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Myocardial Force and Intracellular Ca\textsuperscript{2+} in an Animal Model of Hypertensive Heart Failure

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A thesis submitted in fulfillment of the requirements for the Degree of Doctor of Philosophy

Department of Physiology
Faculty of Medical and Health Sciences,
University of Auckland
2003
Abstract

Hypertensive heart failure has long been associated with diminished cardiac contractile function, yet the underlying cellular mechanisms are not well understood. The aim of this Thesis was to investigate the relationship between intracellular calcium ([Ca$^{2+}$]$_i$) and isometric force during the relatively narrow time frame in which long-standing compensated hypertrophy progresses to decompensated end-stage heart failure in an animal model of human essential hypertension. In order to carry out this aim, left ventricular trabeculae were utilized from failing hearts of spontaneously hypertensive rats (SHR) and their normotensive Wistar-Kyoto (WKY) controls.

At a physiological stimulation frequency (5 Hz), and temperature (37 °C), the peak stress of SHR trabeculae was significantly reduced compared to WKY, although no differences in the time-course of the twitch were detected. Measurements using fura–2/AM as an index of intracellular [Ca$^{2+}$] showed that, for SHR, both the peak of the Ca$^{2+}$ transient and the resting [Ca$^{2+}$]$_i$ were increased and the decay of the Ca$^{2+}$ transient was prolonged compared to WKY.

This unexpected result, i.e., depression of twitch force despite an increased Ca$^{2+}$ transient, was investigated further by utilizing experimental protocols known to affect [Ca$^{2+}$]$_i$ and force. Varying extracellular calcium ([Ca$^{2+}$]$_o$) between 0.5 and 5 mM showed that the reduction of force development by SHR trabeculae was not associated with reduced myofilament Ca$^{2+}$ sensitivity, since, although peak [Ca$^{2+}$]$_i$ continued to increase with increasing [Ca$^{2+}$]$_o$, peak stress reached a plateau. Investigation of the force-frequency response between 0.2 and 10 Hz showed that the mismatch in peak Ca$^{2+}$ and peak force was apparent across all frequencies for SHR.

A consistent finding of studies that have made measurement of [Ca$^{2+}$]$_i$ in failing myocardium is that the decay of intracellular Ca$^{2+}$ following SR release is prolonged. Additionally, expression levels of the SR Ca$^{2+}$-ATPase have been reported as reduced, in conjunction with increased expression of the sarcolemmal Na$^+$/Ca$^{2+}$ exchanger. Although the decay of fluorescence was slower for SHR in this study, no experimental evidence was found to suggest that sarcolemmal Ca$^{2+}$ extrusion was increased in SHR in comparison to WKY. The
re-circulation fraction of activator Ca\(^{2+}\) during recovery from potentiation was not different between rat strains, indicating that SL Ca\(^{2+}\) extrusion was not increased in SHR. Additionally, the decay of fluorescence remained slower for SHR even when the SR Ca\(^{2+}\)-ATPase contribution was functionally removed. Inhibition of the SL Ca\(^{2+}\)-ATPase, together with the functional removal of the SR, removed the differences in the decay of fluorescence between rat strains. A decrease in the sarcolemmal extrusion of [Ca\(^{2+}\)]\(_i\) by the Ca\(^{2+}\)-ATPase might therefore explain the observed differences in the resting [Ca\(^{2+}\)]\(_i\) and in the amplitude of the Ca\(^{2+}\) transient between rat strains.

In summary, this study has provided the first measurements of [Ca\(^{2+}\)]\(_i\) and isometric force carried out at physiological temperature and stimulation frequency in LV trabeculae from failing SHR hearts and their age-matched, normotensive, WKY controls. Most importantly, for this animal model the contractile dysfunction typical of heart failure is not associated with reduced availability of [Ca\(^{2+}\)]\(_i\). Instead it is suggested that contractile function is compromised in these LV trabeculae by the increased collagen, and its three-dimensional organisation.
Communications

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Papers in Edited Conference Proceedings:


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University of Auckland Bioengineering Research Seminar, 8th April 2003. *Altered Ca$^{2+}$ transport fails to explain contractile dysfunction in an animal model of hypertensive heart failure.*


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<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>ANP</td>
<td>atrial natriuretic peptide</td>
</tr>
<tr>
<td>AP</td>
<td>action potential</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>ATPase</td>
<td>adenosine triphosphatase</td>
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<td>BNP</td>
<td>brain natriuretic peptide</td>
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<tr>
<td>BW</td>
<td>body weight</td>
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<td>CaM</td>
<td>calmodulin</td>
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<td>CaMKII</td>
<td>calmodulin kinase II</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCD</td>
<td>charge-coupled device</td>
</tr>
<tr>
<td>CE</td>
<td>carboxy eosin</td>
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<tr>
<td>CICR</td>
<td>calcium-induced calcium release</td>
</tr>
<tr>
<td>CNP</td>
<td>C natriuretic peptide</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>EC</td>
<td>excitation-contraction</td>
</tr>
<tr>
<td>Em</td>
<td>membrane potential</td>
</tr>
<tr>
<td>FKBP</td>
<td>FK-506 binding proteins</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>G proteins</td>
<td>heterotrimeric GTP binding proteins</td>
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<td>Gs</td>
<td>stimulatory G protein</td>
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<tr>
<td>HF</td>
<td>heart failure</td>
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<tr>
<td>ICaL</td>
<td>L-type Ca^{2+} current</td>
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<tr>
<td>INa</td>
<td>sodium current</td>
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<tr>
<td>IP3</td>
<td>inositol trisphosphate</td>
</tr>
<tr>
<td>K-H</td>
<td>Krebs-Henseleit</td>
</tr>
<tr>
<td>LV</td>
<td>left ventricle</td>
</tr>
<tr>
<td>NCX</td>
<td>sodium-calcium exchanger</td>
</tr>
<tr>
<td>P1</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>PIP2</td>
<td>phosphatidylinositol-(4,5)-biphosphate</td>
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PKA  protein kinase A
PKC  protein kinase C
PLB  phospholamban
PSR  picrosirius red
$R^2$ square of the correlation coefficient
RF  re-circulation fraction
RR  ruthenium red
RV  right ventricle
RyR  ryanodine receptor
SA  sino-atrial
SERCA sarco(endo)plasmic reticulum ATPase
SHR  spontaneously hypertensive rat
SHR-HF  SHR in heart failure
SHR-NF  non-failing SHR
SL  sarcolemmal
SR  sarcoplasmic (or sarco(endo)plasmic) reticulum
TL  tibial length
Tm  tropomyosin
TnI  inhibitory troponin
TnC  troponin C
TnT  troponin T
t-tubules  transverse tubular system
$[X]_i$ intracellular concentration of ion X
$[X]_o$ extracellular concentration of ion X
WKY  Wistar-Kyoto (control) rat
Chapter I

Background

1.1 Chapter Overview

Of the many forms of cardiovascular disease, hypertension is the most prevalent. In developed countries hypertension affects about one in five adults, and progression to hypertrophy and heart failure is commonly the end result (Kannel et al. 1972; Levy et al. 1996). In its advanced state heart failure has poor prognosis, despite modern clinical management, and inevitably leads to premature death. Although heart failure is a multi-faceted disease, with many different aetiologies and sequelae, ultimately the contractile performance of the heart is compromised to such an extent that it is no longer capable of pumping sufficient blood to meet the body’s needs. This study focuses on investigation of the cellular mechanisms that underlie the contractile dysfunction during heart failure in an animal model, the spontaneously hypertensive rat (SHR).

The cyclical contraction and relaxation essential for the heart to function as a pump is determined by the rise and fall of myocyte intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]). The first section of this Chapter provides an introduction to cardiac muscle structure and function, with the emphasis on the role of intracellular Ca\(^{2+}\) in excitation-contraction (EC) coupling. An overview of the hypertrophic response of the myocardium in response to sustained pressure overload is then given. The next section introduces the animal model of hypertrophy and failure chosen for this study, the spontaneously hypertensive rat (SHR), and its control, the Wistar Kyoto rat (WKY). The historical background to the establishment of the animal model is outlined, and the typical progression from hypertension to hypertrophy, and ultimately failure, is described. There follows a review of previous studies that have investigated cellular Ca\(^{2+}\) transport mechanisms and contractile function during heart failure, including those that have utilized the SHR as a model. Finally, the use of fluorescent indicators to measure
intracellular Ca\textsuperscript{2+} concentration is reviewed, and the isolated cardiac preparation used for this investigation is then briefly described.

1.2 Cardiac Muscle Structure and Function

The cardiac myocyte

Cardiac muscle is comprised of many different cell types, including smooth muscle, autonomic nerves, fibroblasts and myocytes. Of all these cell types it is the myocytes that are the main functional unit, since they make up the bulk of the myocardium, and contain the contractile proteins responsible for the development of force (for review see Sommer and Johnson (1979)). Cardiac myocytes are elongated cells of about 10-20 μm in width, and about 80-100 μm in length, frequently described as “rod-shaped” when enzymatically isolated. The myocytes are connected to one another at specialised areas, known as the intercalated disks (Sjöstrand et al. 1958). These areas are rich in gap junctions, so that there is both physical and electrical connectivity throughout the myocardium (e.g. Unwin and Zampighi 1980, Hoyt, Cohen et al. 1989). Some of the important cellular structures and mechanisms that determine the development of force, and subsequent relaxation, are outlined below. The focus will be on structures and mechanisms directly responsible for determining Ca\textsuperscript{2+} homeostasis. Many of the important structures characteristic of cardiac muscle are shown in Fig. 1.1. Note the regular pattern of the myofilaments, the abundance of mitochondria, the extensive sarcoplasmic reticulum (SR), and the interdigitation of cells at the intercalated disks.
Figure 1.1 Electron micrograph showing the ultrastructure of cardiac muscle in longitudinal section. The junction of two cells at the intercalated discs (Int. disc) is shown. There is an abundance of mitochondria (Mito), aligned along the length of the myofibrils. In cardiac muscle the myofibrils appear to branch and rejoin, and are of variable widths. Sarcomeres run from Z disc to Z disc, and clearly show the M lines at the centre of the A zone.
Extracellular matrix, sarcolemma and t-tubules

The extracellular matrix has an important role in supporting and aligning the myocytes and blood vessels, and maintains myocardial mass, structure and function. This network was first described by Holmgren in 1907 (cited in Caulfield and Borg 1979), and was later found to consist of extracellular collagen struts that linked myocytes with myocytes, and myocytes with capillaries (Caulfield and Borg 1979). Trans-sarcolemmal connection of the intracellular myofilaments and cytoskeleton to the extracellular matrix occur primarily at the intercalated disks, and on the lateral surfaces at focal points to a lesser extent. The anchor points between the myofibrils and the sarcolemma are known as the costameres (for review see Ervasti 2003). These form rib-like bands in the sarcolemma that overlay the Z-bands, and are made up of multiple proteins. The two major protein complexes forming the trans-sarcolemmal mechanical connection to the extracellular matrix are the integrins and the dystrophins (Stevenson et al. 1998). Essentially, these complexes link the actin filaments, via the Z-lines, to the sarcolemma and the extracellular matrix (Kaprielian et al. 2000), and are crucial to the transmission of force across the cell-to-cell junctions. Additionally, the integrins and dystrophins are able to initiate intracellular cell signaling cascades in response to haemodynamic load, and may be of importance in the hypertrophic response (for reviews see Sugden 1999, Molkentin and Dorn II 2001).

The structure of the myocardium changes with age (for review see Lakatta 2002). Whereas endothelial cells, fibroblasts and smooth muscle cells all continue to undergo mitosis throughout life, myocytes are thought to lose that ability during the perinatal period (reviewed in Soonpaa and Field 1998). It is not surprising, therefore, that changes in the relative proportions of the different cell types occur with aging. Additionally, with senescence the LV wall increases in thickness as individual myocytes increase their dimensions (Gerstenblith et al. 1977), and there is an increase in extracellular matrix collagen and fibronectin. These changes all directly affect the diastolic stiffness of the heart (Weber et al. 1994; Burgess et al. 2001). There is evidence, too, that the total number of myocytes becomes reduced with age (Anversa et al. 1990), and that the remaining myocytes exhibit stress-related phenotypic changes.
Mammalian ventricular myocytes contain an extensive system of tubular membrane invaginations, continuous with the sarcolemma. Since these were initially shown to run at right angles to the sarcolemma, they were termed the transverse-tubular system, or t-tubules (for review see Sommer and Johnson 1979). More recently significant numbers of t-tubules have been shown to run in the axial direction also, as well as transversely (Amsellem et al. 1995). A schematic diagram showing the relationship of the t-tubules to the SR and the myofilaments in cardiac muscle is shown in Fig. 1.2. The t-tubular system is continuous with the surface sarcolemmal (SL) membrane, and therefore is part of the barrier between the intracellular and extracellular environments. The extensive network of the t-tubular system enables a rapid inward spread of electrical excitation throughout the myocyte on activation (Cheng et al. 1994). Moreover, the t-tubular membrane contains more voltage-gated L-type Ca\(^{2+}\) channels per unit area of membrane than does the external sarcolemma (Brandt 1985; Wibo et al. 1991), through which rapid influx of Ca\(^{2+}\) occurs that triggers intracellular Ca\(^{2+}\) release (Cheng et al. 1994; Kawai et al. 1999). Soeller & Cannell (1999) showed that some 3.6% of rat myocyte volume is occupied by the t-tubules. Perhaps more importantly, these authors also estimated that, in rat ventricular myocytes, the t-tubules contribute a surface area of 0.44 \(\mu\text{m}^2\mu\text{m}^{-3}\), compared to a surface area of 0.24 \(\mu\text{m}^2\mu\text{m}^{-3}\) for the external sarcolemma.

Close apposition of the SL L-type Ca\(^{2+}\) channels with the SR Ca\(^{2+}\) release sites (RyRs) is crucial for effective EC coupling. In mammalian cardiac muscle these specialised areas are termed the dyads (Carl et al. 1995; Sun et al. 1995), and are located beneath both the external SL and the SL of the t-tubular system (Page 1968; Page and Surdyk-Droske 1979). Structural evidence for geometric distortion between the SL L-type calcium channels and the RyRs in ventricular myocytes from hypertrophied and failing hearts has been reported (He et al. 2001). These distortions may give rise to defective EC coupling (Gómez et al. 1997), and therefore contribute to the impaired mechanical performance characteristic of hearts in failure.
Figure 1.2 Schematic diagram of the t-tubules and the sarcoplasmic reticulum (SR) surrounding the myofibrils in mammalian cardiac muscle. In comparison to skeletal muscle, cardiac muscle has large t-tubules that penetrate between the myofibrils.
The cardiac action potential

The cardiac action potential (AP) is the initiating event for EC coupling. Propagation of the AP throughout the different regions of the heart is characterised by the membrane channel composition of the underlying region, so that, as a result, the AP waveform is altered depending on its location. In the intact heart the AP begins in the pacemaker region, which is situated at the sino-atrial (SA) node in mammals (DiFrancesco 1986). This region consists of cells with the fastest intrinsic pacemaker activity, and it determines the frequency of membrane depolarisation throughout the rest of the heart. From the SA node the AP is rapidly propagated throughout the atria, and to the atrio-ventricular node cells, where it is slowed considerably. The AP then propagates through the bundle of His, bundle branches, and Purkinje fibres, to ventricular muscle cells. (For review see (Katz 1992).)

For most excitable cells, with the exception of the SA node, it is the Na\(^+\) current (I_{Na}) that is responsible for the rapid depolarisation phase forming the upstroke of the AP. The Na\(^+\) channel is voltage-operated, with channels opening at membrane potentials (E_m) more positive to -60 mV. Opening of the Na\(^+\) channels results in an inward flow of current that rises rapidly and decreases afterwards on a much slower time-course, depending on the channel activation and inactivation kinetics. (For SA node cells it is the Ca\(^{2+}\) current that is important.)

Figure 1.3 shows the changes in membrane potential (Panel A), and the underlying currents associated with the ventricular AP (Panel B) that ultimately give rise to the transient increase in intracellular [Ca\(^{2+}\)] (Panel C) associated with myocyte contraction (redrawn from LabHEART 4.9.4 © software (Donald M. Bers, 2000). In general, the AP in cardiac muscle has five distinct phases (for review see Katz 1992): Phase 0 forms the rapid upstroke, corresponding to depolarisation of the sarcolemma, and is determined by an inward Na\(^+\) current (I_{Na}). Reversal of the membrane potential commences at the peak of the AP, where K\(^+\) permeability is low and Na\(^+\) permeability is high. Termination of the inward Na\(^+\) current is brought about by inactivation of the Na\(^+\) channels, and is controlled by the membrane potential. Phase 1 represents a brief, and rapid, repolarisation, known as the early repolarisation phase. It occurs at the peak of the AP. During Phase 1 there is a fall in Na\(^+\) permeability, and the appearance of two outward currents, a transient K\(^+\) current (I_{to}) and a transient Ca\(^{2+}\)-activated Cl\(^-\) current (I_{Cl}, not shown in Fig. 1.3). Both of these currents
Figure 1.3 The cardiac action potential. A, ventricular action potential, B, major ionic fluxes, and C, intracellular $[Ca^{2+}]$ obtained using the interactive LabHEART (copyright Donald Bers 2000) software package.
influence the duration of the AP, and vary between regions of the heart. Phase 2 is the distinctive plateau phase characteristic of the cardiac AP. It is this phase that is responsible for the duration of the AP, and differs in extent among myocytes depending on their function. Once the initial depolarisation reaches the threshold for Ca\textsuperscript{2+} channel opening, a slow inward Ca\textsuperscript{2+} current appears (I_{CaL}). This Ca\textsuperscript{2+} current is due to the opening of the dihydropyridine sensitive L-type Ca\textsuperscript{2+} channel ("L"-type providing a "lasting" current), and maintains the membrane in a depolarised state during the plateau phase. T-type Ca\textsuperscript{2+} channels also contribute to I_{Ca} (Sipido et al. 1998), but their role is less clear, and they are not thought to have a major role in EC coupling. In ventricular myocytes this plateau phase is longer than in atrial myocytes. Under normal conditions it is the inward L-type Ca\textsuperscript{2+} current that produces the "trigger" Ca\textsuperscript{2+} essential for EC coupling (Grantham and Cannell 1996; Sipido et al. 1998).

There is outward current also at this time, carried by delayed rectifier K\textsuperscript{+} currents. These K\textsuperscript{+} currents are responsive to membrane potential, and their kinetics also influence the duration of the plateau phase. Phase 3, repolarisation, occurs with the outward flux of K\textsuperscript{+} ions through K\textsuperscript{+}-selective membrane channels. I_{K1} is a major current contributing to the late repolarisation of the AP at this time.

Phase 4 represents the resting membrane potential. For the pacemaker cells of the heart, there is a slow spontaneous depolarisation during this phase of the AP (DiFrancesco 1986), but this does not normally occur for cells from other regions. The various ion fluxes that occur during the cardiac AP represent the movement of ions down their electrochemical gradients, and are not accompanied by the flux of a counter ion. The balance of Na\textsuperscript{+}, K\textsuperscript{+}, Ca\textsuperscript{2+} and Cl\textsuperscript{-} ions across the sarcolemma is maintained by the energy expenditure of various ion pumps and (indirectly) exchangers.

The magnitude and the duration of the AP are dependent on the balance of depolarising and repolarising currents (see above), especially those contributing to the plateau phase. A consistent finding of studies that have recorded the AP during cardiac hypertrophy and failure is that its duration is prolonged (Brooksby et al. 1993; Barbieri et al. 1994; Cerbai et al. 1994) (for reviews see Hart 1994, Nabauer and Kaab 1998, Wickenden, Kaprielian et al. 1998). In principle, any increase in inward, or decrease in outward, current during the AP plateau will increase duration. The most prominent of these plateau currents is the I_{CaL}, but this has been reported as either unaltered (in human (Beuckelmann et al. 1992), and in rat (Brooksby et al.
1993; Cerbai et al. 1994; Gómez et al. 1997), or even decreased (for a reviews see Hart 1994, Mukherjee and Spinale 1998) during cardiac hypertrophy or failure. Currents that effect the early repolarisation (phase 1) of the AP strongly influence the influx of Ca$^{2+}$ via L-type Ca$^{2+}$ channels and Na$^{+}$/Ca$^{2+}$-exchange and, therefore, SR Ca$^{2+}$ release. Reductions in I$_{to}$ and I$_{K1}$ have been reported as the currents responsible for the increased AP duration with heart failure (for review see Nabauer and Kaab 1998, Wickenden, Kaprielian et al. 1998). However, a diversity of K$^{+}$ channels exists, with different expression, and different densities between myocardial regions and species. Similar phenotypic prolongations of the AP in cardiac hypertrophy or failure might therefore involve alterations to different AP currents, depending on the particular model of hypertrophy and failure.

Sarcoplasmic reticulum and CICR

The SR is an intracellular membrane-bounded compartment, responsible for sequestration, storage and release of cytosolic Ca$^{2+}$ in all muscle (Ebashi and Lipmann 1962). It is comprised of three distinct interconnected regions, the longitudinal, the corbular, and the junctional SR. Sequestration of cytosolic Ca$^{2+}$ via the SR Ca$^{2+}$-ATPase is thought to occur throughout each of these three regions, whereas Ca$^{2+}$ release occurs primarily at the terminal cisternae of the junctional SR (Jorgensen et al. 1993). The specialised Ca$^{2+}$ release sites, the ryanodine receptors (RyR), lie in close proximity to the SL L-type Ca$^{2+}$ channels (Wibo et al. 1991) (see above). Storage and release of SR Ca$^{2+}$ is controlled by a group of proteins at the junctional SR. The RyR is complexed in cardiac muscle with the FK506-binding protein (FKBP), calsequestrin, triadin 1, and junctin, as well as other proteins (Zhang et al. 1997). Gating of isolated cardiac RyR can be modulated by CaM-dependent phosphorylation (see below), cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) activation (Valdivia et al. 1995) and FKBP, whereas calsequestrin is a high capacity Ca$^{2+}$ binding protein that is contained within the lumen of the SR. Both triadin and junctin appear to anchor calsequestrin to the SR junctional membrane (Müller et al. 2002). Defects in SR Ca$^{2+}$ release, associated with RyRs, may play a role in the pathophysiology of heart failure (for review see Marks 2000), and could contribute to the impaired contractility.
The SL influx of Ca$^{2+}$ via the L-type Ca$^{2+}$ channels, $I_{Ca,L}$, brings about release of Ca$^{2+}$ from the SR in a process termed calcium-induced-calcium-release (CICR). This process was first described for skinned cardiac myocytes by Fabiato & Fabiato (1973). Later, Fabiato (1985) proposed a model to explain the process further. In this model, Ca$^{2+}$ entry via the L-type Ca$^{2+}$ channels binds first to a low affinity site on the RyR, with a high association rate constant. The channel then opens, and SR Ca$^{2+}$ release gives rise to the Ca$^{2+}$ transient. Subsequently, with continued Ca$^{2+}$ availability, Ca$^{2+}$ binds to a second site on the RyR with a high affinity for Ca$^{2+}$, but a low association rate constant. Inactivation of the RyR occurs when Ca$^{2+}$ binds to the site of slower binding, thereby closing the channel. Removal of inactivation was the slowest step of the cycle. Further experiments carried out in a number of different studies (e.g. Cannell, Berlin et al. 1987, Beuckelmann and Weir 1988, Nabauer, Callewaert et al. 1989) have shown that sarcolemmal Ca$^{2+}$ entry appears to be an absolute requirement for the release of SR Ca$^{2+}$ in cardiac muscle under physiological conditions. CICR is extremely rapid ($\ll \) 2 ms (Cheng et al. 1994)), and increases the cytosolic Ca$^{2+}$ concentration approximately ten-fold, from $\sim$100 nM to $\sim$1 $\mu$M. Release of SR Ca$^{2+}$ is altered by both SR Ca$^{2+}$ load, and by influx via $I_{Ca,L}$ (Bassani et al. 1995).

**Sarcolemmal L-type Ca$^{2+}$ channels**

L-type Ca$^{2+}$ channels are present in all myocytes, and are responsible for the bulk of the SL Ca$^{2+}$ entry. Activation of the channels depends primarily on $E_m$, with channels opening at $E_m$ positive to $-40$ mV under physiological conditions. Inactivation of the channels is time-, $E_m$-, and [Ca$^{2+}$]- dependent (Lee et al. 1985; Hadley and Lederer 1991). The [Ca$^{2+}$]- dependent inactivation is sensitive to the sub-sarcolemmal [Ca$^{2+}$]$_i$ adjacent to the channel, and is therefore influenced by both $I_{Ca,L}$ and SR Ca$^{2+}$ release. This [Ca$^{2+}$]$_i$-dependent inactivation therefore provides a feedback mechanism that limits the amount of SL Ca$^{2+}$ entry (for review see Anderson 2001).

It has been shown that CaM is the Ca$^{2+}$ sensor for Ca$^{2+}$-dependent inactivation of Ca$^{2+}$ channels (Peterson et al. 1999). CaM is an essential signaling messenger for almost all cell types, and is an important regulatory protein of EC coupling. Both the L-type Ca$^{2+}$ channel, and the RyR have CaM binding sites on their cytosolic domains, and it is thought that Ca$^{2+}$-CaM modulation is an important phenomenon of ion channels in general. When the myocyte
is in the resting state ($E_m \approx -80 \text{ mV}$), the $Ca^{2+}$ channel is closed, and CaM is bound to the cytosolic side of the channel. Upon depolarisation the channel opens and influx of $Ca^{2+}$ occurs. As $Ca^{2+}$ enters it binds to a high affinity site on the carboxy-lobe of CaM; this, in turn, causes a conformational change to the $Ca^{2+}$-CaM, and channel inactivation. Additionally, $Ca^{2+}$ entry has been shown to facilitate $I_{\text{Cal}}$ independently of the $Ca^{2+}$-dependent channel inactivation above. Facilitation occurs via an effect of $Ca^{2+}$ on CaM kinase II (CaMKII) (Maier and Bers 2002) that progressively slows $Ca^{2+}$-dependent inactivation. Calcium-dependent facilitation is mediated by phosphorylation of CaMKII (Yuan and Bers 1994), resulting in longer single channel openings (Wu et al. 2001).

Beta-adrenergic stimulation is another important mechanism for activation of $I_{\text{Cal}}$ (e.g. Reuter 1967). Agonist binding to the receptor activates a GTP binding protein (G$_i$) that then stimulates adenylate cyclase to produce cAMP. The resulting activation of PKA leads to phosphorylation of a number of key proteins involved with EC coupling (for review see Bers 2001), including the L-type $Ca^{2+}$ channel.

**SR $Ca^{2+}$-ATPase**

Restoration of $[Ca^{2+}]_i$ to its resting value following SR release is brought about by a number of $Ca^{2+}$ transport mechanisms (see Chapter V for a more detailed account), with the SR $Ca^{2+}$-ATPase responsible for 70-85% of $Ca^{2+}$ removal from the cytosol. This ATPase has been sequenced and cloned, and the molecular structure determined (MacLennan et al. 1992; MacLennan and Green 2000). It is a member of the same ion family of transporters as the SL $Na^+/K^+$-ATPase, and the SL $Ca^{2+}$-ATPase. At least three isoforms of the SR $Ca^{2+}$-ATPase have been identified to date, with cardiac muscle, and slow twitch skeletal muscle, containing the SERCA2a isoform. The transport reaction starts with binding of one ATP and two $Ca^{2+}$ to high affinity $Ca^{2+}$ binding sites on the cytoplasmic side. ATP hydrolysis then occurs, and the $Ca^{2+}$ affinity of the ATPase changes, such that $Ca^{2+}$ is then released to the internal lumen of the SR where $[Ca^{2+}]$ is much higher. The pump consumes one ATP for two $Ca^{2+}$ transported (Tada and Katz 1982).
The SR Ca\(^{2+}\)-ATPase is closely associated with the regulatory protein phospholamban. Phospholamban exerts an inhibitory effect on the SR Ca\(^{2+}\)-ATPase, slowing the kinetics of the SR Ca\(^{2+}\)-ATPase once Ca\(^{2+}\) is bound. Phosphorylation of phospholamban removes this inhibitory effect (Tada et al. 1983), enabling the Ca\(^{2+}\) transport rate of the SR Ca\(^{2+}\)-ATPase to increase at least four-fold. The mechanism of the increased Ca\(^{2+}\) transport rate appears to be via an increase in the affinity of the ATPase for Ca\(^{2+}\), rather than in an alteration in the maximal rate of Ca\(^{2+}\) transport. It has been shown that the [Ca\(^{2+}\)] required for half-maximal activation of the Ca\(^{2+}\) pump is reduced by \(-50\%\) after cAMP dependent phosphorylation of phospholamban (Hicks et al. 1979). Phosphorylation of phospholamban by cAMP-dependent, and Ca\(^{2+}\)-CaM-dependent, protein kinases is also the mechanism by which catecholamines increase SR Ca\(^{2+}\) uptake (Li et al. 2000) (for review see Bers 2001). In heart failure the regulation of phospholamban phosphorylation by cAMP-dependent pathways has been reported as altered (Huang et al. 1999; Schwinger et al. 1999), leading to a slowing of the decay of the Ca\(^{2+}\) transient.

The SR contains the low affinity, high capacity, Ca\(^{2+}\) binding protein calsequestrin (see above). This protein is the major SR Ca\(^{2+}\)-binding protein for both cardiac and skeletal muscle, and is concentrated in the terminal cisternae (MacLennan and Wong 1971). Once Ca\(^{2+}\) is transported into the SR, the binding to calsequestrin then acts to reduce the Ca\(^{2+}\) gradient across the SR membrane, thereby allowing the SR ATPase to transport a greater quantity of Ca\(^{2+}\) than the gradient would otherwise permit. Calsequestrin is therefore essential to the Ca\(^{2+}\) buffering, or storage, capacity of the SR. A reduced SR Ca\(^{2+}\) storage capacity may have a role in the patho-physiology of heart disease. Whilst phosphorylation of phospholamban increases the affinity of the ATPase for Ca\(^{2+}\), a reduction in the amount of SR calsequestrin might act to reduce the rate, as well as the extent, of Ca\(^{2+}\) uptake.

As re-sequestration of Ca\(^{2+}\) into the SR progresses the rate of uptake by the Ca\(^{2+}\)-ATPase decreases, so that by the time resting levels of [Ca\(^{2+}\)], are achieved the relative contribution of the SR ATPase to further reduction in [Ca\(^{2+}\)], is low, and the SL transport mechanisms then dominate (Lamont and Eisner 1996).
The sarcolemmal transport of Ca$^{2+}$

For steady-state conditions to prevail in the myocyte, on a beat to beat basis, there needs to be an efflux of Ca$^{2+}$ across the SL in the resting interval that exactly matches the amount of trigger Ca$^{2+}$ entering via the L-type Ca$^{2+}$ channels. The principal SL mechanism responsible for this Ca$^{2+}$ efflux is the Na$^+$/Ca$^{2+}$-exchanger (Reuter 1974; Crespo et al. 1990; Yao et al. 1997), with a smaller contribution from the SL Ca$^{2+}$-ATPase (Bers and Bridge 1989; Choi and Eisner 1999; Choi et al. 2000). The Na$^+$/Ca$^{2+}$-exchanger can move Ca$^{2+}$ either into, or out of, the cell, depending on the net electrochemical driving force. These ion fluxes thus contribute to SL current. It has also been suggested that the exchanger may briefly operate in reverse mode to bring Ca$^{2+}$ into the cell during the early phase of the AP, when the sub-sarcolemmal [Na$^+$], is likely to be high (Mullins 1979; Shattock and Bers 1989). Depolarisation causes $E_m$ to become more positive to the reversal potential for the exchanger ($E_{Na/Ca}$), and $I_{Na/Ca}$ reverses (i.e. outward current) due to the influx of Ca$^{2+}$ in exchange for Na$^+$. In this way the exchanger contributes to the total trigger Ca$^{2+}$ thereby influencing SR Ca$^{2+}$ release (Leblanc and Hume 1990; Wasserstrom and Vites 1996). As [Ca$^{2+}$]$_i$ increases, $E_{Na/Ca}$ shifts to more positive potentials, thus favouring extrusion of Ca$^{2+}$ as repolarisation progresses. The net $I_{Na/Ca}$ during the AP therefore depends on both the duration of the plateau phase of the AP (Crespo et al. 1990), and on the time-course of the Ca$^{2+}$ transient. For review see Bers (2001).

The reversal potential for the exchanger, where Ca$^{2+}$ extrusion (at rest) becomes Ca$^{2+}$ entry, is between -10 and -50 mV in cardiac muscle (for [Na$^+$], between 8 and 14 mM, [Na$^+$]$_o$ of 145 mM, [Ca$^{2+}$]$_i$ around 100 nM, and [Ca$^{2+}$]$_o$ of 1 mM), [Na$^+$]-dependent Ca$^{2+}$-influx occurs when membrane potentials become more positive to $E_{Na/Ca}$. Calcium entry by this mechanism alone is claimed by some to be of sufficient magnitude to trigger SR Ca$^{2+}$ release (Lipp and Niggli 1994), but this is refuted by others (Sham et al. 1992). Many of the experiments investigating a possible role for reverse mode exchange in EC coupling have been carried out under voltage-clamp. These experiments typically involve extensive modification of ion concentrations, and addition of membrane channel blockers in the solutions, so that it is difficult to conclude that reverse mode exchange necessarily has a role physiologically. For an extensive review of Na$^+$/Ca$^{2+}$ exchange, and its physiological implications, see Blaustein & Lederer (1999).
Although the Na\textsuperscript+/Ca\textsuperscript{2+}-exchanger can operate in both forward and reverse modes, it is principally a Ca\textsuperscript{2+} extrusion mechanism (Crespo et al. 1990; Grantham and Cannell 1996). For every Ca\textsuperscript{2+} ion there are 3 Na\textsuperscript{+} ions exchanged (Kimura et al. 1986; Mechmann and Pott 1986; Crespo et al. 1990; Hilgemann 1990; Hinata et al. 2002) (but see Fujioka, Komeda et al. 2000, Dong, Dunn et al. 2002). Since there is a gain of 1 positive charge in the direction of the Na\textsuperscript{+} movement for every cycle, the exchanger is electrogenic. The net direction in which the exchanger moves Ca\textsuperscript{2+} is therefore determined by the Na\textsuperscript{+} gradient, the Ca\textsuperscript{2+} gradient and the membrane potential.

Compared to the SL Ca\textsuperscript{2+}-ATPase, the exchanger has a much lower affinity for Ca\textsuperscript{2+}, but a much higher turnover rate. This ability of the exchanger to transport Ca\textsuperscript{2+} rapidly is particularly important in cardiac muscle for returning the cytosolic [Ca\textsuperscript{2+}] to resting levels following contraction, allowing relaxation to occur. Because of the importance of the Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger as the principal mechanism responsible for SL efflux of Ca\textsuperscript{2+} it has a critical role in determining resting [Ca\textsuperscript{2+}], levels, and the SR Ca\textsuperscript{2+} load (Zhang et al. 2001). Inhibition of Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchange can lead to an overall increase in the SR Ca\textsuperscript{2+} load, or the development of calcium overload.

Since both resting [Ca\textsuperscript{2+}], level, and SR Ca\textsuperscript{2+} load are frequently altered under pathological conditions it is likely that abnormal Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange may be implicated in some disease states. Indeed, a number of studies have linked alterations in Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange with cardiac arrhythmias and heart failure (for a review see Pogwizd and Bers 2002). Expression of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger has been reported as increased in hypertrophy (Pogwizd et al. 2001), and heart failure (Studer et al. 1994; Hobai and O’Rourke 2000) (but see Schwinger, Wang et al. 1999). An increase in Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger activity would shift the balance of cytosolic Ca\textsuperscript{2+} clearance from SR uptake to SL extrusion. Ultimately this could lead to depletion of SR Ca\textsuperscript{2+} stores, compromising systolic function (Schillinger et al. 2000).

A second SL Ca\textsuperscript{2+} extrusion mechanism, the SL Ca\textsuperscript{2+}-ATPase, has been demonstrated in cardiac muscle (Caroni and Carafoli 1980) with a stoichiometry of 1 Ca\textsuperscript{2+} extruded to 1 ATP hydrolysed (Caroni et al. 1983). The role of the SL Ca\textsuperscript{2+}-ATPase in maintaining [Ca\textsuperscript{2+}], has been investigated in rat myocardium (Negretti et al. 1993; Choi and Eisner 1999). Choi & Eisner (1999) showed that when a specific blocker of the SL Ca\textsuperscript{2+}-ATPase was used there was an increase in the resting [Ca\textsuperscript{2+}], and that the magnitude of the systolic Ca\textsuperscript{2+} transient was
increased while its decay was slowed. They concluded that the SL Ca\(^{2+}\)-ATPase had an important role in the control of the resting [Ca\(^{2+}\)], and that it accounted for about 25% of the SL Ca\(^{2+}\) extrusion following activation (the other 75% being via the Na\(^{+}\)/Ca\(^{2+}\)-exchanger).

A summary of the Ca\(^{2+}\) fluxes associated with EC coupling is shown in Fig. 1.4 for a schematic diagram of a myocyte. In this figure the sequence of events is numbered, beginning with the spread of the AP across the sarcolemma (1), and ending with the return of cytosolic Ca\(^{2+}\) to resting levels following triggered SR release. Besides the SR Ca\(^{2+}\)-ATPase (4), and the SL Ca\(^{2+}\) extrusion (5) mechanisms for reducing [Ca\(^{2+}\)], following SR release, there are a number of important buffers of intracellular Ca\(^{2+}\) that also contribute to overall maintenance of Ca\(^{2+}\) homeostasis (e.g. mitochondria (6)).
Figure 1.4 Schematic diagram of a ventricular myocyte summarising Ca^{2+} cycling during EC coupling. During the cardiac AP (1) entry of Ca^{2+} via L-type Ca^{2+} channels triggers SR Ca^{2+} release via the RyR (2). The increased intracellular [Ca^{2+}] then activates the myofilaments (3), initiating cross-bridge cycling and force development. Re-uptake of Ca^{2+} into the SR via the SR Ca^{2+}-ATPase (4), and SL Ca^{2+} extrusion via the Na^+/Ca^{2+}-exchanger and the SL Ca^{2+}-ATPase (5), reduce the [Ca^{2+}], again to resting levels allowing relaxation to take place. Mitochondria (6) also play a role in the intracellular Ca^{2+} fluxes.
Contractile proteins

The myofilaments make up the contractile machinery of the myocyte, and occupy 45-60% of the cell's volume. They consist primarily of thick and thin filaments that interdigitate to form the sarcomeres, the contractile unit of the myocytes (for review see Bers 2001). Sarcomeres are bounded at either end by the Z-lines. The thin filaments meet at the Z-line, and the thick filaments extend in both directions from the M-line at the centre of the sarcomeres (see Fig. 1.1). A third type of myofilament protein in vertebrate striated muscle is the giant elastic protein titin (Granzier and Irving 1995) (also known as connectin). One molecule of this protein spans half a sarcomere, from the Z-line to the M-line, and acts like a giant spring (Linke and Granzier 1998), providing the relaxed myofibrils with elasticity. A number of structural proteins that form connections with the sarcolemma are also associated with the Z-lines. In this way the sarcomeres are anchored into position within the myocytes (see above), and myocyte force development is in the longitudinal direction.

The thick filaments are made up predominantly of myosin (M, 480,000 Da) consisting of two polypeptide chains. The myosin globular head contains the ATP hydrolysis site associated with the cross-bridge cycle. The term cross-bridge applies to the myosin head, whether attached to the actin-binding site, or not.

The thin filaments consist of a backbone made up of two helical strands of the globular protein G-actin, forming the helical filamentous F-actin polymer. Tropomyosin (Tm) lies between the two F-actin polymers, spanning seven of the actin monomers. At one end of the Tm there is an attached troponin complex. This is made up of three subunits: the Tm binding subunit, troponin T (TnT), the Ca$^{2+}$ binding subunit, troponin C (TnC), and the inhibitory subunit, also bound to Tm, troponin I (TnI). Contraction is initiated by a transient increase in cytoplasmic [Ca$^{2+}$], and the subsequent binding of Ca$^{2+}$ to TnC. This removes the inhibitory effects of TnI, allowing actin to bind to myosin, thereby enhancing the ATPase activity of the latter, and initiating the cross-bridge cycle. Cardiac TnC contains three metal binding sites (Holroyde et al. 1980), two of which bind either Ca$^{2+}$ or Mg$^{2+}$ with high affinity, and a third site that is able to bind Ca$^{2+}$ rapidly enough for it to regulate the onset of the cross-bridge cycle. The high affinity sites are thought that they play a role in anchoring the TnC to TnI, rather than initiating the cross-bridge cycle. (For review see (Solaro and Rarick 1998).)
Cross-bridge cycle

The molecular events associated with the cross-bridge cycle can be summarised as follows. During diastole a large number of the cross-bridges are blocked from attaching to the actin binding sites by the position of Tm. On myocyte activation, release of SR Ca\(^{2+}\) increases the cytosolic [Ca\(^{2+}\)] approximately tenfold. Ca\(^{2+}\) binds to the regulatory site on TnC, and conformational changes then occur between Tm and actin proteins. These changes release the blocked state, and promote the transition of weakly bound cross-bridges to the more tightly bound force generating state. Cardiac muscle cross-bridges also exhibit cooperativity, even though cardiac TnC has only one Ca\(^{2+}\)-specific binding site. Cooperative activation is dependent upon thin filament molecular interactions, and the bound cross-bridges, such that the greater the number of force generating cross-bridges the greater the ease with which further cross-bridges bind (Allen and Kentish 1985; Smith and Fuchs 2000). Such a mechanism has an energetic advantage, in that the Ca\(^{2+}\) signal is effectively amplified and the energetic cost of SR Ca\(^{2+}\) transport is reduced.

The contraction of muscle is described by the sliding filament theory (Huxley and Niedergerke 1954; Huxley and Hanson 1954). It is now known that the motive force between filaments is produced by a swinging cross-bridge whose ability to link chemical reactions to mechanical changes was conceptualised by Huxley (1969), and Huxley and Simmons (1971). Biochemical models have also been used to understand the cross-bridge cycle (for review see Geeves (1991), Geeves and Holmes (1999)). These models are based on the notion that there are several states in which actin interacts with myosin, each of which is reversible. At rest myosin (M) is mostly complexed with adenosine triphosphate (ATP), or with adenosine diphosphate and inorganic phosphate (ADP•P\(_i\)), where the ATP has been hydrolysed, but the associated energy has not yet been used. When Ca\(^{2+}\) binds to TnC, the interaction of the myosin complex with actin occurs. Inorganic phosphate (P\(_i\)) is released from the ADP•P\(_i\) complex, and the energetic states associated with the power stroke, and the swivelling of the attached myosin head, follow. The affinity of myosin for actin increases with these steps, until the binding of ATP completes the cycle again with the dissociation of myosin from actin.

The rate of each of the above reactions depends on the contractile protein isoforms present. During cardiac hypertrophy and failure a change in myosin subunit expression has been
identified (Mercadier et al. 1981; Bing et al. 1991; Boluyt et al. 1994) (and reviewed in Schaub, Hefti et al. 1998). The relative portion of the fast (α) isoform to the slow (β) isoform is altered, such that the kinetics of force generation and relaxation is slower. Slower kinetics is thought to improve the economy of the chemo-mechanical transduction process per ATP utilised. The rate of cross-bridge cycling is also affected in vivo by the concentration of intracellular ions such as H+ and inorganic phosphate.

1.3 Hypertension, Hypertrophy and Heart Failure

Hypertension

In healthy individuals blood pressure is a regulated variable, with arterial systolic pressure at rest typically between 100 and 120 mmHg. In some individuals, however, blood pressure is consistently elevated above the accepted “normal” level, increasing the risk of heart failure. The American Heart Association (http://www.americanheart.org) considers a person to be hypertensive when their systolic blood pressure is 140 mmHg or above, and/or their diastolic pressure is ≥ 90 mmHg (as well as including those that rely on anti-hypertensive medication to maintain normal blood pressure levels). The incidence, origin, and sequelae of hypertension vary between individuals. A number of signal transduction pathways are activated early in the disease, and they act initially to enhance contractile function. In the absence of intervention, however, there is an inevitable progression of the disease from compensated hypertrophy to heart failure.

The volume of blood ejected from the ventricles during systole is dependent not only on the force of contraction, but also on the after-load, i.e. the load against which the blood must be ejected. In hypertension, the pressure in the systemic circulation is consistently elevated above normal. The ventricles therefore must develop sufficient force during systole to eject the ventricular volume against the increased after-load. Failure to do so results in incomplete emptying during systole, increasing the pre-load (i.e. the volume of blood in the ventricle at the end of diastole) and, in turn, increasing the ventricular wall tension during diastole. In
healthy myocardium, sympathetic stimulation, and subsequent release of catecholamines, produces an increase in both the force, and the frequency, of contraction. In this way preload is again reduced to normal levels.

The Frank-Starling mechanism also ensures that when venous return is increased (i.e. when there is increased ventricular filling during diastole) there is a concomitant increase in the volume of blood ejected during systole. In this way cardiac output is matched to venous return. At the cellular level, the Frank-Starling relationship is explained, in part, by the length-force relationship of striated muscle (Gordon et al. 1966; ter Keurs et al. 1978; ter Keurs et al. 1980). Increased pre-load gives rise to increased cell (and therefore sarcomere) length in the ventricular walls. As sarcomere length increases over the physiological range (~1.7-2.3 μm), force development also increases (Allen et al. 1974). This results primarily from an increased sensitivity of the myofilaments to Ca$^{2+}$ with increasing length (Allen and Kentish 1985; Kentish et al. 1986; Akella et al. 1997) brought about by an increase in the affinity of Ca$^{2+}$ for the regulatory site on the TnC subunit.

The influence of the sympathetic nervous system

Sympathetic stimulation is the principal physiological means by which the inotropic, lusitropic, and chronotropic states of the heart are altered. Sympathetic nerve endings and adrenergic receptors are widely distributed throughout the myocardium, and the interaction of catecholamines with both the α- and β-adrenergic receptors triggers many cellular mechanisms that directly enhance contractility in healthy myocardium (for review see Bers 2001). Membrane depolarisation in the presence of a β-agonist increases the amplitude of the Ca$^{2+}$ transient, and decreases its time-course (Spurgeon et al. 1990). PKA activation also enhances the contractile response (Hoh et al. 1988), but not to the same extent as the transient, since PKA both decreases the myofilament Ca$^{2+}$ sensitivity (by its phosphorylation of TnI) and produces an increase in the off-rate of Ca$^{2+}$ for TnC (Hoh et al. 1988; Okazaki et al. 1990) (thereby increasing the rate of relaxation).

Inotropy is also modulated by α-adrenergic receptors in cardiac muscle (Capogrossi et al. 1991; O'Rourke et al. 1992). Activation of the α-adrenergic receptors is less important in
modulating short-term cardiac function, but plays a major role in the development of the hypertrophic response in cardiac myocytes (Long et al. 1989).

Increased levels of circulating catecholamines are found with hypertrophy and failure (for reviews see Anker 1998, Ferrari, Ceconi et al. 1998), but down-regulation and loss of cell surface β-adrenergic receptors reduce the myocardial responsiveness with heart failure (Bristow et al. 1982). Sympathetic nerve activity is one clearly identifiable extrinsic factor associated with the regulation of cardiac remodeling (see below). Experimentally, exposure to increased levels of noradrenaline in vitro stimulates the growth of cardiac myocytes, and increases the expression of many foetal genes (Feldman et al. 1993).

These two compensatory mechanisms, the response to ventricular dilatation and sympathetic activation, involve different, but complementary Ca\(^{2+}\)-dependent inotropic pathways. Sympathetic activation increases the delivery of Ca\(^{2+}\) to the myofilaments (Spurgeon et al. 1990; Capogrossi et al. 1991; O'Rourke et al. 1992), whereas ventricular dilatation enhances sensitivity of the myofilaments to Ca\(^{2+}\) (Allen and Kentish 1985). Both mechanisms result from an increase in the internal stress on the ventricular walls during diastole.

Myocardial Remodeling

Central to the progression to heart failure is the remodeling of the myocardium that occurs in response to a number of factors associated with the pathophysiology. These factors include mechanical stress due to pressure (or volume) overload, increased sympathetic activity, and increased levels of angiotensin. Important alterations in the structure and function of the myocardium occur as a result of these factors. Ventricular hypertrophy, myocyte apoptosis, alterations in the myocardial phenotype, and changes in the quantity and composition of the extracellular matrix all develop with time (for review see Wilson and Colucci 1997). More recently, other mechanisms have been suggested as mediators of remodeling, such as oxidative stress, inflammatory cytokines, nitric oxide, endothelin and peptide growth factor.

At the molecular level, remodeling can be divided into two distinct responses, those adaptive responses that act to improve cardiac economy, such as the re-expression of foetal genes in
response to mechanical overload, and those responses that produce phenotypic alterations, such as fibrosis and apoptosis. In general the phenotypic alterations are detrimental to cardiac function.

When the myocardium is exposed to a variety of neurohumoral factors, or to sustained increases in ventricular wall tension, there is an adaptive response to increase the muscle mass of the ventricles. This is achieved by initiation of a complex hypertrophic response, involving a number of intracellular signaling pathways, such that individual myocytes undergo hypertrophic growth. (For a detailed review of the signaling pathways that regulate cardiac hypertrophy see Molkentin & Dorn II (2001).) Development of the hypertrophic response can also be initiated by stimuli other than sustained pressure or volume overload. These factors include the catecholamines, insulin-like growth factor, endothelin, angiotensin II and others (for review see Wilson and Colucci 1997).

The natriuretic peptides, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP), are important in the maintenance of body fluid balance and electrolyte homeostasis. Their regulation and action are summarised in Fig. 1.5. These peptides act on specific cell receptors to cause natriuresis, diuresis and vasodilation, thereby counteracting the effects of the renin-angiotensin-aldosterone system (see below), and protecting against fluid overload, hypertension and excessive vasoconstriction. When cardiac function is normal the plasma levels of ANP are about six times that of BNP, but a sustained increase in wall stress leads to increased secretion of ANP and BNP, with the plasma levels of BNP very much more increased than those of ANP (Yoshibayashi et al. 1996). The plasma concentrations of natriuretic peptides are potentially useful as an indicator of the clinical severity of heart failure (Davidson et al. 1996; Yamamoto et al. 1996).
Figure 1.5 The regulation and action of natriuretic peptides. Modified from Cogan (1991). ADH, antidiuretic hormone. GFR, glomerular filtration rate.
Figure 1.6 A summary of the regulation and actions of the renin-angiotensin system. The flow diagram illustrates the importance of this system in the development of hypertension. Modified from Cogan (1991). ADH, antidiuretic hormone. GFR, glomerular filtration rate.
As well as the fast-responding natriuretic peptide system, there are also much slower systems involved in cardiovascular homeostasis. These include those based on the response of the autonomic or central nervous system, and the renin-angiotensin-aldosterone system. The sequence of events following secretion of renin by the kidneys in response to a fall in arterial pressure, or in response to a decrease in [Na\(^+\)] in the distal tubule, is summarised in Fig. 1.6. Normally this system is the major long-term regulator of both arterial blood pressure and electrolyte balance (for review see Laragh and Sealey 1973). Elevated plasma renin is a common finding in heart failure (Kluger et al. 1982; Levine et al. 1982).

The natriuretic peptides, and the renin-angiotensin-aldosterone system therefore have opposite effects (see Figs 1.5 & 1.6). Secretion of the natriuretic peptides is modulated by distension of the chamber walls of the heart, and by high intraventricular pressures, whereas activation of the renin-angiotensin-aldosterone system follows a decrease in plasma volume, and a fall in renal perfusion. In addition, the natriuretic peptides enhance natriuresis, lower blood pressure and decrease plasma volume, whereas the renin-angiotensin-aldosterone system acts to maintain plasma volume and arterial pressure (for a review see Richards (1996)).

**Loss of compensatory mechanisms and progression to end-stage heart failure**

Continuous exposure to increased arterial pressure eventually leads to the loss of endogenous compensatory mechanisms initiated in the cardiac stress response (for review see Dubus, Samuel et al. 1993). The hypertrophic response is compromised, and the ability of the heart to normalise wall stress is restricted. Loss of the load-reducing compensatory mechanisms of the heart increases the dependence on endogenous inotropic processes to maintain cardiac function. But long-term activation of these functions also leads to their loss of effectiveness on contractility. The contractile response to increased ventricular volume is lost, and the positive inotropic effects of the endogenous and exogenous catecholamines are diminished. There is down-regulation of β-receptors (mainly β-1) (DiPaola et al. 2001), and uncoupling from their effector enzyme, adenylate cyclase. In the failing heart, the ventricles are resistant to increases in preload, and are highly susceptible to increases in afterload. Although the responsiveness of the failing heart to β-adrenergic stimuli is reduced (Bristow et al. 1982), this is offset in part by an increase in the responsiveness of the peripheral blood vessels to α-
1.4 Animal Models of Hypertension and Heart Failure

Investigation of the patho-physiology of human heart failure would ideally be carried out with experiments using human tissue. Indeed, studies that utilize human tissue are increasingly being reported (e.g. Gwathmey, Copelas et al. 1987, Pieske, Kretschmann et al. 1995, Dipla, Mattiello et al. 1999, Kaprielian, Stevenson et al. 2000, Di Paola, Sweet et al. 2001). For these studies tissue is typically obtained from biopsy during surgical procedures, or from diseased hearts removed prior to transplantation. Whilst the relevance of studying human conditions is clearly high, there are disadvantages in the use of human tissue. The availability (both of abnormal, and age-matched healthy control tissue) is limited, and tissue comes from many different cardiomyopathies. In addition, human tissue is likely to have been subjected to various drug treatments. Much of our existing knowledge about the cellular mechanisms associated with hypertrophy and heart failure has therefore arisen from studies in which animal models of heart failure were used.

Chronic hypertension induced experimentally in an animal was first reported by Goldblatt in 1934. In his experiments, he placed a clip around the renal artery of a dog thereby producing a persistent elevation in systolic pressure (cited in Pinto, Paul et al. 1998). Since Goldblatt’s report, a large number of animal models of hypertension have been developed, encompassing a wide range of species and aetiologies. Frequently, small mammals are used as animal models, since they are both readily available and relatively inexpensive.
The spontaneously hypertensive rat (SHR)

The SHR is a genetic model of essential hypertension developed by Okamoto and Aoki in 1963. Initially, inbred Wistar rats from the Wistar Laboratory in the United States were sent to Tokyo University in 1938. From there they were sent to Hokkaido University in 1944, and then to the Animal Centre Laboratory, Kyoto University in 1951. During this time the colony was “closed” to the introduction of rats from other colonies. Systolic blood pressure measurements were made on hundreds of animals in the colony, and in 1959 Okamoto and Aoki were rewarded with the discovery of a male rat with a systolic blood pressure persistently in the range of 150-175 mm Hg (Okamoto and Aoki 1963). This animal was then bred with a female that had an above average systolic blood pressure (130-140 mm Hg) for the colony. Brother-sister breeding was then carried out amongst the offspring. After three generations of inbreeding all descendents of the F₁ and F₂ generations developed systolic blood pressures greater than 150 mm Hg (a level designated as hypertensive by Okamoto and Aoki). The hypertensive strain of rats were then made commercially available after approximately 20 generations of inbreeding, and different colonies were set up around the world. Rat strains that have been inbred for at least twenty generations can be thought of as being genetically homologous, with the genetic complement of a strain becoming relatively fixed. Since this time many different SHR colonies have been established, and it is likely that there are now genetic differences between colonies worldwide.

The normotensive Wistar Kyoto control

The development of a truly co-genic normotensive control strain for the SHR did not take place, and would now require genetic engineering techniques to achieve. Inbred normotensive strains were used as controls to SHR initially, with the NIH-Wistar (established around 1966) giving rise to most of the commercially available normotensive WKY rats at that time. When it was realised that there was no normotensive control strain for SHR, brother/sister inbreeding of Wistar rats from Kyoto was begun (since they were thought to be a more appropriate control than the Wistar-NIH). However, this did not occur until more than a
decade after the establishment of the SHR. Thus while the genetic complement of the SHR strain had become progressively fixed between 1959 and 1969, the Wistar colony in Kyoto had undergone continuous outbreeding. In addition, it is now thought that WKY were made commercially available as early as the F4 generation. It is therefore likely that there are considerable genetic differences existing between WKY control animals used in different laboratories world-wide. For a review see Louis & Howes (1990).

**Developmental progression of hypertension and hypertrophy in SHR**

A number of longitudinal studies have mapped the progress of the disease with age in SHR, and related this to the development of hypertension and hypertrophy in humans. The SHR slowly develop left ventricular hypertrophy in association with increased systolic arterial pressure and peripheral resistance (Pfeffer et al. 1979). Dickhout & Lee (1998) found an elevation in systolic blood pressure in 4 week old SHR when measured by the indirect tail cuff method, and an increased heart rate at an even earlier age. The alteration in heart rate did not appear to be in response to increased sympathetic activity in these animals, as there was no elevation in plasma levels of adrenaline or noradrenaline found (Dickhout and Lee 1998). Tomanek & Hovanec (1981) found that, whereas systolic blood pressure was elevated in 6 week old SHR, it was not considered to be hypertensive (> 150 mm Hg) until SHR were between 8 and 10 weeks old. These authors also found that an increase in LV mass (indexed as the LV weight /body weight ratio) preceded the onset of hypertensive levels of systolic pressure. In agreement with these results, Delbridge et al. (1996) found systolic blood pressure at 11 to 12 weeks of age for male SHR was significantly higher than for WKY (175 mm Hg vs 135 mm Hg, respectively).

Measurements made on isolated myocytes by Delbridge et al. (1996) showed that there was no difference in resting cell length between the two strains, nor was there a difference in the mean resting sarcomere length. Membrane capacitance measurements from isolated myocytes provide a comparative index of cell surface area between SHR and WKY. Cerbai et al. (1994) found increased capacitance of myocytes isolated from 3 month old SHR, and Brooksby et al. (1993) found that capacitance was 37.3 % greater in 16 week old SHR when compared to WKY controls. Cerbai et al. (1994) also showed that capacitance continued to increase
between 3 months and 18 months of age in SHR, unlike in WKY, indicative of the hypertrophic cellular response in SHR.

Tomanek & Hovanec (1981) showed that the myocardial changes characteristic of ageing appeared at an earlier age in SHR than in WKY, and were accentuated. They found that at 1 month of age SHR had a greater LV mass when compared to their WKY controls, and that hypertrophy continued to develop between 1 and 7 months of age. By 15 months of age the cell size was stable in SHR. In contrast, cell growth during this period in WKY was slow. After 15 months parallel cell growth occurred in both SHR and WKY, which these authors attributed to senescence. Some structural abnormalities were observed in the SHR that were not present in even the oldest WKY, and the authors concluded that these were in response to the prolonged period of pressure-overload and hypertrophy. These abnormalities included a “honey-combed” t-tubular system found in SHR older than 22 months (Tomanek and Hovanec 1981), similar to that described previously for an aortic-banded model of rat hypertension (Page and McCallister 1973).

The increased LV mass of SHR in the first eighteen months of life has been associated with enhanced ventricular function, a normal ejection fraction, and development of increased peak systolic pressures (Brooksby et al. 1992), but see (Delbridge et al. 1996). However, adaptive left ventricular hypertrophy in SHR eventually progresses to development of impaired contractile performance. The onset of overt heart failure in SHR develops between 18 and 24 months of age (Bing et al. 1995; Conrad et al. 1995) (Li et al. 1997). Criteria used for identifying the development of heart failure in these studies include the onset of laboured respiration, the presence of left atrial thrombi, the identification of pleural and pericardial effusions, and the development of right ventricular hypertrophy. In this age group echocardiography studies have shown LV diameter is increased, with reduced fractional shortening during systole (Bing et al. 1995; Ward et al. 2003). Increased myocardial apoptosis, and neurohumoral changes typical of heart failure have also been reported in aged SHR (Li et al. 1997).
Connective tissue changes with ageing in SHR

Adaptive changes that occur in the myocardium of SHR with the development of LV hypertrophy and the progression to end-stage heart failure have been documented (Pfeffer et al. 1979; Engelmann et al. 1987; Conrad et al. 1995). Extracellular matrix components are increased, and increases in collagen with ageing have been reported (Pfeffer et al. 1979; Engelmann et al. 1987). Conrad et al. (1995) compared age matched SHR, with or without overt signs of heart failure (SHR-HF and SHR-NF), to WKY controls. They concluded that the connective tissue changes had a role in the progression to heart failure in these animals. Histological examination of ventricular tissue showed increased interstitial fibrosis in SHR, with those animals in heart failure showing an even greater amount. In addition, the SHR in heart failure showed increased hydroxyproline content (an index of collagen content), and a reduction in the fractional myocyte area in conjunction with the increased fibrosis. Experiments using LV papillary muscle from these animals also showed that active tension was decreased, and myocardial stiffness was increased, in SHR in heart failure (Conrad et al. 1995).

1.5 Alterations in Excitation-Contraction Coupling with Heart Failure

A universal criterion of heart failure is that the development of force during systole is insufficient to provide an adequate cardiac output to meet the metabolic requirements of the tissues. This reduction in contractility is also evident when isolated preparations from hearts in failure are studied, whether the preparations are myocytes (Gómez et al. 1997), papillary muscles (Conrad et al. 1991), ventricular strips (Perreault et al. 1990) or isolated perfused hearts (Brooks et al. 1994). Investigations of the contractile dysfunction have identified abnormalities associated with EC coupling, yet frequently the conclusions are not consistent between studies. This is perhaps not surprising since a wide variety of species and models of heart failure have been used experimentally. In addition, the isolated preparations used, and the experimental conditions themselves, may give rise to variability.
Brooksby et al. (1993) investigated the AP in voltage-clamped myocytes from 16 week old SHR and WKY and found a prolonged repolarisation phase for SHR in comparison to their WKY controls. These investigators found a reduction in the magnitude of the I_K1 at negative potentials, and suggested that it was this current that was responsible for AP prolongation. Cerbai et al. (1994) also investigated the ionic basis of the AP in SHR and WKY of different ages. They found no reduction in I_{K1}, but found that the density of I_{to}, the main current controlling the early repolarisation of the AP in rat, was reduced in SHR from 3 months of age, and that it continued to reduce further with age.

As described previously, the amount of SR Ca^{2+} released is dependent on the magnitude and rate of the I_{Ca_l} influx, the amount, and availability, of Ca^{2+} stored in the SR, and the kinetics of the SR release channels (RyRs). Early in the disease process, long before the development of heart failure, the increased AP duration might be expected to increase the amplitude of the Ca^{2+} transient. In support of this, increased Ca^{2+} transient amplitude with hypertrophy has been reported (Shorofsky et al. 1999). It has been suggested that this might be an early compensatory response to increase force production in response to elevated pressures (Wickenden et al. 1998). In contrast, some studies report reduced Ca^{2+} transients with heart failure (Pieske et al. 1995; Pogwizd et al. 2001; Jiang et al. 2002). However, as mentioned above, reduced Ca^{2+} transients might arise from reduced SR Ca^{2+} stores, or from RyR kinetics, rather than from alterations in I_{Ca_l}. It therefore seems likely that important alterations in Ca^{2+} homeostasis might occur with the progression of hypertrophy to end-stage heart failure.

1.6 Measurement of Intracellular Calcium in Cardiac Muscle Preparations

The development of a new family of fluorescent indicators by Roger Tsein and co-workers in 1985 vastly improved the ability to make accurate measurement of intracellular calcium in biological preparations (Grynkiewicz et al. 1985). These indicators act as divalent metal ion chelators that change fluorescent properties upon binding Ca^{2+}. Intracellular Ca^{2+}
concentration ([Ca$^{2+}$]) is inferred indirectly by means of the law of mass action. Typically, these indicators are available either as a K$^+$ salt, introduced into cells via a patch pipette, or by microinjection, or as the membrane-permeable acetoxyethyl (AM) form. The AM indicators were initially favoured for measurement of [Ca$^{2+}$]$_i$ in muscle preparations, since they are easily introduced into cells. For fura-2/AM, the indicator used in this study, the molecule has five acetoxyethyl groups linked to the COO$^-$ groups by ester bonds. Once inside a cell, endogenous, non-specific esterases cleave the lipophylic-blocking COO$^-$ groups from the fura-2 molecule. A negatively charged form of fura-2 that resists crossing cellular membranes then remains.

A number of potential disadvantages in using the AM form of an indicator have been identified. The most important of these is the possibility that the membrane permeable fura-2/AM molecule might permeate into intracellular compartments if the molecule is not rapidly cleaved of its acetoxyethyl groups once inside the cell. Under some conditions, significant compartmentation of indicator has been demonstrated (Di Virgilio et al. 1988; Blatter and Wier 1990; Miyata et al. 1991), compromising the ability to measure the cytosolic [Ca$^{2+}$] accurately. The mechanism by which fura-2 is compartmentalised is uncertain (Roe et al. 1990). The membrane-permeable, esterified form of fura-2 might move directly from the cytosol into organelles (with subsequent de-esterification occurring in the organelles). Alternatively, movement into organelles might occur by transportation of the negatively charged, de-esterified form, via an organic anion system (thought to account for loss of indicator from the cells at high temperatures (Di Virgilio et al. 1990)). That this latter process may occur is confirmed by reports that compartmentation is reduced using a blocker of anionic transport (e.g. probenecid) (Di Virgilio et al. 1989).

Loading of trabeculae with fura-2/AM at room temperature, and use of an anion transport blocker, such as probenecid, minimise the amount of compartmentation in cardiac tissue (Di Virgilio et al. 1988).
The pentapotassium salt of fura-2

An alternative way to introduce fura-2 into cells is to use iontophoretic loading of the pentapotassium salt form of the indicator. This method eliminates some of the disadvantages associated with AM loading, and was first reported for cardiac trabeculae by Backx and ter Keurs (1993). A sharp microelectrode, previously back-filled with solution containing the K⁺ salt form of fura-2, was used to penetrate the sarcolemma of a myocyte, and loading was by microinjection. Once the indicator was loaded into an impaled cell, it was free to diffuse via gap junctions throughout the entire preparation. This technique has been reported to be successful in trabeculae with a single stable impalement (Backx and ter Keurs 1993), but more frequently involves cell membrane penetrations at a number of sites along the length of the preparation (e.g. Layland and Kentish 1999).

The major advantage of iontophoretic loading reported was that the indicator was contained only within myocytes, and that it remained completely within the cytosol (Backx and ter Keurs 1993). However, Backx and ter Keurs (1993) reported no experiments in their paper in which comparative measurements of compartmentation between the two loading techniques were made.

Other influences on fluorescence

Because the influence on fluorescence of non-calcium related factors differs between preparations, an in vivo calibration is typically undertaken for accurate measurement of [Ca²⁺]. These factors include effects of temperature (Shuttleworth and Thompson 1991), ionic strength, pH and viscosity (Uto et al. 1991).

Ratiometric fluorescent indicators

Fluorescent indicators require excitation at a particular wavelength, supplied by an external source (such as an incandescent lamp, or a laser). Absorption of photons by the fluorophore
briefly produces an excited state (1-10 ns), with subsequent emission of fluorescence of longer wavelength and a return to the ground state (Molecular Probes 1992-94). Indicators that shift the excitation wavelength peak (or the emission wavelength peak in the case of indo-1) upon binding Ca\(^{2+}\) are suitable for making ratiometric measurements. This has the advantage that [Ca\(^{2+}\)] can be measured independent of indicator concentration. For fura-2 the peak excitation wavelength is reduced on binding Ca\(^{2+}\). It is therefore suitable for ratiometric measurements using two wavelengths, \(\lambda_1\) and \(\lambda_2\). For fura-2, \(\lambda_1\) (340 nm) is the excitation wavelength producing maximal fluorescence when Ca\(^{2+}\) is bound, and \(\lambda_2\) (380 nm) is the wavelength producing maximal fluorescence for the Ca\(^{2+}\)-free form of fura-2. Equally, ratiometric measurements could be made using the isosbestic point (360 nm) as \(\lambda_2\). This would simplify the calibration equation by removing the need for the \(\beta\) term, since the wavelength at the isosbestic point does not change for Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free forms of the indicator. This is not typically done, however, as full use of the spectral range of the indicator is made if \(\lambda_1\) and \(\lambda_2\) are either side of the isosbestic point (Gryniewicz et al. 1985), therefore maximising the signal. For any [Ca\(^{2+}\)], fura-2 exists in both the Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free forms, depending on the effective dissociation constant for the indicator. In choosing a suitable indicator, the \(K_d\) must be compatible with the range of ion concentrations expected. In general, the [Ca\(^{2+}\)] of interest should lie within 0.1-10 x \(K_d\) (Molecular Probes 1992-94)).

The ratio (R) of the dye’s fluorescence intensities at two different excitation wavelengths (\(\lambda_1/\lambda_2\)) is sufficient to calculate [Ca\(^{2+}\)] independent of total dye concentration, path length, or absolute sensitivity of the measurement system (Gryniewicz et al. 1985). Assuming that the fluorescence contribution from either the fura-2\(_{\text{bound}}\) or the fura-2\(_{\text{free}}\) is proportional to their concentrations, then there are four proportionality coefficients (S) in total, i.e. two excitation wavelengths (\(\lambda_1\) (1), and \(\lambda_2\) (2)), and two forms of dye (bound (b), and free (f)). These four factors are readily measured, in principle, by use of calibration solutions containing known concentrations of Ca\(^{2+}\). Gryniewicz et al. therefore determined that the ratio has the following relationship:

\[
R = ([\text{fura-2}_{自由}]S_1 + [\text{fura-2}_{结合}]S_b)/([\text{fura-2}_{自由}]S_2 + [\text{fura-2}_{结合}]S_{自由})
\]  
(Eq. 1.1)

Calcium concentration is then related to the concentration of the bound and free forms of fura-2 in the following way:
\[ [\text{Ca}^{2+}] = ([\text{fura-2\text{-bound}}]/[\text{fura-2\text{-free}}]) \times K_d \]  
(Eq. 1.2)

Combining equations (1.1) and (1.2) yields the classical equation for determination of \([\text{Ca}^{2+}]\) (Gryniewicz et al. 1985):

\[ [\text{Ca}^{2+}] = K_d \beta \left( \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \right) \]  
(Eq. 1.3)

where:

\[ \beta \] is the ratio of the fluorescence of the \(\text{Ca}^{2+}\) \text{free} form to the \(\text{Ca}^{2+}\) \text{bound} form at \(\lambda_2\) (380 nm) excitation;

\[ K_d \] is the dissociation constant;

\[ R_{\text{min}} \] is the limiting value of \(R\) at zero \([\text{Ca}^{2+}]\), i.e. \(S_R/S_{R2}\);

and, \(R_{\text{max}}\) is the limiting value of \(R\) at saturating \([\text{Ca}^{2+}]\), i.e. \(S_0/S_{b2}\).

Ideally, values for \(R_{\text{min}}\) and \(R_{\text{max}}\) should be determined \textit{in vivo} for every preparation, but generally this is not practicable.

1.7 \textbf{Isolated Cardiac Muscle Preparations}

\textit{Choice of experimental preparation}

A variety of cardiac muscle preparations are used experimentally to examine aspects of muscle function, ranging from the isolated perfused whole-heart, to isolated myocytes, and sub-cellular fractions. Each preparation has specific advantages and disadvantages that make it suitable, or otherwise, for a particular study. A major problem in studying isolated cardiac muscle in a preparation that is not perfused is the need to prevent metabolic demand exceeding supply. For this reason, experiments are commonly carried out at low temperatures and low stimulation frequencies. However, cellular processes observed under these conditions may not be representative of those \textit{in vivo}. Because of their small dimensions, the need to
reduce stimulation frequency and temperature in order to avoid metabolic insufficiency can be obviated by the use of cardiac trabeculae. In addition, ventricular trabeculae are a suitable preparation for simultaneous measurement of isometric force and intracellular Ca\(^{2+}\) at a sarcomere length comparable to that maximally attained at the end of diastolic filling in vivo (Rodriguez et al. 1992).

*Left ventricular trabeculae*

Trabeculae are structurally homologous to ventricular wall tissue composed almost entirely of myocytes (Johnson and Sommer 1967; Hanley et al. 1999), with a thin outer layer of endothelial cells. Preparations dissected from the left ventricle were used in this study to ensure that measurements were carried out in tissue that was representative of the hypertrophied SHR left ventricular wall. Whereas RV trabeculae are consistently found near the tricuspid valve, with one end attached to the base of the atrio-ventricular ring and the other attached to the RV free wall, LV trabeculae are seldom found near the valve. Instead, LV trabeculae typically form cylindrical muscle attachments between ventricular ridges on the LV free wall.

The isolated trabecula relies on diffusion for its supply of oxygen and other metabolic substrates, and for removal of the end products of metabolism. Using the equation derived by Hill (1928, 1965) for radial diffusion of oxygen in a cylindrical preparation, Daut and Elzinga (1989) found that, at rest, the critical radius at which an anoxic core develops in a cylindrical cardiac preparation was 330 µm, when the fraction of O\(_2\) was 100 % and the preparations were adequately superfused with metabolic substrates. Until recently (Janssen et al. 2003) studies in which force and intracellular Ca\(^{2+}\) have been measured in SHR have utilised LV papillary muscles (Bing et al. 1991; Conrad et al. 1995), but papillary muscles frequently exceed the critical radius for development of an anoxic core at rest (e.g. LV preparations of 0.7 mm diameter were used by Conrad et al. (1995)). LV trabeculae typically have a maximum diffusion distance of about 100 µm, and frequently substantially less, therefore making them more suitable for studies of isolated cardiac muscle.
Chapter II

General Methods

2.1 Chapter Overview

This chapter introduces the general methods utilised in order to investigate the relationship between intracellular \([\text{Ca}^{2+}]\), and isometric force in an animal model of human essential hypertension, the spontaneously hypertensive rat, and its age-matched normotensive controls.

2.2 Experimental Animals and Haemodynamic Measurements

**SHR and WKY**

Animals used in this study were obtained from a colony of SHR and WKY animals first established at the Animal Resources Unit, Faculty of Medical and Health Sciences, University of Auckland, in 1992 by Associate-Professor Sheila Doggrell. In establishing the colony, a number of breeding pairs of WKY and Okamoto SHR were purchased from the Animal Resources Centre, Perth, Western Australia. Since that time the animals have been subjected to inbreeding for many generations, such that both the SHR and WKY used for this study can be considered as genetically homologous for their strains. Animals (male, and female) were purchased at 6 weeks old, and allowed to age without interruption of the natural life cycle. Food and water were provided *ad libitum*, and room temperature was maintained at 20-21 °C, with a 12-hour light and dark cycle.
The development of heart failure in SHR occurs from around 18 months of age. Regular observation of animals as they aged allowed detection of the onset of end-stage heart failure: lethargy, poor grooming, rapid irregular breathing and a loss of body weight. A number of animals, both SHR and WKY, died suddenly before obvious signs of ill health were detected. These sudden deaths comprised 19% of the total population for WKY (18% female and 20% male), and 27% of the total for SHR (20% female and 34% male).

Once identified as being in end-stage heart failure, SHR were removed from the Unit for measurement of systolic blood pressure and heart rate (see below), and used for experimentation within 48 hours. WKY control animals of a similar age were also selected for experimentation, and subjected to measurement of blood pressure and heart rate.

*Measurement of systolic arterial blood pressure using tail cuff sphygmomanometry*

Systolic arterial pressure and heart rate were determined in animals prior to experimentation, using a digital blood pressure monitor (Model 179, IITC Life Science, California, USA) and tail cuff sphygmomanometry. Previous studies have shown that the indirect tail-cuff technique is an accurate and reliable method for measurement of systolic arterial pressure in SHR and WKY (Pfeffer et al. 1971). This measurement technique is very sensitive to animal movement, and measurements were repeated until at least 5 traces without movement artifact were obtained. Although the instrument produced a digital reading of pressures and heart rate (see RHS Fig. 2.1), this was always verified by direct measurements taken from the records (systolic pressure obtained from dotted lines in Fig. 2.1A, corresponding to arrow in Fig. 2.1B). Heart rate was determined from the tail pulse records by increasing chart speed for a 6 s period, after detection of the pulse. Diastolic records were not used in this study, since they were much more difficult to detect accurately.
Figure 2.1 Chart record of blood pressure measurement. A, pressure (mm Hg) in the cuff surrounding the rat's tail over a period of 30 s. The cuff was rapidly inflated to about 240 mm Hg (at time 0) and then automatically deflated at a constant rate. B, continuous output from the photodiode throughout the measurement. Note the onset of pulsatile blood flow in the tail arteries (at arrow) once cuff pressure had decreased to 139 mm Hg. Heart rate was determined by increasing chart speed in the middle of the recording and counting the number of inter-beat intervals in a 6 s period.
2.3 Dissection

*Left ventricular trabeculae dissection*

Animals selected for experimentation were anaesthetised by halothane inhalation and immediately decapitated. Hearts were then rapidly excised following a mid-sternal thoracotomy, and plunged into chilled dissection solution (see below for details of solutions used). The heart was then transferred to a dissection dish, attached via the aorta to a horizontal cannula, and perfusion of the coronary circulation with oxygenated dissection solution commenced. The time from decapitation to commencement of coronary perfusion was typically less than 2 min. Dissection was carried out at room temperature, with the heart totally immersed in oxygenated dissection solution, whilst continuously perfusing the coronary circulation. An incision was made down the length of the LV free wall close to the septum. The LV free wall was then reflected back, and a suitable preparation selected for removal. The trabecula was dissected free with a small block of ventricular tissue at either end, and transferred to the experimental muscle chamber.

*Determinaton of hypertrophy and oedema*

After removal of a suitable trabecula the heart was then carefully dissected further for estimations of hypertrophy and oedema. The RV free wall, septum, LV free wall and atria were separated, firmly blotted twice with tissue, and wet weights recorded. The diameter of the aorta was measured using callipers. Wet weight was also obtained for the lungs and the liver. Tissue was then placed in an oven (50 °C) overnight and dry weights obtained the following day. Tibial length was measured, for both left and right hind limbs, and was used as an index of body size. The ratios of weight (wet) to body weight, and weight (wet) to tibial length were used to determine the presence or absence of hypertrophy. Weights (wet and dry) relative to body weight, and weights (wet and dry) relative to tibial length were also obtained for the lungs and the liver. The ratio of wet weight to dry weight for all tissues was used as an indication of tissue oedema.
2.4  Measurement of Isometric Force

Experimental muscle chamber and superfusion system

The muscle chamber consisted of a rectangular Perspex bath (length 2 mm, width 0.5 mm, volume 450 μL), shown in Fig. 2.2A, clamped to the stage of an inverted microscope (Nikon Diaphot 300, Japan) as described previously (Hanley and Loiselle 1998). A borosilicate microscope glass cover-slip (22 x 40 mm, thickness No. 1.5) was used for the base of the bath, sealed in place using medical grade silicon grease (Unimed, Fine Science Tools, Vancouver). Platinum wire stimulating electrodes were mounted along the length of the bath against either wall. The perfusate entered the bath, via gravity feed, through a stainless steel tube (inner diameter 3 mm) after being pumped into an open stainless steel well (outer diameter 1.4 cm, inner diameter 4 mm, height 3 cm) to remove any pump-induced pulsatility in the flow of solution through the bath. Meniscus perturbations from the bath outflow were avoided by placement of a suction nozzle behind a weir at the down-stream end of the bath. The experimental muscle chamber and superfusion system are shown in Fig. 2.2B. Flow of superfusate through the bath during experiments was maintained at 7-8 mL min⁻¹ by means of a four-channel roller pump (Minipuls 2, Gilson, France). The total volume contained by the tubing between the reservoir and the bath was less than 3.5 mL, so that solution changes took approximately 30 s to achieve.

Mounting trabeculae in the force transducer

The ventricular block at one end of the trabecula was mounted in a wire hook that extended from the silicon beam of a force transducer (model AE801, SensoNor, Horten, Norway). The block at the other end of the trabecula was held in a monofilament nylon snare (diameter 30 μm) protruding from a stainless steel tube (length 25 mm; i.d. 100 μm). A schematic diagram of a trabecula held between the hook and the snare is shown in Fig. 2.2C. The snare and the force transducer were each attached to 3-axis micromanipulators mounted on the microscope stage, allowing fine adjustment of trabeculae length and position.
Figure 2.2 Muscle chamber and schematic diagram of a trabecula mounted between the hook and the snare. A, view from the top showing the muscle chamber, and B, the muscle chamber clamped to the microscope stage with the force transducer (5) in position. Solutions were pumped to an open well (1), then gravity fed to the muscle chamber via the chamber inlet (2). A suction nozzle (3) placed behind a weir at the end of the chamber ensured that the level of fluid in the chamber remained constant. Constancy of flow was determined by the pump and the open well. Two platinum wire stimulating electrodes (4) ran either side of the chamber along its length. The position of the force transducer (FT, 5) above the bath is shown in B. C, schematic diagram of a trabecula, mounted between the hook and the snare.
The response of each force transducer used throughout this study was checked for linearity after attaching the hook to the beam. A two-point calibration was carried out, using a 100 mg mass applied to the hook of the transducer in air (i.e. a force of approximately 1 mN), so that displacement was at right angles to the silicon beam. The output of the force transducer remained constant for temperatures between 20 and 38 °C.

After mounting in the force transducer, trabeculae were continuously superfused with oxygenated Krebs-Henseleit (K-H) solution throughout the experiment. Preparations were allowed to stabilise for a period of at least 15 min, during which they were superfused with dissection solution (see below) at room temperature. Trabeculae were field-stimulated at a frequency of 0.1 Hz with 5 ms square wave pulses (± 5 V) delivered by a Digitimer D100 (Digitimer, Welwyn Garden City, Hertfordshire, UK). The stabilisation period allowed trabeculae to recover from handling during dissection and mounting. Following this period the solution was then changed to the standard K-H solution, and the [Ca²⁺]₀ was increased to 1 mM.

Once a trabecula was producing stable twitches in response to stimulation, its length was adjusted. The central portion of the trabecula was viewed, using the x 40 objective (NA 0.55) and a charge-coupled device (CCD) camera connected to a video monitor. At this magnification, muscle striations were clearly visible. Since twitch amplitude and duration have previously been shown to be sarcomere-length dependent in cardiac muscle (Allen and Kentish 1985; Backx and ter Keurs 1993), trabeculae were extended to give a sarcomere length of 2.1-2.2 μm. At this sarcomere length twitch force is at the peak of the length-tension relationship for cardiac muscle (Allen et al. 1974; ter Keurs et al. 1980). This length was then maintained throughout the experiment.

A calibrated eyepiece objective was used to determine trabeculae dimensions. Cross-sectional area was calculated by assuming a circular cross-section for each preparation, and force was normalised to cross-sectional area for each trabecula (i.e. stress, mN mm⁻²). Dimensions obtained for LV trabeculae (n = 56) used in this study were: length 1.9 ± 0.1 mm and cross sectional area 0.038 ± 0.003 mm² (corresponding to an average radius of ~110 μm). Statistical analysis showed no difference in either the average cross-sectional area of preparations, or overall length between SHR and WKY.
Trabeculae were maintained at room temperature until after loading of the fluorescent indicator was completed (see Loading of Fura-2/AM, below). Addition of 1 mM probenecid was then made to all solutions to maximize retention of the indicator in the cytoplasm at 37 °C (Di Virgilio et al. 1990). A heat exchange system, mounted on the microscope stage immediately prior to the bath inlet port, was used to maintain the bath superfusate at 37 °C. Increasing bath temperature from that of the room (~20 °C) took about 30 min. Measurement of bath temperature was made intermittently by use of a thermocouple-based thermometer (Digi-thermo, GSA Industries, Australia). The slow temperature equilibration was necessary to allow the considerable heat sinks associated with the well, muscle bath and microscope (stage and objective) to reach the desired temperature. After this initial period, the temperature remained constant (±0.5 °C) for the duration of the experiment, provided that the superfusate flow rate was maintained constant.

Left ventricular trabeculae used in this study typically had radial dimensions less than half the critical value calculated for quiescent cardiac preparations (i.e. before O2 supply to the core is compromised) (Daut and Elzinga 1989). However, care was taken to limit the exposure time of trabeculae to metabolically demanding experimental protocols (i.e. high external [Ca2+], or high stimulation frequencies).

2.5 Solutions and Chemicals.

A modified Krebs-Henseleit (K-H) solution was used as the standard control solution for superfusion of trabeculae, with the following composition (in mM): 118 NaCl, 4.75 KCl, 1.18 MgSO4.7H2O, 1.18 KH2PO4, 24.8 NaHCO3, and 10 D-glucose. [Ca2+] was adjusted by addition of CaCl2 from a 1 M stock solution. All solutions were made using distilled water and analytical grade chemicals. The K-H superfusate was continuously bubbled with 95% O2 and 5% CO2, at pH 7.4. For experiments at 37 °C, the solutions were oxygenated in a 100 mL reservoir at room temperature, before passing through the heat exchange system. The K-H dissection solution contained 20 mM 2,3-butanedione monoxime (BDM), and 0.25 mM [Ca2+]o, in order to reduce cutting injury and prevent contraction, thereby reducing energy expenditure (Mulieri et al. 1989).
To avoid the use of a CO2-buffering system in Na+-free experiments, an alternative phosphate-buffered solution (Tyrode) was used. The composition of the control phosphate-buffered solution was (in mM): 141.8 NaCl, 6 KCl, 1.2 MgSO4·7H2O, 1.2 Na2HPO4, 10 Hepes, 10 Glucose. The pH was adjusted to 7.4 with the addition of Tris. A Na+-free solution was obtained by equimolar substitution of LiCl for NaCl in the control solution, and the use of K2HPO4·3H2O in the place of Na2HPO4. The phosphate-buffered solutions were oxygenated by bubbling with 100% O2, and [Ca2+] was adjusted by addition of CaCl2 from a 1 M stock solution, as required.

2.6 Measurement of Intracellular Calcium

Fluorescence system

Trabeculae were illuminated using a 75 W xenon arc lamp and a spectrophotometric system (Cairn Research, Faversham, Kent, UK) provided rapidly alternating excitation wavelengths with a rotating filter wheel. The following interference filters were contained in the filter wheel: 3 x 340, 1 x 360 and 2 x 380 nm (10 nm passband). The frequency at which the wheel was rotated was usually 47 Hz (but could be increased to higher frequencies when required) providing a temporal resolution of approximately 10 ms. A combiner module provided a continuous average of the emitted fluorescence for each of the excitation wavelengths as each filter was positioned in the light path. Ratiometric measurements for the 340/380 excitation wavelengths for fura-2 fluorescence were then obtained; i.e. the ratio of fura-2 fluorescence for Ca2+ bound/Ca2+ free. An adjustable rectangular window restricted the area of illumination over the central portion of the trabeculae to approximately 100 μm x 250 μm, depending on the dimensions of individual preparations. Emitted fluorescence was collected by the objective and directed via a 600 nm dichroic mirror and a 480 nm long pass filter to a photomultiplier tube (EMI 9124B, Thorn EMI Electron Tubes, UK).
Autofluorescence

It has previously been reported that a significant portion of trabecular autofluorescence on excitation with UV light originates from mitochondrial NADH and oxidised flavoproteins (Brandes and Bers 1996; Hanley and Loiselle 1998). NADH has a broad emission spectrum with a peak at 447 nm when excited with UV light (Eng et al. 1989). Because of this source of contamination, subtraction of tissue autofluorescence measured early in the experiment was made for both the 340 and 380 nm wavelengths. This method assumes that autofluorescence remains constant throughout the experiment.

Loading of Fura-2/AM

After measurement of background bath fluorescence, and tissue autofluorescence, loading of trabeculae with fura-2/AM was carried out. The loading solution consisted of 10 mL of K-H superfusate, with 100 μg of fura-2/AM (Texas Fluorescent Laboratories, USA) previously dissolved in 30 μL of freshly prepared anhydrous dimethyl sulphoxide (Aldrich), and 5% wt/vol pluronic F-127 (Texas Fluorescent Laboratories, USA), yielding a 10 μM final concentration of fura-2/AM. Solubility of the pluronic F-127 was enhanced by warming the mixture for 5 min at 40 °C prior to adding to the fura-2/AM. Since pluronic F-127 is a detergent, 15 μL of Antifoam A (Sigma Chemical Co., St Louis, USA), a 30% aqueous solution of non-ionic emulsifiers, was added to the 10 mL loading solution to prevent foaming.

To load the fura-2 into the myocytes, trabeculae were superfused with oxygenated loading solution for a period of about 2 hours. Loading was carried out at room temperature (20–22 °C), with a stimulation frequency of 0.1 Hz, and with a superfusate [Ca²⁺] of 1 mM. The 10 mL of loading solution was continuously circulated between the bath, containing the trabecula, and a reservoir. To maintain adequate oxygenation, and a pH of 7.4, the solution was bubbled in the reservoir with 95% O₂ and 5% CO₂.
Iontophoretic loading of fura-2

To compare the use of the membrane-permeable fura-2/AM form of the indicator with that of its membrane-impermeable potassium salt, a number of right ventricular trabeculae from young Wistar rats were loaded via iontophoresis using a method modified from Backx et al. (1993). Micropipettes were made from borosilicate glass capillaries (GC 120F-15, Clark Electromedical Instruments, Reading, U.K.), loaded with fura-2 pentapotassium salt solution (1 mM), and backfilled with 140 mM KCl. Micropipette resistances were typically 150-250 MΩ when placed in K-H buffer solution (i.e. tips < 0.2 µm diameter). Measurement of membrane potential, and injection of hyperpolarising current, was carried out using an Axoclamp-2A bridge amplifier (Axon Instruments, USA). The stimulator was turned off during membrane impalement, to minimise any trabecula movement that might damage the sharp electrode tip. On obtaining a stable membrane potential recording, the Axoclamp-2 was switched to current injection mode, and fura-2 was loaded into the myocyte by passing a -15 nA current for about 30 min. Fluorescence was monitored throughout the loading procedure. Once successfully loaded into the impaled myocyte, fura-2 was free to diffuse via gap junctions throughout the entire trabecula. Some trabeculae required a number of impalements along their length before successful loading was achieved.

Compartmentation of indicator

For a number of AM-loaded trabeculae (n = 6 at 37 °C, and n = 5 at 20 °C) the extent of fura-2 compartmentation was determined at the end of experimentation. This was carried out by first permeabilizing the sarcolemmal membranes with 25 µM digitonin, followed by permeabilization of the subcellular compartment membranes with 2% Triton X-100. To account for any changes that occur in autofluorescence with this technique, three unloaded trabeculae were also subjected to the sequential permeabilisation steps whilst autofluorescence at 360 nm was measured.

Similar determination of the amount of fura-2 within intracellular compartments was made for three iontophoretically-loaded RV trabeculae for comparison.
2.7 Calibration of Fura-2

*In vivo calibration*

Calibration in terms of absolute values of $[Ca^{2+}]$ has been described previously according to equation 1.3. However, since the $Ca^{2+}$-binding of fluorescent probes is affected to some extent by the internal milieu, a full *in vivo* calibration procedure is usually carried out. In order to do this, preparations are exposed to a number of solutions of different $[Ca^{2+}]$ in the presence of a $Ca^{2+}$ ionophore (Haworth and Redon 1998). With time, the intracellular $[Ca^{2+}]$ then becomes equal to the extracellular $[Ca^{2+}]$, and the 340/380 fluorescence ratio can be related directly to the $[Ca^{2+}]$.

For multicellular preparations, such as cardiac trabeculae, *in vivo* calibration requires long equilibration times at each of the $[Ca^{2+}]$ concentrations, such that the time required for four different $[Ca^{2+}]$ is in excess of 3 hours. For fura-2, at 37°C, this is further complicated by loss of indicator with time, even in the presence of an anionic transport blocker, so that it is necessary to take this into account in determining $\beta$. For these reasons, a full *in vivo* calibration procedure was not undertaken, but measurements of $R_{min}$ and $R_{max}$ were made at 37°C. A $K_a$ value of 371 nM for fura-2 in cardiac myocytes (Haworth and Redon 1998) was subsequently used. Use of this $K_a$ value yielded calculated $[Ca^{2+}]$, comparable to other reports in the literature for rat myocytes at 37°C (e.g. Backx and ter Keurs 1993; Bers and Berlin 1995; Maier *et al.* 1998; Janssen *et al.* 2002).

As mentioned above, during experiments at 37°C, about 20% of intracellular indicator is lost per hour, even in the presence of the anionic transport blocker, probenecid (1 mM). While this effect is of little consequence when making ratiometric measurements, when *in vivo* calibrations are performed the value of $\beta$ in Eq. (2.1) is affected by the indicator loss (since equilibration to the minimum and maximum $[Ca^{2+}]$ can each take in excess of 30 min in these multicellular preparations). A method similar to that of Jiang and Julian (1997) was therefore used to account for time-dependent changes in the 380 nm signal due to the loss of indicator during measurement of $R_{max}$. This was carried out by multiplying the $R_{max}$ 380 nm fluorescence by the ratio of the fluorescence at the isosbestic wavelength (360 nm) for fura-2 at the beginning ($R_{min}$) and at the end ($R_{max}$) of the calibration procedure:
\[ \beta = \frac{380R_{\text{min}}}{(380R_{\text{max}})(360R_{\text{min}}/360R_{\text{max}})} \quad (\text{Eq. 2.2}) \]

Values for \( R_{\text{min}} \), \( R_{\text{max}} \) and \( \beta \) were obtained by exposing RV trabeculae from Wistar rats first to a \( \text{Ca}^{2+} \)-free (\( R_{\text{min}} \)) solution, followed by a saturating \( \text{Ca}^{2+} \) (\( R_{\text{max}} \)) solution. A number of additions were made to the calibration solutions in order to eliminate the cell’s ability to control intracellular \( \text{Ca}^{2+} \).

Calibration solutions contained the following (in mM). \( R_{\text{min}} \): 118 NaCl, 6 KCl, 1.18 MgSO\(_4\), 1.18 KH\(_2\)PO\(_4\), 24.8 NaHCO\(_3\), 10 glucose, 10 caffeine, 10 BDM, 10 ethylene glycol-bis (\( \beta \)-aminoethyl ether)-N, N', N'-tetraacetic acid (EGTA), 0.1 2,5-di(tert-butyl)-1,4-benzohydroquinone (TBQ) and 1 ouabain. \( R_{\text{max}} \): 120 LiCl, 1.18 MgSO\(_4\), 1.18 KH\(_2\)PO\(_4\), 20 NaHCO\(_3\), 4.82 KHCO\(_3\), 10 glucose, 10 caffeine, 10 BDM, 0.1 TBQ, 1 ouabain and 0.01 Br-A23187. The SR was functionally eliminated by addition of caffeine and TBQ (an inhibitor of the SR \( \text{Ca}^{2+} \)-ATPase). Ouabain was added to eliminate the sarcolemmal Na\(^+\)-K\(^+\) ATPase, and BDM was added to prevent energy expenditure due to cross-bridge cycling. \( R_{\text{max}} \) was obtained by using a low [Na\(^+\)] to enhance \( \text{Ca}^{2+} \) influx via reverse mode Na\(^+\)-Ca\(^{2+}\) exchange (Cooper et al. 2001), together with a \( \text{Ca}^{2+} \) ionophore in 2 mM [Ca\(^{2+}\)]\(_o\).

**\( \text{Ca}^{2+} \)-independent effects on fura-2 fluorescence**

The kinetics of binding of fura-2 and \( \text{Ca}^{2+} \) are altered to some extent by the surrounding environment, as outlined in Chapter I (1.6). It is for this reason that \textit{in vivo} calibration is carried out to determine [\( \text{Ca}^{2+} \)] accurately, rather than simply obtaining values for \( \beta \), \( R_{\text{min}} \) and \( R_{\text{max}} \) using the much simpler \textit{in vitro} calibration procedure. In order to ascertain whether [\( \text{Ca}^{2+} \)]-independent influences affected fluorescence measurements between rat strains in this study, the relationship of emitted fluorescence for 380 nm versus the 340 nm excitation wavelengths was plotted for a number of SHR and WKY trabeculae (Bakker et al. 1993). The slope of this relationship was determined for each trabecula by fitting a linear regression line to a randomly chosen 1 s selection of fluorescence data, and to data from \textit{in vitro} calibration procedures. Analysis showed no differences in the slope of the regression lines between rat strains (SHR: \(-0.39 \pm 0.06 \) (\( n = 14 \)), WKY: \(-0.35 \pm 0.04 \) (\( n = 9 \)), or \textit{in vitro} calibration solutions: \(-0.37 \pm 0.04 \) (\( n = 8 \))).
2.8 General Experimental Protocol

Experimental protocols differed depending on the experimental series. Details are therefore given, as appropriate, in each Chapter. Unless otherwise stated, measurements were made under steady-state conditions, and at 37 °C. For alterations of \([\text{Ca}^{2+}]_o\), or of stimulus rate, all measurements were obtained once steady-state had been reached, at least three minutes after the onset of the intervention.

Fixation of trabeculae and staining for collagen

A number of preparations were fixed for morphological examination on completion of experiments. Trabeculae were first stretched by 5% (to allow for shrinkage during the fixation process), and then fixed with Bouin’s solution, as previously described (Hanley et al. 1999). Investigation of the distribution and content of collagen in these trabeculae was then carried out by Adèle J. Pope as a student summer project (Ward et al. 2003). Trabeculae were cut in half, and one half stained with picrosirius red (PSR) (0.1% w/v sirius red F3BA in saturated picric acid) for 2 hours. Both halves were then resin-embedded, and the second half was stained with methyl blue (1%) for myocytes and basic fuchsin (1%) for the extracellular matrix. The PSR stained preparations were imaged using a confocal microscope (Leica TCS 4D, Heidelberg, Germany) with a x25 objective (0.75 NA). By summing a series of confocal sections, extended-focus images to a depth of 50 μm were obtained for qualitative analysis of the longitudinal collagen distribution. Transmitted light images of the transversely sectioned trabeculae were captured with a digital camera (Zeiss ProgRes 3008) mounted on a light microscope (Leica DM R, Wetzla, Germany) with a x100 objective (1.4 NA). Images were then analysed to determine the relative proportions of myocytes and collagen using NIH Image (http://rsb.info.nih.gov/nih-image/). Total cross-sectional area calculated in this way excluded unstained areas (such as the interior of blood vessels).
2.9 Data Analysis

Data acquisition

Continuous force records were made using a flatbed chart recorder (Graphtec Linear Recorder Mk IV). In addition, emitted fluorescence at 510 nm from each of 340, 360 and 380 nm excitation wavelengths, the 340/380 ratio, force and stimulus voltage were acquired at selected times using custom-written software (LabView 3.1.1, National Instruments, Austin, TX, USA), for analysis off-line (see Fig. 2.3).

Un-biased measurement of Ca\(^{2+}\) transient and isometric force parameters were obtained from data averaged over 16-19 cardiac cycles, using a custom-written programme (IDL, Research Systems Inc., Boulder, CO, USA), as shown in Fig. 2.4. Averaging was carried out using autocorrelation based on the stimulus. A five-parameter exponential function (Eq. 2.3) was adopted to describe the Ca\(^{2+}\) transient and its kinetics:

\[
y = A_0\exp(-t/A_1)/(1 + \exp(-(t + A_2)/A_3)) + A_4
\]

(Eq. 2.3)

Typical values of parameters found using Eq. 2.3 were: \(A_0\) 1.3 (maximum fluorescence 340/380 ratio change); \(A_1\) 0.1 s (time constant of exponential decline during relaxation); \(A_2\) 0.001 s (offset in time between rising and decaying phases of the Ca\(^{2+}\) transient); \(A_3\) 0.003 s (the time constant of rise of the transient); \(A_4\) 0.4 (minimum fluorescence before stimulation). No correction was made for the delay with which fura-2 tracks changes in [Ca\(^{2+}\)].

Peak systolic fluorescence, minimum resting fluorescence, time-to-peak fluorescence, and the maximum rate-of-rise of fluorescence (from the differential), were obtained from both the averaged and fitted data (see Fig. 2.4). Note that resting Ca\(^{2+}\) was taken as the minimum of the fluorescence in the interval between transients. Minimum fluorescence was obtained for both the averaged data, and for the curve fitted to the averaged data. The two methods of obtaining the minimum fluorescence were in close agreement for all experiments, with the exception of those carried out at high [Ca\(^{2+}\)]. The fitted curve did not then follow the slow elevation of [Ca\(^{2+}\)], in the interval between stimuli, typical of "calcium overload". Force was also averaged
for the same number of cardiac cycles, and measurements made of the peak twitch (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.

Statistical analysis of data

Results from the averaged data were imported directly from the IDL analysis program into SAS statistical analysis software (SAS Institute, Cary, NC, USA) where analyses of variance (ANOVA) were carried out using its general linear model procedure. Data are expressed as mean ± S.E.M., and statistical significance as P ≤ 0.05. In total, 27 SHR and 34 WKY were used in this study, but not all hearts yielded a viable left ventricular trabecula. Not more than one trabecula per heart was utilized. Paired or unpaired t-tests were occasionally used instead of ANOVA for comparison of two means, as appropriate.

Just as with the practice of applying multiple t-tests, multiple ANOVAS performed across many variables run the possibility of inflating α, the Type I Error Rate (i.e. the probability of falsely rejecting a true Null Hypothesis). This occurs because some of the variables (peak twitch force and peak fluorescence, for example) may be highly correlated. Hotelling’s $T^2$ (the two-sample equivalent of Multivariate Analysis of Variance) prevents this possibility by examining the effects on all dependent variables simultaneously. However, use of this test requires that $p < n_1 + n_2 - 1$, and Hotelling’s $T^2 \rightarrow F_p, n_1 + n_2 - p - 1$ (where $p$ is the number of dependent variables, and $n_1$ and $n_2$ refer to the number of SHR and WKY trabeculae, respectively). Whereas over all experiments, $n_1 = 27$ and $n_2 = 34$, for individual interventions there were seldom more than 8 animals per group, although the behaviour of some 14 variables were of interest (see, for example, Table 3.5). Hence, with a single exception (see Chapter IV, Section 4.5), for practical (i.e. “numerical”) reasons multiple ANOVAS were performed with the consequence that the putative error rate may have become inflated. This is not likely to have influenced the overall results of this study, though, since there were few significantly different variables between rat strains.
Figure 2.3 Example of data acquired using LabView software over 15 consecutive cardiac cycles from a representative trabecula. Note that, as intracellular \([\text{Ca}^{2+}]\) increased, the emitted fluorescence at the 340 nm excitation wavelength increased, and the fluorescence at the 380 nm wavelength decreased, whereas there was no change in emitted fluorescence at the 360 nm (isosbestic) excitation wavelength. The ratio of the 340/380 wavelength (top trace) was used as an index of intracellular \([\text{Ca}^{2+}]\).
Figure 2.4 Example of data analysed using IDL software. A, shows 19 consecutive Ca^{2+} transients obtained from a representative trabecula at a stimulation frequency of 5 Hz ([Ca^{2+}]_o 2 mM, 37 °C), vertically aligned for comparison. B, shows the average of the Ca^{2+} transients in A (solid line), fitted with an exponential function (dotted line). C, an overlay of the averaged Ca^{2+} transient (dark line), with the averaged twitch (green line). D, shows the differential of the averaged transient, from which the maximum rate-of-rise of fluorescence was obtained, and E, shows the differential of the fitted transient (dotted line in B). F, phase plots of the averaged force, plotted as a function of averaged fluorescence.
Chapter III

Force and \([Ca^{2+}]_i\) at Physiological Temperature

3.1 Chapter Overview

It is well known that the temperature at which experiments are carried out has profound effects on force and \([Ca^{2+}]_i\) in isolated muscle preparations. A reduction in temperature from 37 °C has a positive inotropic effect on isolated cardiac muscle (Langer and Brady 1968; Shatlock and Bers 1987; de Tombe and ter Keurs 1990). The increase in peak force can be partially explained by the prolonged availability of cytosolic Ca\(^{2+}\) for activation of the myofilaments at lower temperature, since the Ca\(^{2+}\) transport mechanisms that reduce cytosolic [Ca\(^{2+}\)] are slower. Decreasing temperature also produces a decrease in myofilament Ca\(^{2+}\) sensitivity (Harrison and Bers 1989), but this effect appears to be overcome by the slowed return of cytosolic Ca\(^{2+}\) to resting levels.

Since cellular processes are not all affected to the same extent by a reduction in temperature, there is the possibility that the contribution of intracellular Ca\(^{2+}\) transport to heart failure might be misinterpreted if experiments are carried out at non-physiological temperatures. Consider, for example, two Ca\(^{2+}\) transport systems that work in parallel, such as SL Ca\(^{2+}\) extrusion via Na\(^+\)/Ca\(^{2+}\) exchange, and Ca\(^{2+}\) uptake by the SR Ca\(^{2+}\)-ATPase. These two systems have different Q\(_{10}\); the exchanger has a Q\(_{10}\) ~4 (Kimura et al. 1987; Niggli and Lederer 1993) compared to the Ca\(^{2+}\)-ATPase Q\(_{10}\) of ~2.6 (Shigekawa et al. 1976). A decrease in temperature from 37 °C would therefore disproportionately reduce SL Ca\(^{2+}\) extrusion via the Na\(^+\)/Ca\(^{2+}\)-exchanger, and result in a greater fraction of the total Ca\(^{2+}\) being accumulated by the SR (subsequently making more Ca\(^{2+}\) available for release with each contraction (Puglisi et al. 1996; Eisner et al. 1998)).

However, experiments are generally carried out at reduced temperatures to minimise metabolic compromise, and to increase the retention of fluorescent indicators within the
cytosol. In this study, the use of LV trabeculae of very small diameter reduced the risk of an anoxic core developing (see Chapter II). Indicator retention in the cytosol was maximised by addition of probenecid to all solutions following fura-2 loading (see Chapter II), and measurements of fura-2 fluorescence and isometric force were carried out at 37 °C.

3.2 Cardiovascular Changes Associated with Heart Failure

Physical signs of heart failure in SHR

After reaching 18 months of age, SHR were examined regularly for signs of heart failure. Typically, animals in heart failure were observed to have lost physical condition and appeared lethargic. They also had reduced appetite, and were poorly groomed. In addition, their breathing was rapid and shallow when compared to age-matched WKY animals. Fig. 3.1 shows a representative SHR in heart failure, and an age-matched normotensive WKY control. On performing thoracotomy, it was frequently noted that the chest cavity of SHR in failure was awash with clear fluid, and that the lungs were yellowish in appearance. These observations were not made for any of the WKY animals. The lungs from SHR also frequently contained numerous dark brown spots, of up to 2 mm in diameter, assumed to be localised haemorrhages. Compared to WKY, the hearts were all markedly enlarged in SHR, and had visible fibroses (i.e. a white-marbled appearance rather than uniformly dark red). The relative difference in the size of the hearts between rat strains is shown in Fig. 3.2.
Figure 3.1 Animals representative of those used in this study. A, an SHR with typical signs of failure, and B, an age-matched, normotensive, WKY. The SHR has a lower body weight (266 g in comparison to 310 g for the WKY), a poorly groomed coat, and is relatively uninterested in its surroundings.
Figure 3.2 Representative hearts from SHR in failure (A & B) and age-matched WKY (C & D), illustrating the difference in size. Hearts are shown suspended in dissection solution in panels A & C, and with the coronary vessels perfused via a cannula inserted into the aorta in panels B & D.
Patho-physiological evidence of hypertrophy and heart failure

Physical evidence supporting the presence of heart failure and hypertrophy in SHR is summarized in Tables 3.1, 3.2 and 3.3, together with corresponding data for the WKY control animals. Measured values of systolic blood pressure confirmed that the SHR were hypertensive, and that the control WKY animals were normotensive. Both male and female animals were used in this study, and analysis of data showed the expected gender differences relating to body size. For both strains of rat, heart rate was higher in females than in males. Heart rate was also increased in SHR when compared to WKY, but the sex differences in heart rate were the same for both rat strains. Body weight (BW) was lower in SHR (for both male and female), but tibial length (TL, an index of body size) did not differ between SHR and WKY.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WKY Male</th>
<th>WKY Female</th>
<th>SHR Male</th>
<th>SHR Female</th>
<th>P ≤ 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Animals</td>
<td>22</td>
<td>12</td>
<td>17</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Age (months)</td>
<td>23.0 ± 0.6</td>
<td>24.1 ± 0.4</td>
<td>21.0 ± 0.4</td>
<td>23.2 ± 0.3</td>
<td>*$</td>
</tr>
<tr>
<td>Body Wt (g)</td>
<td>422.5 ± 7.8</td>
<td>280.6 ± 10.4</td>
<td>381.0 ± 14.6</td>
<td>236.7 ± 6.5</td>
<td>*$</td>
</tr>
<tr>
<td>Systolic Pressure (mm Hg)</td>
<td>130.5 ± 3.5</td>
<td>127.0 ± 3.8</td>
<td>188.7 ± 5.4</td>
<td>197.1 ± 13.7</td>
<td>$</td>
</tr>
<tr>
<td>Heart Rate (min⁻¹)</td>
<td>348.8 ± 10.9</td>
<td>374.2 ± 6.9</td>
<td>406.4 ± 10.3</td>
<td>444.7 ± 6.8</td>
<td>*$</td>
</tr>
<tr>
<td>TL (cm)</td>
<td>5.3 ± 0.1</td>
<td>4.8 ± 0.0</td>
<td>5.5 ± 0.1</td>
<td>5.0 ± 0.1</td>
<td>*</td>
</tr>
<tr>
<td>Body Wt/TL (g/cm)</td>
<td>81.1 ± 2.0</td>
<td>58.7 ± 2.0</td>
<td>69.3 ± 3.0</td>
<td>48.9 ± 2.0</td>
<td>*$</td>
</tr>
</tbody>
</table>

Table 3.1 Changes associated with heart failure in SHR. Values are means ± S.E.M. WKY: normotensive Wistar-Kyoto rats, SHR: spontaneously hypertensive rats with heart failure, Wt: weight, TL: tibial length. Symbols denote P ≤ 0.05: * differences between sex, $ differences between rat strain. Measurements were made on awake animals immobilized in a restraining tube (apart from tibial length, which was carried out post mortem).

The ratio of heart wet weight to body weight was significantly higher in SHR compared with WKY, as were values for the ratio of the LV free wall to BW, the septum to BW, and the RV free wall to BW. However, since body weight was also lower for SHR, using it as the denominator in the ratio could bias the conclusion that hypertrophy was present. For this reason the ratio of wet weight to TL (which was not different between rat strains) is also
presented (see Table 3.2). When this ratio was used as the index of hypertrophy, the difference between SHR & WKY for the RV free wall to TL was no longer significant.

The outer diameter of the aorta was also significantly larger in SHR, presumably as a results of elevated systolic pressure from early adulthood. Ratios of both the lung wet weight to BW, and the lung wet weight to lung dry weight (an indicator of tissue fluid retention) were elevated in SHR, consistent with pulmonary congestion and oedema. Liver wet weight to BW was also greater in SHR than in WKY (i.e. hepatomegaly), but the wet weight to dry weight ratio for liver was not different between strains.

<table>
<thead>
<tr>
<th>Variable</th>
<th>WKY Male</th>
<th>WKY Female</th>
<th>SHR Male</th>
<th>SHR Female</th>
<th>P ≤ 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV(wet) Wt (g)</td>
<td>0.73 ± 0.02</td>
<td>0.49 ± 0.04</td>
<td>0.94 ± 0.07</td>
<td>0.78 ± 0.05</td>
<td>*§</td>
</tr>
<tr>
<td>RV(wet) Wt (g)</td>
<td>0.24 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.25 ± 0.03</td>
<td>0.18 ± 0.01</td>
<td>*</td>
</tr>
<tr>
<td>Septum(wet) Wt (g)</td>
<td>0.45 ± 0.02</td>
<td>0.41 ± 0.03</td>
<td>0.77 ± 0.05</td>
<td>0.52 ± 0.05</td>
<td>*§ #</td>
</tr>
<tr>
<td>Atria(wet) Wt (g)</td>
<td>0.25 ± 0.02</td>
<td>0.17 ± 0.01</td>
<td>0.35 ± 0.06</td>
<td>0.25 ± 0.03</td>
<td>*§</td>
</tr>
<tr>
<td>Aorta Outer Diameter (mm)</td>
<td>2.7 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>3.6 ± 0.1</td>
<td>3.4 ± 0.1</td>
<td>§</td>
</tr>
<tr>
<td>Heart Wt/ BW (mg/g)</td>
<td>3.95 ± 0.10</td>
<td>4.41 ± 0.12</td>
<td>6.11 ± 0.32</td>
<td>7.28 ± 0.42</td>
<td>*§</td>
</tr>
<tr>
<td>LV(wet) Wt/ BW (mg/g)</td>
<td>1.73 ± 0.06</td>
<td>1.76 ± 0.12</td>
<td>2.50 ± 0.18</td>
<td>3.29 ± 0.19</td>
<td>*§ #</td>
</tr>
<tr>
<td>RV(wet) Wt/ BW (mg/g)</td>
<td>0.57 ± 0.02</td>
<td>0.61 ± 0.03</td>
<td>0.65 ± 0.06</td>
<td>0.75 ± 0.04</td>
<td>§</td>
</tr>
<tr>
<td>Septum(wet) Wt/ BW (mg/g)</td>
<td>1.06 ± 0.04</td>
<td>1.42 ± 0.12</td>
<td>2.04 ± 0.12</td>
<td>2.20 ± 0.24</td>
<td>*§</td>
</tr>
<tr>
<td>Heart Wt/ TL (g/cm)</td>
<td>0.32 ± 0.01</td>
<td>0.26 ± 0.01</td>
<td>0.43 ± 0.03</td>
<td>0.37 ± 0.02</td>
<td>*§</td>
</tr>
<tr>
<td>LV(wet) Wt/ TL (g/cm)</td>
<td>0.14 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>*§</td>
</tr>
<tr>
<td>RV(wet) Wt/ TL (g/cm)</td>
<td>0.05 ± 0.00</td>
<td>0.04 ± 0.00</td>
<td>0.05 ± 0.01</td>
<td>0.04 ± 0.00</td>
<td>*</td>
</tr>
<tr>
<td>Septum(wet) Wt/ TL (g/cm)</td>
<td>0.09 ± 0.00</td>
<td>0.09 ± 0.01</td>
<td>0.14 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>*§ #</td>
</tr>
</tbody>
</table>

Table 3.2 Indices of cardiac hypertrophy. Values are means ± S.E.M. WKY: normotensive Wistar-Kyoto rats, SHR: spontaneously hypertensive rats with heart failure, Wt: weight, LV: left ventricular, RV: right ventricular, TL: tibial length. Symbols denote P ≤ 0.05: * differences between sex, § differences between rat strain, # differences between the interaction of sex and rat strain.
Physiological measurements were made on several parameters including lung weight, liver weight, and heart weight, expressed as a ratio of wet weight to body weight (Wt/BW) or wet weight to dry weight (Wt/dry). The results are presented in Table 3.3.

Table 3.3 Indices of oedema and size. Values are means ± S.E.M. WKY: normotensive Wistar-Kyoto rats, SHR: spontaneously hypertensive rats with heart failure, Wt: weight. Symbols denote P ≤ 0.05: * differences between sex, § differences between rat strain.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WKY Male</th>
<th>WKY Female</th>
<th>SHR Male</th>
<th>SHR Female</th>
<th>P ≤ 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lungs (wet) Wt/BW (mg/g)</td>
<td>4.03 ± 0.34</td>
<td>4.58 ± 0.15</td>
<td>6.67 ± 0.56</td>
<td>6.90 ± 0.43</td>
<td>§</td>
</tr>
<tr>
<td>Lungs Wt wet/dry</td>
<td>4.96 ± 0.03</td>
<td>4.87 ± 0.06</td>
<td>5.59 ± 0.18</td>
<td>5.60 ± 0.16</td>
<td>§</td>
</tr>
<tr>
<td>Liver Wt wet/BW (mg/g)</td>
<td>35.19 ± 1.68</td>
<td>33.81 ± 1.10</td>
<td>43.31 ± 1.49</td>
<td>44.43 ± 1.57</td>
<td>§</td>
</tr>
<tr>
<td>Liver Wt wet/dry</td>
<td>3.12 ± 0.09</td>
<td>3.34 ± 0.06</td>
<td>3.28 ± 0.07</td>
<td>3.37 ± 0.07</td>
<td>§</td>
</tr>
<tr>
<td>Heart Wt wet/dry</td>
<td>0.19 ± 0.00</td>
<td>0.19 ± 0.00</td>
<td>0.19 ± 0.01</td>
<td>0.18 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>Atria Wt wet/dry</td>
<td>4.16 ± 0.15</td>
<td>4.29 ± 0.24</td>
<td>4.70 ± 0.35</td>
<td>5.61 ± 0.20</td>
<td>*§</td>
</tr>
</tbody>
</table>

Sex-related differences were not found for any of the variables associated with either the mechanical, or the fluorescence measurements. Data from both male and female animals were therefore combined for experiments involving subgroups of the total population.

**Use of Fura-2/AM**

Following washout of loading solution, most trabeculae showed a restitution of force to pre-loading levels, and the mean fluorescence at 360 nm excitation was increased to 5.4 ± 0.3 times the autofluorescence value. The relative amount of indicator compartmentalized in intracellular organelles was determined by sequential membrane permeabilisation for a number of trabeculae, as shown in Fig. 3.3. Fura-2 trapped within the intracellular compartments was found to contribute less than 15% of the total fluorescence by the end of an experiment (illustrated for trabeculae at room temperature in Fig. 3.3 as (a + b) x 100), with no temperature-dependent differences found. During these experiments autofluorescence was decreased by approximately 25% on treatment with digitonin, and was decreased further after treatment with Triton. Changes in autofluorescence probably reflect decreased [NADH] on membrane permeabilisation.
Data shown in Fig. 3.3 also indicate the amount of indicator 'lost' during experimentation, represented by the difference between condition 2 (directly after loading) and condition 3 (at the end of the experiment). At room temperature, the loss of fura-2 was \(2.7 \times 10^{-3}\) fluorescence units (V) min\(^{-1}\), and at 37 °C was \(17 \times 10^{-3}\) fluorescence units (V) min\(^{-1}\) (in the presence of 1 mM probenecid). Without the use of probenecid at 37 °C complete loss of indicator occurred in less than 20 min (data not shown), i.e. at a rate of about \(217 \times 10^{-3}\) fluorescence units (V) min\(^{-1}\).

The degree of compartmentation of fura-2 was also estimated for RV preparations loaded by iontophoresis (n = 3, data not shown). Prior to loading, the mean autofluorescence recorded with the 360 nm excitation wavelength was 0.7 V. Loading increased the 360 nm reading in these three trabeculae 5-fold, which was reduced to only 1.5 times the autofluorescence by the end of the experiment. Subsequent treatment with 25 μM digitonin then reduced the 360 nm reading to 0.6 V, slightly less than the original autofluorescence prior to loading. Whilst these results imply that compartmentation of indicator was negligible for iontophoretically-loaded trabeculae, they do not provide conclusive evidence, since little indicator remained within the cytosol immediately prior to the digitonin treatment.

**Comparison of fura-2 loading forms**

In general, the degree of loading (indexed as the increase in fluorescence above the autofluorescence reading at 360 nm excitation) was less for iontophoretically-loaded trabeculae (3.7 ± 1.0, for n = 5 trabeculae) than for AM-loaded. Comparison of Ca\(^{2+}\) and force transients obtained from RV trabeculae loaded using either the AM form of the indicator, or the K\(^+\) salt yielded similar results (data not shown).

It was observed that multiple electrode impalements during iontophoretic loading frequently produced signs of damage at impalement sites, with spontaneous waves of contraction in the impalement area (i.e. areas of localised ‘writhing’). Since results of AM-loaded trabeculae were qualitatively similar to those of iontophoretically-loaded preparations, the AM form of loading was the method of choice because of its simplicity.
Figure 3.3 Estimation of the amount of compartmentalised fura-2. Data are for RV trabeculae at 20 °C (blue bars, n = 6) and at 37 °C (red bars, n = 5). The contribution of autofluorescence is shown as the cross-hatched area for each condition. Determination of the relative amount of indicator contained within intracellular compartments for room temperature experiments was made by dividing a by b.
Effect of acute β-adrenergic blockade in adult WKY

Isolated cardiac preparations, such as trabeculae, no longer receive neural input. In order to determine an appropriate physiological stimulation frequency for trabeculae *in vitro*, the effects of acute β-adrenergic blockade on heart rate and blood pressure were determined for four conscious WKY. Tail-cuff systolic blood pressure and heart rate measurements were made immediately before, and 15 minutes following, intra-peritoneal injection of 10 mg kg⁻¹ DL-propranolol HCl in sterile saline. Results are shown in Table 3.4.

Fifteen minutes after injection of DL-propranolol, resting heart rate was reduced from 368 min⁻¹ to 269 min⁻¹ in four WKY (2 male and 2 female, mean age 24.5 months). From these data, it appears that a stimulation rate of 5 Hz for isolated trabeculae should be close to physiological for LV trabeculae from both SHR and WKY at 37 °C in the absence of β-adrenergic stimulation.

<table>
<thead>
<tr>
<th></th>
<th>Pre-Propranolol</th>
<th>15 min Post-Propranolol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Systolic Pressure (mm Hg)</td>
<td>Heart Rate (min⁻¹)</td>
</tr>
<tr>
<td>WKY (M) 1</td>
<td>112</td>
<td>372</td>
</tr>
<tr>
<td>WKY (M) 2</td>
<td>125</td>
<td>372</td>
</tr>
<tr>
<td>WKY (F) 1</td>
<td>108</td>
<td>395</td>
</tr>
<tr>
<td>WKY (F) 2</td>
<td>114</td>
<td>333</td>
</tr>
<tr>
<td>AVERAGE</td>
<td>115 ± 4</td>
<td>368 ± 13</td>
</tr>
<tr>
<td>Frequency (Hz)</td>
<td>6.1 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4 Effect of Acute β-adrenergic Blockade. Systolic blood pressure and heart rate measurements made immediately before, and 15 minutes following, intra-peritoneal injection of 10 mg kg⁻¹ DL-propranolol HCl for four WKY rats (mean age 24.5 months). Heart rate decreased from ~6 Hz to ~4.5 Hz following drug administration, and systolic blood pressure was increased.
Force and $[Ca^{2+}]_i$ at 37 °C and at 5 Hz stimulation frequency

Fig. 3.4 shows examples of data obtained from a representative WKY trabecula at a stimulation frequency of 5 Hz, and at 37 °C with $[Ca^{2+}]_o$ of 2 mM. Panel A shows raw data for fluorescence (top trace) and force (bottom trace) collected for a 4 s time period. Note that, although force appears to have relaxed back to resting levels between stimuli, the $[Ca^{2+}]_i$ appears not to have reached a plateau. However, resting $[Ca^{2+}]_i$ remained at the same level during the 3 min continuous stimulation at 5 Hz, for all trabeculae (including SHR). Insufficient time for uptake of SR Ca$^{2+}$ between stimuli at 5 Hz might have been expected to produce a steady increase in the resting $[Ca^{2+}]_i$, and ultimately to have decreased the peak amplitude of the Ca$^{2+}$ transients as SR Ca$^{2+}$ stores were depleted. However, this appears not to have been the case.

Examples of averaged data from the same trabecula are shown in Fig. 3.4B: averaged Ca$^{2+}$ transient with the fitted transient (dotted lines) superimposed, and averaged force, C: the derivative of the Ca$^{2+}$ transient, with the derivative of the fitted transient superimposed (dotted lines), and D: averaged data shown as a phase plot for $[Ca^{2+}]_i$ and force.

Results of averaged data from left ventricular trabeculae at 5 Hz stimulation, and at 37 °C, are shown in Table 3.5. Under these conditions, the peak stress of SHR trabeculae was significantly reduced compared to WKY (8 ± 1 mN mm$^{-2}$ (n=8) versus 21 ± 5 mN mm$^{-2}$ (n=8), respectively). The maximum rate-of-rise of stress, obtained from the time-derivative of the twitch force, was lower in SHR, but the time-to-peak force, and the times to 50 % and 90 % relaxation of force, were not different. For SHR both the peak of the Ca$^{2+}$ transient, and the resting $[Ca^{2+}]_i$ were higher compared to WKY (peak: 0.70 ± 0.08 μM vs 0.51 ± 0.08 μM (P < 0.1) and resting: 0.19 ± 0.02 μM vs 0.09 ± 0.02 μM (P < 0.05), SHR vs WKY, respectively). The decay of the Ca$^{2+}$ transient was prolonged in SHR, with a time constant of 0.063 ± 0.002 s vs 0.052 ± 0.003 s in WKY.

In summary, at 5 Hz stimulation, and at 37 °C, resting $[Ca^{2+}]_i$ was higher in SHR trabeculae, as was peak systolic $[Ca^{2+}]_i$ (P < 0.1), but, despite this, twitch force was substantially lower. Peak systolic stress was only 39 % of that in WKY, with no difference in resting stress between rat strains.
Figure 3.4 Example of data obtained from a representative WKY LV trabecula stimulated at 5 Hz. A, raw data records for 340/380 ratio (top) and force (bottom) for a 4 s period. Note that fluorescence and force both return to diastolic levels in the interval between stimuli. B, C, and D, same data as in A, following analysis; averaged data are shown as solid lines, and the fitted data as dotted lines.
<table>
<thead>
<tr>
<th>Variable</th>
<th>WKY</th>
<th>SHR</th>
<th>P ≤ 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Trabeculae cross-sectional area (mm^2)</td>
<td>0.050 ± 0.004</td>
<td>0.059 ± 0.008</td>
<td></td>
</tr>
<tr>
<td>Time-to-peak 340/380 ratio (s)</td>
<td>0.035 ± 0.003</td>
<td>0.040 ± 0.002</td>
<td></td>
</tr>
<tr>
<td>Time constant of decay (s)</td>
<td>0.052 ± 0.003</td>
<td>0.063 ± 0.002</td>
<td>*</td>
</tr>
<tr>
<td>Resting 340/380 ratio</td>
<td>0.43 ± 0.03</td>
<td>0.60 ± 0.03</td>
<td>*</td>
</tr>
<tr>
<td>Resting [Ca^{2+}] (μM)</td>
<td>0.09 ± 0.02</td>
<td>0.19 ± 0.02</td>
<td>*</td>
</tr>
<tr>
<td>Peak 340/380 ratio</td>
<td>0.98 ± 0.08</td>
<td>1.16 ± 0.06</td>
<td>P = 0.1</td>
</tr>
<tr>
<td>Peak [Ca^{2+}] (μM)</td>
<td>0.51 ± 0.08</td>
<td>0.69 ± 0.08</td>
<td>P = 0.1</td>
</tr>
<tr>
<td>Max. 340/380 ratio rise (ms^-1)</td>
<td>0.08 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Max. stress rise (mN mm^-2 ms^-1)</td>
<td>0.98 ± 0.21</td>
<td>0.41 ± 0.11</td>
<td>*</td>
</tr>
<tr>
<td>Time-to-peak force (s)</td>
<td>0.067 ± 0.002</td>
<td>0.068 ± 0.004</td>
<td></td>
</tr>
<tr>
<td>Time-to-50% relax. (s)</td>
<td>0.031 ± 0.001</td>
<td>0.032 ± 0.001</td>
<td></td>
</tr>
<tr>
<td>Resting stress (mN mm^-2)</td>
<td>3.9 ± 1.4</td>
<td>3.6 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Peak systolic stress (mN mm^-2)</td>
<td>20.9 ± 4.8</td>
<td>8.2 ± 1.1</td>
<td>*</td>
</tr>
<tr>
<td>Active stress (mN mm^-2)</td>
<td>16.9 ± 4.6</td>
<td>4.6 ± 1.4</td>
<td>*</td>
</tr>
<tr>
<td>Peak [Ca^{2+}] to peak force (s)</td>
<td>0.032 ± 0.004</td>
<td>0.028 ± 0.004</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.5 Comparison of [Ca^{2+}] and force between LV trabeculae from SHR in heart failure and normotensive WKY. Data are mean ± S.E.M. from LV trabeculae at 37 °C, stimulated at a frequency of 5 Hz, and with [Ca^{2+}]o of 2 mM. [Ca^{2+}], and force were averaged for a number (~16) of consecutive cardiac cycles, and the Ca^{2+} transient fitted according to Eq 2.3 (see Chapter II). Variables in the Table associated with [Ca^{2+}], were derived from the fitted equation, and were not different from those measured from the averaged Ca^{2+} transient (not shown). Symbols denote P ≤ 0.05: * differences between rat strain.
3.4 The Effect of Temperature on Force and \([Ca^{2+}]_i\)

In order to determine whether low experimental temperatures could explain, in part, the differences between results (Table 3.5) obtained in this study and those of others, averaged responses from SHR (n = 9) and WKY (n = 7) trabeculae were compared at room temperature and at 37 °C. Fig. 3.5 shows results for a representative LV trabecula from an SHR, first at room temperature, then at 37 °C. Stimulation frequency was 0.1 Hz and 1 Hz at both temperatures. Fig. 3.5A shows the response at room temperature (~22 °C). At 0.1 Hz there was a complete return to resting levels between stimuli for both fluorescence and force. Increasing the stimulation frequency to 1 Hz, at room temperature, resulted initially in a decrease in peak force that then recovered to reach a steady state level after 18 twitches that was higher than peak force at 0.1 Hz. On increasing stimulus frequency to 1 Hz, the resting [Ca2+] level increased immediately. The peak of the Ca2+ transient increased to a new steady-state level that was higher than at 0.1 Hz, with a similar time-course to the increase in peak force. For this SHR trabecula, it appeared that, at a stimulation frequency of 1 Hz, there was insufficient time between stimuli for complete return of [Ca2+] to resting levels at room temperature. Both peak [Ca2+]i and peak force showed positive Treppe at room temperature for this trabecula. Individual twitches and Ca2+ transients are shown for each frequency beneath the continuous records. Fig. 3.5B shows the subsequent response from the same trabecula to a change in frequency when the temperature was 37 °C. Note that the increase in temperature had very little effect on the amplitude of the Ca2+ transient (Panel A compared with Panel B), but that the kinetics of the decay of both fluorescence and force were markedly increased at the higher temperature. Peak force was very much reduced, and Treppe was negative, on increasing stimulation frequency at 37 °C.

Averaged data from trabeculae at both temperatures are shown in Table 3.6. Experiments were carried out using an extracellular Ca2+ of 1 mM, and a low stimulation rate (1 Hz) to avoid the possibility of irreversible damage from sustained high levels of [Ca2+]i at room temperature. No correction for temperature was made to the \(K_d\) for fura-2 in calculating the [Ca2+]i at room temperature. Whereas experiments carried out by Shuttleworth and Thompson (1991) found that use of \(K_d\) values calculated at 37°C for fura-2 at room temperature underestimated the calculated [Ca2+]i, by about 25%, similar experiments carried out by Groden et
al. (1991) predict that [Ca\(^{2+}\)]\(_i\) would be over-estimated by about 20% if the apparent Ca-EGTA association constant was adjusted for ionic strength, pH and temperature.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WKY Room</th>
<th>WKY Body</th>
<th>SHR Room</th>
<th>SHR Body</th>
<th>P ≤ 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>22.6 ± 0.3</td>
<td>36.5 ± 0.2</td>
<td>22.3 ± 0.3</td>
<td>36.7 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Number of Animals</td>
<td>7</td>
<td>7</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Time-to-peak fluorescence (s)</td>
<td>0.063 ± 0.002</td>
<td>0.039 ± 0.003</td>
<td>0.078 ± 0.005</td>
<td>0.045 ± 0.002</td>
<td>* §</td>
</tr>
<tr>
<td>Time constant of decay (s)</td>
<td>0.232 ± 0.014</td>
<td>0.090 ± 0.007</td>
<td>0.276 ± 0.016</td>
<td>0.116 ± 0.008</td>
<td>* §</td>
</tr>
<tr>
<td>Resting 340/380 ratio</td>
<td>0.37 ± 0.02</td>
<td>0.42 ± 0.03</td>
<td>0.47 ± 0.03</td>
<td>0.49 ± 0.03</td>
<td>* §</td>
</tr>
<tr>
<td>Peak 340/380 ratio</td>
<td>0.76 ± 0.07</td>
<td>0.77 ± 0.04</td>
<td>0.86 ± 0.07</td>
<td>0.88 ± 0.055</td>
<td></td>
</tr>
<tr>
<td>Max. 340/380 ratio rise (ms(^{-1}))</td>
<td>0.06 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.11 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Resting [Ca(^{2+})] (μM)</td>
<td>0.19 ± 0.04</td>
<td>0.27 ± 0.05</td>
<td>0.36 ± 0.06</td>
<td>0.39 ± 0.05</td>
<td>* §</td>
</tr>
<tr>
<td>Peak [Ca(^{2+})] (μM)</td>
<td>1.00 ± 0.17</td>
<td>1.00 ± 0.11</td>
<td>1.29 ± 0.22</td>
<td>1.31 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>Resting stress (mN mm(^{-2}))</td>
<td>4.03 ± 1.37</td>
<td>3.63 ± 1.19</td>
<td>6.58 ± 1.84</td>
<td>4.94 ± 1.30</td>
<td>*</td>
</tr>
<tr>
<td>Peak stress (mN mm(^{-2}))</td>
<td>15.30 ± 2.30</td>
<td>11.79 ± 3.46</td>
<td>18.40 ± 2.89</td>
<td>9.88 ± 1.74</td>
<td>*</td>
</tr>
<tr>
<td>Time-to-peak stress (s)</td>
<td>0.195 ± 0.011</td>
<td>0.079 ± 0.004</td>
<td>0.226 ± 0.010</td>
<td>0.078 ± 0.004</td>
<td>* #</td>
</tr>
<tr>
<td>Max. stress rise (mNmm(^{-2})ms(^{-1}))</td>
<td>0.309 ± 0.048</td>
<td>0.459 ± 0.117</td>
<td>0.282 ± 0.056</td>
<td>0.310 ± 0.056</td>
<td></td>
</tr>
<tr>
<td>Time-to-50% relax. (s)</td>
<td>0.137 ± 0.008</td>
<td>0.036 ± 0.002</td>
<td>0.178 ± 0.012</td>
<td>0.038 ± 0.002</td>
<td>* § #</td>
</tr>
<tr>
<td>Time-to-90% relax. (s)</td>
<td>0.266 ± 0.020</td>
<td>0.189 ± 0.120</td>
<td>0.375 ± 0.052</td>
<td>0.090 ± 0.008</td>
<td>*</td>
</tr>
<tr>
<td>Peak [Ca(^{2+})] to peak force (s)</td>
<td>0.132 ± 0.011</td>
<td>0.041 ± 0.005</td>
<td>0.148 ± 0.011</td>
<td>0.033 ± 0.004</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.6 The Effect of Temperature. Data are mean ± S.E.M. from LV trabeculae at room temperature and at 37 °C, stimulated at a frequency of 1 Hz, and with [Ca\(^{2+}\)]\(_o\) of 1 mM. Symbols denote P ≤ 0.05: * Temperature, § Rat strain, # Interaction between Temperature and Rat strain.
Figure 3.5 The effect of temperature on 

\([\text{Ca}^{2+}]_o\) and force in a representative SHR LV trabecula. \(A\), 22 °C, at 0.1, and 1 Hz stimulus frequency. \(B\), 37 °C, at 0.1, and 1 Hz stimulus frequency. Note that peak force was greater at the higher stimulus frequency at room temperature, but not at 37 °C.
Analysis of data from WKY (n = 7), and SHR (n = 9), showed that the ~15 °C temperature difference had no effect on the maximum rate-of-rise of either [Ca^{2+}], or stress, or on the peak systolic [Ca^{2+}]. All other parameters measured were altered with temperature. Results are shown graphically in Fig 3.6. The kinetics of both the Ca^{2+} transient, and the twitch, were slower at 22 °C when compared to 37 °C for both rat strains, with a greater temperature-dependent effect on the relaxation of force evident for SHR (i.e. the interaction term in the statistical analysis, Table 3.6). Temperature-dependent differences were also found in the resting levels of both [Ca^{2+}], and stress (Fig.3.6). Resting [Ca^{2+}], was higher at body temperature for both SHR and WKY, but, paradoxically, resting stress was lower for both rat strains.

These results differ to those from studies that have investigated myosin isoforms in SHR and WKY. Development of hypertrophy and heart failure, in both human (Nakao et al. 1997) and rat models (Mercadier et al. 1981; Boluyt et al. 1994), have been shown to be accompanied by a change in the relative expression of the left ventricular myosin heavy chain isoforms, from the predominantly fast (adult) V₁ to the predominantly slower (foetal) V₃ type. Bing et al. (1991) found, in experiments carried out at 30 °C, that the peak rate of force development was slower in SHR-F, with no difference in the time-to-peak force, and that the time-to-50% relaxation was prolonged in SHR-F. In addition they reported an increase in the relative proportion of the slower V₃ myosin isoform in these animals (Bing et al. 1991). However, there was no difference in the time-to-peak force, or in the maximum rate-of-rise in stress, between SHR and WKY at 37 °C in this study, although the time-to-peak force (Fig. 3.6B) and relaxation rate (Fig. 3.6F) were slower for SHR at room temperature.

Effect of temperature on the amplitude of the Ca^{2+} transient

Results showed no effect of temperature on the amplitude of the Ca^{2+} transient for either SHR or WKY trabeculae. This result is not in agreement with previous studies utilizing either isolated myocytes from rabbits, ferrets and cats (Puglisi et al. 1996), or RV trabeculae from rats (Janssen et al. 2002). In those studies, the peak [Ca^{2+}] at room temperature was more than 200% that at 37 °C. Puglisi et al. (1996) also used fura-2, although they loaded cells iontophoretically, whereas Janssen et al. (2002) relied on AM loading of Indo-1 (rather than
fura-2). Janssen et al. (2002) found peak isometric force decreased by 67% on rapidly changing from room temperature to 37 °C, whereas for LV trabeculae in this study it was much less than this (peak stress decreased by only 23% in WKY, and by 45% in SHR). It is unlikely that such profound differences can be attributed to either the indicator or the loading technique. It may instead be due to the different time-courses of changing temperature. Both groups effected a rapid temperature change (in the case of Janssen et al., less than 2 s). In contrast, in this study temperature was increased slowly from room to 37 °C, allowing at least 30 min between obtaining results at each temperature. Additionally, room temperature always preceded the increase to 37 °C. It is therefore uncertain as to whether differences between results of this study and those of Puglisi et al. (1996), and Janssen et al. (2002), arise from differences in experimental conditions, preparations, rat strains, or animal age.
Figure 3.6 Differences between $[Ca^{2+}]$, and force at room and body temperatures. Response to temperature of LV trabeculae from SHR (open bars: n = 9) and WKY (solid bars: n = 7) at 1 Hz stimulus frequency, $[Ca^{2+}]_o$, 1 mM. Symbols denote $P \leq 0.05$: * rat strain; # temperature.
3.5 **Response to Altering Extracellular [Ca\(^{2+}\)]**

Changes in [Ca\(^{2+}\)]\(_{o}\) also have profound effects on force production and intracellular Ca\(^{2+}\) homeostasis (Allen *et al.* 1976; Allen and Kurihara 1980; de Tombe and ter Keurs 1990). Increasing [Ca\(^{2+}\)]\(_{o}\) results in an increase in the amount of Ca\(^{2+}\) released from the SR (Bers 1989), and in the magnitude of the Ca\(^{2+}\) transient, but has recently been shown to have little effect on SR Ca\(^{2+}\) content (Trafford *et al.* 2001). I therefore examined the effect of altering [Ca\(^{2+}\)]\(_{o}\) over a range of concentrations encompassing those used for most other experiments reported in the Literature. To minimise the possibility of Ca\(^{2+}\) overload, [Ca\(^{2+}\)]\(_{o}\) was increased for each trabecula only until the magnitude of the twitch started to fall, at which point no further increase was applied. For trabeculae from WKY, the [Ca\(^{2+}\)]\(_{o}\) at which this occurred was lower than for SHR. This resulted in only 4 WKY trabeculae being exposed to 5 mM [Ca\(^{2+}\)]\(_{o}\). A stimulation frequency of 1 Hz was used for these experiments (to mimic the low rates that have generally been used by other investigators).

Averaged mechanical results obtained after 3 min exposure to each [Ca\(^{2+}\)]\(_{o}\) are shown in Fig. 3.7. Over the full range of [Ca\(^{2+}\)]\(_{o}\) used, from 0.5 to 5.0 mM, resting stress was constant and did not differ between rat strains. In contrast, peak twitch stress showed a sigmoidal dependence on [Ca\(^{2+}\)]\(_{o}\) and was quite different between strains (Fig. 3.7A). The maximum active twitch stress was some 63\% higher in WKY than in SHR, and occurred at a lower [Ca\(^{2+}\)]\(_{o}\). With increasing [Ca\(^{2+}\)]\(_{o}\), the maximum rate-of-rise of stress showed a 10-fold increase for both SHR and WKY (data not shown), becoming maximal at a [Ca\(^{2+}\)]\(_{o}\) concentration of 3 mM in WKY, and 3.5 mM in SHR, but was lower in SHR for all [Ca\(^{2+}\)]\(_{o}\). (This result differs from that obtained for the above experiments comparing body temperature and room temperature. This might be due to the relatively low [Ca\(^{2+}\)]\(_{o}\) used for the temperature comparison.) No difference was found in the time-to-50\%, or in the time-to-90\%, relaxation between SHR and WKY, nor was the time-to-50\% relaxation altered with [Ca\(^{2+}\)]\(_{o}\). The time-to-90\% relaxation showed differences with [Ca\(^{2+}\)]\(_{o}\), but not with rat strain. Relaxation was prolonged at very low concentrations of [Ca\(^{2+}\)]\(_{o}\), but this might reflect only the difficulty in measurement when twitch amplitude was very small: at 0.5 mM [Ca\(^{2+}\)]\(_{o}\) peak active stress was 1.6 ± 0.2 mN mm\(^{-2}\) for SHR and 2.3 ± 0.7 mN mm\(^{-2}\) for WKY (see Fig. 3.7A).
Data showing the response of intracellular \([\text{Ca}^{2+}]\) to alterations in extracellular \([\text{Ca}^{2+}]_o\) are shown in Fig. 3.7B. Resting \([\text{Ca}^{2+}]_i\) increased with increasing \([\text{Ca}^{2+}]_o\) for both SHR and WKY, and was greater in SHR compared to WKY for all \([\text{Ca}^{2+}]_o\) examined. Peak systolic \([\text{Ca}^{2+}]_i\) was also higher in SHR than in WKY across all \([\text{Ca}^{2+}]_o\). Whereas the amplitude of the \([\text{Ca}^{2+}]_o\) transient (i.e. the difference between the peak and the resting \([\text{Ca}^{2+}]_i\)) increased monotonically with \([\text{Ca}^{2+}]_o\) in SHR trabeculae, it was significantly depressed at the highest concentration examined (5 mM) in the WKY preparations (\(n = 4\)). In WKY, increasing \([\text{Ca}^{2+}]_o\) above 2 mM resulted in no further increase in the peak stress, or the magnitude of \([\text{Ca}^{2+}]_i\), and resting levels remained constant for \([\text{Ca}^{2+}]_o\) between 0.5 and 4.0 mM. Despite the increased magnitude of the \([\text{Ca}^{2+}]_o\) transients in SHR, peak systolic stress was reduced when compared to WKY under the same experimental conditions, with no difference in the time-course of either force development or relaxation.

These pronounced differences in inotropic (Fig. 3.7A) and \([\text{Ca}^{2+}]_i\) (Fig. 3.7B) responses to \([\text{Ca}^{2+}]_o\) are shown as typical phase plots for representative trabeculae in Fig 3.7C, and for averaged data in Fig. 3.7D where peak stress development is plotted as a function of the peak \([\text{Ca}^{2+}]_i\). It is noteworthy that there is no overlap between the relationships in either Figs 3.7C or 3.7D. Comparable results obtain for the relationship between rat strains at the physiological stimulation frequency of 5 Hz (see Table 3.5).

Although the time-to-peak fluorescence was not affected by altering \([\text{Ca}^{2+}]_o\), for either rat strain, the maximum rate-of-rise of fluorescence increased with \([\text{Ca}^{2+}]_o\) in a similar way for both SHR and WKY (data not shown). The representative examples of fluorescence traces shown in Fig. 3.8A (\(a\): original data; \(b\): same data as in \(a\), but normalised to the peak of the transient) suggest a difference between rat strains in the time constant of decay of the \([\text{Ca}^{2+}]_o\) transient. This is confirmed by the data of Fig. 3.8C that display the mean time constants as a function of peak systolic \([\text{Ca}^{2+}]_i\), for both SHR and WKY. Linear regression analysis showed that, although the time constant of decay was larger in SHR for all \([\text{Ca}^{2+}]_o\), there was no difference between SHR and WKY in the dependence of the time constant on \([\text{Ca}^{2+}]_i\).
Figure 3.7 The effect of [Ca$^{2+}$]$_o$ on [Ca$^{2+}$]$_o$ and stress at 37°C and 1 Hz stimulation frequency. Mean ± S.E.M., (A, B, & D) and representative (C) data for SHR (open symbols) and WKY (solid symbols). A and B: SHR (n = 6) and WKY (n = 5 for 4.0 mM [Ca$^{2+}$]$_o$, n = 4 for 5.0 mM [Ca$^{2+}$]$_o$, n = 6 otherwise). A, peak systolic (squares) and diastolic (circles) stress as a function of [Ca$^{2+}$]$_o$. B, peak systolic and resting [Ca$^{2+}$] as a function of [Ca$^{2+}$]$_o$. Resting [Ca$^{2+}$]$_o$ (circles) was elevated in SHR (open circles) compared to WKY (filled circles) over all [Ca$^{2+}$]$_o$ (P ≤ 0.05). Peak systolic [Ca$^{2+}$]$_o$ (squares) was higher in SHR when compared to WKY for all [Ca$^{2+}$]$_o$ (P ≤ 0.05). C, data from typical experiments shown as phase plots for two different [Ca$^{2+}$]$_o$: an intermediary concentration (WKY: 1.5 mM; SHR: 3.5 mM), and the [Ca$^{2+}$]$_o$ at which force was maximal for these trabeculae (WKY: 3.5 mM; SHR: 5 mM). D, peak systolic stress (from A) plotted as a function of peak systolic [Ca$^{2+}$] (from B).
Figure 3.8 The effect of [Ca\[^{2+}\]]_o on the time constant of fluorescence decay. A, averaged fluorescence transients from representative SHR (continuous line) and WKY (dotted line) trabeculae at 1 Hz stimulation frequency (2 mM [Ca\[^{2+}\]]_o, 37 °C). In panel a both the 340 nm/380 nm fluorescence ratio and the corresponding [Ca\[^{2+}\]]_o are shown. Panel b shows the same fluorescence transients as in a normalised to their respective peak values. B, the time constant of decay of fluorescence decreased with increasing [Ca\[^{2+}\]]_o for both SHR (open symbols) and WKY (filled symbols), but was greater in SHR over all [Ca\[^{2+}\]]_o (P ≤ 0.05). Data are means ± S.E.M. from SHR (n = 6) and WKY (n = 5 for 4.0 mM [Ca\[^{2+}\]]_o, n = 4 for 5.0 mM [Ca\[^{2+}\]]_o, n = 6 otherwise). C mean data for the time constant of decay (as in B above) of fluorescence ratio as a function of peak systolic [Ca\[^{2+}\]]. No difference of slopes of linear regression relations between SHR and WKY. Error bars have been omitted for clarity.
3.6 Distribution and Content of Collagen in Trabeculae

Transverse sections of trabeculae were stained (see Methods) to allow examination of collagen within trabeculae. Fig. 3.9 shows data from representative WKY and SHR LV trabeculae of similar diameter. Panels A and D show that, despite comparable fluorescence records, the SHR preparation contracted much more weakly. Cross-sections of the trabeculae are shown in Fig. 3.9B and Fig. 3.9E, where the pink-stained collagen is seen to be more prevalent in the SHR. Analysis of cross-sections showed that average collagen was six-fold higher in LV trabeculae from SHR (18.0 ± 4.6 % (n = 10) vs 2.9 ± 0.9 % (n = 11) in WKY). The longitudinal distribution of collagen can be seen in Fig. 3.9C and Fig. 3.9F. The difference in collagen organization was marked; in WKY the collagen generally formed quite uniform longitudinal bands (aligned in parallel with the myocytes), whereas in SHR the collagen was far more branched with many strands running transversely, forming a mesh-like structure. Since such a mesh would inhibit the ability of cells to shorten at constant volume, these data indicate that matrix remodeling during the progression to heart failure may be a major contributory factor to failure of the myocytes to develop full force, despite availability of adequate calcium. To examine this point further, force was normalized to the proportion of cross-sectional area occupied by myocytes (rather than to total cross-sectional area). When this correction was applied, the difference in force production at 5 Hz stimulation frequency remained reduced for SHR: peak systolic stress (8.1 ± 1.8 versus 22.3 ± 5.6 mN mm\textsuperscript{-2}myocyte\textsuperscript{-2}), with no difference in diastolic stress (4.4 ± 1.4 versus 3.6 ± 2.3 mN mm\textsuperscript{-2}myocyte\textsuperscript{-2}) between SHR (n = 4) and WKY (n = 5), respectively. In other words, it cannot be the collagen per se which is responsible for diminished contractile performance, but rather the complex three-dimensional structure of the collagen.
**Figure 3.9 Morphometric analysis of LV trabeculae.** Examples of force, $[\text{Ca}^{2+}]$, (5 Hz frequency, 2 mM $[\text{Ca}^{2+}]_0$, 37 °C, sarcomere length: 2.1 μm), and collagen distribution in representative LV trabeculae of similar dimensions from WKY (top) and SHR (bottom). *A* and *D*, averaged 340/380 ratios and force. *B* and *E*, transversely sectioned trabeculae, stained blue for myocytes and pink for collagen. WKY trabecula: 2 % collagen, SHR trabecula: 7 % collagen. *C* and *F*, trabeculae in longitudinal section (extended focus to a depth of 50 μm) stained for collagen.
3.6 Chapter Summary and Discussion

Diagnosis of Heart Failure in SHR

Strong evidence supports the diagnosis of heart failure in SHR used in this study. SHR animals had hypertrophy which had progressed to the RV (typical of development of pulmonary hypertension in response to increased LV filling pressure), an increased ratio of lung wet weight to dry weight, and an increased lung wet weight to body weight ratio. Although it might appear strange that failing hearts may support hypertension, the Framingham heart study showed that 75% of all patients with congestive heart failure were hypertensive (Braunwald 1988). Ultimately, though, systolic pressure will fall, and death will rapidly ensue. Prior to experimentation, SHR exhibited rapid, irregular breathing, and were in poor condition (of those not used for experimentation, 20% of the animals had already died by 22 months, and no SHR survived past 25 months). The livers of SHR were found to be enlarged (indexed as liver wet weight/body weight ratio), a symptom frequently seen in right heart failure (Braunwald 1988). When one considers the poor condition of the animals, the lung oedema, and the frequent presence of left atrial thrombi, the condition of “failure” appears unequivocal. Furthermore, a low ejection fraction in SHR displaying the same overt signs of heart failure as SHR used in this study has been measured (Ward et al. 2003). It is generally accepted that the onset of heart failure in SHR occurs from 18 months, while heart failure per se is a continuum that progresses to end-stage failure and death between 18 and 24 months of age (Bing et al. 1991; Brooks et al. 1993; Doggrell and Brown 1998; Emanuel et al. 1999).

Comparison of haemodynamic variables for animals used in this study with those of other studies (Bing et al. 1991; Brooks et al. 1993; Emanuel et al. 1999) revealed some differences that might arise, in part, from genetic differences in SHR and WKY colonies worldwide (as discussed in Chapter I). For example, animals used in this study were of lower body weight than reported for other studies; e.g. Bing et al. (1991) reported BW for male WKY as 690 ± 16 g, and male SHR with heart failure as 418 ± 7 g (Bing et al. 1991), whereas male animals in this study had body weights of 424 ± 8 g and 381 ± 15 g respectively. Systolic
blood pressure was not different from that reported by Bing et al. for SHR in failure, but the indices of hypertrophy (chamber wall wet weight to body weight ratios) were smaller for animals in this study. Whilst the possibility exists that the SHR hearts were indeed smaller, these differences might also arise from differences in the way the measurements were made, since the values for WKY quoted by Bing et al. (1991) are also higher than WKY used for this study (Bing et al. 1991). For animals used in this study, ventricular and septal weights were obtained after prolonged saline perfusion of the hearts during trabeculae dissection. Tissues were then blotted firmly on clean dry tissue paper before wet weights were obtained. Another difference between SHR in this study and those of Conrad et al. (1991) was that the lung wet weight to dry weight ratio was higher for SHR in this study. This difference is not likely to be due to different measurement techniques, though, as the ratio was not different for WKY between studies.

In this study, at 5 Hz stimulation frequency, and at 37 °C, peak systolic stress in SHR trabeculae was reduced to ~40% of that from age-matched WKY trabeculae. This result is similar to those of others for SHR in failure, for papillary muscle (Bing et al. 1991; Conrad et al. 1991), isolated perfused hearts (Brooks et al. 1994), and isolated myocytes (Gómez et al. 1997).

No alteration in the amplitude of the Ca²⁺ transient was found by Bing et al. (1991) or by Brooks et al. (1994), and resting [Ca²⁺]ᵢ was reported as decreased in SHR with heart failure by these authors. Gomez et al. (1997), using patch clamp techniques, found no difference in the L-type Ca²⁺ current density between SHR and WKY, but reported that the same current had a reduced ability to trigger SR release (indexed as Ca²⁺ spark amplitude) in failing SHR. Emanuel et al. (1999) report a reduced SR Ca²⁺ content for failing SHR (determined using caffeine to release SR stores). However, their study provided no data from age-matched WKY for comparison with failing and non-failing SHR myocytes. In addition, Emanuel et al. (1999) did not find that contractility was decreased in hypertrophied and failing myocytes when compared to myocytes from young WKY (6 and 12 months old). Their experiments were carried out at room temperature, with an extracellular [Ca²⁺] of 1.0 mM, and a stimulation rate of 0.5 Hz. Indo-1/AM was used for measurement of [Ca²⁺].

Differences in AP duration between rat strains might provide an explanation for the increased magnitude of the [Ca²⁺]ᵢ in SHR, since increased action potential duration increases the influx
of trigger $\text{Ca}^{2+}$ via the voltage-gated $\text{Ca}^{2+}$ channels (Sah et al. 2001). In support of this suggestion, electrophysiological studies have shown that prolongation of the membrane depolarization delays the return of $\text{Ca}^{2+}$ to resting levels (Cannell et al. 1987). Previous studies have shown that AP duration is prolonged with hypertrophy and heart failure in SHR (Brooksby et al. 1993; Cerbai et al. 1994; Doggrell et al. 1999).

The Influence of Experimental Conditions on $[\text{Ca}^{2+}]_i$ and Force

As expected, the kinetics of both the $\text{Ca}^{2+}$ transient and the twitch were slowed by reducing temperature, but the extent of this slowing was different between rat strains. At room temperature, the time constant of decay was 260% larger for WKY, and 238% larger for SHR, than at 37 °C, whereas the time from peak force to 50% relaxation was 380% larger for WKY, and 468% larger for SHR. Decreasing temperature appeared, therefore, to have a greater effect on the time taken for relaxation of force, rather than on mechanisms responsible for removal of cytosolic $\text{Ca}^{2+}$, and this effect was more pronounced in SHR. Although the time constant of decay of $[\text{Ca}^{2+}]_i$ was larger for SHR than for WKY at both experimental temperatures, I found no difference in the rate of relaxation of force at 37 °C. This result is consistent with a recent report by Janssen et al. (2002) who showed that under experimental conditions resembling those that prevail in vivo, relaxation rate was limited by cross-bridge cycling, but at lower temperatures it was the decay of $[\text{Ca}^{2+}]_i$ that determined the relaxation rate. Since the relaxation rate was not different between WKY and SHR at 37 °C, this would imply that the increase in the time-to-50% relaxation in force for SHR at room temperature reflects altered $\text{Ca}^{2+}$ handling (consistent with the longer time constant of fluorescence decay for SHR) and not a slower rate of cross-bridge cycling.

The response to alterations in extracellular $[\text{Ca}^{2+}]_o$ was also investigated by Bing et al. (1991) and Brooks et al. (1994). In both studies there was a positive inotropic response to increasing $[\text{Ca}^{2+}]_o$ for both WKY and SHR. Between 0.625 mM and 5 mM $[\text{Ca}^{2+}]_o$, peak isometric force increased 143% in WKY and 158% in SHR papillary muscles (Bing et al. 1991), whereas LV pressure of isolated perfused hearts increased 184% in WKY and 188% in SHR between 0.5 mM and 3 mM $[\text{Ca}^{2+}]_o$ (Brooks et al. 1994). For LV trabeculae at 37 °C in this study, the increase in peak systolic stress between 0.5 mM and 5 mM extracellular $[\text{Ca}^{2+}]$ was much
greater than reported for either of the above studies. Peak systolic stress was increased 263% in SHR trabeculae over this range of extracellular $[Ca^{2+}]$ and 533% in WKY trabeculae. That increases of this magnitude were not seen by Bing et al. (1991), or Brooks et al. (1994), for a similar range of extracellular $[Ca^{2+}]$ almost certainly reflects the different temperature between studies. At 37 °C, $Ca^{2+}$ is very rapidly removed from the cytosol under physiological conditions (compare the time constants of decay for 22 °C and in 37 °C in Table 3.6). As extracellular $[Ca^{2+}]$ was increased, the time constant of decay became smaller at 37 °C (see Figure 3.8B), and the magnitude of the $Ca^{2+}$ transient was increased (see Fig. 3.7D), although to a lesser extent in WKY trabeculae. Both of these responses to $[Ca^{2+}]_o$ are suggestive of increased SR $Ca^{2+}$ load. As previously explained, the Q10 of the SR $Ca^{2+}$-ATPase and that of the Na+/Ca2+-exchanger are different, such that at lower temperatures there is less SL $Ca^{2+}$ extrusion and relatively more SR $Ca^{2+}$ loading. A change in extracellular $[Ca^{2+}]$ would therefore not be expected to produce as great an increase in SR $Ca^{2+}$ load at room temperature as at 37 °C since the SR would reach saturation sooner. Another important difference to consider is the likelihood of an anoxic core developing in the papillary muscles with high perfusate $[Ca^{2+}]$, as in the Bing et al. (1991) study, although this should be less likely to occur in perfused hearts.

Since the reduced contractile force in SHR trabeculae cannot be explained simply by a reduction in the amplitude of the $Ca^{2+}$ transient, explanations based on changes in the time-course of the $Ca^{2+}$ transient need to be considered. The rate of SR $Ca^{2+}$ uptake is strongly influenced by the peak amplitude of the $Ca^{2+}$ transient in cardiac muscle (as discussed in Chapter I), with the SR $Ca^{2+}$ transport rate high when $[Ca^{2+}]_i$ is high. Despite the increased peak amplitude of the $Ca^{2+}$ transient for SHR in this study, under a number of different experimental conditions, the rate of decay in fluorescence was slower for SHR trabeculae. This is consistent with studies of human (Pieske et al. 1995), and animal (Kimura et al. 1989; Ren et al. 1999) hypertrophied myocardium. The influence of $[Ca^{2+}]_i$ on the rate of decay is shown in Fig. 3.8C where linear regression lines fitted to mean data showed no difference in slope between rat strains, although for any particular peak $[Ca^{2+}]$ the decay was slower for SHR. The slower decay in fluorescence for SHR is not simply attributable to saturation of the SR $Ca^{2+}$-ATPase, as even at high $[Ca^{2+}]_i$ the data were well fitted by a linear regression line. No corresponding difference in the time-course of relaxation of stress at 37 °C or in the kinetics of the $Ca^{2+}$ transient were found for either rat strain, consistent with the finding of Janssen et al. (2002) mentioned above (see Section 3.4).
Temperature, stimulation frequency, and extracellular [Ca\textsuperscript{2+}] all influence both the Ca\textsuperscript{2+} transient and twitch force, in isolated cardiac preparations. Therefore, when comparing results between studies, or making conclusions about the ways in which [Ca\textsuperscript{2+}]\textsubscript{i} and force might be altered with heart failure, the experimental conditions must also be considered. Whereas it is difficult to explain, the increased magnitude of the Ca\textsuperscript{2+} transient in SHR trabeculae was a consistent finding across the different experimental conditions outlined in this Chapter, as was the increased level of resting [Ca\textsuperscript{2+}]\textsubscript{i} and the decreased peak systolic stress.
Chapter IV

Interval-Dependent Alterations of Force & $[Ca^{2+}]_i$

4.1 Chapter Overview

An increase of heart rate in vivo usually produces an increase in the force of ventricular contraction, and a decrease in its duration, i.e. a stronger, faster, heartbeat. These changes arise, in part, from the actions of neurotransmitters, hormones and intracellular second messengers in response to physiological demand. Isolated cardiac muscle preparations usually show a similar response when stimulation frequency is increased, even in the absence of neuro-humoral input (for a review see McDonald, Pelzer et al. 1994). This property of cardiac muscle is commonly referred to as a positive force-frequency relationship, or positive Treppe. Experiments described in this Chapter investigate the effect of alterations in stimulation interval on intracellular $[Ca^{2+}]_i$ and force during heart failure in SHR.

4.2 The Response of Cardiac Muscle to Stimulus Rate

Cellular mechanisms underlying the positive force-frequency response

As described in Chapter I, membrane depolarisation results first in a rapid influx of Na$^+$ ions ($I_{Na}$), followed by the opening of the L-type Ca$^{2+}$ channels. Influx of Ca$^{2+}$ ions ($I_{CaL}$) subsequently triggers Ca$^{2+}$ release from the SR (i.e. CICR), and produces the transient rise in intracellular Ca$^{2+}$ that initiates muscle contraction. When the number of membrane depolarisations per unit time is increased, the net influx of Ca$^{2+}$ into the cells is also increased, until some new steady-state is achieved where SL influx again matches SL efflux. The increased net influx of Ca$^{2+}$ increases the amount of Ca$^{2+}$ stored in the SR, producing
augmented Ca$^{2+}$ transients (Han et al. 1994; Bassani et al. 1995), and greater force production. This change in SR Ca$^{2+}$ load occurs within a few beats, producing a rapid inotropic response for isolated muscle preparations. A second, much slower, response to increased stimulus frequency has been identified in Purkinje fibres (from sheep or dog) at 37 °C (Boyett et al. 1987). The slower response takes several minutes to establish, and is associated with a gradual increase in the intracellular [Na$^+$] from the increased frequency of opening of voltage gated Na$^+$ channels per unit time (reviewed in (Langer 1983)). Increased intracellular [Na$^+$] produces a secondary net gain of Ca$^{2+}$, due to a slowing of Ca$^{2+}$ extrusion by the Na$^+$/Ca$^{2+}$-exchanger.

Frequency-dependent modulation of SL Ca$^{2+}$ entry has been demonstrated also. An increase in the frequency of depolarisations produces up-regulation of L-type Ca$^{2+}$ channels (Piot et al. 1996), an increase in the amplitude of the $I_{CaL}$ (Zygunt and Maylie 1990; Peineau et al. 1992), and a prolongation of the current decay (Tiao et al. 1994). The overall effect is a substantial increase in the amount of Ca$^{2+}$ influx, increased SR Ca$^{2+}$ release, and increased twitch force. Application of isoproterenol further enhances these effects.

**Cellular mechanisms opposing a positive force-frequency response**

The force-frequency response is not always positive in mammalian cardiac muscle. As described below, there are species-dependent, as well as patho-physiological, differences in the response to stimulus frequency. At the cellular level, the dominant factor in determining whether the response is either positive or negative is the effect stimulus frequency has on SR Ca$^{2+}$ stores. Factors that contribute to a reduction in the SR Ca$^{2+}$ store (and hence a reduced Ca$^{2+}$ transient amplitude) at high stimulation frequencies include: (i) insufficient time between stimuli for SR re-filling, (ii) insufficient time for recovery of SR Ca$^{2+}$ release mechanisms (such as recovery of RyR from inactivation), and (iii) alterations in [Na$^+$].

Early investigators of the force-frequency response proposed that the time required for translocation of Ca$^{2+}$ within the SR was sufficiently long to explain a reduced [Ca$^{2+}$] transient at high rates of stimulation (Yue et al. 1985). Later studies showed that the distance from uptake sites in the longitudinal SR to the release sites in the junctional SR was of the order of
1 µm (as quoted in Bers (2001)). Diffusion of Ca^{2+} over this distance would therefore take only a few ms. Hence the time taken for translocation of Ca^{2+} was unlikely to account for decreased SR release at high frequencies, unless there was some other mechanism operating within the SR preventing the immediate availability of recently sequestered Ca^{2+} for release.

Gating of the SR Ca^{2+} release channels is central to cardiac EC coupling. It is thought that RyR gating is controlled in some way by an increase in [Ca^{2+}] in the sub-sarcolemmal space immediately surrounding the dyad (Cannell et al. 1995). It has been suggested that continuous regeneration of CICR is prevented by inactivation of the SR Ca^{2+} release channels (RyRs) (Fabiato 1985; Sham et al. 1998), although the mechanism of inactivation is not fully understood. It is therefore possible that, at very high stimulation rates, decreased SR release might result from insufficient time for recovery of RyRs. Cheng et al. (1996) found that the localised time-dependent recovery of an evoked Ca^{2+} transient following a Ca^{2+} wave had a half-time of 450 ms, and Sham et al. (1998) reported an absolute refractoriness of ~150 ms for voltage-clamped rat ventricular myocytes at room temperature. Inactivation time intervals of such magnitudes could therefore explain reduced SR release at high frequencies, although only a small percentage of the total number of RyR are utilised during an electrically-evoked Ca^{2+} transient (Sham et al. 1998). In addition, it has been shown by use of caffeine pulses, and rapid cooling contractures, that the total amount of SR Ca^{2+} available for release is considerably larger than the actual amount released during activation (Bassani et al. 1995).
4.3 The Influence of Stimulus Interval in Rat Myocardium

The force-frequency response

Whereas heart muscle from most mammals shows a positive force-frequency relationship, isolated rat myocardium has historically been described as having a negative relationship, e.g. Koch-Weser and Blinks 1963; Orchard and Lakatta 1985; Capogrossi, Kort et al 1986; Bers 1989; Bouchard and Bose 1989; Shattock and Bers 1989; Maier, Pieske et al, 1997 (but see below). This is paradoxical, since many of the processes associated with increased stimulus rate should also increase force, e.g. rate-dependent increases in [Na⁺], (Maier et al. 1997) and [Ca²⁺], (Orchard and Lakatta 1985) (from the increased number of depolarisations per unit time). Rat myocardium differs from that of many other species in that the duration of the ventricular AP is shorter, with, consequently, a greater dependence on SR Ca²⁺ for activation of the myofilaments (Shattock and Bers 1989; Yuan et al. 1996; Terracciano and MacLeod 1997). As a result, the net SL Ca²⁺ influx at higher frequencies (i.e. from increased L-type channel openings per unit time) will be less in rat, as will the frequency-dependent increase in net SR Ca²⁺ content.

Other studies have suggested a biphasic (Borzak et al. 1991; Kassiri et al. 2000) response for rat myocardium, where at high stimulus frequencies the response is positive. The difference between these studies and reports of negative force-frequency responses might be explained by metabolic compromise (Schouten and ter Keurs 1986), but this does not explain the negative component of the biphasic response at lower frequencies. Finally, some studies have reported a positive (Henry 1975; Schouten and ter Keurs 1986; Layland and Kentish 1999; Janssen et al. 2002) force-frequency relationship for rat myocardium.

Layland et al. (1999) found that when the experimental conditions produced a positive force-frequency response, SR Ca²⁺ load was increased with increasing stimulus frequency. Janssen et al. (2002) also found a positive force-frequency relationship in RV trabeculae at 37 °C, for preparations of ≤ 100 μm diameter, but only when preparations were selected on the basis that their maximum stress exceeded 50 mN mm⁻² at some stage of their experimental protocol.
When temperature was lowered to 22.5 °C these authors saw a slight negative response to increasing frequency in the 0.1 to 1 Hz range. It is notable that Frampton et al. (1991) found both positive and negative force-frequency responses for myocytes isolated from the same hearts. The population of myocytes (~60%) that showed a positive response to increasing frequency (i.e. an increase in the degree of unloaded shortening) also had increased [Na\(^+\)]; activity, increased resting and peak [Ca\(^{2+}\)], and increased SR Ca\(^{2+}\) content. Cells showing a negative force-frequency response showed little or no change in [Na\(^+\)], in the amplitude of the Ca\(^{2+}\) transient, or in SR Ca\(^{2+}\) content. With increasing stimulus frequency, both populations of cells exhibited a decrease in the duration of the Ca\(^{2+}\) transient. These authors concluded that the negative force-frequency response did not result from frequency-dependent changes to the SR Ca\(^{2+}\) load, but that the altered Ca\(^{2+}\) transients might arise from either changes in the AP duration, or in the trigger for SR Ca\(^{2+}\) release (i.e. I\(_{\text{Cal}}\)).

As stimulus frequency is increased there is an acceleration in relaxation regardless of the slope of the force-frequency relationship (Frampton et al. 1991; Layland and Kentish 1999; Kassiri et al. 2000). The frequency-dependent acceleration of relaxation has been linked to the effect of phospholamban and/or CaMKII on regulation of the SR ATPase (Bluhm et al. 2000; DeSantiago et al. 2002) (but see Layland and Kentish 1999; Kassiri, Myers et al. 2000).

Rest potentiation

For rat myocardium the first stimulus following a period of rest produces a twitch of increased peak force, a phenomenon known as rest potentiation. This is generally considered a special feature of rat cardiac muscle. However, in the light of the previous discussion, rest potentiation could be put within the framework of very low frequency of stimulation, such that there is a continuum from a frequency of ~0 to higher rates. The predicted force-frequency response would therefore be bi-phasic, with some uncertainty as to the frequency at which force is minimal.

Following the potentiated twitch, each subsequent twitch is then reduced in amplitude by a similar amount (Bers and MacLeod 1985; Capogrossi et al. 1986; Borzak et al. 1991; Bassani and Bers 1994). Early papers suggested that rest potentiation of contraction was due to an
increase in SR Ca^{2+} stores during the rest period (Schouten et al. 1987). In support of this, Lamont and Eisner (1996) found sodium-independent SL entry of Ca^{2+} during rest periods in rat cardiac trabeculae for experimental conditions where [Ca^{2+}]_o was high. In contrast, Bassani and Bers (1994) found that post-rest potentiation occurs in rat in the absence of an increase in SR Ca^{2+} content, suggesting that it was the slow recovery of EC coupling that accounted for the potentiation. The gradual recovery of RyR from inactivation during the rest interval has been suggested in explanation of rest potentiation (Fabiato 1985. However, it seems unlikely that this could be the only mechanism, since: (i) rest potentiation occurs in rat myocardium but not in other species (such as guinea-pig), suggesting that the duration of RyR inactivation would need to differ between species (rather than being a property of the molecular structure of the cardiac RyR), (ii) not all of the total RyRs available participate in SR Ca^{2+} release associated with each influx of trigger Ca^{2+}, and (iii) rest potentiation is observed even when the preceding stimulus frequency is low.

Another possible explanation of rest potentiation might be that there is an increase in I_{CaL} immediately following a rest period. This could be explained if the gradient for Ca^{2+} entry across the SL were increased following a period of rest, as a result of a continuous reduction in cytosolic [Ca^{2+}]_i during the rest interval. Comparison between voltage clamped myocytes of rat and guinea-pig estimated that SL Ca^{2+} entry during a transient was only 3.5% of SR content for rat, but was 30-50% of the SR content for guinea-pig (Bassani et al. 1994; Terracciano and MacLeod 1997). Therefore any given change to the trigger Ca^{2+} in rat may have a greater influence on the amount of Ca^{2+} released by the SR.

Previous studies have shown that the rat ventricular AP is modulated by the Ca^{2+} transient (Mitchell et al. 1984; Mitchell et al. 1985; Schouten and ter Keurs 1985; Mitchell et al. 1987; Mitchell et al. 1987; DuBell et al. 1991) such that the AP is prolonged when the magnitude of the Ca^{2+} transient is increased. Experiments carried out by Du Bell, Boyett et al. (1991), using isolated rat myocytes at room temperature, showed that when the myocytes were stimulated following a rest period, the duration of the plateau phase of subsequent APs then decreased along with the beat-by-beat decrease in Ca^{2+} transient amplitude, during the recovery period. They found also that the Ca^{2+} transient influence on the AP manifested itself early in the plateau phase, and that at least a portion of the AP modulating current could be attributed to electrogenic Na^-Ca^{2+} exchange.
It has been observed that the twitch-by-twitch recovery of potentiated peak force to pre-rest control levels follows an exponential time-course (ter Keurs et al. 1990). It is commonly assumed, therefore, that a constant fraction of the total SR Ca\(^{2+}\) released during a potentiated twitch is then re-circulated via the SR to produce the next Ca\(^{2+}\) transient (Schouten et al. 1987), with the remainder extruded via the Na\(^+\)/Ca\(^{2+}\)-exchanger (and therefore unavailable for the subsequent Ca\(^{2+}\) transient). The decrease in peak force with each successive twitch following potentiation eventually results in twitch amplitude being less than the pre-rest (control) twitch amplitude (Schouten et al. 1987). Over a longer time period (several minutes) peak force then slowly increases back to the pre-rest control level.

As previously described for the force-frequency response, there are species differences in the myocardial response to periods of rest. Guinea-pig myocardium, for example, shows a decrease in peak force with increasing rest interval duration, whereas human myocardium has a biphasic response. In species, such as rat, that demonstrate rest potentiation of force, it might also be predicted that the force-frequency relationship should also be negative. Although it is commonly assumed that rest potentiation of force results from an increase in SR Ca\(^{2+}\) stores (as a result of continuous Ca\(^{2+}\) sequestration during the rest interval), an increased trigger for SR Ca\(^{2+}\) release might also contribute.

Paired-pulse stimulation

Systolic pressure is increased in vivo for one or more beats following an early diastolic extrasystole (Hoffman et al. 1956), a phenomenon termed post-extrasystolic potentiation. A similar occurrence is observed in isolated cardiac preparations when a premature electrical stimulus is applied early after the preceding stimulus. Whereas the contraction associated with the premature stimulus is weak (or even absent), the following contraction is enhanced. The usual explanation is that the additional Ca\(^{2+}\) influx due to the extra stimulus increases SR Ca\(^{2+}\), subsequently producing an increased Ca\(^{2+}\) transient and a potentiated contraction. Paired-pulse stimulation can be used experimentally to increase net SL Ca\(^{2+}\) entry, thereby increasing SR Ca\(^{2+}\) load, and potentiating twitch force. A train of paired-pulse stimuli increases the SR Ca\(^{2+}\) load substantially, increasing peak force until some new steady-state level is reached.
4.4 Force-Frequency Response in Heart Failure

Notwithstanding marked differences in AP configuration between human and rat, it is worth recalling the force-frequency response in failing human hearts, which has been well studied. It is well known that failing human myocardium, both in vivo (Feldman et al. 1988; Hasenfuss et al. 1994; Kass 1998) and in vitro (Beuckelmann et al. 1992; Mulieri et al. 1992) (Pieske et al. 1995; Sipido et al. 1998; Munch et al. 2000), no longer responds to increased rate with an increased contractile response. The clinical consequence of this for patients with end-stage heart failure is an inability to match cardiac output with demand, resulting in breathlessness and fatigue even at low levels of exercise. At the cellular level, altered Ca\textsuperscript{2+} homeostasis (Pieske et al. 1995) is thought to account, at least in part, for the altered force-frequency response. The slower rate of decay of the Ca\textsuperscript{2+} transient with hypertrophy and heart failure suggests that at high stimulation frequencies insufficient time for SR filling might result in a decreased Ca\textsuperscript{2+} transient. In the absence of alterations to SL Ca\textsuperscript{2+} transport, an increase in resting [Ca\textsuperscript{2+}], levels will ensue, reducing the driving force for L-type Ca\textsuperscript{2+} entry, and decreasing the trigger Ca\textsuperscript{2+} for SR release.

Both a prolonged [Ca\textsuperscript{2+}], transient, and an elevated resting Ca\textsuperscript{2+} level have been reported for tissue from failing human hearts (Gwathmey et al. 1987; Beuckelmann et al. 1992; Sipido et al. 1998). Reduced expression of SR Ca\textsuperscript{2+}-ATPase in human heart failure, at the mRNA and protein levels (Studer et al. 1994; Hasenfuss et al. 1999; Munch et al. 2000), has also been described. Studer et al. (1994) investigated gene expression and protein levels for both the SR Ca\textsuperscript{2+}-ATPase, and the Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger, and compared the relative levels of each in the same tissue samples. They found an increase in gene expression of the Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger, in conjunction with elevated protein levels. In addition, a low SR Ca\textsuperscript{2+}-ATPase mRNA, accompanied by a decrease in the Ca\textsuperscript{2+}-ATPase protein levels, was found. When data from both failing and control human hearts were examined by these authors, there was a positive correlation between SR Ca\textsuperscript{2+}-ATPase and Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger mRNA. The relationship for failing myocardium was shifted to the right, with a negative slope to the fitted linear trend line. They concluded that, for human heart failure in conjunction with dilated
cardiomyopathies, there was a decrease in the density of SR Ca$^{2+}$-ATPase, with an increased abundance of Na$^+$/Ca$^{2+}$-exchanger mRNA and protein levels.

Other cellular mechanisms might also contribute to alterations in the force-frequency response during heart failure. Schillinger et al. (1998) investigated whether inadequate energy supply accounted for the decreased rate of the SR Ca$^{2+}$-ATPase in human heart failure. They recorded the relationship between the force-time integral and myocardial oxygen consumption in isolated muscle strips from control and failing hearts, but found no evidence of impaired energy supply.

Response to altering stimulus interval during hypertrophy and heart failure in SHR

Brooks et al. (1994) investigated the force-frequency response of failing and non-failing hearts of SHR, and WKY (Langendorff-perfused [Ca$^{2+}$]o 1.0 mM, 30 °C). These authors found that both the peak aequorin luminescence and the peak LV pressure were markedly decreased between 2.4 and 3.0 Hz in failing SHR hearts, whereas they were maintained in normotensive WKY. More recently, Janssen et al. (2003) have examined the force-frequency response (between 0.5 and 10 Hz) in both left and right ventricular trabeculae from spontaneously hypertensive heart failure rats (SHHF) and age-matched Wistar controls at 37 °C. They found a biphasic response for normotensive control trabeculae, and for RV SHHF trabeculae. However, these authors found that the response was negative for LV SHHF trabeculae, and was associated with reduced Ca$^{2+}$ transient amplitudes at high frequencies.

Rested-state contractions (the first contraction following a rest period of 15 min), and rest potentiation (the first contraction following a rest period of 1 min), were investigated in papillary muscles from 6 month old SHR and WKY ([Ca$^{2+}$]o 1.3 mM, 37 °C) (Perez et al. 1993), with differences in the response found between rat strains. In normotensive animals, for each protocol, the contraction following the rest interval was potentiated when compared to the pre-rest contraction. For SHR (hypertrophic, but not in failure) there was no contractile potentiation following the rested-state, and the relative rest potentiation was reduced when compared to WKY. These authors attributed their results to diminished SR Ca$^{2+}$ release in SHR when compared to WKY (Perez et al. 1993). Similar conclusions were reached in a
study by Lammerich et al. (1995) in papillary muscles from ~5 month old SHR & WKY, but [Ca\textsuperscript{2+}], measurements were not made in either study to confirm their conclusions. Rest potentiation has not been examined in failing SHR myocardium.

4.5 Experimental Methods

Force-frequency response

Experimental conditions strongly influence the relationship between peak systolic force, and frequency of stimulation for rat myocardium (see Section 4.2 above). Therefore, in addition to examining the force-frequency relationship between LV trabeculae from SHR and WKY, the force-frequency relationship was also examined in trabeculae from young adult Wistar rats. This allowed comparison of results presented here with those of others, in the absence of effects due to age, ventricular preparation, or pathophysiology.

Dissection of RV preparations was carried out following the procedure previously described by Hanley and Loiselle 1998. RV trabeculae were then mounted in the muscle chamber in the same manner as described for LV trabeculae (see Chapter II), and subjected to the same experimental protocols.

Experiments for all three rat strains were carried out at 37 °C, with [Ca\textsuperscript{2+}]\textsubscript{o} of 2 mM. Each trabecula was subjected to six stimulation frequencies between 0.2 Hz and 10 Hz. For SHR (n = 6) and WKY (n = 6) the order in which the frequencies were presented was determined using a 6 x 6 Latin Square design (Edwards 1963) (see Table 4.1). In this design each stimulation frequency appeared at one of six times for each trabecula (i.e. “row”), the sequence being different for each preparation. This protocol was chosen to separate any effect of preceding stimulus frequency from the effects of the frequency being tested. In addition, each of the six frequencies examined was preceded by a 3 min period of stimulation at 1 Hz. Trabeculae were exposed to each stimulation frequency for 3 min, to ensure that a steady-state
response had been reached. At the end of the 3 min, data were obtained for analysis from 10-20 consecutive cardiac cycles, as outlined in Chapter Two.

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<td>2 Hz</td>
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<tr>
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<td>0.2 Hz</td>
<td>1 Hz</td>
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<td>1 Hz</td>
<td>10 Hz</td>
<td>0.5 Hz</td>
<td>0.2 Hz</td>
</tr>
<tr>
<td>Trabecula 6</td>
<td>0.2 Hz</td>
<td>0.5 Hz</td>
<td>10 Hz</td>
<td>1 Hz</td>
<td>5 Hz</td>
<td>2 Hz</td>
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Table 4.1 Latin Square used for force-frequency experiments. Rows show the order each frequency was presented for a trabecula. A 3 min period of stimulation at 1 Hz was carried out between each of the test frequencies.

*Autofluorescence*

The influence of autofluorescence on measurement of fluorescence was investigated by subjecting a trabecula, not loaded with fura-2, to the force-frequency protocol whilst monitoring emitted fluorescence. In order to detect any possible influence on the fura-2 Ca\(^{2+}\) signal, comparison was made of emitted autofluorescence at the end of a 3 min rest period with that recorded at the end of 3 min of stimulation at 5 Hz. At room temperature, this comparison was expected to represent extremes in the mitochondrial concentrations of the reduced nicotinamide adenine nucleotides (NADH and NADPH), the prime source of cellular autofluorescence (Aubin 1979). Small changes only in autofluorescence were detected. The ratio of emitted autofluorescence at the end of a 3 min rest period (when [NADH] should have been high) to that at the end of a 3 min stimulation at 5 Hz (i.e. at low [NADH]) was: ratio 340/380 excitation wavelengths, +1.6 %; 340 nm, 0 %; 360 nm, -0.7 %; 380 nm, -1.3 %. It was therefore concluded that any influence due to autofluorescence alterations was negligible for these experiments, and that metabolic compromise was not problematic.
Rest potentiation and paired-pulse potentiation

For each of the potentiation protocols, trabeculae were stimulated at 1 Hz and then subjected to the intervention (see Fig. 4.4A) for a 30s period. The paired-pulses were delivered at 1 Hz, with an 80 ms interval between pairs. The number of paired-pulses before maximum force was reached was determined for each trabecula. Recovery of force following both potentiation protocols was quantified using a modified version of the IDL analysis program (see Chapter II) for both rat strains. The modification allowed the same measurements to be obtained for [Ca^{2+}]_i and twitch force, but from a single stimulus, rather than from averaged steady-state data. Data for analysis were obtained only from trabeculae that showed good recovery of both force and [Ca^{2+}]_i following potentiation procedures.

The re-circulation fraction was determined by plotting the peak stress of each twitch during recovery from potentiation against the peak stress of the preceding twitch, for SHR (n = 5) and WKY (n = 5) trabeculae. Linear trend lines were fitted to the data arising from each trabecula under both protocols. Multivariate Analysis of Variance was then carried out on the resulting paired data comparing the gradient and the intercept between potentiation protocols and rat strains.

4.6 Results

The force-frequency response

The response to alterations of stimulus frequency for RV trabeculae from young (~ 2 months of age) adult Wistar rats (n = 6) is shown in Figs 4.1A and 4.1B. A bi-phasic response was found for both peak [Ca^{2+}]_i (Fig. 4.1A) and peak stress (Fig. 4.1B) in response to stimulus frequencies between 0.2 and 10 Hz. It was notable that for Wistar rats, peak stress at 5 Hz was essentially the same as that at 0.2 Hz (20.7 ± 3.5 and 20.3 ± 6.8 mN mm^-2 respectively), but that the peak systolic [Ca^{2+}]_i at 5 Hz was much greater than that at 0.2 Hz (1.03 ± 0.29 and 0.61 ± 0.10 µM respectively). These apparently paradoxical results can be explained by
consideration of the frequency-dependent effects on the kinetics of fluorescence decay, illustrated for a representative Wistar RV trabecula in Fig. 4.1C. Data are shown as normalised Ca$^{2+}$ transients for stimulus frequencies of 0.2, 1, 5 and 10 Hz, and are overlaid for comparison. The influence these stimulus frequencies have on peak stress is shown for a representative Wistar trabecula in Fig. 4.1D.

Although peak [Ca$^{2+}$]$_i$ showed a pronounced biphasic response to stimulus frequency for Wistar trabeculae, the response over the same frequencies was only slightly biphasic for SHR and WKY (Fig. 4.2A). The minimum peak [Ca$^{2+}$]$_i$ value occurred at 2 Hz and 1 Hz for SHR and WKY respectively (c.f. 0.5 Hz for Wistar trabeculae). Resting [Ca$^{2+}$]$_i$ was increased at the highest frequency for all three rat strains.

For frequencies of 0.2 to 1 Hz the force-frequency response was negative for all rat strains, with a similar relative decrease in the peak stress. The bi-phasic peak stress response to stimulus frequency found for Wistar trabeculae (Fig. 4.1B), was not found for either SHR, or WKY (Fig 4.2B) trabeculae.
Figure 4.1 The response to stimulus frequency in RV trabeculae from young adult Wistar rats. Average (n = 6) peak systolic (closed symbols) and resting (open symbols) [Ca^{2+}] (μM). A, and stress B. C and D: data from a representative trabecula illustrating the effect of stimulus frequency on the time-course of fluorescence decay, C, and on peak systolic force, D.
Figure 4.2 The response to stimulus frequency for SHR (red diamonds, n = 6) and WKY (blue squares, n = 6) LV trabeculae. A, resting (open symbols) and systolic (closed symbols) \([Ca^{2+}]\), (µM). B, resting (open symbols) and peak systolic (closed symbols) stress. C, time-to-peak fluorescence (open symbols) and time-to-peak force (closed symbols). D, time constant of decay in fluorescence (closed symbols) and time-to-50% relaxation of force (open symbols).
Figure 4.3 Comparison of SHR (▲), WKY (■) and Wistar (⟳) at 0.2 and 5 Hz. Symbols denote P ≤ 0.05: * frequency, # rat strain, § interaction between rat strain and frequency. Note that the time constant of fluorescence decay (τ) decreased with increasing frequency for all rat strains, and that peak [Ca^{2+}]_{i} was increased at 5 Hz when compared to 0.2 Hz only for Wistar. Combination of the frequency-dependent effects on peak [Ca^{2+}]_{i} and the time constant of decay offers an explanation of the positive Treppe for Wistar, and its absence for both SHR and WKY.
Data for the three rat strains at stimulus frequencies of 0.2 and 5 Hz are summarised in Fig. 4.3. It is notable that peak stress at 0.2 Hz was comparable between SHR and Wistar trabeculae, but was much less than that for WKY at the same frequency. Yet at the higher frequency, whilst peak stress was the same as at 0.2 Hz for Wistar, it was very much less for SHR and WKY (41% and 69% respectively). The relationship between the peak \([\text{Ca}^{2+}]\), and the time constant of decay in fluorescence at 0.2 and 5 Hz was different for SHR & WKY when compared to Wistar trabeculae (Fig. 4.1D). Although the frequency-dependent decrease in the time constant of fluorescence decay was similar for all three rat strains, peak \([\text{Ca}^{2+}]\), was not increased to the same extent at 5 Hz for either SHR or WKY. Consequently the force-frequency response was not biphasic for either SHR or WKY trabeculae.

Rest potentiation and paired-pulse potentiation in SHR and WKY

The responses to a 30 s train of paired-pulses (delivered at 1 Hz, with 80 ms delay between pair-pulses), and to a 30 s rest period, were investigated in SHR (n = 5) and WKY (n = 5) trabeculae. Fig. 4.4A shows representative data from an SHR trabecula, with individual transients, and associated twitches shown in Fig. 4.4B for: (i) control, (ii) the peak of the paired-pulse response, and (iii) the first pulse following a 30 s rest interval. Note that the peak force obtained from a series of paired-pulses was equal to the peak force obtained immediately following the rest interval, but that the duration of both the \(\text{Ca}^{2+}\) transient and the twitch were prolonged with the paired-pulse protocol (Fig. 4.4B (ii)). The same peak force for each of the potentiation protocols was frequently seen in trabeculae from both SHR and WKY. When not observed, it was noted that the peak force from the paired-pulse protocol was inevitably less than that from rest potentiation.

The influence of the preceding stimulation frequency on the potentiation of force following a rest period is shown in Fig. 4.5 for a representative LV trabeculae from SHR. Panel A shows a 30 s rest period following a prolonged period of stimulation at 1 Hz, i.e. the protocol used for the above experiments. For this preparation, the amplitude of the twitch was 3 times that of the pre-rest twitch at 1 Hz. Panel B shows the response from this same preparation when a 5 Hz stimulus frequency preceded the rest interval. The amplitude of the twitch following the 30 s rest period was then 3.5 times that of the same 1 Hz control force from the pre-rest period in Fig. 4.5A. The time-course of the initial recovery following the rest period was similar for
Figure 4.4 Response to potentiation protocols for a representative SHR LV trabecula. A, continuous records of 340/380 fluorescence ratio (top), force (middle) and stimulus voltage (bottom). B, individual Ca<sup>2+</sup> transients (top), twitches (middle), and stimuli from the continuous records shown in A, as indicated by the arrows and labels.
Figure 4.5 The influence of preceding stimulus frequency on potentiation following a 30 s rest period for a representative SHR trabecula. A, 1 Hz followed by 30 s rest. B, 5 Hz, followed by 30 s rest.
both pre-rest protocols, but for Panel B peak force then remained steady over the time interval shown.

For the paired-pulse potentiation protocol SHR trabeculae required fewer paired-pulses delivered at 1 Hz (with an 80 ms delay between pulses) before peak stress reached a new (potentiated) steady-state (SHR (n = 7): 10.6 ± 1.3, and WKY (n = 9): 17.5 ± 2.9, P ≤ 0.05). The mean amplitude of Ca\(^{2+}\) transients immediately following paired-pulse stimulation was increased similarly for both rat strains when normalised to their pre-potentiation control amplitudes (Fig. 4.6B), with no difference evident in the time-course of recovery between rat strains. However, the mean amplitude of the twitch was potentiated by 200 % for SHR (n = 5), and 150 % for WKY (n = 4), and the recovery followed a different time-course between rat strains (i.e. the interaction between rat strain and time was statistically significant (P ≤ 0.01)). Whereas SHR trabeculae returned to the pre-intervention control amplitude by the 17\(^{th}\) twitch and remained at that level, the amplitude of the twitch was slower to reach the pre-potentiation control amplitude for WKY trabeculae (Fig. 4.6D).

The time-course of recovery of resting [Ca\(^{2+}\)], and of the time constant of fluorescence decay were not different between rat strains following paired-pulse stimulation, although resting [Ca\(^{2+}\)], was initially high following the paired-pulses for both rat strains (data not shown). The paired-pulse protocol increased resting [Ca\(^{2+}\)], to a greater extent in SHR, and, although there was initially some recovery towards the control level following the first few single pulses, the resting [Ca\(^{2+}\)], in SHR remained higher than the pre-potentiation control level even after 50 s.

Fig. 4.7A shows recovery of the potentiated amplitude of the Ca\(^{2+}\) transient to its pre-rest control Ca\(^{2+}\) (represented by the dotted lines for the normalised transients in Fig. 4.7B). This differed between rat strains (P ≤ 0.05), although the relative increase in the amplitude of the first Ca\(^{2+}\) transient following the rest period was similar for SHR and WKY, and the recovery time-course followed the same trend for both rat strains. The relative increase in the amplitude of the first potentiated twitch (Fig. 4.7D) was similar for SHR and WKY, but analysis revealed that the time-course of twitch amplitude recovery was more rapid for SHR. Fig. 4.7D shows these data normalised to the pre-rest control amplitudes (shown as the dotted line). Neither the recovery of the time constant of fluorescence decay, nor the resting [Ca\(^{2+}\)], was different between rat strains following recovery from rest (data not shown).
Figure 4.6 The time course of recovery following 30 s of paired pulse stimulation ([Ca\textsuperscript{2+}]\textsubscript{i}, 2 mM, 1 Hz). Open symbols SHR (n = 5), solid symbols WKY (n = 4). A, mean amplitude of the Ca\textsuperscript{2+} transients during recovery. B, amplitude of the Ca\textsuperscript{2+} transients when normalized to the pre-potentiation control amplitude (dotted line). C, amplitude of the twitches during recovery. Standard error bars shown only for representative mean values. D, peak stress amplitude, normalized to the pre-potentiation control peak stress value (dotted line).
Figure 4.7 The time course of recovery from a 30 s rest period ([Ca$^{2+}$], 2 mM, 1 Hz). Open symbols SHR (n = 5), solid symbols WKY (n = 4). A, mean amplitude of Ca$^{2+}$ transients during recovery. B, data shown in A, normalized to the average amplitude of pre-potentiation control transients. C, twitch amplitude during recovery. Standard error bars are not shown on all twitches for clarity. D, peak stress normalized to pre-rest control peak stress.
Comparison of the recovery following the two different potentiating protocols for both rat strains (i.e. examination of the statistical interaction of rat strain, potentiation protocol, and time) showed differences for peak [Ca$^{2+}$], peak stress, and twitch amplitude. This was examined further by determining the re-circulation fractions during recovery from each protocol.

The re-circulation fraction

The recovery of twitch force back to control values following either a period of rest or some other potentiating intervention follows an exponential time-course (see Section 4.3 above). By plotting the amplitude of the $(n+1)^{th}$ twitch against the amplitude of the preceding $(n^{th})$ twitch a straight line relationship is obtained. The slope of this trend line is said to represent the fraction of activator Ca$^{2+}$ from the preceding transient that is re-sequestered into the SR between twitches (ter Keurs et al. 1990), and is therefore available for subsequent release with the next stimulation. It is assumed that the balance of [Ca$^{2+}$]i is then extruded via the SL Ca$^{2+}$ transport mechanisms, or removed from the “activator pool” by other processes. Previous estimations of the re-circulation fraction (RF) for SHR and WKY have been obtained only following rest potentiation in young adult animals (Perez et al. 1993). Data are plotted for recovery from potentiation following 30 s of paired-pulses (Fig. 4.8A and 4.8B), and following a 30 s rest interval (Fig. 4.8C and 4.8D) for SHR (open symbols) and WKY (solid symbols). The fit of linear trend lines to the mechanical data, for both potentiating protocols, gave $R^2$ values of 0.99. Data obtained from the amplitude of the Ca$^{2+}$ transients were much more variable, as a result of the inherent noisiness of the fluorescence data obtained from single transients in comparison to the mechanical data.

For each data set shown in Fig 4.8, the line-of-identity is shown as a dotted line. Deviation from the line-of-identity is attributed to that relative portion of Ca$^{2+}$ that is not re-circulated via the SR between stimuli (ter Keurs et al. 1990); i.e. the closer the trend line is to the line-of-identity the greater the estimated RF. The values of RFs determined from the recovery of peak stress following potentiation are summarised in Table 4.2 below. The estimated RF was slightly less for SHR than for WKY (consistent with the larger time constant of decay of fluorescence for SHR, see Chapter III) for both potentiating protocols, although the
Figure 4.8 Re-circulation fractions for recovery from potentiation. The amplitude of the (n + 1)<sup>th</sup> versus the n<sup>th</sup> Ca<sup>2+</sup> transient, A and C, and twitch, B and D, during recovery from potentiation. The slopes of linear regression lines fitted to the data give an estimate of the re-circulation fraction of SR Ca<sup>2+</sup>. The dotted lines show the lines of identity. The greater the deviation of the regression lines from the line of identity, the greater the net loss of SR Ca<sup>2+</sup> between stimuli. Open symbols SHR (n = 5), solid symbols WKY (n = 5). A and B, recovery from 30 s of paired pulses at 80 ms delay; C and D, recovery from 30 s rest.
differences were not significant (statistical analysis of the linear trend lines fitted to recovery data yielded the following P values for rat strain: rest potentiation: 0.19, and paired pulses: 0.26). The fraction of the activator Ca\(^{2+}\) "lost" from the activator pool with each subsequent twitch during recovery is therefore given by the following relationship:

\[ RF = 1 - \text{activator Ca}^{2+} \text{ "lost"} \]  
(Eq. 4.1)

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Stress</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>SHR (n = 5)</td>
</tr>
<tr>
<td>30 s Rest</td>
<td>0.65 ± 0.05</td>
</tr>
<tr>
<td>30 s Paired-pulses</td>
<td>0.82 ± 0.03</td>
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Table 4.2 Summary of results for re-circulation fractions determined during the recovery from potentiation. Multivariate Analysis of Variance showed no difference between rat strains for either protocol, but that the RF was higher using the paired-pulse protocol.

Determination of the RF assumes that the contribution of SL L-type Ca\(^{2+}\) to the transient is very small, and does not vary between stimuli, and that the relationship between SR Ca\(^{2+}\) release and peak force is linear. If the RF were invariable, then it would be predicted that the slope of the trend lines would not differ between potentiation protocols. Since this is not the case, it can be concluded that the decay of [Ca\(^{2+}\)]\(_i\) is influenced by other factors, and that the relative contribution of the various Ca\(^{2+}\) transport mechanisms is altered with experimental protocol.
A bi-phasic force-frequency relationship was observed for Wistar RV trabeculae, similar to that reported for rat RV trabeculae by Kassiri et al. (2000) under different experimental conditions (room temperature, versus 37 °C, and [Ca^{2+}]_{o}, 1 mM, versus 2 mM). At very low stimulus frequencies, peak systolic stress decreased with increasing frequency, but, as frequency was increased further, peak stress began to increase again, until at 5 Hz it was similar to that at 0.2 Hz. Factors associated with rest potentiation in rat myocardium will predominate at low stimulation frequencies (Section 4.3, above). As the interval between stimuli is decreased, the “rest” interval is decreased, and the degree of “potentiation” of force is reduced.

For frequencies above 2 Hz the force-frequency response was positive for Wistar RV trabeculae. An increased number of depolarisations per unit time at high frequencies, together with a decreased time interval for SL ion transport and increased [Ca^{2+}]_{o}-dependent SR uptake (Kimura et al. 1997), might be expected to increase SR Ca^{2+} loading, in turn producing an increase in the amplitude of the Ca^{2+} transient. Previous studies that have reported a positive force-frequency relationship for rat (see Section 4.3 above) have concluded that experimental conditions need to be close to physiological, and that the preparation needs to be of very small diameter for a positive relationship. However, these criteria do not explain either a bi-phasic response over a wide range of frequencies, or the rest potentiation of force. The observed increase in peak force for RV trabeculae for frequencies above 1 Hz, up to and including 10 Hz, would suggest that neither diffusion distance, nor experimental conditions was limiting for these preparations. A similarly biphasic force-frequency response was recently reported for RV trabeculae from mice (Stuyvers et al. 2002). Stuyvers et al. (2002) found that application of 0.1 μM nifedipine, a blocker of L-type Ca^{2+} channels, eliminated the positive phase of the force-frequency response at frequencies above 1 Hz, and that application of 0.1 μM ryanodine, to increase RyR opening probability, eliminated the negative phase of the response at low frequencies. Whilst these results are interesting, it should not be assumed that
explanation of a biphasic force-frequency response resides only in terms of net L-type Ca\(^{2+}\) entry and SR loading. Examination of Fig. 4.1C, and 4.1D shows also that the frequency-dependent time-course of the Ca\(^{2+}\) transient influences peak force also. Acceleration in the decay of the Ca\(^{2+}\) transient with increasing stimulus frequency has been associated with CaMKII-dependent stimulation of SR Ca\(^{2+}\) transport (DeSantiago et al. 2002), although Kassiri et al. (2000) suggest that SR uptake is enhanced by increased [Ca\(^{2+}\)]\(_{SR}\)-dependent phosphorylation of phospholamban.

**Force-frequency response in SHR and WKY**

At low stimulus frequencies, the force-frequency responses for LV trabeculae from both SHR and WKY were negative, and similar to that of Wistar RV trabeculae (see Fig 4.2B). However, for frequencies above 2 Hz, the responses were different to the positive response found for Wistar trabeculae. It should be emphasised that the Wistar animals were young adults (of about 2 months of age), and that their trabeculae were from the right ventricle. The lack of a positive force-frequency response at higher frequencies for either SHR or WKY might therefore be related to myocardial changes due to senescence, although the peak stress at 5 Hz (i.e. close to the in vivo heart rate for rats) was similar for Wistar and WKY trabeculae (Wistar: 20.7 ± 3.5, WKY: 24.8 ± 5.5 mN mm\(^{-2}\)). The positive response in Wistar trabeculae at higher frequencies, and the flattened response for SHR at these same frequencies, might be explained in terms of SR Ca\(^{2+}\) loading (see Section 4.2 & 4.3 above). The slower rate of fluorescence decay for SHR (see Chapter III) is frequently attributed to decreased SR Ca\(^{2+}\)-ATPase activity (Cuneo and Grassi de Gende 1988). If this were so, at high frequencies, incomplete SR uptake between stimuli would be expected to reduce SR Ca\(^{2+}\) load in SHR in comparison to healthy tissue. Consistent with this interpretation, examination of the Ca\(^{2+}\) transient amplitudes (Fig. 4.1A) show that they were increased at high frequencies for Wistar trabeculae, but were not increased to the same extent for SHR (Fig. 4.2A). However, paradoxically, the mean amplitudes of the Ca\(^{2+}\) transients were lower for WKY trabeculae than for either SHR or Wistar across all frequencies (despite comparable peak stress values between WKY and Wistar at a stimulus frequency of 5 Hz). Age-related changes in the time-course of the twitch (Capasso et al. 1983), and in the amplitude and rate of decay of the Ca\(^{2+}\) transient have been observed previously (for reviews see Swynghedauw, Besse et al. 1995; Lakatta and Sollett 2002). Therefore, one possible explanation of the observed Ca\(^{2+}\) transient
differences between WKY (at ~24 months), and Wistar (~2 months), might be the difference in age. Age-related decreases in the amplitude and kinetics of the Ca\(^{2+}\) transient have been associated previously with reduced force production. Ca\(^{2+}\) transients for SHR (at ~24 months, and from failing hearts) had increased amplitudes in comparison to age-matched WKY controls (Fig. 3.6 B and D), yet force was still reduced.

At high frequencies, diminished SR uptake during the interval between stimuli, in the absence of any increase in the amount of Ca\(^{2+}\) extruded via SL mechanisms, would be expected to produce an increase in the resting [Ca\(^{2+}\)]. However, resting [Ca\(^{2+}\)] was increased in SHR in comparison to both WKY (Fig. 4.2A) and Wistar trabeculae (Fig. 4.1A), for all frequencies examined. In addition, the resting [Ca\(^{2+}\)] was increased at 5 Hz and 10 Hz for all rat strains, suggesting that the interval between stimuli did not allow a complete return to resting levels at these frequencies (despite the apparent return of force to resting levels between stimuli for WKY shown in Fig. 3.3A).

Whereas the peak [Ca\(^{2+}\)]-frequency response was biphasic for Wistar, and mirrored the shape of the force-frequency response, it was not obviously so for either SHR or WKY. Studies that have measured [Ca\(^{2+}\)] and force over a range of stimulation frequencies in myocardium from failing human hearts report a flattening of the (normally positive) response at high frequencies (Pieske et al. 1995). A similar result obtains when comparison is made between SHR and Wistar preparations, but not between SHR and WKY. Although the contractile response was reduced for SHR in comparison to WKY (see Fig. 4.2B), the shape of the force-frequency response was similar for both rat strains. The force-[Ca\(^{2+}\)] response was also similar in shape between SHR and WKY strains, but with the amplitude of the Ca\(^{2+}\) transients bigger for SHR. One possible interpretation of these results is that the force-frequency response is affected by aging (if Wistar versus WKY are compared), but this would need to be tested using LV trabeculae from young WKY. It should be noted that very often in human studies tissue from failing and control and hearts is poorly age-matched (see Chapter I), and might account for the different force-frequency response found by Pieske et al. (1995) in their study.

Results from Janssen et al. (2003) for LV and RV trabeculae from SHHF and age-matched normotensive controls over the same range of stimulus frequencies yielded different results to those described in this Chapter. Janssen et al. (2003) found that peak stress was higher for LV trabeculae from SHHF at 0.5 Hz than for either RV SHHF, or control trabeculae, at this
frequency, but that peak stress was similar at 4 Hz for all trabeculae. The reduced peak force at higher frequencies for LV trabeculae from SHHF in the Janssen et al. (2003) study was associated with reduced Ca\(^{2+}\) transient amplitudes. This is in contrast with results from SHR and WKY trabeculae described here. Some of these differences may have arisen from the experimental protocols used, such as the manner in which frequency changes were carried out, the time interval following the stimulus frequency change before measurements were obtained, and the order in which frequency changes were presented. In addition, there are small differences in the ionic composition of solutions (e.g. \([\text{Ca}^{2+}]_o\) (1 mM vs 2 mM), and \([\text{HCO}_3^-]_o\) (25 mM vs 20 mM)) that might also contribute to the differences in results between studies.

**Force potentiation: paired-pulses versus rest potentiation**

In rat myocardium, prolonged rest and paired-pulse stimulation protocols both increase twitch force, but via different mechanisms. Additional SL Ca\(^{2+}\) influx during a train of paired-pulses increases SR Ca\(^{2+}\) content with Ca\(^{2+}\) of extracellular origin (arising from the increased number of depolarisation-induced L-type channel openings with time). In contrast, any net increase in SR Ca\(^{2+}\) content during the rest interval preceding rest potentiation will be predominantly of intracellular origin (Bers et al. 1993). A train of paired-pulse stimuli will increase the relative saturation of intracellular Ca\(^{2+}\) buffers, in addition to increasing the SR Ca\(^{2+}\) load, whereas these buffers are likely to be depleted during a prolonged rest interval.

The mechanism by which force is potentiated following rest in rat myocardium (as opposed to guinea-pig myocardium, for example) is less well understood. Several explanations are suggested in the literature (see Section 4.1 above). Bers and Stiffel (1993) proposed that during the interval associated with rest potentiation, as cytosolic [Ca\(^{2+}\)] is reduced (primarily by sequestration of Ca\(^{2+}\) into the SR), other buffers of intracellular Ca\(^{2+}\) (e.g. mitochondria) release Ca\(^{2+}\) back to the cytosol. The Ca\(^{2+}\) released is then free either to be taken up by the SR, or transported out of the cell (via Na\(^+\)/Ca\(^{2+}\)-exchanger and SL Ca\(^{2+}\)-ATPase). Since rat myocardium favours SR uptake over SL efflux (Terracciano and MacLeod 1997; Cooper et al. 2001), the net SR Ca\(^{2+}\) store is therefore increased during the rest period. Others have proposed that the rest interval allows for full recovery of RyRs from inactivation, therefore
making more receptors available for participation in SR release immediately following the rest interval (Satoh et al. 1997).

Increased SR Ca\(^{2+}\) load appears to account for at least some of the force potentiation following a rest interval, since a higher stimulus frequency preceding the rest interval increases the degree of potentiation in force (see Fig. 4.5, and especially the decrease in resting [Ca\(^{2+}\)]; during the rest interval in panel B). For the data shown (SHR) the resting [Ca\(^{2+}\)]; was increased with stimulation at 5 Hz (as shown by the step in [Ca\(^{2+}\)]; at the beginning of the rest period in Fig. 4.5B), favouring an increase in SR Ca\(^{2+}\) load during the rest interval. Even a relatively small increase in the SR Ca\(^{2+}\) content increases fractional SR release upon stimulation (Bassani et al. 1995).

It should be noted, though, that for either potentiation protocol, the enhanced Ca\(^{2+}\) transient did not restore stress to the control WKY level in SHR. When the potentiated force was normalised to the pre-potentiation control value, however, there was a greater relative increase for SHR trabeculae. This was most obvious with the paired-pulse protocol, and seemed to be the result of a reduced response to paired-pulse stimulation in WKY, since the relative increases were similar for SHR with both protocols.

*Net movement of Ca\(^{2+}\) following potentiation*

Since the source of the Ca\(^{2+}\) contributing to the increased SR load following the two potentiation protocols is different, the time-course of the recovery back to control force levels might be expected to differ also. Following rest potentiation, the initial decline to steady-state control responses should be faster, since depleted intracellular buffers, in addition to SR uptake, will contribute to reducing cytosolic Ca\(^{2+}\) levels between stimuli. Likewise, [Na\(^{+}\)]; during recovery might also differ between protocols. Any accumulation of [Na\(^{+}\)]; during paired-pulse stimulation would act to reduce net SL Ca\(^{2+}\) extrusion via the Na\(^{+}\)/Ca\(^{2+}\)-exchanger, whereas during a prolonged rest period [Na\(^{+}\)]; should remain at “normal” resting levels. Comparison of the estimated SR re-circulation fractions during recovery from both potentiation protocols (Table 4.2) suggests that a greater fraction of activator Ca\(^{2+}\) is re-
circulated between stimuli for both rat strains with the paired-pulse protocol (consistent with a possible increase in [Na⁺]i).

Results of experiments described in Chapter III showed an increased resting [Ca²⁺]i for SHR in comparison to WKY at near physiological stimulation frequencies. This result was also found for experiments described in this Chapter, even when the stimulation frequency was very low (0.2 Hz). The increased resting [Ca²⁺]i for SHR can therefore not be explained by the slower decay of the Ca²⁺ transient alone. However, the SR Ca²⁺-ATPase contributes less to the removal of cytosolic Ca²⁺ when [Ca²⁺]i is low (Choi and Eisner 1999), and SL Ca²⁺ transport mechanisms are then of more importance in maintaining resting [Ca²⁺]i. Although no difference was found in the RF between rat strains (as might be expected, for example, if expression levels of Na⁺/Ca²⁺-exchanger were increased in this animal model of heart failure), the SR Ca²⁺-ATPase was the dominant mechanism for removal of cytosolic Ca²⁺ for each of the potentiation protocols. This might therefore have masked any differences in SL transport contributing to the increased resting [Ca²⁺]i in SHR. Previously it has been shown that, when Na⁺/Ca²⁺-exchange is inhibited, resting [Ca²⁺]i is still regulated, but at a higher level (Allen et al. 1983). Experiments described in the following Chapter were therefore carried out to determine whether a difference in the relative contribution of the SL Ca²⁺ transport mechanisms might exist between WKY and SHR in failure, in the functional absence of the SR Ca²⁺-ATPase.
In healthy cardiac myocytes, intracellular [Ca\textsuperscript{2+}] rapidly returns to resting levels following the depolarisation-induced release of Ca\textsuperscript{2+} from the SR. This restoration of [Ca\textsuperscript{2+}], to resting levels is essential for the heart to function as a pump, since relaxation requires a low [Ca\textsuperscript{2+}], level. Removal of Ca\textsuperscript{2+} from the cytosol is brought about largely by four Ca\textsuperscript{2+} transport systems: the SR Ca\textsuperscript{2+}-ATPase, the sarcolemmal (SL) Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger, the SL Ca\textsuperscript{2+}-ATPase, and the mitochondrial Ca\textsuperscript{2+} uniporter. Of these four mechanisms, the SR Ca\textsuperscript{2+}-ATPase, and the SL Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger are known as the ‘primary’ mechanisms, since they are responsible for removing the bulk of Ca\textsuperscript{2+} from the cytosol (Bers and Bridge 1989; Crespo et al. 1990; Negretti et al. 1993). Triggered release of Ca\textsuperscript{2+} from the SR is very rapid, but, in contrast, the processes that remove Ca\textsuperscript{2+} from the cytosol act more slowly. Typically the peak of the Ca\textsuperscript{2+} transient appears ~30 ms after stimulation (i.e. time-to-peak fluorescence) in trabeculae at 37 °C, whereas the time taken for [Ca\textsuperscript{2+}], to return to resting levels is almost 10-fold longer. Experiments described in this Chapter investigate the relative contribution of the four Ca\textsuperscript{2+} transport mechanisms to the maintenance of resting [Ca\textsuperscript{2+}], during heart failure.
5.2 Mechanisms responsible for maintenance of resting \([Ca^{2+}]_i\)

Transport of cytosolic \(Ca^{2+}\)

As discussed previously, the return of \([Ca^{2+}]_i\) to resting levels following SR release is primarily due to re-uptake by the SR \(Ca^{2+}\)-ATPase. The SR \(Ca^{2+}\)-ATPase therefore influences not only the rate and extent of myocardial relaxation, but also the rate and amplitude of contraction, since this is directly dependent on the amount of \(Ca^{2+}\) stored in the SR, and on the gradient between the SR and the cytosol at the time of \(Ca^{2+}\) release. Initially, following release, SR uptake of \(Ca^{2+}\) is rapid, but as \([Ca^{2+}]_i\) is reduced closer to the resting level, the rate of SR \(Ca^{2+}\) sequestration is also reduced. The contribution of other \(Ca^{2+}\) transport mechanisms then dominates (Choi and Eisner 1999).

Experiments described in Chapters III and IV revealed differences between SHR and WKY in the rate of decay of fluorescence following triggered SR release, and in the level of resting \([Ca^{2+}]_i\). A slower rate of fluorescence decay is consistent with previous reports of hypertrophy and heart failure (see Chapter II), and has been attributed to a decrease in SR \(Ca^{2+}\)-ATPase activity. It has been suggested that this results in reduced SR \(Ca^{2+}\) stores and diminished \(Ca^{2+}\) transients, thus explaining the contractile dysfunction typical of heart failure (Pieske et al. 1995; Pogwizd et al. 2001). Experiments described in Chapter IV also showed that, although the recovery of peak stress to steady-state levels following potentiation differed between SHR and WKY, the slower rate of SR uptake in SHR trabeculae did not compromise the fraction of activator \(Ca^{2+}\) re-circulated via the SR between twitches (at 1 Hz) during the recovery.

The amount of \(Ca^{2+}\) transported by the SR \(Ca^{2+}\)-ATPase, relative to that transported by other mechanisms, differs between species (Bassani et al. 1994; Terracciano and MacLeod 1997), with maturity (Bassani and Bassani 2002), and with disease (Hasenfuss et al. 1994). Since the hypertrophic response increases foetal gene expression (Parker et al. 1990; Razeghi et al. 2001) (for review see Swynghedauw 1986), and, in general, \(Na^+/Ca^{2+}\)-exchanger expression is increased in the foetus compared to mature myocardium, such a change in expression might
explain alterations in time-course of the Ca\textsuperscript{2+} transient (Studer et al. 1994; Hasenfuss et al. 1999; Pogwizd et al. 1999).

**Trans-sarcolemmal transport of intracellular Ca\textsuperscript{2+}**

The electrochemical gradient for Ca\textsuperscript{2+} entry across the SL is large, requiring an active transport system, or a system coupled to energy supply for maintenance of resting [Ca\textsuperscript{2+}]i levels. Under steady-state conditions, the net SL Ca\textsuperscript{2+} efflux (via the Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger and the SL Ca\textsuperscript{2+}-ATPase) is balanced by the net influx of trigger Ca\textsuperscript{2+} during depolarisation. Imbalance of these trans-sarcolemmal transport systems would result in a net gain or loss of [Ca\textsuperscript{2+}]i, thereby preventing steady-state conditions. Experimentally, it is relatively easy to perturb steady-state conditions, by altering [Ca\textsuperscript{2+}]o (Chapter III) or stimulation frequency (Chapter IV), for example. When this is carried out, a new steady state Ca\textsuperscript{2+} balance is achieved usually within just a few contractions. This rapid return to some new level is brought about by alterations in the relative contributions of the different transport mechanisms. Beat-to-beat regulation also occurs in vivo as the heart matches output to demand, such that, even in heart failure, the heart is usually able to function adequately under non-stressful conditions. Of the two SL Ca\textsuperscript{2+} transport mechanisms, the Ca\textsuperscript{2+}-ATPase has the higher affinity for Ca\textsuperscript{2+}, but a lower maximum velocity for Ca\textsuperscript{2+} transport (Caroni and Carafoli 1980) (but see Lamont and Eisner 1996).

In general, Ca\textsuperscript{2+} is very highly buffered in cardiac myocytes, as with other cells. For a cytosolic free [Ca\textsuperscript{2+}] of 1 \mu M, as might occur at the peak of the Ca\textsuperscript{2+} transient, the total cytosolic [Ca\textsuperscript{2+}] for a myocyte is estimated to be in the order of 120 \mu M (Hove-Madsen and Bers 1993). The relative importance of individual ligands to the overall decay of the Ca\textsuperscript{2+} transient will depend on their cytosolic abundance, and on their equilibrium dissociation constants (K\textsubscript{d}). Many factors, such as [H\textsuperscript{+}], and [Mg\textsuperscript{2+}], for example, are known to influence cytosolic Ca\textsuperscript{2+} buffering by shifting the K\textsubscript{d} for Ca\textsuperscript{2+}-binding.
Mitochondria as sources and sinks of intracellular Ca\(^{2+}\)

Mitochondria are located in close proximity to the SR in cardiac cells, resulting in their exposure to high [Ca\(^{2+}\)] immediately following SR release. Some studies have shown that the Ca\(^{2+}\) content of the mitochondria changes in response to beat-to-beat changes in cytosolic [Ca\(^{2+}\)], (Isenberg et al. 1993; Ohata et al. 1998), although at physiological levels of [Ca\(^{2+}\)], mitochondrial uptake is thought to be minimal (Fry et al. 1984). Intra-mitochondrial free [Ca\(^{2+}\)] has an important role in the regulation of respiration (Denton et al. 1988) (for reviews see Balaban 1990; Gunter et al. 1994). Therefore the location of mitochondria at the SR release sites in cardiac muscle probably contributes to close matching of energy supply and demand in coordination with E-C coupling. Mitochondria comprise about 30% of cardiac myocyte volume (Delbridge and Loiselle 1981; Barth et al. 1992), and are therefore capable of substantial Ca\(^{2+}\) buffering (Zhou et al. 1998).

Entry of Ca\(^{2+}\) into the mitochondria is via a Ca\(^{2+}\) uniporter in the outer mitochondrial membrane. The rate at which the uniporter transports Ca\(^{2+}\) is dependent on the mitochondrial membrane potential, maintained through the extrusion of protons by the electron transport chain. Any reduction in the mitochondrial membrane potential therefore reduces the rate of Ca\(^{2+}\) uptake via the uniporter (Bowser et al. 1998).

Mitochondrial efflux of Ca\(^{2+}\) is via a non-electrogenic 2Na\(^+\)/Ca\(^{2+}\)-exchanger, and a non-electrogenic Ca\(^{2+}\)/2H\(^+\) exchanger (for a review see (Gunter et al. 1994)). The 2Na\(^+\)/Ca\(^{2+}\)-exchanger is sensitive to [Na\(^+\)], and could also work to extrude mitochondrial Na\(^+\) and take up Ca\(^{2+}\). The [Na\(^+\)] dependence of this exchanger has a half maximal Ca\(^{2+}\) extrusion at an intracellular [Na\(^+\)] of ~5-8 mM (Fry et al. 1984). Mitochondrial accumulation of Ca\(^{2+}\) is likely to play a significant role under some in vitro conditions where intracellular [Na\(^+\)] is significantly perturbed, such as when solutions containing 0 mM [Na\(^+\)]\(_o\) are used.

Excessive accumulation of mitochondrial Ca\(^{2+}\) can result in the selective permeabilisation of the mitochondrial membrane into a large proteinaceous pore, providing an additional pathway for Ca\(^{2+}\) efflux. This pore has been implicated as playing a central role in cell death pathways, and is likely to exist in patho-physiological states (for review see Gunter and Pfeiffer 1990).
5.3 The Ca$^{2+}$ transport mechanisms

The relative roles of the major Ca$^{2+}$ transport mechanisms to the decay of [Ca$^{2+}$]$_i$ following SR release have been investigated previously in experiments using isolated myocytes (Bassani et al. 1992; Bassani et al. 1994; Bassani 1994; Choi and Eisner 1999; Choi et al. 2000) or RV trabeculae (Lamont and Eisner 1996). Commonly, these experiments have involved manipulation of extracellular ion concentrations, and/or exposure to pharmacological agents that block one or more of the Ca$^{2+}$ transport mechanisms.

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

Caffeine is used experimentally as a pharmacological agent capable of totally releasing the SR Ca$^{2+}$ stores in cardiac muscle (Bers et al. 1987; Callewaert et al. 1989). The application of caffeine to quiescent cardiac muscle causes a transient increase in both [Ca$^{2+}$]$_i$ and force (Allen and Kurihara 1980; Kitazawa 1988; Smith et al. 1988), that, even with continuous application, eventually relaxes back to resting levels. Caffeine, as with other methylxanthines, activates the RyRs by binding at a specific site. Rapid application of ~5 mM caffeine to muscle preparations decreases the Ca$^{2+}$ dependence of RyR gating, so that even at resting [Ca$^{2+}$], there is release of SR Ca$^{2+}$ stores (Rousseau and Meissner 1989; Sitsapesan and Williams 1990). The time-course of the caffeine-induced response is prolonged in comparison to a response evoked by electrical stimulation, because of the “leakiness” of the SR as a Ca$^{2+}$ storage site.

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$^{2+}$]$_i$ is used as a measure of the amount of releasable Ca$^{2+}$ stored in the SR. It is less acceptable to use caffeine in this way to determine SR Ca$^{2+}$ stores in multicellular preparations that are superfused (rather than perfused, via the coronary circulation) though, since Ca$^{2+}$ release by this means necessarily relies on the diffusion of caffeine to the release sites (RyR). With multicellular preparations, such as trabeculae, those cells near the periphery will release their SR contents before those in the...
centre of the preparation. Therefore caffeine-induced Ca\(^{2+}\) release is not synchronised for multicellular preparations, unlike the case for electrically evoked SR Ca\(^{2+}\) transients.

Caffeine also modifies Ca\(^{2+}\)-activated force production by increasing the sensitivity of the myofilaments to Ca\(^{2+}\) (Wendt and Stephenson 1983), and is therefore not suitable for use when an unperturbed value of force is required. The effects of caffeine are completely reversible for the concentrations commonly used experimentally, unlike ryanodine, for example, which is also used to release SR Ca\(^{2+}\) in a similar manner to caffeine.

When a sustained application of caffeine is made to an isolated muscle preparation, the contribution of the SR as a Ca\(^{2+}\) storage site is functionally eliminated. Although the SR Ca\(^{2+}\)-ATPase still transports Ca\(^{2+}\), the RyRs remain in an open state, so that there is no net Ca\(^{2+}\) storage. A decrease in [Ca\(^{2+}\)]\(_i\) under these conditions must therefore be via other Ca\(^{2+}\) transport mechanisms.

The use of carboxyeosin to block the sarclemmal Ca\(^{2+}\)-ATPase

Caroni and Carafoli (1980) first demonstrated a sarclemmal Ca\(^{2+}\)-ATPase for cardiac muscle. They suggested that this SL Ca\(^{2+}\) transporter had an important role in the maintenance of resting [Ca\(^{2+}\)]. Determining the relative contribution of the SL Ca\(^{2+}\)-ATPase to Ca\(^{2+}\) transport was initially difficult, since there was no specific inhibitor of the ATPase. Some studies made use of a thermodynamic approach (Bassani et al. 1992; Negretti et al. 1993), by exposing preparations to a high [Ca\(^{2+}\)]\(_o\) (10 mM). It was reasoned that Ca\(^{2+}\) extrusion by the SL ATPase was effectively eliminated in this manner, since the concentration gradient against which the pump was required to work was increased about ten-fold. Later, carboxyeosin (CE) was identified as a specific inhibitor of the SL Ca\(^{2+}\)-ATPase (Gatto and Milanick 1993), with no effect on the SL Na\(^{+}\)/Ca\(^{2+}\) exchanger. It has been used since to investigate the role of the SL Ca\(^{2+}\)-ATPase in extrusion of cytosolic Ca\(^{2+}\) in isolated cardiac myocytes (Bassani et al. 1995; Choi and Eisner 1999).
Inhibition of the mitochondrial Ca\(^{2+}\) uniporter

A commonly used inhibitor of the mitochondrial Ca\(^{2+}\) uniporter is the polysaccharide dye ruthenium red (Gunter and Pfeiffer 1990). Ruthenium red is not specific for the uniporter, and has also been shown to inhibit SR Ca\(^{2+}\) transport (Kargacin et al. 1998) in a dose-dependent manner from 5 - 20 \(\mu\)M. At low concentrations, \(~1\ \mu\)M, ruthenium red prevents accumulation of Ca\(^{2+}\) in the mitochondria (Vasington et al. 1972), and has a cardio-protective effect against hypoxic injury in isolated-perfused rat hearts (Park et al. 1990).

5.4 Methods

Investigation of the Ca\(^{2+}\) fluxes contributing to the decay of \([\text{Ca}^{2+}]_i\) was made for a number of SHR and WKY LV trabeculae. This was done by first functionally eliminating SR Ca\(^{2+}\) storage with 10 mM caffeine, followed by subsequent exposure to 5,6-carboxy eosin diacetate (CE, Molecular Probes), and 0 mM \([\text{Na}^+]_o\) (see below) combined with 10 mM Ni\(^{2+}\), to inhibit the SL Ca\(^{2+}\)-ATPase and Na\(^+\)/Ca\(^{2+}\) exchange, respectively (Choi and Eisner 1999). Trabeculae were loaded for 30 min with 20 \(\mu\)M CE from a freshly made 10 mM stock solution of CE in DMSO. Loading was followed by a washout period of ten minutes in fresh control solution, to ensure that CE was completely removed from the extracellular space. Finally, addition of 1 \(\mu\)M ruthenium red was made to the superfusate, to prevent mitochondrial accumulation of cytosolic Ca\(^{2+}\) by the uniporter. Exposure to caffeine was then carried out again, whilst inhibiting both SL Ca\(^{2+}\) transport mechanisms (as above).

For these experiments, a Tyrode solution was used, in place of the bicarbonate-buffered Krebs-Henseleit solution used previously. The Tyrode solution was composed of the following (in mM): 141.8 NaCl, 6 KCl, 1.2 MgSO\(_4\).7H\(_2\)O, 1.2 Na\(_2\)HPO\(_4\), 10 HEPES, 2 CaCl\(_2\) and 10 D-glucose, adjusted to pH 7.4 with NaOH. A Na\(^+\)-free and Ca\(^{2+}\)-free Tyrode solution was obtained by equimolar substitution of NaCl with LiCl, and the use of K\(_2\)HPO\(_4\).3H\(_2\)O in the place of Na\(_2\)HPO\(_4\). Ca\(^{2+}\)-free Tyrode solution was used as the superfusate to eliminate any SL Ca\(^{2+}\) influx during the evaluation of SL Ca\(^{2+}\) extrusion (O'Neill and Eisner 1990). To ensure that the solution was effectively Ca\(^{2+}\) free, CaCl\(_2\) was replaced with 1 mM EGTA, and
the pH was adjusted with KOH. Tyrode solutions were continuously bubbled with 100% oxygen. These experiments were carried out at room temperature (20-22 °C).

Data analysis

LabView text files of 100 s in duration, from 10 s before the onset of the caffeine transient, were created for analysis using a custom-written IDL programme (see Section 2.9, Chapter II). The kinetics of the decay phase of the caffeine-induced fluorescence were determined by fitting a three-parameter exponential function from 90% of the peak fluorescence (see Chapter II, Section 2.9). Comparisons of the time constants of decay in fluorescence, for rat strains, and experimental conditions, were then made. Note: it was not appropriate to compare the time-to-peak for these caffeine-induced transients, since the time taken for diffusion of caffeine throughout trabeculae was variable for these multicellular preparations. Likewise, the magnitude of the caffeine transient was not necessarily representative of the SR Ca\(^{2+}\) store. No difference was found between rat strains for the average diameter of trabeculae used for these experiments, and the peak fluorescence of the caffeine transient was not significantly different between rat strains (data not shown). This allowed inferences to be made about Ca\(^{2+}\) transport mechanisms between SHR and WKY by comparison of the time constant of decay in fluorescence (Bers and Berlin 1995).
5.5 Results

The response to caffeine

Fig. 5.1A shows fluorescence records from a representative trabecula in response to application of 10 mM caffeine at room temperature. Comparison of averaged results from SHR and WKY trabeculae (Panel B) showed that the time constant of decay was greater for SHR.

The effect of Carboxy eosin on the Ca\(^{2+}\) transient

Previously, CE has been used to inhibit the cardiac SL Ca\(^{2+}\)-ATPase in isolated myocytes (Bassani et al. 1995; Choi and Eisner 1999), but not in multicellular cardiac preparations. Since the effectiveness of CE as an inhibitor of the SL Ca\(^{2+}\)-ATPase relies both on its diffusion throughout the preparation, and on its de-esterification within the cytosol, averaged data for Ca\(^{2+}\) transients and force obtained immediately prior to CE loading were compared with those obtained after loading, following a 10 minute period in CE-free solution. Fig. 5.2A shows the fluorescence and force obtained from an LV trabecula before, during and after CE loading. Although the CE loading solution was highly fluorescent (see Fig. 5.2A) replacement with CE-free Tyrode solution showed an immediate decrease in both the resting, and peak, fluorescence.

Comparison of averaged, electrically-evoked, Ca\(^{2+}\) transients before and after CE loading showed increases in the following parameters with CE: resting [Ca\(^{+}\): SHR, 124%, and WKY, 127% (NS, P = 0.08); peak [Ca\(^{2+}\): SHR, 111%, and WKY, 108%; time constant of fluorescence decay: SHR, 107%, and WKY, 103%; peak force: SHR, 114%, and WKY, 111%; and time to 90% relaxation in force: SHR, 117%, and WKY, 115%. Paired t-tests showed these increases were significant for resting [Ca\(^{2+}\)], in SHR only, and for peak systolic [Ca\(^{2+}\)], in both SHR and WKY (P ≤ 0.05). These data are displayed graphically in Fig. 5.3.
Since resting and peak $[Ca^{2+}]_i$ were both increased following CE loading, and, given the highly fluorescent nature of the CE loading solution (see Fig 5.2A), it might be assumed that any increase in fluorescence following loading arose from intracellular CE, rather than from increased $[Ca^{2+}]_i$. In order to test for non-$Ca^{2+}$ related changes in fluorescence, emitted fluorescence at 380 nm was plotted against that at 340 nm (see Chapter II, section 2.7) before and after CE loading. For each trabecula, data were obtained from before, and after, CE loading, and linear regression lines fitted. Paired t-tests carried out on the gradients of the regression lines revealed no $Ca^{2+}$-independent differences in fluorescence after CE loading. Therefore it was assumed that any increase in fluorescence following CE loading was due to alterations in $[Ca^{2+}]_i$, and not to CE per se.
Figure 5.1 The response to caffeine. *A*, fluorescence from a representative trabecula before, during, and after application of 10 mM caffeine, at 22 °C. Red arrows indicate reduction of $[Ca^{2+}]_o$ to 0 mM: grey bars indicate duration of the caffeine exposure. *B*, average time constants of fluorescence decay for SHR (hatched bars) and WKY (solid bars).
Figure 5.2 The effect of carboxy eosin (CE) loading on the electrically-evoked Ca^{2+} transient for a representative trabecula. A, fluorescence (top trace) and force (bottom trace) before, during and after CE loading. B, overlaid Ca^{2+} transients, and twitches from before and after (red arrows) CE loading for representative WKY and SHR trabeculae.
Figure 5.3 Summary of the effect of carboxy eosin on electric ally-evoked Ca²⁺ transients. Data are means and S.E.M. for SHR (n = 5, open bars), and WKY (n = 5, solid bars) trabeculae. P<0.05: *.
The relative contribution of all four Ca$^{2+}$ transport mechanisms

Fig. 5.4 shows the experimental protocol used to determine the relative contribution of the Ca$^{2+}$ transport mechanisms to the decay in fluorescence for a representative SHR trabecula at room temperature. Panel A shows application of 10 mM caffeine to a quiescent trabecula in the nominal absence of extracellular Ca$^{2+}$. Note that, on elimination of Ca$^{2+}$ from the superfusate, there was a decrease in fluorescence. Application of caffeine caused a transient rise in fluorescence, that then decayed slowly back to pre-caffeine levels. Caffeine was effective in functionally eliminating the SR as a Ca$^{2+}$ storage site, since electrical stimulation, begun prior to removal of superfusate caffeine, did not evoke an increase in either Ca$^{2+}$, or force, even in the presence of extracellular Ca$^{2+}$ (data not shown). Removal of caffeine (Fig. 5.4A), and re-introduction of [Ca$^{2+}$]$_o$, caused an immediate increase in the resting level of fluorescence, with a slow recovery of the Ca$^{2+}$ transient over several minutes.

During sustained exposure to caffeine, it is the Na$^+$/Ca$^{2+}$-exchanger that is primarily responsible for removal of Ca$^{2+}$ from the cytosol, with some contribution from the SL Ca$^{2+}$-ATPase and intracellular buffers of Ca$^{2+}$ (such as mitochondria). As discussed previously (see Chapter I, Section 1.2), the exchanger has a stoichiometry of 3 Na$^+$ ions transported into the cytosol for every Ca$^{2+}$ ion extruded. It is therefore likely that Na$^+$ accumulates in the cytosol during caffeine exposure, especially at room temperature when the Na$^+$/K$^+$ ATPase operates at a lower rate. The increase in fluorescence seen in Fig. 5.4A immediately following caffeine withdrawal is therefore consistent with Na$^+$-dependent influx of Ca$^{2+}$ into the cells via reverse mode Na$^+$/Ca$^{2+}$-exchange on re-introduction of Ca$^{2+}$ to the superfusate. This was frequently seen in both SHR and WKY trabeculae (e.g. see Fig. 5.1A).

Following exposure to caffeine, in the presence of 143 mM [Na$^+$]$_o$, trabeculae were then loaded with CE, and a repeat caffeine exposure was carried out to determine the relative contribution of the SL Ca$^{2+}$-ATPase to removal of cytosolic Ca$^{2+}$. Elimination of both SL transport mechanisms was then made by removal of [Na$^+$]$_o$ (following removal of [Ca$^{2+}$]$_o$) and addition of 10 mM NiCl$_2$ (see Fig. 5.4B). Whereas studies utilizing isolated myocytes frequently achieve effective blocking of the SL Na$^+$/Ca$^{2+}$-exchanger simply by withdrawal of [Na$^+$]$_o$ (usually in 0 mM [Ca$^{2+}$]$_o$ solution, e.g. Choi and Eisner 1999), this is more difficult to achieve with multicellular preparations such as trabeculae. To ensure a complete block of the
addition of Ni\(^{2+}\) to the 0 mM [Na\(^{+}\)]_o solutions was therefore made, since Ni\(^{2+}\) has previously been shown to be a rapid, and reversible, blocker of the Na\(^{+}/Ca^{2+}\)-exchanger (Kimura et al. 1987; Beuckelmann and Wier 1989; Niggli and Lederer 1991; Hinde et al. 1999). Nickel also blocks the L-type Ca\(^{2+}\) channel (Hobai et al. 2000), but this was not problematic here since trabeculae were quiescent during these experiments. Evidence that the exchanger was adequately blocked under these experimental conditions is seen by the absence of any increase in resting [Ca\(^{2+}\)]_i following removal of caffeine and return to the control solution in Fig. 5.4B (compare with Fig. 5.4A).

Comparison of the time constants of fluorescence decay for each condition was made in order to obtain an estimate of the relative contribution of each of the Ca\(^{2+}\) transport systems (see Table 5.1), in a manner similar to that carried out by Choi and Eisner (1999). Analysis showed that the SR Ca\(^{2+}\)-ATPase accounted for removal of ~ 97% of [Ca\(^{2+}\)]_i following release from the SR at room temperature, and that the other transport mechanisms account for ~ 3% of cytosolic Ca\(^{2+}\) removal. For both SHR, and WKY, the SL Na\(^{+}/Ca^{2+}\)-exchanger was the second most important mechanism for returning [Ca\(^{2+}\)]_i to resting levels, accounting for 70 % and 58 % of the non-SR Ca\(^{2+}\) respectively. However, it should be noted that the relative proportion of SL Ca\(^{2+}\) extrusion was similar between rat strains: SHR, 70 %, and WKY, 74 %.

One difficulty in determining the relative contribution of the SR Ca\(^{2+}\)-ATPase, in comparison to other transport systems, from these data is that the experimental conditions were different between the electrically-evoked transients, and the caffeine transients. The latter were carried out in the nominal absence of [Ca\(^{2+}\)]_o, whereas, for electrically-evoked transients, SL influx of Ca\(^{2+}\) is obligatory in order to trigger SR release; therefore experiments were carried out with [Ca\(^{2+}\)]_o of 2 mM, and a stimulus frequency of 0.1 Hz. Results from earlier experiments, presented in Chapters III and IV, illustrate the importance of experimental conditions on the time constant of fluorescence decay. Therefore the contribution of the SR Ca\(^{2+}\)-ATPase to fluorescence decay from my experiments is likely to be an over-estimate of the true value under physiological conditions.
Figure 5.4 The experimental protocol used to determine the relative contribution of the Ca\textsuperscript{2+} transport mechanisms to the decay of fluorescence for a representative SHR LV trabecula. Each panel shows fluorescence (top trace), and stimulus (lower trace), with superfusate concentration changes shown schematically beneath. The transient increase in fluorescence following caffeine application was fit by an exponential function (not shown) to determine the time constant of decay of fluorescence for each condition. A, functional elimination of the SR with 10 mM caffeine. B, functional elimination of SR, and SL Ca\textsuperscript{2+} transport.
Table 5.1 The relative contribution of Ca\(^{2+}\) transport mechanisms to the decay of [Ca\(^{2+}\)].
Relative contributions were determined by comparison of the time constants of decay in fluorescence (\(\text{Tau}\)) for the caffeine-induced Ca\(^{2+}\) transients arising from sequential blockade of the Ca\(^{2+}\) transport mechanisms. CE carboxyxyeosin, RR ruthenium red.

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<td>9.1 ± 0.6</td>
<td>0 ± 0.2 SL ATPase</td>
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<td>9.1 ± 0.6</td>
<td>2.2 ± 0.3 Na(^{+})/Ca(^{2+}) exchanger</td>
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<td>97.2 ± 0.02 SR ATPase</td>
</tr>
<tr>
<td>Caffeine</td>
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<td>0.4 ± 0.02 SL ATPase</td>
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<td>CE &amp; Caffeine</td>
<td>4</td>
<td>9.7 ± 1.4</td>
<td>1.7 ± 0.03 Na(^{+})/Ca(^{2+}) exchanger</td>
</tr>
<tr>
<td>0Na, CE &amp; Caffeine</td>
<td>4</td>
<td>33.8 ± 4.0</td>
<td>~ 0 ± 0.03 Mitochondrial</td>
</tr>
<tr>
<td>RR, CE, 0Na &amp; Caffeine</td>
<td>3</td>
<td>33.2 ± 3.2</td>
<td>0.7 Unaccounted</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td>0.7 Unaccounted</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.2 The relative contribution of Ca\(^{2+}\) transport mechanisms to the decay of [Ca\(^{2+}\)]\(_i\) in the absence of the SR Ca\(^{2+}\)-ATPase.

<table>
<thead>
<tr>
<th>Ca(^{2+}) transporter</th>
<th>SHR (%)</th>
<th>WKY (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL ATPase</td>
<td>~ 0</td>
<td>14.3 ± 0.9</td>
</tr>
<tr>
<td>Na(^{+})/Ca(^{2+})-exchanger</td>
<td>71.0 ± 8.8</td>
<td>60.7 ± 1.1</td>
</tr>
<tr>
<td>Mitochondrial uptake</td>
<td>6.5 ± 9.4</td>
<td>~ 0 ± 0.03</td>
</tr>
<tr>
<td>Other</td>
<td>22.6 ± 10.0</td>
<td>25.0 ± 11.4</td>
</tr>
</tbody>
</table>

Results from experiments described in Chapters III of this Thesis clearly show that the decay in fluorescence is influenced by the [Ca\(^{2+}\)]\(_i\) itself (see Fig. 3.5C). Conclusions made from experiments such as those described in this Chapter about the relative contributions to the transport of [Ca\(^{2+}\)]\(_i\) must therefore take this into account. Comparison of the decay rates
should be made over the same range of $[\text{Ca}^{2+}]_i$ (Bers and Berlin 1995) in order to look at the relative contributions of the transporters between rat strains, although this is not necessary if the decay is exponential. Although the decays of caffeine-induced fluorescence were well fitted by an exponential function, this would not be true for $[\text{Ca}^{2+}]_i$. However, Figs 5.5A and 5.6B show that the range of $[\text{Ca}^{2+}]_i$ were nearly the same for all manoeuvres.

Statistical analysis of data obtained for caffeine transients at room temperature, with the SL and mitochondrial transport mechanisms uninhibited, revealed no difference between rat strains for $[\text{Ca}^{2+}]_i$, at the peak of the caffeine transient (SHR: $0.241 \pm 0.034 \, \mu\text{M} \, (n = 8)$, versus WKY: $0.189 \pm 0.178 \, \mu\text{M} \, (n = 8)$). Nor was there a difference in the resting $[\text{Ca}^{2+}]_i$, measured $10 \, \text{s}$ before the onset of the caffeine transient (SHR: $0.090 \pm 0.007 \, \mu\text{M} \, (n = 8)$, versus WKY: $0.078 \pm 0.012 \, \mu\text{M} \, (n = 8)$). Therefore the amplitude of the caffeine transients encompassed $[\text{Ca}^{2+}]_i$ of comparable size between rat strains, allowing inferences to be drawn about the kinetics of $\text{Ca}^{2+}$ transport based on the comparison of the time constants of fluorescence.

Results from experiments in previous Chapters showed an increase in the resting $[\text{Ca}^{2+}]_i$ for SHR in comparison to WKY (see Chapters III and IV), yet there was no difference in the resting $[\text{Ca}^{2+}]_i$, immediately prior to the onset of the caffeine transient between rat strains. This result is of interest, and might shed light on the origin of the increased resting $[\text{Ca}^{2+}]_i$ in SHR trabeculae previously detected. Measurement of resting $[\text{Ca}^{2+}]_i$ for experiments described in this Chapter was carried out in quiescent trabeculae, at room temperature, and following 1 minute of the $\text{Ca}^{2+}$-free superfusion. Such conditions would minimise stimulation rate-dependent differences in SR uptake between rat strains, and increase the driving force for SL $\text{Ca}^{2+}$ transport mechanisms.
Figure 5.5 Sequential elimination of the Ca\(^{2+}\) transport mechanisms for representative trabeculae. Condition 1, elimination of the SR as a storage site for Ca\(^{2+}\) by use of 10 mM caffeine. Condition 2, following CE loading to inhibit the SL Ca\(^{2+}\)-ATPase. Condition 3, following block of Na\(^+/Ca\(^{2+}\)-exchanger. Condition 4, following addition of ruthenium red to block the mitochondrial uniporter. A, raw data. B, data from an SHR trabecula following averaging, and normalized. Note that there was no change in time-course of the decay of fluorescence following CE loading for this SHR trabecula.
Figure 5.6 Summary of sequential elimination of Ca$^{2+}$ transport mechanisms. SHR: open bars, WKY: solid bars. Condition 1, elimination of the SR as a storage site for Ca$^{2+}$ by use of 10 mM caffeine. Condition 2, following CE loading. Condition 3, following block of Na$^+$/Ca$^{2+}$-exchanger, and Condition 4, following addition of ruthenium red to block the mitochondrial uniporter. A, shows the mean time constant of decay of fluorescence for each condition. B, shows the mean amplitude of the caffeine transients.
5.6 Discussion

The purpose of experiments described in this Chapter was to determine the relative contribution of the four main Ca\(^{2+}\) transport mechanisms to the decay of [Ca\(^{2+}\)]\(_{i}\) between LV trabeculae from failing SHR hearts and those from WKY. Previously, experiments of this type have been performed using isolated myocytes, where diffusion of caffeine across the SL is rapid. The caffeine-induced SR Ca\(^{2+}\) release is synchronised under these conditions, and the amplitude of the resulting caffeine evoked Ca\(^{2+}\) transient is typically much greater than that of an electrically evoked Ca\(^{2+}\) transient. This occurs since caffeine totally empties the SR of releasable Ca\(^{2+}\) (Rousseau et al. 1988), whereas only a portion of the total SR Ca\(^{2+}\) is released with electrical stimulation. For multicellular trabeculae, used in the experiments described here, the amplitude of the caffeine transient was always less than that of the electrically evoked transients (e.g. see Fig. 5.1). Although the [Ca\(^{2+}\)]\(_{o}\) was different for each condition (2.0 mM versus 0 mM, respectively), this does not explain the difference in amplitude between electrically-evoked and caffeine-evoked transients.

Experiments were carried out at room temperature, rather than at 37 °C, for the following reasons: (i) to maximise the amplitude of the caffeine-induced Ca\(^{2+}\) transient by slowing the time-course of Ca\(^{2+}\) transport compared to caffeine diffusion across the multicellular trabeculae, (ii) to minimise any possible difference in SR Ca\(^{2+}\) buffering during futile cycling in the presence of caffeine, and (iii) to increase the relative contribution of the SL Ca\(^{2+}\)-ATPase compared to Na\(^{+}/Ca^{2+}\) exchange. The latter was important because the SL Ca\(^{2+}\)-ATPase contribution is usually an order of magnitude smaller than that of the Na/Ca\(^{2+}\)-exchanger, and therefore harder to detect.

*Inhibition of all four Ca\(^{2+}\) transport mechanisms*

The use of pharmacological agents to block sequentially cytosolic Ca\(^{2+}\) transport mechanisms in experiments such as those described in this Chapter assumes that the agent has been effective. This is even more important for multicellular preparations, since diffusion might
limit the actual intracellular concentration of the agent at the core of the preparation. Evidence that caffeine released all available SR Ca\(^{2+}\), and allowed no net accumulation of Ca\(^{2+}\) throughout the exposure period, was demonstrated by the lack of response to electrical stimulation at the end of the exposure period, even in the presence of [Ca\(^{2+}\)]\(_i\) (data not shown).

Analysis of electrically evoked Ca\(^{2+}\) transients indicated changes following CE loading that were consistent with an effect on the SL Ca\(^{2+}\)-ATPase (refer Section 5.4 above). In similar experiments, Choi and Eisner (1999) showed that CE was not specific for the SL Ca\(^{2+}\)-ATPase, producing a greater effect on the electrically-evoked Ca\(^{2+}\) transient in comparison to the caffeine transient. They postulated that CE partially inhibited the SR Ca\(^{2+}\)-ATPase also. Such an effect appears to be evident with data for WKY trabeculae, in that CE had a greater relative effect on the time constant of fluorescence decay for the electrically evoked transients than it did for the caffeine transients.

Whereas removal of both Ca\(^{2+}\) and Na\(^+\) from the superfusate is sufficient to block Na\(^+\)/Ca\(^{2+}\)-exchange for isolated myocytes (e.g. Bassani, Bassani et al. 1994, Choi and Eisner 1999), for trabeculae it was necessary to include 10 mM NiCl\(_2\) in the superfusate also. This enabled an effective block of the exchanger in a shorter time period than could otherwise have been achieved.

Fig. 5.6 A (Condition 4) clearly shows that the time constant of fluorescence decay was greater following ruthenium red exposure for SHR trabeculae, but not for WKY. Whilst there is evidence in the Literature to suggest that mitochondrial uptake of Ca\(^{2+}\) is important in myocytes when [Ca\(^{2+}\)]\(_i\) is pathologically elevated (for a review see Gunter, Gunter et al. 1994), interpretation of the WKY results must recognise the possibility that the mitochondrial uniporter was not completely blocked by ruthenium red. A recent paper by Zhou and Bers (2002) found that an incubation time of 30 min for ruthenium red (50 \(\mu\)M) was required in order to inhibit mitochondrial uptake significantly in intact myocytes.

Another confounding factor in the determination of the mitochondrial contribution to decay of [Ca\(^{2+}\)]\(_i\) is that mitochondrial efflux of Ca\(^{2+}\) is via a Na\(^+\)/Ca\(^{2+}\)-exchanger in the outer membrane (Crompton et al. 1976). Therefore, under experimental conditions that increase [Na\(^+\)]\(_i\), such as during exposure to caffeine in the absence of an SL Na\(^+\)/Ca\(^{2+}\)-exchanger block, net
mitochondrial Ca\textsuperscript{2+} uptake should be minimal. However, when the SL Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger is blocked, and [Ca\textsuperscript{2+}]\textsubscript{i} is high, the mitochondria are likely to take up large amounts of Ca\textsuperscript{2+} (Bers \textit{et al.} 1993). Since both the resting [Ca\textsuperscript{2+}]\textsubscript{i} and the amplitude of the caffeine transients were greater for SHR than for WKY (see Fig. 5.6B), it is likely that blocking mitochondrial Ca\textsuperscript{2+} entry might have affected SHR more than WKY, thereby providing an explanation of the larger time constant of decay for SHR trabeculae.

\textit{Comparison with other studies}

Others have examined the relative contribution of the Ca\textsuperscript{2+} transport mechanisms to the decay of fluorescence in isolated myocytes (Negretti \textit{et al.} 1993; Bassani \textit{et al.} 1994), and RV trabeculae (Lamont and Eisner 1996), from rat heart. Despite the differences between preparations (trabeculae versus isolated myocytes, and old versus young) for my experiments and those of others, there is close agreement in the relative contribution of the SR Ca\textsuperscript{2+}-ATPase. Negretti \textit{et al.} (1993) found SR contributes 87\% of the total cytosolic Ca\textsuperscript{2+} extrusion in rat myocytes (indo/AM loaded, caffeine transients, 27 °C, 1 mM [Ca\textsuperscript{2+}]\textsubscript{o}), and Bassani \textit{et al.} (1994) reported that SR uptake accounted for 92\% (indo/AM loaded, caffeine transients, 22-23 °C, 1 mM [Ca\textsuperscript{2+}]\textsubscript{o}). Results shown in Table 5.2 show that the SR accounted for about 97\% of the cytosolic extrusion for both SHR and WKY. (As discussed above, this result is influenced by the difference in [Ca\textsuperscript{2+}]\textsubscript{o} between the electrically-evoked and caffeine-evoked transients, perhaps accounting for the comparatively higher SR contribution.)

Choi & Eisner (1999) used similar experimental techniques to those described here to determine the relative role of the SR and SL Ca\textsuperscript{2+} transport mechanisms, but for isolated myocytes from young rats. These authors showed that fluorescence decay was eliminated completely when CE-loaded cells were exposed to a Ca\textsuperscript{2+}- and Na\textsuperscript{+}-free solution (> 30 s trace shown), implying that mitochondrial uptake did not contribute to the decay of fluorescence. Lamont & Eisner (1996), using a different technique, looked at SL transport mechanisms in trabeculae. They obtained a synchronised increase of [Ca\textsuperscript{2+}]\textsubscript{i} by first inhibiting the SR with ryanodine (10 \mu M), and then applying a 1-5 s burst of 10 Hz stimulation. This method resulted in similar increases of [Ca\textsuperscript{2+}]\textsubscript{i} to those observed in my experiments. These authors found that Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchange contributed around 77\% to Ca\textsuperscript{2+} extrusion in the absence of SR, close to that found for myocytes by Choi & Eisner (1999). Results from my experiments
yielded lower values for both SHR (70%) and WKY (58%). Bassani & Bassani (2002) showed that the relative contribution of the SL transport mechanisms decreased between birth and 14 months in rats. It therefore seems reasonable that in rats of ~24 months the relative contribution might have decreased further.

5.7 Chapter Summary

The principal finding from experiments described in this Chapter was that, in the functional absence of the SR as a storage site for Ca\(^{2+}\), the rate of decay of caffeine-induced fluorescence was slower for SHR than for WKY, but that this difference between rat strains was no longer apparent when both the SR and SL Ca\(^{2+}\)-ATPases were inhibited simultaneously. A difference in the SL Ca\(^{2+}\)-ATPase-dependent extrusion of Ca\(^{2+}\) might therefore account for the difference in resting [Ca\(^{2+}\)]\(_i\) between rat strains, since the SL Ca\(^{2+}\)-ATPase has previously been shown to have an important role in the control of resting [Ca\(^{2+}\)]\(_i\) in rat myocytes (Choi and Eisner 1999; Bers 2001).
Heart failure essentially involves two different biological processes, one associated with the altered cell growth that occurs with the development of hypertrophy, and the other associated with the contractile dysfunction. This Thesis has concentrated on the second of these processes, investigation of the underlying mechanisms that might give rise to the decreased contractile function typical of hearts in failure. An animal model of hypertension that progresses to hypertrophy and heart failure with age was utilized for this study, together with age-matched normotensive control animals. The spontaneously hypertensive rat (SHR) is a widely studied animal model of systemic hypertension that progresses to heart failure in a manner paralleling the human disease. Despite the large number of studies using SHR, few have examined both force and intracellular $[\text{Ca}^{2+}]$ when hypertension has progressed to failure. Consequently, it was previously unclear whether altered $\text{Ca}^{2+}$ homeostasis had a role in the development of contractile failure. Therefore the primary aim of this Thesis was to make simultaneous measurements of intracellular $[\text{Ca}^{2+}]$ and isometric force in trabeculae from SHR hearts in failure, and from age-matched WKY controls. To carry out these aims, the ratiometric $\text{Ca}^{2+}$-fluorophore, fura-2/AM, was used as an index of $[\text{Ca}^{2+}]_i$ under experimental conditions that were close to physiological.

Results confirmed that peak systolic stress was decreased in LV trabeculae from SHR in heart failure, consistent with previous reports of mechanical dysfunction (e.g. Gwathmey, Copelas et al. 1987; Mattiello, Margulies et al. 1998; Bing, Brooks et al. 1991; Brooks, Bing et al. 1994; Gómez, Valdivia et al. 1997; Dipla, Mattiello et al. 1999). However, in contrast to other studies (Bing et al. 1991; Brooks et al. 1994; Janssen et al. 2003), this study found that the amplitude of the $\text{Ca}^{2+}$ transient was larger in SHR than in WKY, even though there was a
marked reduction in the force of contraction. Additionally, the Ca\textsuperscript{2+} transient was found to decay more slowly for SHR, while the resting level of [Ca\textsuperscript{2+}]\textsubscript{i} was greater than for WKY.

A slower decay of the Ca\textsuperscript{2+} transient has previously been reported for many models of hypertrophy and heart failure. Most frequently, the slowing has been attributed to a reduced rate of SR Ca\textsuperscript{2+}-ATPase activity (for a review see Movsesian (1998) and references cited therein). There is some evidence, however, that a slower rate of SR Ca\textsuperscript{2+}-ATPase activity was not responsible for the prolonged decay of the Ca\textsuperscript{2+} transient found in this study, as suggested by the following factors. (i) The amplitude of the Ca\textsuperscript{2+} transient was greater in SHR, which is not consistent with decreased SR loading (due to slower uptake during diastole). (ii) The resting level of intracellular [Ca\textsuperscript{2+}] was higher in SHR even at low stimulation rates, when the increased interval between stimuli should allow reduction of [Ca\textsuperscript{2+}] to its steady state level. (iii) The inverse relationship between the time constant of decay of the Ca\textsuperscript{2+} transient and intracellular [Ca\textsuperscript{2+}] was the same for both SHR and WKY. The activity of the SR Ca\textsuperscript{2+}-ATPase is strongly influenced by the peak amplitude of the Ca\textsuperscript{2+} transient in cardiac muscle, with the rate of decay being increased with increasing [Ca\textsuperscript{2+}]. That this relationship was unaltered between SHR and WKY suggests that the sensitivity of the SR Ca\textsuperscript{2+}-ATPase to [Ca\textsuperscript{2+}], was not different between strains. (iv) Finally, quantification of the SR Ca\textsuperscript{2+}-ATPase, using Northern blot analysis, carried out by Boluyt et al. (1994) did not show a significant difference in the level of the mRNA between WKY and SHR in heart failure.

If the SR pumping capacity is unaltered, then a slowing in the decay of the Ca\textsuperscript{2+} transient might be explained by a difference in the calcium buffer capacity of the myocytes, perhaps as a result of the increased amount of contractile machinery present. In other words, if the ratio of SR Ca\textsuperscript{2+}-ATPase to calcium binding proteins were decreased by only 10%, then the observed slowing of the Ca\textsuperscript{2+} decay could be explained. This hypothesis should be testable in the future with measurements of buffer capacity of the type described by Diaz et al. (2001). However, an increase in the Ca\textsuperscript{2+} buffering capacity of the myocytes would also reduce the observed peak of the Ca\textsuperscript{2+} transient. This explanation therefore also requires a comparable increase in the amount of SR calcium released. Since resting Ca\textsuperscript{2+} was increased, such an increase in SR release appears reasonable.

Many studies have shown alteration of the force-frequency relationship during the progression to heart failure. Hence, examination of the force-frequency response between stimulus
frequencies of 0.2 and 10 Hz was carried out. Since the force-frequency response for rat myocardium is strongly influenced by the experimental conditions, the response was also investigated for RV trabeculae from young Wistar rats. A biphasic force-frequency relationship obtained for Wistar trabeculae, in agreement with recently published data for rat RV trabeculae (Kassiri et al. 2000). However, the force-frequency relationship for both SHR and WKY trabeculae was different to that of RV trabeculae from young Wistar rats. For both SHR and WKY the response was negative at low stimulus frequencies, similar to that observed in Wistar, but, for frequencies above 1 Hz, there was no positive phase to the response for either SHR or WKY. The absence of a positive phase to the force-frequency response in SHR, for frequencies close to those found in vivo, might therefore be linked to the age of the animals, rather than to heart failure per se, since age-matched WKY trabeculae also lacked a positive phase to the response. Peak systolic stress remained reduced for SHR trabeculae, in comparison to WKY, for the range of frequencies tested, despite the amplitude of the Ca$^{2+}$ transient being greater for SHR.

Recovery from force potentiation back to steady state levels relies on a re-distribution of Ca$^{2+}$ between the SR, the intracellular buffers, and the extracellular environment. Two different protocols were used to increase peak force, and the time-course of recovery back to pre-potentiation control levels was examined for each protocol. When peak [Ca$^{2+}$], and peak stress were normalised to their pre-potentiation values, there was a difference between rat strains only in the time-course of recovery of peak stress following paired-pulse stimulation.

The relative contribution of the Ca$^{2+}$ transport mechanisms to the recovery of resting fluorescence levels subsequent to SR release was investigated. Comparison of the time constant of fluorescence decay for caffeine-transients following sequential blockade of the Ca$^{2+}$ transport mechanisms showed that, in the functional absence of the SR, the decay of fluorescence remained slower for SHR when compared to WKY. Since removal of cytosolic Ca$^{2+}$ under these conditions is primarily by the SL Na$^+/Ca^{2+}$-exchanger and SL Ca$^{2+}$-ATPase, this result implies that the contribution of the SL transport mechanisms to cytosolic Ca$^{2+}$ extrusion was less for SHR. Such a difference might arise as a result of the hypertrophy of SHR myocytes, rather than a difference in Ca$^{2+}$ transport mechanisms per se, since the surface area to volume ratio might be different between SHR and WKY myocytes (but see Delbridge, Satoh et al. 1997). However, the difference in fluorescence decay was eliminated when the SL Ca$^{2+}$-ATPase was inhibited, and the Na$^+/Ca^{2+}$-exchanger was primarily responsible for
cytosolic Ca\(^{2+}\) extrusion. This result suggests that the contribution of the Na\(^+\)/Ca\(^{2+}\)-exchanger to maintenance of cytosolic [Ca\(^{2+}\)]\(_i\) is similar between the two rat strains.

6.2 Conclusions

In conclusion, the principle finding of this study is that for experimental conditions in which the temperature was maintained at 37°C, and at stimulation frequencies approximating those found in vivo, left ventricular trabeculae from failing SHR hearts demonstrated an elevated resting [Ca\(^{2+}\)]\(_i\) and an increased peak systolic [Ca\(^{2+}\)]\(_i\), but a decreased peak systolic stress, with respect to their WKY controls. These results indicate that the reduced contractile response of LV trabeculae from SHR in heart failure is not due to reduced myofilament Ca\(^{2+}\) availability, in contrast to previous studies of heart failure. Rather, alterations to the Ca\(^{2+}\) transient that, in healthy myocytes, would favour increased force development were found, namely increased peak [Ca\(^{2+}\)]\(_i\) and slowed rate of decay. Furthermore, unlike many previous reports of heart failure in human (Studer et al. 1994; Gaughan et al. 1999; Hasenfuss et al. 1999; Pieske et al. 1999) and animal (Hobai and O'Rourke 2000; Bers et al. 2002) studies, there was no functional evidence that Na\(^+\)/Ca\(^{2+}\)-exchange was increased in this animal model of heart failure.

Prior to the onset of heart failure, increased left ventricular mass is accompanied by enhanced ventricular function (Conrad et al. 1991; Brooksby et al. 1992; Mitchell et al. 1997). Furthermore, hypertrophied, but non-failing, SHR have been reported as having increased SR Ca\(^{2+}\) release, but in the absence of any reduction in contractility (Bing et al. 1991; Lammerich et al. 1995; Ren et al. 1999; Shorofsky et al. 1999). It might therefore seem reasonable to conclude that the progression to heart failure in this animal model is not accompanied by any further changes in Ca\(^{2+}\) homeostasis, and that explanation of the contractile dysfunction resides elsewhere.

Morphological examination of trabeculae showed that the collagen content is increased in SHR compared to WKY, and that the longitudinal distribution of collagen contains extensive branching. In SHR, the progression from stable hypertrophy to heart failure develops slowly
over a period of months, unlike many other experimental models of hypertrophy and failure. The observed alterations in the Ca$^{2+}$ transient may therefore have developed in this animal model of hypertensive failure as a compensatory response to changes in the extracellular matrix that compromise contractility.


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