_i$ relationship was also carried out in an additional group of trabeculae ($n = 4$) in the presence of KN-93, a blocker of CamKII, before (control) and during EPAC activation. Figure 5.6 (A) shows representative data from a quiescent trabecula during superfusion with high $[K^+]_o$ solution. The dashed rectangle represents the area from which data was obtained for the force-$[Ca^{2+}]_i$ relationship. Figure 5.6 (B) shows data from the single trabecula before and during EPAC activation fitted with Hill plots. EPAC activation shifted the force-$[Ca^{2+}]_i$ relationship towards the left, indicating increased myofilament calcium sensitivity. Figure 5.6 (C) shows mean ± SEM for EC50 values for set of experiments with the significant drop in EC50 value for ratio 340/380 during EPAC activation.
Figure 5.6. Myofilament Ca\(^{2+}\) sensitivity. A, representative data from a trabecula superfused with 142 mM [K\(^+\)]\(_o\), 0.5 mM [Ca\(^{2+}\)]\(_o\), and 10 mM caffeine. 340/380 ratio (top), and force (bottom) with solution changes shown underneath. The data within the dotted rectangle were used as a measure of myofilament Ca\(^{2+}\) sensitivity. B, stress versus 340/380 ratio from a representative trabecula. Data were fitted by Hill plot before (control) and
during cpTOME. The dashed lines show EC\textsubscript{50} values. C, mean ± SEM EC\textsubscript{50} (340/380 ratio) from n = 5 trabeculae. * \( p \leq 0.05 \) (One way ANOVA for repeated measures).

### 5.7. Myofilament Ca\textsuperscript{2+} sensitivity in presence of CamKII inhibition

To investigate the mechanism responsible for the increased myofilament Ca\textsuperscript{2+} sensitivity during EPAC activation at low [Ca\textsuperscript{2+}]	extsubscript{o} we repeated the high [K\textsuperscript{+}]	extsubscript{o} experiments in KN-93 (1 \( \mu \)M), a competitive inhibitor of calmodulin binding to Cam kinase with a reported K\textsubscript{i} of \(~0.37\ \mu\text{M}\) (Sumi et al., 1991). Application of KN-93 abolished the EPAC-dependent increase in Ca\textsuperscript{2+} sensitivity. Figure 5.7 (A) shows data from a representative trabecula during CaMKII inhibition, with mean ± SEM EC\textsubscript{50} values (n = 3) before and during cpTOME shown in figure 5.7 (B).

![Figure 5.7](image.jpg)

**Figure 5.7.** CaMK-II inhibition and myofilament Ca\textsuperscript{2+} sensitivity. A, the relationship between stress and 340/380 ratio fitted by Hill plots during CaMKII inhibition with 1 \( \mu \)M KN93 (142 mM [K\textsuperscript{+}]	extsubscript{o}, 0.5 mM [Ca\textsuperscript{2+}]	extsubscript{o} and 10 mM caffeine) with and without cpTOME. B, mean ± SEM EC\textsubscript{50} (340/380 ratio) from n = 3 trabeculae.
5.8. Caffeine evoked Ca\textsuperscript{2+} transients in isolated trabeculae

5.8.1. Measurement of SR Ca\textsuperscript{2+} by caffeine

A modified protocol with caffeine was used to measure the SR Ca\textsuperscript{2+} load in trabeculae. In this protocol, the stimulation frequency was turned off after recording [Ca\textsuperscript{2+}]\textsubscript{i} and force (0.2 Hz, 0.5 mM [Ca\textsuperscript{2+}])\textsubscript{o} at T\textsubscript{room}. Then 0 mM [Ca\textsuperscript{2+}])\textsubscript{o} K-H buffer was introduced for 30 s before switching to 20 mM caffeine solution made in K-H buffer. Caffeine is used to functionally eliminate Ca\textsuperscript{2+} storage in the SR. The caffeine exposure was followed by washout with caffeine-free 0.5 mM [Ca\textsuperscript{2+}]\textsubscript{o} K-H buffer. This intervention produces a contracture in force and [Ca\textsuperscript{2+}]\textsubscript{i} recordings and was repeated in the presence of cpTOME (at ~20 min of incubation) also.

5.8.2. Estimation of SR Ca\textsuperscript{2+} load before and after EPAC activation

The aim was to investigate whether the low [Ca\textsuperscript{2+}]\textsubscript{o} affects the SR Ca\textsuperscript{2+} load during EPAC activation. Figure 5.8 (A) shows an example of [Ca\textsuperscript{2+}]\textsubscript{i} transient (ratio 340/380) during caffeine application and (B) the mean amplitude of [Ca\textsuperscript{2+}]\textsubscript{i} obtained during 20mM caffeine application with and without cpTOME. My results showed that EPAC activation with the application of 10 µM cpTOME at low [Ca\textsuperscript{2+}]\textsubscript{o} significantly decrease the SR Ca\textsuperscript{2+} load in trabeculae.
Figure 5.8. Estimation of SR Ca\(^{2+}\) load. (A), representative caffeine-evoked [Ca\(^{2+}\)]\(_i\) transients (340/380 ratio) recorded in a single trabecula at 0.5 mM [Ca\(^{2+}\)]\(_o\); (B), mean ± SEM amplitude of [Ca\(^{2+}\)]\(_i\) obtained during 20mM caffeine application with and without cpTOME from n = 5 trabeculae, * \(p \leq 0.05\) (Two-tailed paired t-tests for the means).
5.9. Chapter Summary and Discussion

My data shows that EPAC activation did not alter Ca\(^{2+}\) transients or isometric force in cardiac trabeculae under control conditions of 1.5 mM [Ca\(^{2+}\)]\(_o\) (Figure 5.2). However, in agreement with Pereira et al., (Pereira et al., 2007), an increase in the Ca\(^{2+}\) spark frequency in isolated myocytes was observed that reduced over time (Figure 5.4). From the observations obtained from the single cell experiments, it can be assumed that EPAC activation decreases the SR Ca\(^{2+}\) content via diastolic leak in the form of Ca\(^{2+}\) sparks (Figure 5.4). This is further supported by the results obtained from isolated trabeculae experiments (only observed for depotentiated trabeculae) where a decrease in SR Ca\(^{2+}\) load was observed with EPAC activation. Consistent with these findings, Pereira (2007) and his group also found that cpTOME increased spark frequency with reduced SR Ca\(^{2+}\) load in a PKA-independent manner in isolated myocytes. In contrast, Oestreich and colleagues found that the Ca\(^{2+}\) transient amplitude was increased in field stimulated mouse ventricular myocytes in a Rap1 and PLC-\(\varepsilon\)-dependent manner (Oestreich et al., 2009). The reason for these discrepancies is still unclear. Figure 5.9 displays a schematic of the proposed mechanism of action under EPAC activation.

![Figure 5.9](image)

**Figure 5.9.** Mechanism of proposed EPAC activation. EPAC activation causes the phosphorylation of RyRs and increases the probability of their opening. The opening of the RyR channels releases Ca\(^{2+}\) and ultimately reduces the SR Ca\(^{2+}\) load. The release of Ca\(^{2+}\) from the RyRs is observed in the form of “calcium sparks” in myocytes. Modified from (Oestreich et al., 2009).
Under depotentiated conditions (by lowering the superfusate [Ca\(^{2+}\)], cpTOME application increased force and increased the sensitivity of myofilaments which was related to CaMKII activation. No effect of cpTOME was observed on resting Ca\(^{2+}\) in trabeculae, suggesting the observed (short term) increase in Ca\(^{2+}\) spark rate was not sufficient to change the cytosolic [Ca\(^{2+}\)] per se. A possible RyR2 related increase in opening probability with EPAC activation was not investigated. The increase in Ca\(^{2+}\) spark rate is in accord with the results of Pereira et al. (2007) who also observed a decrease in the amplitude of the Ca\(^{2+}\) transient (Figure 5.2). It is possible that RyR2 phosphorylation might explain the short term effect of EPAC on isolated myocytes, but this was not investigated further. The short term effect of EPAC activation on isolated myocytes is clearly shown in Figure 5.4C as an increased rate in Ca\(^{2+}\) sparks. My findings of the force-[Ca\(^{2+}\)], relationship in intact trabeculae exhibiting increased myofilament Ca\(^{2+}\) sensitivity with cpTOME are concordant with previous studies of Cazorla and his group (Cazorla et al., 2009). Similar findings were also reported by the Pereira group (2007). It is also known from previous studies that PKA-dependent phosphorylation of cTnI reduces the myofilament Ca\(^{2+}\) sensitivity (Solaro et al., 2008). Here, my data shows that EPAC activation increased myofilament Ca\(^{2+}\) sensitivity, suggesting it is PKA-independent. It was also demonstrated that CamKII inhibitor abolished the effect of cpTOME on myofilament Ca\(^{2+}\) sensitivity by blocking the phosphorylation of sarcomeric proteins, suggesting that CamKII is involved in EPAC signalling pathway. Consistent with this observation, two independent groups also reported similar observations, suggesting the role for CamKII in phosphorylating cTnI and cMyBP-C proteins (Cazorla et al., 2009; Oestreich et al., 2009). Curran and colleagues reported that β-AR stimulation increase SR Ca\(^{2+}\) leak in ventricular myocytes in a CamKII-dependent manner (Curran et al., 2007), while Oestreich and colleagues reported the increase in Ca\(^{2+}\) transient amplitude with 8-cpTOME was Rap1 and PLC-ε-dependent (Oestreich et al., 2009). However, the possibility of RyR2-dependent CamKII phosphorylation was not investigated in my study.

In order to summarise the results obtained from this study, a schematic outline of Ca\(^{2+}\) signalling pathway via EPAC proteins in a cardiac tissue is proposed (figure 5.10).
Figure 5.10. Proposed schematic outline of Ca^{2+} signalling pathway via EPAC proteins in a cardiac tissue. In response to β-AR stimulation, increase in cAMP result in activation of EPAC or either directly by cpTOME (EPAC agonist) which ultimately activates CaM and CamKII. CamKII mediated phosphorylation of RyR causes the SR Ca^{2+} release and myofilament Ca^{2+} sensitivity.

Although lowering extracellular [Ca^{2+}] unmasked an effect of EPAC on the myofilament Ca^{2+} sensitivity, the lack of any visible response in trabeculae at a more physiological [Ca^{2+}] was still puzzling. One of the possible reasons to explain this discrepancy in isolated myocytes and trabeculae is the difference in sarcomere length. Experiments in isolated trabeculae were done at fixed sarcomere length of ~2.1-2.2 µm, whilst isolated myocytes are at short sarcomere length of 1.8 µm. The results from this chapter 3 have already shown that there are differences in amplitude of Ca^{2+} transients recorded from isolated myocytes and trabeculae at same sarcomere length (1.8 µm). Cardiac trabeculae are multicellular preparations, similar in composition to the ventricular free wall, with an outer layer of
endothelial cells in direct contact with the superfusate and containing a number of different cell types: fibroblasts, vascular smooth muscle, neurons and vascular endothelial cells (Hanley et al., 1999). It is possible therefore that EPAC activation might increase signalling between myocytes and other cell types, since EPAC is ubiquitously expressed inducing many diverse cAMP actions (Breckler et al., 2011).

In conclusion, the results from the isolated myocyte experiments suggest that cpTOME decreases SR Ca\(^{2+}\) content due to increased diastolic leak (via Ca\(^{2+}\) sparks), and reduced Ca\(^{2+}\) re-uptake, with some recovery of the Ca\(^{2+}\) transient amplitude on prolonged treatment. Reducing the Ca\(^{2+}\) current (low [Ca\(^{2+}\)]\(_o\)) revealed an increase in myofilament Ca\(^{2+}\) sensitivity with cpTOME that was removed by CamKII inhibition. Hence, this work indicates that EPAC contributes to Ca\(^{2+}\) signalling in a CamKII-dependent fashion in cardiac tissue.
Chapter 6. Thesis Summary

6.1. Summary

In this thesis, the role of the ECM as a potential modulator of myocyte Ca\(^{2+}\) handling was investigated in multicellular and single cell preparations isolated from rat hearts. Isometric force and intracellular [Ca\(^{2+}\)] (indexed as the fura-2 340/380 fluorescence ratio) were recorded continuously in multicellular ventricular trabeculae that were subjected to an experimental protocol that mimicked the cell isolation procedure. Following experimentation, the enzyme treated trabeculae were fixed and immunolabelled for key ECM proteins to measure the loss of ECM components. Fixed cardiac tissue was also examined for evidence of primary cilium, which might contribute to trans-sarcolemmal Ca\(^{2+}\) entry in cardiac tissue. Finally, the EPAC signalling pathway was investigated in trabeculae and isolated cells, and its contribution to Ca\(^{2+}\) signalling determined.

Experiments outlined in Chapter 3 confirmed that the smaller amplitude Ca\(^{2+}\) transients obtained from isolated myocytes in comparison to intact trabeculae did not result from a difference in sarcomere length between preparations. Ca\(^{2+}\) transients recorded from trabeculae at short sarcomere length of 1.8 µm still remained bigger in amplitude to those recorded from isolated myocytes. This finding directed the study to look for other differences between isolated myocytes and intact trabeculae that might underlie the altered Ca\(^{2+}\) transients.

An important difference between isolated myocytes and intact trabeculae is the loss of ECM during the standard myocyte isolation process. This study investigated the Ca\(^{2+}\) transients in intact trabeculae under experimental conditions that mimicked the cell isolation procedure. It was found that resting and peak Ca\(^{2+}\) was altered by enzyme treatment. Partially digested trabeculae had smaller amplitude transients, with an increase in resting Ca\(^{2+}\) and stress observed. The amplitude of Ca\(^{2+}\) transients was similar to those of isolated cells, but the Ca\(^{2+}\) transients from partially digested trabeculae showed signs of Ca\(^{2+}\) overload with a characteristic "undershoot". Moreover, the Ca\(^{2+}\) transients remained altered following exposure to the enzyme solution, even after a "wash" period when the trabeculae were superfused again with control (pre-enzyme) solution, and the enzyme digestion halted. Preliminary data from SR Ca\(^{2+}\) load determined in quiescent trabeculae exposed to 20 mM caffeine showed the decay of the caffeine transients was slower post-enzyme treatment, due
to slower $\text{Ca}^{2+}$ extrusion via the NCX. Results presented in Chapter 3 revealed the interaction between contracting cardiomyocytes and their close neighbours, non-myocyte components that make up the extracellular matrix. The enzymes, such as collagenase and protease, used for ECM dismantling not only enhanced the ECM degradation but also affect the physical interaction of myocytes with the ECM. At the same time, the enzyme treatment apparently made the myocyte cell membranes leakier, observed as $\text{Ca}^{2+}$ overload in partially digested trabeculae. Taken together, this chapter concludes the ECM has an important role in myocyte $\text{Ca}^{2+}$ signalling in intact trabeculae. The structural changes in post-enzyme treated trabeculae were observed using optical coherence tomography scanning, carried out by Mr Ming Cheuk (Auckland Bioengineering Institute). The optical scans of two trabeculae before and after enzyme treatment confirmed the loss of structure. These trabeculae were subsequently fixed and examined for the loss of key ECM proteins by immunohistochemistry. Immuno-histological examination revealed reduced endothelial cell labelling (RECA-1) without any change in collagen I, laminin, vimentin, and Cxn-43 labelling. The loss of physical links between the ECM and the myocytes explains the reduced force measured in partially digested trabeculae.

The ECM is known to release paracrine factors such as ET-1, PGF$_{2\alpha}$, and Angiotensin II that alter $\text{Ca}^{2+}$ transients when applied directly to cardiac muscle preparations (Brutsaert et al., 1998; Yew et al., 1998; Calaghan & White, 2001; Brutsaert, 2003). Hence, Chapter 3 reports the results of experiments investigating the exposure of intact trabeculae and single cells to ET-1. An initial increase in the amplitude of the $\text{Ca}^{2+}$ transients was observed in trabeculae and in isolated myocytes with direct application of ET-1, which is in agreement with published data for rabbit pulmonary artery smooth muscle cells (Ko et al., 2005). However, results from enzyme treated trabeculae showed no change in force or in the $\text{Ca}^{2+}$ transients with ET-1 application. One possible explanation could be that the trabeculae myocytes were already overloaded with $\text{Ca}^{2+}$ post-enzyme treatment.

The endothelial and vascular smooth muscle cells of the ECM might also release prostaglandins that target myocyte $\text{Ca}^{2+}$ handling. The relative contribution of PGF$_{2\alpha}$ to force and the $\text{Ca}^{2+}$ transients was therefore investigated in intact trabeculae. Direct application of PGF$_{2\alpha}$ to isolated trabeculae increased active force, with a small increase in $\text{Ca}^{2+}$ transient amplitude. These results are in agreement with the published data of Yew et al. (1998) and Ward et al. (2014). The trabeculae response to PGF$_{2\alpha}$ was reminiscent of the response to β-adrenergic stimulation, but without any change in the time course of $\text{Ca}^{2+}$ transient decay.
Chapter 4 investigated whether primary cilia were present in adult cardiac tissue, and might therefore contribute to Ca\(^{2+}\) homeostasis. We therefore looked for evidence in fixed tissue and cell using acetylated-\(\alpha\)-tubulin and ARL13B antibodies. However, the acetylated-\(\alpha\)-tubulin antibody labelled neurons (tubulin) which were abundant in the cardiac tissue sections, as well as labelling the cytoskeletal tubulin in single cells. There was no evidence of ARL13B labelling in adult rat tissue or isolated cells. Therefore, primary cilia do not account for any trans-sarcolemmal Ca\(^{2+}\) entry that might explain the observed Ca\(^{2+}\) transient differences between isolated myocytes and intact trabeculae. At the same time, I investigated ARL13B labelling in fixed tissue from embryonic, neonatal and young rat hearts. Sections from these hearts showed evidence of primary cilia suggesting a role for primary cilia during the developmental stages of the heart, as reported by others using electron microscopy (Willaredt et al., 2012; Koefoed et al., 2014). This chapter also investigated whether the cilia were associated with cardiac myocytes, or with other cell types present in the ECM. Confocal imaging results showed there was a close association between the labelled primary cilia and the myocytes, in accordance with studies from different species (Myklebust et al., 1977; Gerhardt et al., 2013). Primary cilia therefore contribute to signalling pathways involved in morphogenesis and maturation of the heart. Such processes are apparently no longer required in mature adult hearts.

Experiments outlined in Chapter 5 investigated the EPAC signalling pathway in isolated cardiac myocytes and trabeculae. Experiments showed that prolonged activation of EPAC had no effect on either the Ca\(^{2+}\) transients, or isometric force in multicellular cardiac trabeculae under "control" conditions of 1.5 mM [Ca\(^{2+}\)]\(_o\), 0.2 Hz stimulation, and at Troom. On the other hand, EPAC activation in isolated myocytes increased the Ca\(^{2+}\) spark frequency which was accompanied by a steady increase in the resting Ca\(^{2+}\) level throughout cpTOME exposure in isolated myocytes. This suggests that EPAC activation increased SR Ca\(^{2+}\) leak by increasing ryanodine receptors open probability. These results were supported by (Pereira et al., 2007; Cazorla et al., 2009). Given the isolated cell results, we expected Ca\(^{2+}\) handling in our multicellular trabeculae to be modulated by cpTOME exposure. Instead, EPAC activation showed a lack of response in trabeculae. This was not due either to insufficient \(\beta\)-adrenergic stimulation, or to PKA signalling masking any EPAC effect. In order to better match the experimental conditions between isolated myocytes and the trabeculae and was achieved by lowering extracellular [Ca\(^{2+}\)] to 0.5 mM. Under these depotentialized conditions, there were again no changes in either the Ca\(^{2+}\) transients, or the resting Ca\(^{2+}\). However, force was increased by EPAC activation, suggesting it altered the contractile properties by increasing the myofilament Ca\(^{2+}\) sensitivity. It is well known that
PKA-dependent phosphorylation of TnI reduces myofilament Ca\(^{2+}\) sensitivity (Hofmann et al., 1995). However, this study showed that EPAC activation increased myofilament Ca\(^{2+}\) sensitivity, suggesting a PKA-independent effect that was abolished by inhibition of CamKII. Consistent with this observation, a role for CamKII following EPAC activation of isolated cardiac myocytes has previously been reported by (Pereira et al., 2007; Cazorla et al., 2009; Oestreich et al., 2009). In conclusion, the results from multicellular trabeculae revealed an increase in myofilament Ca\(^{2+}\) sensitivity with cpTOME that was removed by CamKII inhibition by reducing the Ca\(^{2+}\) current (low \([\text{Ca}^{2+}]_o\)). Therefore, this study shows the contribution of EPAC to Ca\(^{2+}\) signalling in a CamKII-dependent manner.

6.2. Conclusions

My study has shown that myocyte Ca\(^{2+}\) handling is modulated either directly or indirectly by the ECM and its components. Ca\(^{2+}\) transients were irreversibly altered in trabeculae exposed to enzymes, as used routinely in cell isolation protocols, suggesting that important linkages (structures and proteins) were lost between the ECM and the cell membrane during the isolation process. A reduction in endothelial cells from the surface of enzyme-treated trabeculae was found, strongly implicating them as modulators of myocyte Ca\(^{2+}\) transients.

Much of our existing understanding of excitation-contraction coupling and Ca\(^{2+}\) handling for the literature has come from experiments performed on voltage-clamped, isolated myocytes. My thesis has shown that studies of Ca\(^{2+}\) handling in isolated cells show important differences in comparison to myocytes from multicellular trabeculae. They should therefore be interpreted with care.
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