CARDIAC SARCOPLASMIC RETICULUM FUNCTION IN HEART FAILURE

A thesis submitted in fulfilment
of the degree of
Doctor of Philosophy

by

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Abbreviations

ADR..... Adriamycin treated rabbit

ATP..... Adenosine Triphosphate.

$[Ca^{2+}]$..... Calcium concentration

cAMP..... cyclic adenosine monophosphate.

CIC..... Caffeine-Induced Contracture

CICR..... Calcium-Induced Calcium Release

CO..... Cardiac Output

C_{max}..... Maximum calcium activated force.

CrP..... Creatine Phosphate.

DIC..... Differential Interference Microscopy.

EF..... Ejection Fraction

EGTA..... Ethylene Glycol bis(b-aminoethyl ether)N,N,N′,N′ tetra acetic acid.

Iso-OP..... Rabbit infused with Isoprenaline by mini-Osmotic Pump.

LIG..... Coronary ligation rabbit.

LVEDD..... Left Ventricular End-Diastolic diameter.

LVEDP..... Left Ventricular End-Diastolic pressure.

LVSP..... Left Ventricular Systolic pressure.

P_i..... Inorganic Phosphate.

SAL..... Saline-injected (adriamycin) control rabbit

Sal-OP..... Rabbit infused with Saline by mini-Osmotic Pump.

SH..... Sham-operated (ligation) rabbit.

SR..... Sarcoplasmic reticulum.

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Publications

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SUMMARY

Heart failure is characterised by abnormalities of myocardial systolic and diastolic function. While there are changes in the structure of the heart which could account, in part, for these abnormalities, there is strong evidence to suggest that the regulation of calcium within the failing cardiac myocyte is abnormal. A diminished ability to release and sequester calcium could give rise to such contractile abnormalities and so the sarcoplasmic reticulum (SR), as the main site for calcium uptake and release, is the major focus of study in this thesis.

Two rabbit models of heart failure were studied: adriamycin-induced cardiomyopathy and coronary artery ligation. The adriamycin-treated animals were studied 2 weeks after stopping 8 weeks of chronic intravenous administration. The coronary ligation animals were studied in two groups : 8 weeks after ligation and 15 weeks after ligation. The effects of down-regulation of adrenoreceptors on SR function was studied in a rabbit model following 7 days continuous administration of isoprenaline by mini-osmotic pump. Lastly, human ventricular trabeculae were studied from patients undergoing cardiac transplantation and are presented as a separate chapter.

The severity of heart failure was assessed in each rabbit by echocardiography and by invasive measurement of cardiac output and left ventricular pressures.

The preparation chosen to study the SR was the saponin-treated cardiac ventricular trabecula. This preparation has a number of advantages in that it leaves the SR anatomically undisrupted, the SR can load and leak calcium in a physiological way, the permeabilised sarcolemma allows free access to drugs and chemicals and the myofilaments may be assessed both in association with the SR and independently following treatment with the detergent Triton-X100. Furthermore, in our hands, this technique is very

reliable and reproducible which is extremely important since very few animals which started the protocol, for any of the models, failed to produce results from the saponin-treated fibres. This has important implications for animal suffering.

Sarcoplasmic reticulum function was assessed in the rabbit model of adriamycin-induced cardiomyopathy. The method for inducing cardiomyopathy is described in chapter 2 (methods 1) and the results are described in chapter 3. Unfortunately few animals with significant left ventricular dysfunction were produced but those with ejection fractions of less than 45% demonstrated enhanced SR Calcium loading compared with saline-treated controls and with adriamycin treated animals with higher ejection fractions. The ability of the myofilaments to produce force was unaffected by chronic treatment with adriamycin and the calcium responsiveness of the myofilaments was also unaffected by adriamycin treatment.

8 weeks after coronary ligation, rabbits with significant left ventricular dysfunction demonstrated enhanced SR calcium uptake compared with controls. A group of ligation animals with less severe left ventricular dysfunction, assessed by a scoring system, also demonstrated enhanced SR calcium loading but the difference between this group and sham-operated controls was less marked. Additionally , this enhanced SR calcium loading was correlated with a number of indices of heart failure including ejection fraction, left ventricular end-diastolic pressure and resting cardiac output. Enhanced SR calcium loading was associated with an increased predisposition to calcium overload, revealed as spontaneous tension oscillations. These oscillations were observed more frequently in the ligated animals and at lower loading calcium concentrations than in the control animals. This pattern of SR loading was observed in both left and right ventricular preparations although the greatest increase in SR loading was

observed in the left ventricle. The ability of the myofilaments to produce force was not altered in the left ventricle but there was a fall in the maximum force produced by trabeculae from the right ventricle in the ligated group. The calcium responsiveness of the myofilaments was not different between groups.

Chapter 4 examines SR calcium loading 15 weeks after coronary ligation. In these animals there was no significant difference in SR calcium loading ability between ligation and control trabeculae. The ligated animals demonstrated significant left ventricular dysfunction but this was not more severe than that observed in 8 week animals. Myofilament force production was unaltered as was calcium responsiveness of the myofilaments.

Chapter 6 examines possible changes in the SR calcium release mechanism. There was no difference in the sensitivity of the mechanism of caffeine-induced calcium release in ligation animals compared with controls. Trabeculae from ligation animals appeared more sensitive to the calcium channel blocking agent, ryanodine, when applied during sustained oscillations. In a separate set of experiments SR calcium leak was assessed. There was a tendency for ligation trabeculae to demonstrate less SR calcium leak compared with controls.

Chapter 7 examines sarcoplasmic reticulum calcium loading in rabbits infused with isoprenaline for a period of 7 days. There was evidence of β-receptor down-regulation in isoprenaline treated animals assessed in-vivo by invasive measurements of cardiac output, left ventricular pressures and heart rate response to isoprenaline. In isolated saponin-treated ventricular trabeculae, there was no significant difference in SR calcium loading ability between isoprenaline and saline infused animals. Myofilament force production was unaltered and calcium responsiveness was also unaffected.

Human right ventricular trabeculae were obtained from hearts of patients undergoing transplantation. Following saponin-treatment, SR function was

found to be inversely correlated with the severity of heart failure as assessed by a range of haemodynamic variables and by radio nuclide left ventricular ejection fraction. Patients with moderately impaired LV function demonstrated greater SR calcium loading ability than patients with severely impaired LV function. Patients with idiopathic dilated cardiomyopathy demonstrated reduced SR calcium loading compared with patients with moderately impaired LV function. Myofilament force production and calcium responsiveness was not correlated with severity of heart failure.

Chapter 9 compares the response of SR calcium loading to cAMP in ligation rabbits, isoprenaline-infused rabbits and failing human trabeculae. Of all the preparations studied, human trabeculae demonstrated the greatest response to cAMP while isoprenaline-infused rabbits demonstrated a significantly greater response than their respective saline-treated controls. Ligation rabbits demonstrated a slightly greater response than the sham-operated controls but this was less marked than the isoprenaline-infused rabbits and the human trabeculae.

In the concluding chapter these results are considered together. The adriamycin-induced cardiomyopathy may represent a more complicated model of left ventricular dysfunction since this drug has specific effects on the SR in addition to possible secondary changes resulting from heart failure. The enhanced calcium loading ability in ligation animals at 8 weeks may represent a compensatory phase, in the rabbit, resulting from ventricular overload following myocardial infarction. This phenomenon may have implications for arrhythmogenesis early in the development of heart failure. With time, SR calcium loading ability reduces such that at 15 weeks there is no significant difference from controls. β-receptor down-regulation appears to play no direct role in the SR calcium pump changes observed in the rabbit. In the human, SR calcium loading ability diminishes with increasing severity of heart failure. The level of phosphorylation of the SR calcium

pump may play a major part in the observed SR calcium loading ability in saponin-treated cardiac trabeculae in this study.

CHAPTER 1

General Introduction

INTRODUCTION

Heart failure: a definition

There are a number of definitions of heart failure which take account of the force of contraction of the heart, oxygen delivery to the tissues and the response of this system to increased demands of the body. The simplest, by far, is that of Wood (1950): *"A state in which the heart fails to maintain an adequate circulation for the needs of the body despite a satisfactory filling pressure "*. However, this definition takes no account of wider and more generalised changes resulting from chronic heart failure. A more holistic and progressive definition of heart failure was proposed by Poole-Wilson :

" A clinical syndrome caused by an abnormality of the heart and recognised by a characteristic pattern of haemodynamic, renal, neural and hormonal responses ".

A brief historical account of heart failure

The earliest recorded diagnosis and attempted treatment of heart failure was that of the fatal illness of Alexius I, ruler of the Byzantine Empire from 1081-1118. His daughter noted that the attending physicians *"felt his pulse and found all kinds of irregularities..... he was unable to lie on either side .....for never for one moment could he breath freely. He was forced to sit upright to breath at all.....but when his stomach was visibly enlarged and his feet also swelled up, some of the doctors with scant regard for the fever had recourse to cauterisation"* (Lutz, 1988).

Theophile Bonet (1620-1689) published detailed accounts of the clinico-pathology of patients with valvular heart disease and heart failure and linked the increase in size of the cardiac chambers to the symptoms of dyspnoea and oedema. Francesco Albertini (1662-1738) described pulmonary oedema and associated this with dyspnoea. Jean Baptiste de Senac (1693-1770) also drew attention to the association of dyspnoea, orthopnoea and oedema

as a manifestations of heart failure (East, 1958). Perhaps the most famous therapeutic advance of that era was made by William Withering (1741-1791) when he discovered the benefits of the extract of the Foxglove (Willius & Keys, 1941).

The nineteenth century saw a number of advances in the understanding of heart failure. Pierre Potain (1825-1901) described the origin of *gallop rhythm* while Ludwig Traube (1818-1876) described *pulsus alternans* in severe heart failure. The underlying cause of heart failure remained obscure although valvular heart disease was the major contribution. Peter Malunsten (1811-1883) provided evidence that coronary occlusion was associated with myocardial infarction and Charles Fagge (1838-1883) described areas of focal fibrosis in a series of patients dying from heart failure.

Therapeutic options in heart failure were extremely limited until the twentieth century. Venesection, starvation, and purgation were commonly prescribed remedies. Reginald Southey (1835-1899) advocated the use of his eponymous tubes for the relief of peripheral oedema introduced through the skin and allowing fluid to drain directly from the tissues. The discovery of mercurial compounds as diuretics in the early twentieth century was the most significant pharmacological advance in the treatment of heart failure since Withering had discovered digitalis (Baldry, 1971). Following this, a series of less toxic compounds with potent diuretic effects were manufactured over the next fifty years. Ernest Starling (1866-1927), in 1914, provided an understanding of the physiological mechanism by which the concept of altering the load on the heart, using vasodilators, was based. In the last twenty years the use of drugs which produce changes in pre-load and after-load have improved the symptoms of heart failure but have failed to reduce mortality which has remained as high as 50% for one year. Only in recent years have we been able to see a true improvement in the survival from

heart failure brought about by the use of angiotensin converting enzyme inhibitors.

Heart failure in the 1990's

"During the last decade heart failure has evolved into the most important public health problem in cardiovascular medicine " (Garg, Packer, Pitt & Yusuf, 1993). In the United Kingdom it is estimated that the cost of treating people with heart failure is now in excess of £300 million per year which represents 1.2% of the NHS budget (McMurray & Hart, 1993). In the United States heart failure is estimated to affect 1.5% of the population at an annual cost to the nation of $8 billion. The problem has arisen as a result of a number of factors but mainly due to increasing longevity. The Framingham study clearly indicated that the incidence of heart failure rises dramatically with age, with a tenfold increase between 59 and 80 years (Ho, Anderson, Kannel, Grossman & Levy, 1993). Increased life expectancy, as a result of improvements in health care and general living conditions, has largely been responsible for this epidemic (Pitt, 1993).

A number of studies, using large numbers of patients in carefully designed protocols, have yielded important clinical information both of the clinical course of the disease and of the appalling prognosis even when treated with conventional drug therapy. In the Framingham study a diagnosis of heart failure, based on clinical criteria, was associated with a median survival time of 1.7 years in men and 3.2 years in women, with a five year survival of 25% and 38% for men and women respectively (Ho *et al*, 1993). Disappointingly, the age adjusted incidence of heart failure has reduced by only 11% per calendar decade in men and by 17% per calendar decade in women in the last 40 years despite the advances in treatment of ischaemic heart disease and hypertension which were the major underlying causes of heart failure in the Framingham population. In the SOLVD registry (6273 patients with radio nuclide ejection fractions of less than 45%) the most common cause of heart

failure was ischaemic heart disease (70%) with hypertension contributing in 32% of blacks and 4% of white cases. The 1 year total mortality rate was 18% with 19% having at least one hospital admission with a diagnosis of heart failure during the first year of follow-up. The most significant factor influencing endpoints of death and hospital admission with heart failure was age. The event rate was low under the age of 55 and increased progressively thereafter.

Several large studies have now addressed the question of whether drugs can alter outcome in heart failure. The combination of vasodilators, hydralazine and isosorbide dinitrate, was found to significantly reduce mortality (Cohn, Johnson, Ziesche *et al*, 1986) with further benefit obtained by the use of enalapril (Cohn, Johnson, Ziesche *et al*, 1991). There are now a number of studies which indicate that ACE inhibitors can significantly reduce mortality in patients with severe heart failure (CONSENSUS), patients with reduced ejection fraction and mild to moderate heart failure (SOLVD trial, 1991, Cohn *et al*, 1991) and patients with asymptomatic left ventricular dysfunction (SAVE, 1992, SOLVD, 1992). These trials are often associated with only small reductions in mortality but because mortality is so high in heart failure, even a small reduction results in a significant number of lives saved.

Despite the therapeutic benefits conveyed by ACE inhibition, the overall effect on mortality is modest and mortality from heart failure remains high even with treatment - possibly around 20% in 1 year. There is, therefore, a need for improved understanding of the pathophysiological processes involved in heart failure to allow the development of new clinical therapeutic strategies.

The pathophysiology of heart failure

In patients with heart failure cardiac output is often normal at rest but typically fails to increase with exercise or stress so that vasoconstriction must occur to maintain blood pressure. This is brought about by activation of a number of physiological mechanisms which act to maintain or increase blood pressure including the renin/angiotensin system and the sympathetic nervous system. The physiological effects of this are widespread and affect the peripheral, renal and pulmonary circulations. Virtually every major organ of the body, with the exception of the brain, is affected in heart failure with changes occurring in the kidney, lung, skeletal muscle and, of course, the heart.

Anatomical and structural changes in the failing heart

Gross structural changes occur in the heart as a result of heart failure which have profound effects on function. A reduction in cardiac muscle mass as a result of myocardial infarction is the most common clinical cause of myocyte loss although apoptosis secondary to viral infection or toxic damage may play a role in the muscle loss observed in dilated cardiomyopathy.

Ventricular wall tension is described by the law of Laplace :

$$T \propto r \, . \, P$$

where P is the transmural pressure, r is the radius of the chamber and T is wall tension. It is clear from this relationship that an increase in end-diastolic volume and end-diastolic pressure, such as occur in heart failure, will have profound effects on the tension within the wall of the ventricle. This increase in wall stress is thought to initiate the process of cellular hypertrophy (Pfeffer & Braunwauld, 1990). Nuclear imaging has allowed repeated assessments of ventricular function in patients with heart failure over a period of 1-2 years (Pouleur, Konstam, Udelson & Rouseau, 1993). This study indicated that, with time, systolic ejection fraction decreases and

the ventricle dilates producing an increases in wall tension. As the ventricle dilates, there follows a period of compensation afforded by the Starling mechanism and compensatory cellular hypertrophy. This maintains the ejection fraction at a relatively stable level despite further dilatation. However, if the ability of the heart to compensate is overcome by progressive dilatation then ejection fraction falls further and clinical heart failure will follow. Throughout this period of remodelling inco-ordinate contraction resulting from infarction or abnormal electrical activation may also contribute to LV dysfunction.

There are additional abnormalities of cellular architecture in the failing heart which may give rise to contractile dysfunction. The myocardium is composed of a number of different cell types. The largest tissue component, by far, is that of the cardiac myocyte which makes-up approximately 75% of the total mass but represents only one third of the total number of cells (Zak, 1973). The remaining two thirds include fibroblasts, vascular smooth muscle cells and endothelial cells. Changes in these, such as occur during remodelling, will dramatically alter the structure and function of the heart. The relative proportions of type III to type I collagen are altered in hypertrophied rat heart (Doering, Jalil, Janicki *et al*, 1983) and a similar alteration in the production of collagen can be reproduced in cultured cardiac fibroblasts exposed to mechanical stretch (Carver, Nagpal & Nachtigal *et al*, 1991). These changes in collagen production to less elastic forms tends to reduce the compliance of the ventricle. The stiffness of the myocardium is also affected by medial thickening of intra-myocardial coronary arteries although the overall contribution of this is probably relatively small compared to that of collagen (O'Brien & Moore, 1966). While these factors will clearly influence relaxation they may have additional influences on the ability of the heart to develop force. A stiffer extracellular matrix may diminish the ability of the myocyte to generate force as a result of being

"encased in concrete". Myocardial cell slippage may cause abnormal cellular alignment which will affect the ability of the syncytium to develop tension and may also affect the mechanism and co-ordination of cellular activation (Schaper & Schaper, 1983, Weber & Brilla, 1991).

In addition to biophysical factors which may alter the structure of the failing heart, there are a number of neurohormonal factors which may also play a role. Angiotensin II is known to have powerful direct effects on myocardial cell growth and function (Lindpainter & Ganten, 1991). Local tissue production of angiotensin II may be important and some of the benefits of ACE inhibitors in heart failure may be mediated by a reduction in tissue levels of the ACE (Lindpainter *et al*, 1991). Although angiotensin II is thought to have inotropic effects on the heart, the effects of the renin-angiotensin system on fibroblast activity, mediated by aldosterone (Weber & Brilla, 1991), may be more important.

Cellular aspects of failing myocardium

The intrinsic ability of isolated cardiac myofilaments to produce force is unaltered in heart failure (Newman, 1983) and although significant shifts in myosin isoenzymes have been described in animal models (Greenen, Malhotra & Scheuer, 1989) such changes may be less important in the failing human myocardium (Solaro, Powers, Gao & Gwathmey, 1993). Mechanisms underlying contractile dysfunction have, therefore, been attributed to a number of cellular processes such as shifts in adrenoreceptor populations (Bristow & Grunsburg, 1982), reduced levels of high energy phosphates (Conway, Allis, Hardy *et al*, 1991) and abnormalities of Ca^{2+} regulation (Morgan, 1992).

Myofilament changes in heart failure

A number of changes have been described in the structure and function of the cardiac myofilaments during the development of heart failure. One consistent biochemical finding, for both small mammal and human heart failure, is a depression of Ca^{2+}-dependent MgATPase activity (Pagani, Alousi, Grant *et al*, 1988, Geenen, Malhotra & Scheuer, 1989). In small mammals this has been attributed to a change in the ventricular myosin heavy chain (MHC) with a shift from faster forms (V1) to slower more efficient (V3) forms (Mercadier *et al*, 1981). However, there have been no reported changes in the expression of MHC in human heart failure and so an alternative mechanism to explain the observed depression of Mg^{2+}-ATPase activity has been sought. Familial cardiomyopathy has been shown to be associated with a mutation on the myosin molecule which could alter the ATPase rate (Feldman, Ray, Silan*et al*, 1991). The ATPase rate may also be affected by loss of myosin light chain-2 (MLC-2) in human heart failure (Margossian, White, Caulfield *et al*, 1992).

There is growing evidence that the expression of thin filament proteins may change in heart failure (Anderson, Malouf, Pagani & Allen, 1991, Hunkeler, Kullman & Murphy, 1991). There are a number of phosphorylation sites on the thin filaments which can alter Mg^{2+}-ATPase activity of myosin (England, 1976) and can also alter Ca^{2+} responsiveness (Herzig, Kohler, Pfitzer, Ruegg, Wolffe, 1981). Solaro *et al* (1993) have proposed an additional mechanism by which an alteration in co-operativity between weakly and strongly bound cross-bridges may influence myosin ATPase rate and play a role in the changes observed in the failing human heart.

These molecular changes have been supported in some studies by evidence indicating a functional difference in myofilaments in heart failure. Increased Ca^{2+} sensitivity, which fails to increase further with stretch, has been reported in ventricular strips isolated from patients with heart failure (Schwinger, Bohm, Koch *et al*, 1994). These authors proposed that this lack of response to stretch represents an inability of the failing heart to use the Frank-Starling mechanism. However, since they failed to measure sarcomere length directly in these preparations it is not clear whether this was a consequence of reduced compliance or a true loss of the mechanism of length sensitivity of the myofilaments. In another study from the same group no difference was found in the Ca^{2+} sensitivity of failing human ventricular fibres (Wankerl, Bohm, Morano *et al*, 1990). A number of other studies have also failed to demonstrate a difference in steady state Ca^{2+} sensitivity of the myofilaments (D'Agnolo, Luciano, Mazucco, Gallucci & Salviati, 1992, Gwathmey & Hajjar, 1990). There is, therefore, no consistent change in myofibrillar responsiveness to Ca^{2+} in heart failure. However, one consistent finding is that the maximal response of the myofilaments to Ca^{2+} is unchanged in heart failure in the human (Hajjar, Grossman & Gwathmey, 1992) and in a number of animal models (see review by Gwathmey & Davidoff, 1993).

Adrenoreceptor changes in heart failure

Heart failure induces widespread activation of the neurohormonal system which results in high circulating levels of adrenaline and noradrenaline. Spill-over of noradrenaline from the failing heart, which is not taken up by myocardial nerve endings, accounts for 30-50% of the circulating pool (Hasking, Esler, Jennings *et al*, 1986). This chronic stimulation results in down-regulation of β_1-receptors (reduction in receptor density) with no significant down regulation of β_2-receptors but with a modest uncoupling of the β_2-response (Bristow, Hershberger, Port, Rasmussen, 1989). This uncoupling of response occurs between the β-receptor and adenylate-cyclase by increased activity of a G-inhibitory (G_i) peptide with no significant change in the G-stimulatory (G_S) peptide (Feldman, Gates, Veazey *et al*, 1988). This results in diminished production of cyclic adenosine monophosphate which attenuates the activation of protein kinase C. Interestingly, this diminished response to β-receptor stimulation occurs predominantly in the failing ventricle and does not appear in the non-failing ventricle (*e.g.* LV in primary pulmonary hypertension) despite high circulating levels of catecholamines (Bristow, Minobe, Rasmussen *et al*, 1992).

The α_1-response of human heart is not as marked as that of the rabbit (Bing *et al*, 1990). However, there is evidence for an increased response to α-agonists in the failing human heart and this has been supported by evidence indicating an increase in the levels of α_1-receptor m-RNA (Bristow, 1993). This may be brought about by so-called cross-regulation between β-receptors and α_1-receptors.

The overall effect of this redistribution of the adrenoreceptor population is decreased numbers of β_1-receptors, uncoupling of the β_2-response and an increase in the α_1-response.

Excitation contraction coupling in the normal heart

Before discussing abnormalities of excitation contraction coupling in heart failure it would seem appropriate to review the main features of the process as it occurs in the normal heart. In lower vertebrates, such as the frog, Ca^{2+} influx during the action potential raises intracellular Ca^{2+} sufficiently to produce contraction (Morad & Goldman, 1973). In mammalian cardiac muscle, Ca^{2+} entry triggers a further release of Ca^{2+} from an intracellular store, the sarcoplasmic reticulum. The mechanism which allows this is a Ca^{2+}-activated Ca^{2+} release channel in the SR membrane - the ryanodine receptor (Fabiato, 1983). There is considerable experimental evidence to support this mechanism including studies using photolytic release of caged Ca^{2+}. Flash photolysis causes a small, step-increase in intracellular $[Ca^{2+}]$ which stimulates the SR to release stored Ca^{2+} thus producing contraction. In the presence of ryanodine this process fails to produce contraction (Naebauer & Morad, 1990).

The Ca^{2+} transient

During a single activation Ca^{2+} rises and falls within the cytosol and this transitory rise in Ca^{2+} is now commonly termed the Ca^{2+} transient. However, although the Ca^{2+} transient, as an experimental measurement, is now discussed widely in the literature as a standard measure of Ca^{2+} release within the cell, in fact, there are a number of factors which may influence its amplitude and duration. The conditions under which the Ca^{2+} transient is measured vary widely from study to study and from preparation to preparation. In the intact cell, Ca^{2+} entry via the slow inward current and SR Ca^{2+} release contribute to the rising phase while SR Ca^{2+}-reuptake and the Na^{+}/Ca^{2+} exchanger contribute to the decline phase. The sarcolemmal Ca^{2+} pump, mitochondral Ca^{2+} uptake and binding of Ca^{2+} to intracellular sites (troponin C, calmodulin, (Cheung, 1980)) will also act to reduce $[Ca^{2+}]_i$.

Possible variation in these factors is often ignored in the description of the Ca^{2+} transient.

The sarcoplasmic reticulum

The sarcoplasmic reticulum (SR) is the main source of activator Ca^{2+} in mammalian myocytes and contributes most to the Ca^{2+} transient (Fabiato, 1983, Naebauer *et al*, 1990, Marban & Weir, 1985). The SR has a well characterised ATP-dependent Ca^{2+} pump that is distinct from the sarcolemmal Ca^{2+} pump. There appears to be two skeletal SR Ca^{2+}-pump proteins with 84% homogeneity. One is from fast twitch skeletal muscle and the other, similar to that of cardiac muscle, is from slow twitch skeletal muscle (MacLennan, Brandl, Bozena & Green, 1985). The transport reaction occurs with two Ca^{2+} ions transported and one ATP molecule hydrolysed by a process involving -

(i) binding of Ca^{2+} to high affinity sites on the cytosolic surface (ii) transfer of the terminal phosphate of the ATP molecule to an aspartate residue on the SR pump (iii) reduced affinity of the receptor for Ca^{2+} on the luminal side of the SR and extrusion of Ca^{2+} into the lumen.

Regulation of the SR Ca^{2+}-pump

The main difference between the skeletal muscle SR Ca^{2+}-pump and that of cardiac muscle is that the latter is regulated by the protein phospholamban. When phosphorylated by cAMP-dependent protein kinase this protein stimulates the Ca^{2+}-pump by producing a 3 to 5 fold decrease in the K_m (Ca^{2+}) (Shamoo, Ambudkar & Jacobson *et al*, 1985). Hicks *et al* (Hicks, Shigekawa & Katz, 1990) proposed that phospholamban has an inhibitory effect on the Ca^{2+}-pump which is diminished after phosphorylation thus allowing it to work at rates similar to that observed in skeletal muscle. This enhanced Ca^{2+}-uptake has been shown to be the dominant mechanism by which catecholamines produce an increase in force and relaxation in cardiac muscle

by increasing the total Ca^{2+} content of the SR and the rate of sequestration (Kranias & Solaro, 1982).

The activity of the SR Ca^{2+}-pump is regulated by a number of other factors including [ATP], [ADP], pH and inorganic phosphate (P_i). The K_d for ATP is of the order of 1μM and so during ischaemia ATP would have to fall to very low levels before there was a significant decline in SR Ca^{2+}-pumping. Low pH has also been shown to reduce the activity of the pump in isolated single myocytes (Fabiato & Fabiato, 1978) which is consistent with the effects of acidosis in multicellular preparations. Inorganic phosphate reduces the Ca^{2+} content of the SR probably by inhibiting the Ca^{2+}-pump. This reduction in SR content occurs over a concentration range similar to that observed in isolated rat and ferret hearts during ischaemia (Smith & Steele, 1992). Suko *et al* (Suko, Hellman & Winkler, 1977) have shown, in isolated SR vesicles, that raising the levels of ADP in the bathing solution can reverse the direction of the Ca^{2+}-pump probably by the process of product inhibition.

Calsequestrin

Calsequestrin is a protein of ~45,000 molecular weight localised primarily in the junctional SR (Meissner, 1975). This protein is highly acidic and can bind around 35 to 40 Ca^{2+} ions with an affinity constant of ~500μM (Mitchell, 1988). This corresponds to a total Ca^{2+} binding ability of 70 to 150μmol Ca^{2+} per kg wet weight of tissue, assuming a yield of 40mg/kg wet weight of calsequestrin from cardiac tissue (Campbell, MacLennan, Jorgensen *et al*, 1983). This is obviously a considerable source of Ca^{2+} buffering within the lumen of the SR and acts to maintain low intraluminal [Ca^{2+}].

The SR Ca^{2+} release channel

The SR Ca^{2+} release channel is also described in the literature as the ryanodine receptor because of the specific affinity of this plant derived alkaloid for the channel. The structure of the release channel is described

later in this thesis but there are a few points which are important to discuss at this stage. The activity of the channel is affected by a number of factors including cytosolic [Ca^{2+}], SR luminal [Ca^{2+}], Mg^{2+}, [ATP], caffeine and ryanodine. Under physiological conditions, the release of Ca^{2+} is mediated by Ca^{2+} itself entering the cytosol during the action potential. However, there is evidence for a constant leak of Ca^{2+} from the SR distinct from that occurring during CICR. There are three main sources of this leak -

1. The ryanodine sensitive Ca^{2+} release channel
2. The ryanodine insensitive "Passive leak" channel
3. The SR Ca^{2+}-pump itself (Feher & Briggs, 1983).

The net uptake of Ca^{2+} by the SR, during the cellular equivalent of diastole, is therefore the quantity loaded by the SR Ca^{2+}-pump minus the quantity leaked by the various mechanisms listed above. In the saponin-treated trabecula, studied in this thesis, the relative contributions of leak and uptake to SR Ca^{2+}-content may be important since the time periods involved in loading the SR are longer than those occurring physiologically.

Sarcoplasmic Reticulum function in heart failure

There is now strong evidence suggesting abnormal Ca^{2+} regulation within failing cardiac myocytes both in human tissue and in animal models of hypertrophy and failure (Morgan, 1992). The sarcoplasmic reticulum, as the main site of Ca^{2+} uptake and release within the cell, has been the focus of much attention.

SR function in animal models of heart failure

Ca^{2+} overload has been implicated as a cause of contractile dysfunction in a number of animal models and many of the features of heart failure seen in the cardiomyopathic hamster can be prevented by treatment with the Ca^{2+} antagonist verapamil (Finkel, Shen, Oddis & Romeo, 1993). Studies using isolated SR vesicles from a number of animal models of hypertrophy and failure have demonstrated a variety of changes in the rate of uptake of Ca^{2+} by the SR (Lamers & Stinis, 1992, Afzal & Dhalla, 1979) These studies provide evidence of abnormal cell Ca^{2+} regulation but are not necessarily representative of how the SR is functioning in the intact cell. Studies using bioluminescent Ca^{2+} indicators in intact cells have shown prolonged Ca^{2+} transients suggesting a reduced re-uptake of Ca^{2+} by the SR (Gwathmey, Copelas, McKinnon *et al*, 1987). Such changes suggest that the reduced rate of relaxation seen in pressure-overload hypertrophy and heart failure are related to abnormal intracellular buffering of Ca^{2+}.

Animal studies have largely concentrated on pressure-overload hypertrophy and subsequent transition to heart failure (Bing, Brooks, Conrad *et al*, 1991, Ito, Suko & Chidsey, 1974, Perreault, Bing, Brooks *et al*, 1990). Heart failure following pressure overload hypertrophy is now increasingly rare clinically and might be a different pathophysiological process from heart failure produced by increases in preload alone.

Human SR studies in heart failure

Reduced SR Ca^{2+} uptake in isolated SR vesicles from failing human myocardium has been reported by some authors (Movsesian, Bristow & Krall, 1989), while others have reported no difference from controls (Limas, Olivari, Goldenberg *et al*, 1987). Reduced SR pump function has been supported by evidence demonstrating lower levels of SR Ca^{2+}-ATPase m-RNA in heart failure (Mercadier, Lompre, Duc *et al.*, 1990). Interpretation of studies using human heart failure tissue has been limited by the large size of experimental preparations (Hasenfauss, Mulieri, Leavitt *et al*, 1990, Gwathmey, Copelas, McKinnon *et al*, 1987) and the methods used to prepare the tissue which can damage substantial portions of the preparation (Gwathmey *et al*, 1987).

Further studies are limited by the paucity of normal human control tissue. Many studies have used control tissue from patients with ischaemic heart disease, *post mortem* specimens with long warm-ischaemic times and from variable sites of the myocardium. In addition, tissue used for these studies have, predominantly, come from patients with end-stage heart failure. Further studies are required to evaluate changes earlier in the development of heart failure since mortality remains high even in mild-moderate heart failure when there is, arguably, compensated contractile function (Ho *et al*, 1993). The underlying basis for this may be related to arrhythmogenic mechanisms, which predispose to sudden death and occur before contractile function has deteriorated significantly. There is, therefore, a need to address cellular processes relating to contractile function at all stages in heart failure and not only those occurring in end-stage disease.

Methods of assessing SR function

Some of the differences observed in Ca^{2+} regulation between studies have been related in part to the method used to assess SR Ca^{2+}-uptake and release.

Isolated SR preparations represent homogenised vesicles which are isolated by a crude process by which separation of a mixture of membrane vesicles is brought about by ultra-centrifugation. The limitations of this technique are related to the fact that firstly the SR is anatomically disrupted and secondly that the uptake of Ca^{2+} is 'oxalate-supported'. Oxalate anions, when added to the incubating medium, passes into the SR and binds to Ca^{2+} forming an insoluble precipitate which therefore reduces the effective intra-SR free-$[Ca^{2+}]$. The SR Ca^{2+} pump is never inhibited by a high intra-luminal SR $[Ca^{2+}]$ which therefore allows SR Ca^{2+}-uptake to continue and furthermore does not allow Ca^{2+} to leak *from* the SR, as occurs physiologically. The resulting rate of uptake is therefore a measure of the absolute maximum rate of Ca^{2+}-uptake which is not how the SR functions in the intact cell. Furthermore, when oxalate is applied to a saponin-skinned preparation Ca^{2+}-uptake is initially increased but this is rapidly followed by a fall in tension responses and SR function (DS Steele, personal communication). This result implies that SR vesicles behave differently from *in-situ* SR and may be more stable to internal Ca^{2+} oxalate precipitation. This in turn might suggest an inherent difference in the SR in this isolated state.

Many studies have utilised fluorescent Ca^{2+}-indicators to demonstrate Ca^{2+} transients in failing and non-failing myocardium. In early experiments, micro-pipettes were used to inject aqueorin into cells often damaging a significant part of the preparation. The resulting Ca^{2+} transient therefore consisted of an averaged signal from damaged and undamaged cells. Other earlier techniques employed high concentrations of EGTA in the bathing medium to introduce aequorin into the cells (Gwathmey *et al*, 1987). Bathing the preparation in such strong Ca^{2+} buffering conditions can damage a significant proportion of the muscle fibre.. Use of agents such as Fura-2 and Indo-1 have allowed fluorescent indicators to be introduced into the cell by less damaging chemical means.

Evidence supporting a change in SR Ca^{2+} uptake in heart failure has been obtained by calculating the exponential decay of the Ca^{2+} transient signal that follows activation of the preparation (Vahl, Bonz, Timek & Hagl, 1994). While a delay in the decay phase of the Ca^{2+} transient would suggest reduced Ca^{2+} uptake by the SR there are a number of factors, other than an intrinsic change in SR function (discussed earlier in this chapter), which can give rise to changes in the Ca^{2+} transient.

Multi-cellular vs single cell techniques

Isolation of single cardiac myocytes has provided an important and useful technique to examine cellular function without added complications of changes in the extracellular matrix and problems associated with multi-cellular preparations. However, although this is an advantage in some ways it also represents conditions further removed from physiological conditions. For example, myocytes may function differently when arranged in the cardiac architecture where they may be influenced by a number of local factors such as endothelium derived factors (Wang & Morgan, 1992) compared with in the isolated state. There are problems related to cell survival during the isolation procedure and subsequent selection of viable cells which may also influence experimental findings. Most studies using isolated myocytes have used amplitude of unloaded shortening as a measure of contractile function. The sarcomere length cannot be measured therefore and the amplitude of contraction, rather than the tension response, is assessed. This is a less controlled and less well defined measure of contractile function.

Experimental data obtained from multi-cellular preparations are obviously the collective response of a number of cells which reduces sampling errors to a certain extent. However, even selecting a single trabecula from a viable region of myocardium in a failing heart creates a sampling bias and therefore interpretation of results should not be sweepingly extended to the heart as a whole. There are additional diffusion problems which tend to slow response

times and in larger preparations may be limiting enough to create central areas of anoxia and rigor. In the saponin-treated preparations used in this study the trabeculae are typically elliptical with major diameters in the region of 150-200μm and minor diameters of 60-100μm. The critical size in these preparations, with respect to diffusion, is the minor diameter since a substance (such as ATP) must pass through one half of this distance to reach the centre of the trabecula and therefore in preparations of this size there are no problems with regions of central anoxia.

Ventricular chamber differences in heart failure

Few studies have addressed possible differences in the cellular aspects of contractile function between left and right ventricles in heart failure. This may be more important in ischaemic heart disease where the initial damage occurs in the LV and progresses to produce RV hypertrophy and failure. There is some evidence for a differential response between left and right ventricles during progression to heart failure. Perreault *et al* (Perreault, Bing , Brooks, Ransil & Morgan, 1990) demonstrated a differential response to hypertrophy in the LV and RV of the spontaneously hypertensive rat during the progression to heart failure. In normotensive rats they found an increased Ca^{2+} sensitivity of papillary muscles from the left ventricle. The hypertrophied left ventricle demonstrated an increase in maximal Ca^{2+} activated force which reduced back to control levels as heart failure developed with no change in the sensitivity of the myofilaments for Ca^{2+}. In contrast, right ventricular preparations demonstrated an increase in Ca^{2+} sensitivity and an increase in maximum Ca^{2+} activated force as failure developed. The authors interpreted these findings as progression of LV hypertrophy to LV failure and progression to subsequent RV hypertrophy.

There may also be differences in SR function between ventricular chambers during the development of heart failure. In a rat model of coronary ligation Afzal *et al* (Afzal & Dhalla, 1992) demonstrated that in control animals Ca^{2+}-

uptake was greater in SR vesicles isolated from the LV compared with RV. There was a decrease in SR Ca^{2+}-uptake in the LV over a period of 16 weeks following infarction and an increase in RV Ca^{2+}-uptake at 8 weeks. Ca^{2+}-uptake returned to control values in the RV at 16 weeks after infarction. This differential response between left and right ventricles suggests that we should be more cautious in interpreting experimental findings limited to one chamber and, additionally, that temporal changes in SR function may be important during the development of heart failure.

CHAPTER 2

Methods

METHODS 1

ANIMAL MODELS OF HEART FAILURE

Adriamycin-induced cardiomyopathy in the rabbit

Background

Adriamycin is a widely used anthracycline antibiotic with important clinical uses in the treatment of a range of malignancies. It is useful for both solid and haematological malignancies but unfortunately possesses cardiotoxic properties which limit the total dose to which the patient can be exposed. A dose dependent cardiomyopathy is a well recognised problem in clinical practise. The risk of developing cardiac side-effects increases greatly after a total dose exceeding 550mg/m^2 (Von Hoff, 1979). This toxic side-effect has been utilised in a number of studies to induce chronic heart failure in a number of species including rabbit and rat (Wanless, Anand & Poole-Wilson, 1987, Jensen, Acton & Peters, 1984). The method involves administration of a toxic dose of adriamycin over a prolonged time period sufficient to produce heart failure without inducing further toxic effects which are linked to this drug e.g. renal and hepatic impairment, bone marrow suppression, intestinal malabsorption, mucositis, anorexia and alopecia. The animals must, therefore, be closely observed for these side-effects and a scoring system used to assess the general condition and distress of the animal.

Method

Male New Zealand white rabbits (2.5-3kg) were given adriamycin intravenously 1mg/kg twice weekly for 8 weeks via the marginal ear vein. Injection of adriamycin into the marginal ear vein produced significant local irritation without visible evidence of extra-vasation of the drug during the injection. This was improved by giving the adriamycin into the side-port of a concurrent saline infusion as is recommended in humans. The animals were

assessed three times weekly for signs of toxicity and distress using a scoring system proposed by Morton and Griffiths (Morton & Griffiths, 1985). Specific features noted were anorexia, weight loss, diarrhoea, mucosal ulceration, conjunctivitis, alopecia and nasal discharge. If the animal scored above a threshold level on this scale then it was humanely and promptly killed. Once the 8 week series of injections was complete the animal was allowed to develop chronic heart failure over the next 2-4weeks while the rabbit recovered from the acute effects of the adriamycin. Normally, during this period, the rabbits would regain their appetite and start gaining weight rapidly. Control animals were given saline injections and infusions twice weekly for 8 weeks and observed for a further 4-6 weeks after this.

At the end of this period echocardiography and haemodynamic assessment were performed as explained in later in this chapter.

20 animals were started on adriamycin using this protocol but final data were obtained in only 12. Six animals with high distress scores were killed before the end of the protocol. Two animals died while placing the catheters for haemodynamic assessment. Placement of the LV and femoral catheters was not without risk and so 5 ADR animals, constitutionally less well, were assessed only by echocardiography. These results were in keeping with previous experience of this model in our department (Doherty & Cobbe, 1991).

Coronary ligation in the rabbit

Background

The rabbit coronary ligation model is a well characterised and relatively straightforward model of left ventricular dysfunction. The rabbit has a poorly developed collateral coronary circulation (Maxwell, Hearse & Yellon, 1987), similar to the human and the pig, and ligation of a single coronary artery produces a well defined myocardial infarction. The model is unaffected by additional factors which relate to the method of producing heart failure which can have profound effects on the animals condition such as with adriamycin-induced cardiomyopathy.

The model has some clinical relevance in that it leaves a region of infarction which brings about ventricular remodelling in a similar way to that seen in the human. The severity of LV dysfunction is variable and this is beneficial since it provides a range of severity of LV dysfunction which can be correlated with cellular processes. Furthermore, the rabbit is of a convenient size to allow instrumentation and echocardiography to be performed with relative ease.

Method

Male New Zealand White rabbits (2.5-3.0kg) were pre-medicated with intra-muscular Hypnorm (0.3mg/kg fluanisone(10mg/ml): fentanyl citrate (0.315mg/ml)). The anterior and left lateral chest wall was shaved and cleaned with Hibidil in isopropyl alcohol. The animal was further sedated with intravenous midazolam (0.25-0.5mg/kg) via the marginal ear vein to allow endotracheal intubation (size 3-4). Endotracheal intubation was performed using a Wisconsin paediatric straight blade (size 1) laryngoscope with the rabbit lying on its back and the head allowed to drape, supported, over the edge of the operating table. The tongue was pulled laterally and the laryngoscope introduced to allow visualisation of the vocal chords. Gentle

crichoid pressure was applied and was extremely helpful in allowing clearer vision of the 'vocal' chords. The chords were sprayed with 1% xylocaine and after a few minutes an uncuffed size 3-3.5 endotracheal tube was introduced over a soft-tipped plastic-coated wire introducer. The ET tube was secured by taping to the operating table and was attached to a Harvard small animal ventilator. The animal was ventilated (0.3-0.4L/min/kg) using mild positive end-expiratory pressure (1-2cm H_2O). Anaesthesia was maintained using an inhaled mixture of 1% Halothane/1% nitrous oxide/1% oxygen. No muscle relaxants were given to allow possible distress to be recognised quickly by the operator. The animal was placed on a prewarmed table and body temperature maintained by means of a thermostatically controlled heating mat.

The heart was exposed through a left thoracotomy, the pericardium opened and a tie placed through the cardiac apex to allow easier manipulation of the heart. The coronary artery anatomy in the rabbit is somewhat different from that of other species in that the left anterior descending artery is small and supplies only a small area of the upper interventricular septum (Flores, Davies, Penny *et al*, 1984). The left circumflex is dominant and gives off a large left ventricular branch early in its course in the AV groove. This left marginal branch is easily seen running over the LV surface towards the apex and supplies the lower lateral LV wall, the LV apex and occasionally part of the RV apex. This artery was ligated using 6/0 Ethicon suture with a 4mm curved needle at the midpoint between the atrioventricular groove and the cardiac apex. The infarcted region rapidly becomes blue and is easily seen. The size of this infarcted region could be assessed and if it was not large enough a second suture was placed more proximal to the first until a satisfactory infarct size was obtained.

Occasionally the infarct size was so large that cardiac output fell sufficiently to produce a marked reduction in peripheral pulse volume. Intractable ventricular fibrillation would follow soon after and only by untying the

coronary ligature could there be any hope of the animal recovering. Such animals rarely survived and are not included in analysis. Animals were treated with intravenous quinidine (15mg/kg, marginal ear vein) 5 minutes prior to ligation of the coronary artery as this has been shown to reduce the incidence of arrhythmias with this procedure in rabbits (Connelly, Vogel, Mernandez & Apstein, 1982). Ventricular arrhythmias were most commonly observed 10-12 minutes after coronary occlusion and were treated with DC shock (5-10J) using small, sterilised, curved defibrillation paddles applied directly to the myocardial surface using the apical suture to hold the heart away from the chest wall.

Once the animal was haemodynamically and electrically stable the left lung was examined for regions of lobar collapse. Such non-perfused and non-ventilated regions could be re-inflated by occluding the outlet pipe on the ventilator and carefully observing the region of lung re-inflate with each respiratory cycle. The chest wall was closed using three 2/0 interrupted catgut sutures placed through the chest wall around the ribs adjacent to the wound and drawn firmly together thus splinting the ribs. The subcutaneous layer was closed with 4/0 catgut and the skin closed with continuous Dexon using a subcuticular stitch. Antibiotics were administered at the end of the procedure by intramuscular injection (ampicillin 25mg/kg and cephalexidine 15mg/kg). Intravenous fluids were administered at the end of the procedure (15-20ml normal saline). Post-operative analgesia was administered (0.2mg/kg intra-muscular buprenorphine) at 30 minutes and 6-8 hours after the procedure. Animals showing signs of distress after this period were given further similar doses of analgesia as required. The animal was then allowed to develop chronic heart failure over the next 6-8 weeks. A further group of rabbits were studied at 15 weeks following coronary artery ligation. A typical infarction after 8 weeks is shown in figure 2.1.

Sham-operated controls underwent thoracotomy, the pericardium opened, a tie placed through the apex and the heart manipulated in a similar fashion to the ligation animals but without ligation of the coronary artery. Procedures were performed in pairs with one sham-operated and one ligation animal placed onto the protocol at weekly intervals so that they reached the end of the protocol at the same time.

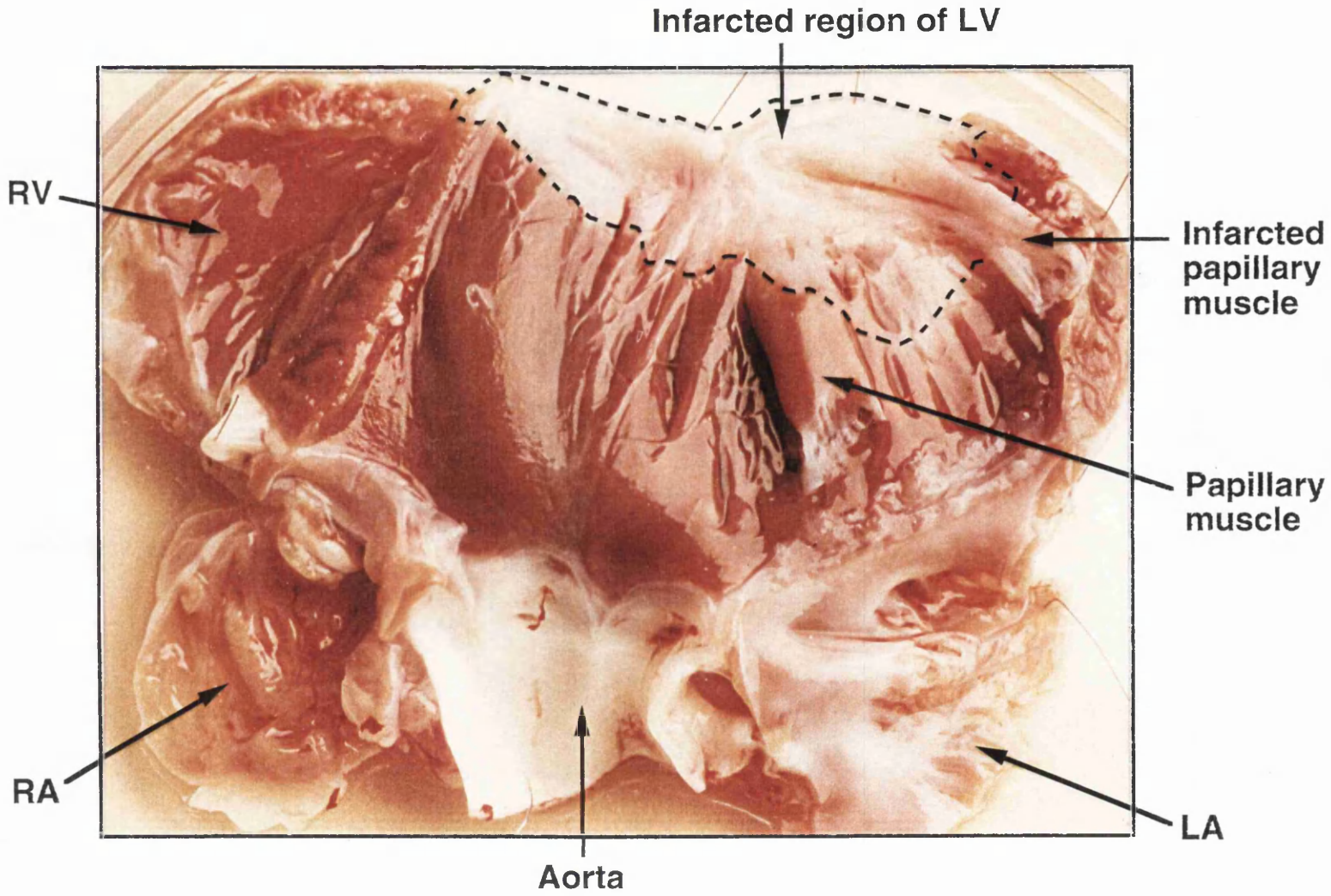

Infarcted region of LV
RV
Infarcted papillary muscle
Papillary muscle
RA
Aorta
LA

Figure 2.1 Photograph of an infarcted rabbit heart.This photograph shows a typical myocardial infarction 8 weeks after ligation of the left marginal coronary artery in the rabbit. The ventricles have been opened along the line of the interventricular septum, the apex is to the top of the picture and the opened aorta to the bottom (middle). The infarct margin is defined by the dotted line. The infarct involves the lower part of the free wall of the LV and the lower part of the interventricular septum. The anterior papillary muscle is completely infarcted and replaced by white fibrous tissue. The base of the posterior papillary muscle is also infarcted but the distal portion remains viable.

Chronic infusion of isoprenaline in the rabbit

Background

Beta-receptor stimulation brings about an increase in the force of contraction by increasing intra-cellular levels of cAMP which activates a specific protein kinase. Protein kinase catalyses phosphorylation of a number of intracellular sites including the sarcolemmal Ca^{2+} channel and the regulatory protein, phospholamban, on the sarcoplasmic reticulum. Force of contraction increases as cytosolic Ca^{2+} rises secondarily to increased influx via the phosphorylated surface membrane Ca^{2+} channel. The increased influx is compensated for by an increase in SR Ca^{2+}-uptake which increases the rate of relaxation. Phosphorylation of sites on Troponin-C increases the 'off-rate' of Ca^{2+} from the binding sites on the myofilaments and in this way also helps to bring about an increase in the rate of relaxation (Solaro, 1992).

Beta-receptor down regulation in myocardial cells is a recognised feature of heart failure (Bristow, 1993). This results from high circulating levels of catecholamines which are part of a wider neurohormonal response to heart failure. This results in a diminished response to adrenaline and noradrenaline and, therefore, loss of an important physiological inotropic mechanism. Diminished response to isoprenaline in heart failure can be demonstrated both clinically and at a cellular level. A reduced number of beta-receptors has been reported but other evidence suggests an uncoupling of the beta-receptor from adenyl-cyclase by a G-inhibitory protein (Bristow, 1993).

Down regulation of adrenoreceptors observed in heart failure can be mimicked in animal models by chronically infusing catecholamines. The model used for this study has been previously validated and has shown evidence of functional down-regulation of adrenoreceptors (Jones, Kirby,

Harding *et al*, 1990). The method used is simple and reliable and produces minimal discomfort to the animal.

Mini-osmotic pumps

Isoprenaline was administered at a dose of 100μg/kg/hr for 7 days. Mini-osmotic pumps were obtained from Alzet, Charles River, UK Ltd. The pumps chosen for this model allowed a final volume of 2ml to be delivered over a period of 7 days at an infusion rate of 10μl/hr. The desired dose of isoprenaline was dissolved in saline with 0.1mM ascorbic acid and injected into the mini-pump under sterile conditions. The rabbit was lightly sedated and a small area on the back, between the scapulae, shaved. A 1% mixture of oxygen/nitrous oxide and halothane was used to maintain brief anaesthesia while a small (2cm) incision was made on the back. A subcutaneous pouch was created by blunt dissection and the mini-pump inserted with the pump outlet toward the inner section of the pouch. The skin was closed by subcuticular stitch and intramuscular antibiotics given at the end of the procedure. This procedure takes only around 10 minutes to perform and the animals recover rapidly after only very brief anaesthesia.

Six days following pump implantation, a fluid-filled catheter was placed in the left ventricle and a thermodilution catheter placed in the femoral artery under general anaesthesia. After a recovery period of 24 hours, cardiac output, left ventricular systolic pressure and left ventricular end-diastolic pressure were measured at rest and after a challenge with a standardised dose of intra-cardiac isoprenaline. In addition the heart rate reponse to increasing doses of isoprenaline was recorded as an index of beta-receptor down regulation. Control animals were implanted with mini-pumps with vehicle (normal saline with 0.1mM ascorbic acid) only for 7 days before being similarly implanted with LV and femoral artery catheters 24 hours prior to sacrifice.

METHODS 2

ASSESSMENT OF LEFT VENTRICULAR FUNCTION

Echocardiographic assessment of LV function

Background

Left ventricular ejection fraction is recognised as a useful clinical measure of LV systolic function (Nelson, Cohn & Gorlin, 1975). Echocardiographic measurement of LVEF has been validated in human studies against radionuclide studies with good cross-correlation. There are numerous methods used to measure EF, some based on single plane, M-mode techniques with others involving more complicated multi-plane methods. For the reasons of convenience and reproducibility, a combined single plane/area trace method was used for this study as it was found to be reliable and minimised inter- and intra-observer variation.

Method

The rabbit was lightly sedated using Hypnorm (0.25mg/kg for ligation animals and 0.3mg/kg for shams) and a small area of the anterior chest wall was shaved to allow a satisfactory echo-window. The animal was placed in the left lateral position and allowed to settle for a period of 5-10 minutes before the examination commenced. All examinations were performed using a Toshiba SSH160A sonograph with a 5MHz short focus, wide angle, phased array, paediatric probe with a small (1 cm) diameter head which allowed full contact with the rabbit chest wall. Images were acquired 'real time' as M-mode and 2D images and analysed at the time of examination and again, from the video after the examination was completed. The clearest echo-window was obtained in region of the lower left sternal edge. A conventional parasternal long axis image could be obtained by angling the ultrasonic beam in a longitudinal/sagittal axis and from this it was possible to obtain clear

views of the left ventricular diameter (systole and diastole) at the tips of the mitral valve (figure 2.2). By rotating the probe through 90° a standard short axis view was obtained and a second measurement of LVEDD was made. Ejection fraction was calculated by acquiring images over a number of cardiac cycles and storing these on a 64 frame cine-loop facility incorporated into the software of the machine. These images could then be reviewed by 'tracking' back and forward with a tracker-ball and selecting a representative end-diastolic and end-systolic frame. The endocardial border was then traced using a best-fit ellipse method to measure end-diastolic and end-systolic area at the tips of the papillary muscles. The ejection fraction is calculated as the percentage area reduction *i.e.* end-systolic area/end-diastolic area x 100% over at least 3 cardiac cycles. Intra-observer variation was assessed by repeated measurements on 6 rabbits performed 2 days apart. Measurements of LVEDD and ejection fraction were made. LVEDD was found to vary by less than 0.1mm while EF was found to vary by up to 5%.

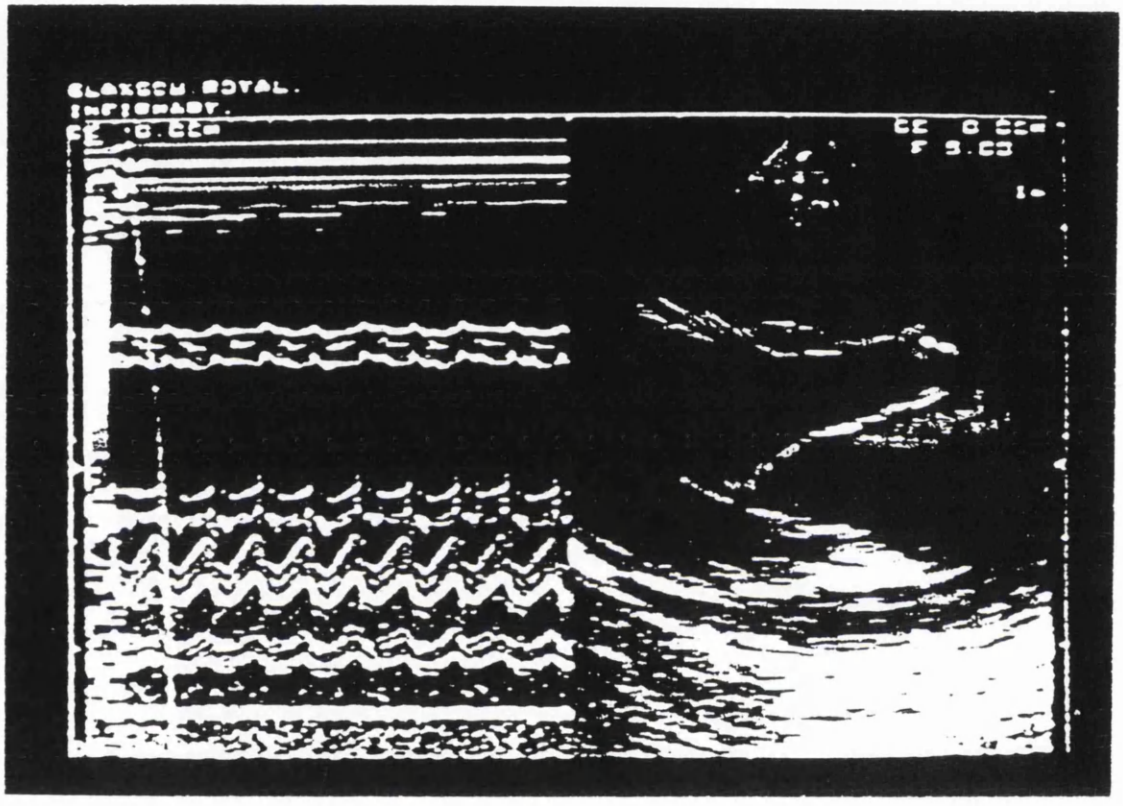


Figure 2.2 Echocardiographic parasternal long-axis view of the heart of a rabbit 8 weeks after myocardial infarction. The left atrium, left ventricle and left ventricular outflow-tract are clearly seen. The cursor is placed across the tips of the mitral valve and the M-mode image on the left panel is used to measure left ventricular end-diastolic diameter.

Measurement of cardiac output

Background

Blood flow is often measured by indicator dilution which is based on a principle first proposed by Adolph Fick in 1870. The extension of this principle to use thermodilution was developed by Fegler in 1953. Finally the clinical application of this was developed by Braithwaite & Bradley (1968). The method encompasses the principle that the temperature of blood is reduced by injection of a quantity of cold fluid. Measurement of the temperature change at a point downstream from the injection site allows the construction of a relationship between temperature and time. This temperature/time curve is the result of firstly, a delay between injection and the time taken for the cold injectate to reach the measuring point, secondly the temperature falling as the cold fluid passes and finally rising again as the cold injectate passes away. By constructing a semilogarithmic plot of temperature(abscissa) against time (ordinate) the integral of this curve will represent the mean temperature change at the sampling site over the sampled time period. The method for calculating blood flow using a non-diffusible indicator can then be applied in modified form *i.e.*

$$Q_b = \frac{60 \times V_i \times d_i \times S_i \times (T_b - T_i) \times k}{d_b \times S_b \times [T_b(t)\, dt]}$$

Where: Q_b = blood flow per minute

V_i = Volume of fluid injected minus catheter dead space outside the body.

d_b ,d_i = density of injectate and blood (respectively)

S_i ,S_b = specific heat of injectate and blood

T_i ,T_b = temperature of injectate and blood

Tb(t) = change in temperature at the sampling site at time Ts

Tb(t) dt = area of thermodilution curve calculated by extrapolation of the upslope.

k = correction factor to allow for loss of thermal indicator due to heat exchange in the injecting catheter.

When 5% dextrose is used as the injectate and the specific heats and densities of blood are assumed then the following expression holds:

$$\frac{ds \bullet Si}{db \bullet Sb} = 1.08$$

In addition, for the purpose of the measurements performed in this study the catheter warming is assumed to be constant between animals (since identical catheters are used each time) and so an empirical correction factor of 0.825 is used for k. The formula can subsequently be modified to :

$$Qb = \frac{56.3 \times Vi \times (Tb - Ti)}{Tb(t)\, dt}$$

This equation makes a number of assumptions:

1. Uniform mixing of the cold injectate with blood
2. Minimal loss of cold injectate between injection and sampling sites
3. Traversal time (at sampling site) of any given portion of the injectate must be representative of the whole injectate.

There are a number of advantages of thermodilution cardiac output measurement. Firstly, a large number of measurements can be made and secondly these can be performed in rapid succession since the recirculation of indicator is negligible.

Apparatus

Previous work in this department has indicated that a 90% response time of 0.15-6 seconds for a temperature change of 1-2 ^{o}C is required to measure blood flow in the descending aorta in rabbits. Clinically used pulmonary capillary wedge catheters have a rapid response time and so a thermistor bead was salvaged from a discarded Swan-Ganz catheter attached to a 30cm length of its insulated connecting wire. This bead and wire were threaded into a 3F Portex catheter and the thermistor bead fixed to the distal end with Araldite. The insulation was stripped from 0.5 cm of the proximal end and the wires soldered to a small plug. The relationship between temperature and resistance in the thermistor beads is not linear and so a small 'linearising' Wheatstone bridge designed in the physics department was used to convert the resistance signal to a linear voltage signal. This provided a linear signal ±1% over a temperature range 32-44 ^{o}C. Each thermistor catheter was calibrated before insertion using two water baths of known temperature to normalise the voltage output between catheters. Each catheter therefore had a value for the slope of the temperature voltage relationship(a) and the point at which it crosses the y-axis (c) which represent the simple algebraic relation:

$$y = ax + c$$

The Wheatstone bridge was connected to a BBC micro computer, installed with a computer program kindly lent by Professor Poole-Wilson. This program calculated the area under the temperature/time curve and, taking the previous mentioned factors into account along with the characteristics of the thermistor, calculated the cardiac output.

Surgical insertion of catheters

The rabbit was premedicated as described previously and the neck and left femoral region shaved. Under general anaesthesia (1% halothane/1% nitrous oxide/1% Oxygen), a small incision was made in the neck over the

right carotid artery and the vessel exposed by blunt dissection. A catheter (4F) was introduced into the artery and advanced downward towards the LV. Arterial pressure was monitored during this period by means of a Gould fluid-filled pressure transducer. The catheter was advanced and gently manipulated until a ventricular pressure trace was obtained. The catheter was then fixed at the proximal end and the wound closed in two layers with interrupted sutures. The catheter was then filled with heparin, a bung placed over the end and this was then taped to the dorsal surface of the rabbit. The left femoral artery was exposed by blunt dissection and carefully separated from the left femoral nerve. A thermodilution probe (3 French gauge) was placed into the artery and advanced 8cm into the descending aorta. The proximal end of the catheter was fixed to the vessel and surrounding tissue and the plug tunnelled subcutaneously onto the dorsal thoracic region through a small incision on the back. Skin incisions were closed by subcuticular sutures and a small jacket placed on the animal over-night to prevent chewing and scratching of the catheter sites.

Method for measuring Cardiac output

Cardiac output was measured by rapidly injecting a bolus (1ml) of cold saline (usually 19-22^{o}C) of known temperature into the left ventricle . This provided a reproducible value for cardiac output with some practise at injecting the cold saline with similar speed and force. This technique was previously validated in rabbits (Wanless, Anand Poole-Wilson & Harris, 1987). A typical temperature/time curve is shown in figure 2.3. A mean value of cardiac output was calculated from five injections.

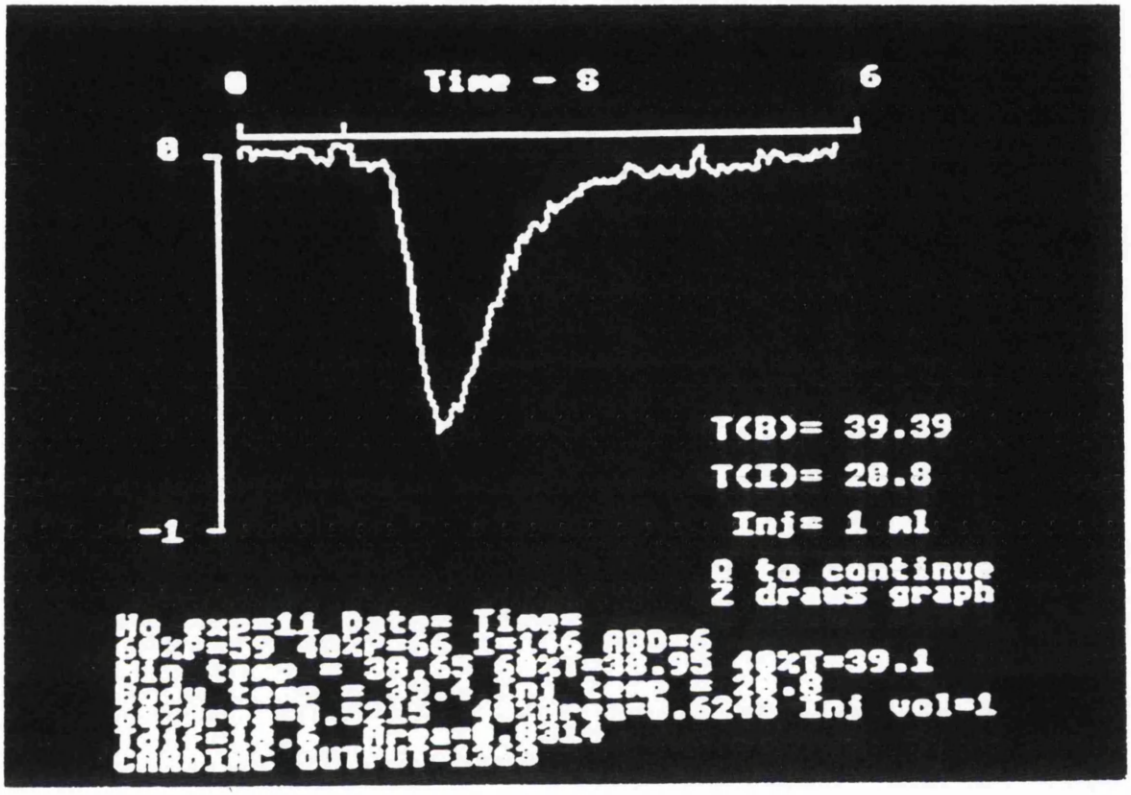


Figure 2.3 - Temperature/time curve used for the calculation of cardiac output. The body temperature (T(B)) is 39.4°C and the injectate temperature is 20.8°C. The sampling time (6 seconds) is given along the x-axis with the relative temperature change on the y-axis. The curve describes the temperature fall and rise as the bolus of cold dextrose passes the thermodilution catheter in the descending aorta. The cardiac output (1363 ml/minute, bottom of the figure) is calculated from the area under the curve. This was then divided by the animals body weight to give cardiac index. Measurement of cardiac output was repeated (mean of five injections) after the same fluid challenge described in the previous section. The percentage increase in cardiac index following fluid challenge is expressed as ΔCO.

Measurement of left ventricular pressures

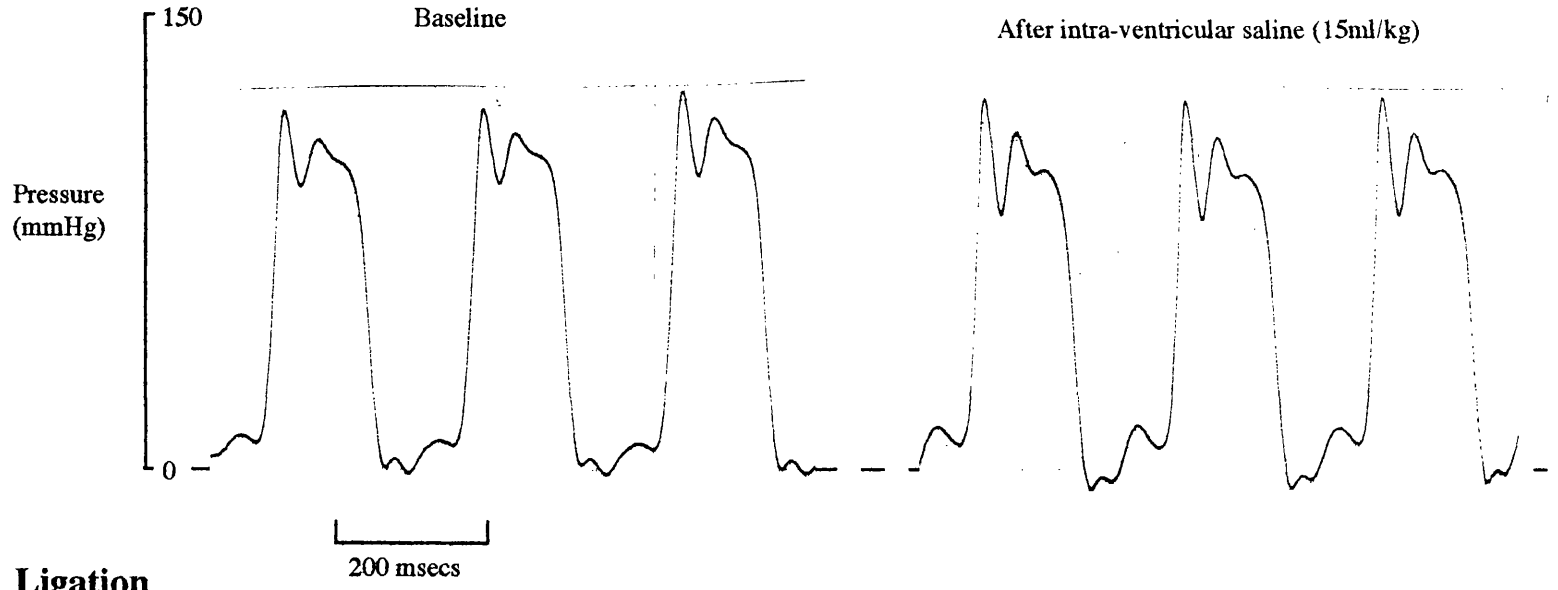
Left ventricular systolic (LVSP) and end-diastolic pressures (LVEDP)

Measurement of these pressures was performed with the rabbit held in a custom made holding-box. This was not obviously distressing for the rabbit but it became clear that a significant period of time was necessary between placing the rabbit in the box and measurement of pressures. Left ventricular systolic pressure (LVSP) could reduce from values of 150mmHg to 110 mmHg simply by allowing the animal to settle and become accustomed to the box. In the majority of cases the rabbit had been in the box on a previous occasion and so was not as stressed by the experience. A further consideration, which became clear, was the fluid-balance status of the animal in this early post-operative period. This could vary depending on fluid loss during the previous days placement of catheters and the intake of fluid during the intervening 24 hours. By assessing the overnight fluid intake from the water bottles on the cages it was clear that some animals would drink very little water during this early post-operative period. This was probably due to difficulties reaching the water bottle while wearing the protective jackets or slow recovery from the anaesthetic. In an attempt to compensate for this, all animals were given a minimum of 30mls of intravenous saline at the end of the catheter placement procedure and on the morning of haemodynamic assessment a further 20mls of saline if there had been little or no water consumption overnight.

As previously indicated, the animal was allowed to recover from the anaesthetic for at least 24 hours before recordings of left ventricular end-diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP) and cardiac output were made. Left ventricular pressure was measured with a fluid filled pressure transducer connected to the catheter by a short fluid filled wide bore tube. The pressure trace was recorded on a Siemens

Mingograph 7 ink-jet chart recorder. Representative traces from sham and ligation animals are shown in figure 2.4. The measurements were repeated following a standard fluid challenge of 15ml/kg of saline administered over 30 seconds into the left ventricle. 4 control animals implanted with an additional right atrial catheter, given this fluid-challenge, demonstrated a twofold increase in right atrial pressure. The trace was analysed, in a blinded fashion, by hand and pressure values calculated by using the recorded calibration marks.

Sham



Ligation

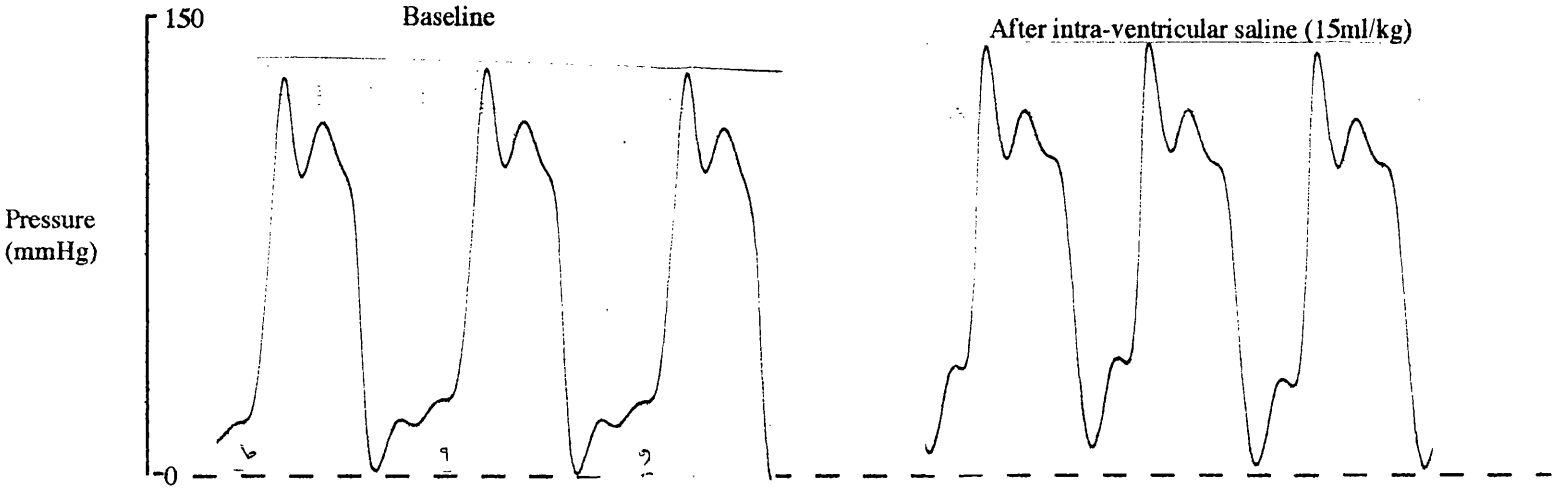


Figure 2.4 - (facing page) Left ventricular pressure trace from a sham and ligation rabbit. The upper panel demonstrates a typical LV pressure trace from a sham operated rabbit at baseline and after infusion of 15ml/kg of normal saline over a period of 30 seconds directly into the left ventricle. The systolic pressure does not change significantly with a small increase in left ventricular end-diastolic pressure. The lower panel shows a similar trace in a rabbit 8 weeks after coronary ligation. The baseline trace shows a higher LVEDP and this rises significantly after infusion of saline. The systolic pressure also rises slightly.

Heart failure score

Each coronary ligation animal was assigned a score reflecting the severity of heart failure produced by myocardial infarction. The score was calculated using the ejection fraction, LVEDD, change in cardiac output in response to a fluid challenge and LVEDP at rest. The categories shown in table 1 are based on the following scheme *i.e.* a LVEDP value for a LIG animal which fell within the normal range (for shams) scored zero points, if the value fell in the lower two quartiles of the LIG data then this scored 1 point and in the upper two quartiles scored 2 points. This principle was applied to each of the four parameters in the table and a total heart failure score calculated for each LIG animal (maximum 8, minimum 0). For *some* aspects of analysis, LIG animals were arbitrarily separated into two groups : moderate heart failure with a score of 1-4 (score≤4) and more severe failure with a score of 5-8 (score≥5).

Table 2.1 - Heart failure scoring system

	LVEDP (mmHg)			ΔCO (% change)			LVEDD (mm)			EF (%)		
	<10	10-15	>15	>26	21-25	<20	<16	16-19	>20	>60	40-55	<40
Score	0	1	2	0	1	2	0	1	2	0	1	2

METHODS 3

ISOLATED VENTRICULAR TRABECULAE EXPERIMENTS

This chapter describes the general protocol followed for the majority of experiments on isolated cardiac trabeculae described within this thesis. Specific details of some experimental conditions and techniques have been described within the appropriate chapters.

Isolation and mounting of trabeculae for force measurement

All animals were killed by a lethal dose of intravenous Euthatal (0.5ml/kg). The heart was rapidly excised via a median sternotomy and placed, still beating, in modified Tyrodes solution (composition: 150mM NaCl, 5mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 5mM HEPES at pH7.0). The right ventricle was opened by a single cut, close to the intraventricular septum, running from the base of the heart to the apex. Experiments were performed on free running trabeculae isolated from both right and left ventricle. The right ventricle is more trabeculated than the left and so free-running trabeculae of suitable proportions were more readily found in the right. However, every attempt was made to find a suitable preparation in the LV and in a few animals trabeculae were used from both LV and RV. Preparations ranged from 1 to 3mm in length and 80 to 200mm in diameter and were mounted for isometric force measurement in an assembly described as follows: the preparation was suspended between a fixed point and an Akers AE 875 force transducer by nylon monofilament snares (figure 2.5). The snares were held within stainless steel tubing (Goodfellows Metals Ltd, Cambridge; o.d. 200μm, i.d. 100μm) and were formed by double threading a 3-4cm length of the stainless steel tube with the 25μm monofilament so that a snare was produced at the base of the tube that could

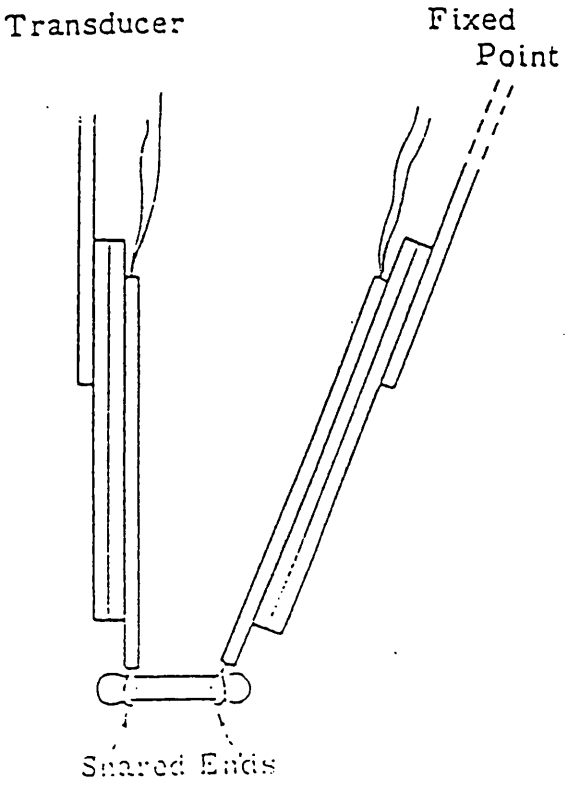


Figure 2.5 Diagram of the system for mounting the trabecula. The trabecula is held by nylon monofilament snares which are threaded through the stainless steel tubes in such a way as to form a loop at one end. The separation between the force transducer and the fixed point can be adjusted by the micro-manipulator which is held firmly by a magnet base.

be tightened by pulling the monofilament from the top of the tube. Additional rigidity was provided to the system by glueing further pieces of tubing to those forming the snares (as shown in figure 2.5). The compliance of this system has previously been calculated and measured at less than 1% of preparation length at maximum tension (for a preparation of 2mm in length producing 60 mg.wt.). The assembly was mounted on a Narashige MM3 micromanipulator. This allowed fine movement in three planes to allow accurate positioning of the muscle within the bath change system and the microscope changer. The force transducer was supported by a further micromanipulator which allowed fine adjustment of separation between the tubing to adjust sarcomere length.

Chemical skinning of ventricular trabeculae

The saponin-treated preparation has been extensively studied and has characteristics which make it ideally suited to assessing possible changes in SR Ca^{2+}-loading resulting from heart failure. Endo and Kitazawa (1978) first described the use of saponin, a plant derived glycoside, to skin cardiac muscle preparations chemically. At low extracellular Ca^{2+} concentrations, saponin combines with cholesterol in the sarcolemma and induces small perforations which allow small ions and molecules (<*c*.2000 Da) to permeate (Noireaud, Bright & Ellis, 1989). However, the sarcoplasmic reticulum and the mitochondrial inner membrane have a lower cholesterol content than the sarcolemma and so the preparation retains functional contractile proteins, sarcoplasmic reticulum and mitochondria. After chemical skinning, the bathing solution surrounding the preparation becomes an extension of the intracellular environment. The SR is anatomically undisrupted and can function in a manner close to that in the intact cell. The conditions imposed by the bathing medium are such that the SR can load and leak Ca^{2+} in a way similar to that *in vivo* and so it is possible to study factors controlling both uptake and release of Ca^{2+}. The conditions of excess ATP and an ATP-

regenerating system in the bathing solution removes possible differences in energy conditions which may underlie contractile abnormalities in heart failure in the intact cell. The permeable nature of the membrane allows free access to ions and small molecules and so addition of drugs or chemicals to the bathing medium allows a direct assessment of their effects on the SR. As a further consideration this method is a relatively cheap and simple way of estimating SR Ca^{2+}-loading since no expensive bioluminescent dyes are required with the tension produced being the index for Ca-release from evoked from the SR.

Treatment with the non-ionic detergent Triton X-100 results in the complete disruption of surface and intracellular membrane diffusion barriers, while the myofilaments are functionally retained (Miller, Elder & Smith, 1985). The residual membrane micelles do not support ATPase activity (Kurebashi & Ogawa, 1991). Therefore, any intervention which influences the contractile response of a Triton-treated preparation can be attributed to a direct action on the myofilaments. A schematic representation of the status of the components relevant in EC coupling as a result of the most commonly used techniques to disrupt muscle membranes is shown in figure 2.6.

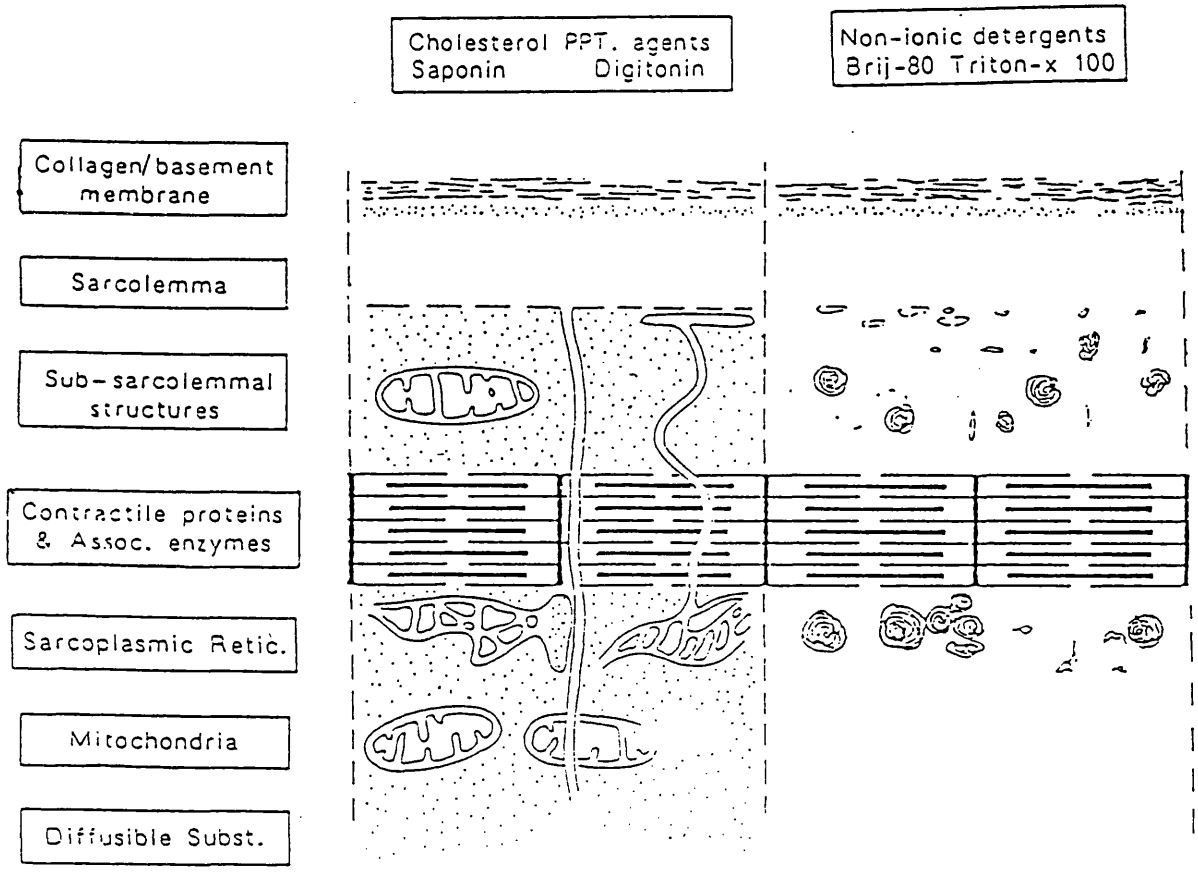


Figure 2.6 Diagram of the cell membrane after saponin and Triton treatment.

Measurement of sarcomere length

It is crucial to measure sarcomere length accurately and consistently in experiments such as these since a number of physiological parameters, particularly Ca^{2+}-sensitivity, are influenced by sarcomere length. In preference to the laser diffraction system used in most other laboratories, a modified Vickers M-16 microscope with Differential Interference Contrast (DIC) optics (Smith, 1969) was used to examine the preparation and set sarcomere length to the desired value (2.1-2.2μm). The microscope lies supine, the stage has been replaced by a mounting for the transducer assembly and an observation chamber (so that the preparation can be lowered into the optical path of the microscope, figure 2.7). The preparation is viewed on a television monitor via a video camera which further enhances the contrast of the DIC image. A graticule was placed in the video camera mounting tube allowing accurate measurement of the preparation from the monitor screen.

Measurement of preparation cross-sectional area

Accurate measurement of cross-sectional area is important in order to be able to express the maximum force production relative to unit cross-sectional area by a given trabecula. Measurement of cross-sectional area was performed using light microscopy with DIC optics. Once the sarcomere length was set at 2.1-2.2μm, the width of the preparation was measured from the graticule displayed on the TV monitor. The depth of the preparation was measured by focusing backwards and forwards through the trabecula and using a micrometer on the microscope stage to assess the distance moved by the preparation. The trabeculae were typically elliptical in cross-section and so, using major (D) and minor (d) diameters, the cross-sectional area was calculated from the formula for the area of an ellipse *i.e.*

$$\text{Area} = (\pi \times D \times d)/4.$$

* (See page 52)

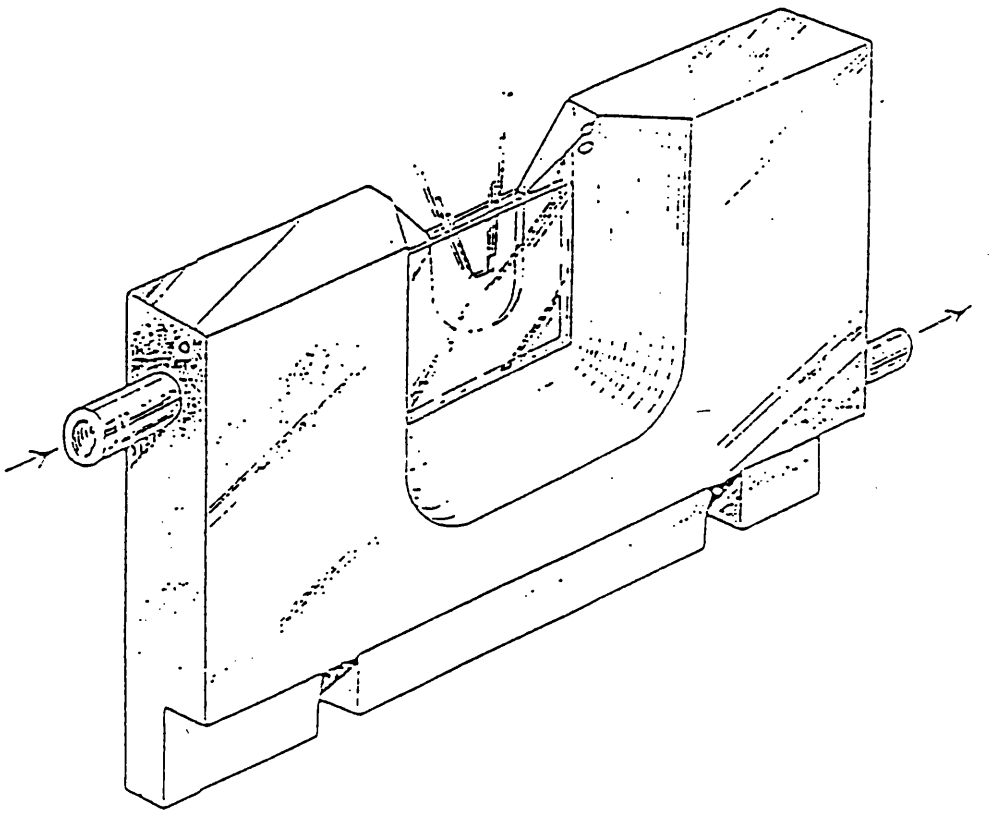


Figure 2.7 Diagram of the chamber used to permit the measurement of sarcomere length. The bath is created using two standard cover-slips sealed at the edges with vaseline. The preparation is lowered into the field of view of the microscope from above.

Automated Solution Change System

Many of the experiments described in this thesis require accurate control of the duration of exposure to the experimental solutions. For this reason a computer-controlled solution change system was employed (Miller, Sinclair, Smith & Smith, 1982). Solutions were contained within a series of wells cut into a perspex block and were continuously stirred by a small stainless steel paddle driven by an electric motor to maintain circulation of the solutions. A solution change was made by lowering the perspex block, moving it horizontally under the preparation and raising it to immerse the preparation in the required solution. Horizontal and vertical movements of this bath were made by two stepper motors under control of an Apple IIe computer. The speed of these stepper motors was such that, optimally, a solution change could be made within 200ms. The software accompanying this system allowed the programming of a large number of accurately timed solution changes. Amongst the many advantages of this solution change system is a reduction of 'carry-over' and, subsequently, contamination of the new experimental solution.

Solution composition

The rationale for solution composition, the method for calculating free ion levels and ionic strength, the choice of ion binding constants for the various ligands, precautions for EGTA purity, Ca^{2+} contamination determination, the measurement of pH and other details of the experimental solution are given in detail elsewhere (Harrison, Lamont & Miller, 1988; Miller & Smith, 1984) but are described briefly here. Components and their respective concentrations in experimental solutions are given in detail in Table 2. These solutions were made by mixing the appropriate volume of the following stock solutions, which were freshly prepared on a regular basis, 1M KCl, 1M $MgCl_2$, 100mM EGTA, 100mM CaEGTA, 100mM HDTA and 500mM HEPES.

The EGTA and HDTA were dissolved by adding twice the molar amounts of KOH, heating to 50-60°C and stirring before adding more distilled water to bring to the desired volume. CaEGTA was prepared in a similar way but with $CaCO_3$ and KOH added to the EGTA resulting in CO_2 being produced during warming. ATP and CrP were added in solid form, at pH 6.5, and the solution titrated to pH 7.0 with KOH. ATP, CrP and all other chemicals were obtained from the Sigma Chemical Company, Poole, Dorset, UK unless otherwise stated. Solutions with different Ca^{2+} concentrations were obtained by mixing solutions A and B. $CaCl_2$ was added to aliquots of solution B to achieve pCa 4.00 which, under our standard conditions, ensures maximal activation. Rapid and uniform activation throughout the cross-section of the preparation was ensured by use of the 'Ca^{2+}-jump' method. This method produces a rapid increase in $[Ca^{2+}]$ by increasing the Ca^{2+} buffer concentration at the same time as the free Ca^{2+} concentration (Miller, 1975 ; Moisescu, 1976; Ashley & Moisescu, 1977) *i.e.* the preparation is moved to the test pCa solution after prior equilibration in solution C (Table 2), which has its ionic balance (0.2M) maintained by addition of HDTA or KCl.

*** Reproducibility of width and depth measurements**

The measurement of width and depth of preparations was repeated by an independent observer on a number of occasions. Variation between observers was found to be less than 10% for both width and depth.

Table 2 Composition of solutions (in mM except where stated; pH 7.0).

Soln	K^{+*}	*Mg*†	*ATP*	*CrP*	*Na*‡	*EGTA*	*Hepes*	*pCa*
A	140	7	5	15	40	10	25	9.03
B	140	7	5	15	40	10	25	4.25§
C	140	7	5	15	40	0.2	25	7.29

* Potassium ions added as KCl and KOH. †Magnesium added as 1M $MgCl_2$; free Mg^{2+}=2.1-2.5mM in solutions A, B and mixtures of these solutions. ‡Sodium ions from the salts Na_2ATP and Na_2CrP. The Ca_{total} in all solutions was 0.02mM, except solution B where it was 10mM. §An additional 0.1mM $CaCl_2$ was added to aliquots of solution B to yield "full activating" solution (pCa 4.0). Total chloride concentrations varied from about 110 to 120mM. ¶1,6 diaminohexane N,N,N',N'-tetraacetic acid (9.8mM) was added to this solution to maintain equivalent ionic strength.

pH measurement and buffering

pH of solutions used in these experiments was accurately measured and tightly buffered. Within our laboratory, separate reference and pH electrodes are used (Ciba Corning Diagnostics Ltd., Essex, England) to give a reliable measure of pH (Illingworth, 1981). The pH of experimental solutions was adjusted using a null point method. The electrodes were allowed to equilibrate in a standard solution (6.08mM KOH, 197mM KCl and 25mM HEPES) calculated to have the same pH_a (pH activity) and ionic strength as the experimental solutions (Harrison, Lamont & Miller, 1988). HEPES (25mM) was included in all experimental solutions, at pH_a 7.0, to minimise changes in pH that may occur during contraction (local acidosis principally resulting from hydrolysis of ATP by myofilaments).

Calculation of free metal concentration and ionic strength

EGTA binds metal ions other than Ca^{2+} and therefore, the affinity constants of EGTA and the other ligands (ATP, CrP and HEPES) in these experimental solutions must be known. In order to calculate free [Ca^{2+}] it is important to take account of the concentration of other ionic species and other experimental variables such as pH and temperature.

The REACT computer program (Smith & Miller, 1985) was used to assess free ion concentrations of experimental solutions. The free metal concentration of the various commonly used mixtures of solutions A and B are given in Table 2. Ionic strength was defined using the following equation (see Miller & Smith, 1984):

$$I_e = {}^1/_2 \Sigma c_j z_j$$

where I_e is ionic strength, defined as the total of the ionic equivalents and c_j is the concentration of the jth ionic species and z_j is its valency.

Simultaneous measurement of Ca^{2+} release from the sarcoplasmic reticulum and tension measurement

The preparation was mounted between a tension transducer (Akers 46136, SensorNor a.s. Norway) and a fixed point by means of nylon monofilament snares in a similar way to that previously described (Figure 2.8). In contrast to the previous method of supporting the preparation, however, the steel pins containing the snares lay in the same plane as the preparation. The preparation was continuously perfused with solutions of low Ca^{2+} buffering capacity, solution C (Table 2), at a flow rate of 1ml/min. This perfusing solution was pumped down the centre bore of a 5mm diameter perspex column into a perspex bath whose base was formed from a glass coverslip. The column was lowered to within 5-10μm from the top of the preparation in order to minimise the volume of the perfusing solution above the preparation. The volume of solution surrounding the preparation was estimated at approximately 4 μl and excess solution was collected from the edge of the column. Aliquots of the perfusing solution containing 20mM caffeine were injected onto the preparation via the manifold at the base of the column. A solenoid-controlled pneumatic system ensured that a uniform volume of the solution (approximately 100μl) was applied to the preparation during a 50ms injection period. The bath was placed on the stage of a Nikon Diaphot inverted microscope.

Ca^{2+} release from the sarcoplasmic reticulum was detected by addition of the Ca^{2+}-sensitive fluorescent probe Fura-2. The preparation was illuminated with light alternately at 340 and 380nm wavelength and the ratio of emitted light intensities (at 510nm) was measured. The light emitted from areas of the microscope's field not occupied by the image of the muscle was reduced by shuttering on the side port of the microscope. These experiments were performed in collaboration with Dr GL Smith and Dr DS Steele.

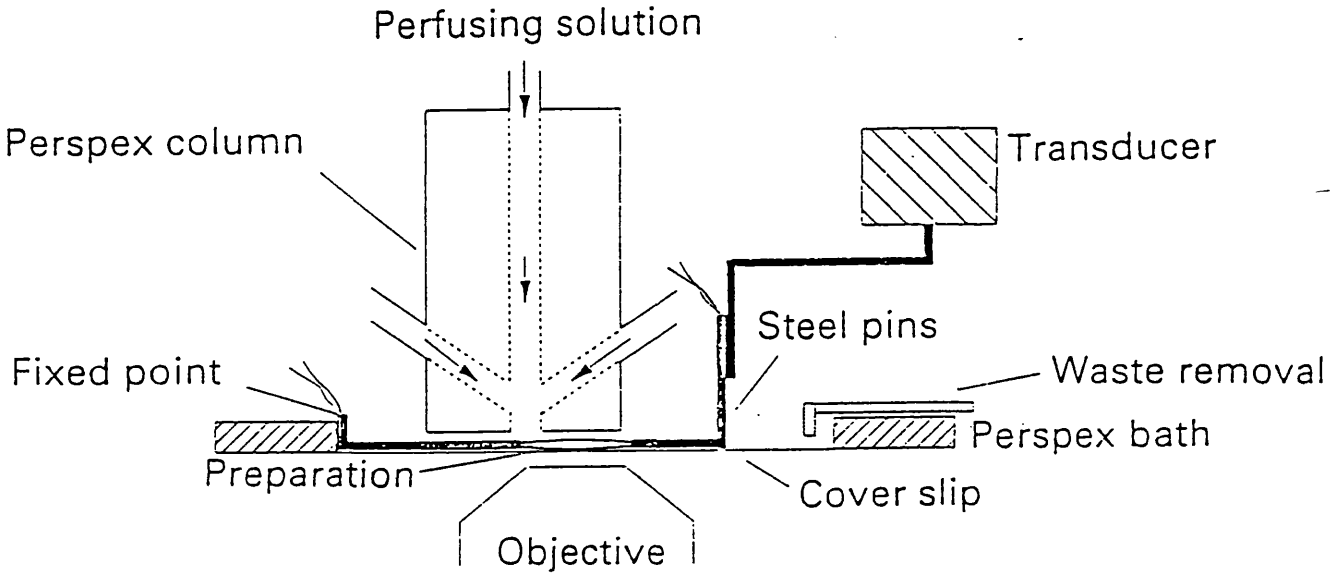


Figure 2.8 Diagram of apparatus used to make simultaneous measurement of Ca^{2+} and tension in saponin-treated trabeculae. The trabecula is mounted between the fixed point and the tension transducer by means of nylon monofilament snares. The perspex column is lowered to within 5-10μm of the upper surface of the muscle. The preparation is perfused by pumping solution through the central bore of the column. This solution could be switched using a series of valves positioned above the column allowing the bath volume to be changed within 10-15 ms.

'Fall-off' in force generation

Cardiac preparations, skinned with non-ionic detergents such as Triton X-100, have previously been shown to deteriorate with time (Jewell & Kentish, 1981). Prolonged exposures to high concentrations of EGTA and low concentrations of Ca^{2+} cause the myofilament lattice to become disorganised and, in skeletal muscle, results in a rapid decay in tension generation (Steele, 1990). In these experiments, therefore, the muscle was bathed in a low power Ca^{2+}-buffering solution for the majority of the time (solution C, Table 2). Saponin-treated fibres rarely exhibit significant functional decay with time and using the protocol described in figure 3.1, the fall-off in tension could be monitored by evoking caffeine contractures under standard control conditions throughout the experiment. The mean fall-off in peak tension response to caffeine averaged 25% over 3-4 hours and was not different in ligation or sham operated animals (figure 2.9).

Effect of temperature

All experiments were done at room temperature which is a steady 20-22°C in this laboratory throughout the year. The rate constants relating to calcium binding and rates of consumption of ATP have previously been well documented at room temperature and have been applied to the interpretation of data in this thesis. Previous studies have suggested that myofilament function is affected by temperature (Harrison & Bers, 1989; see page 241) but that SR calcium release may not be affected significantly between 23°C and 35°C (Vornanen, Shepherd & Isenberg, 1994; see page 241). In this study I cannot exclude the possibility that differences or lack of differences in the parameters measured in chemically-treated trabeculae could have been affected by raising the temperature to physiological levels. However, there are no previous studies that indicate a differential effect of temperature on failing and non-failing myocardium.

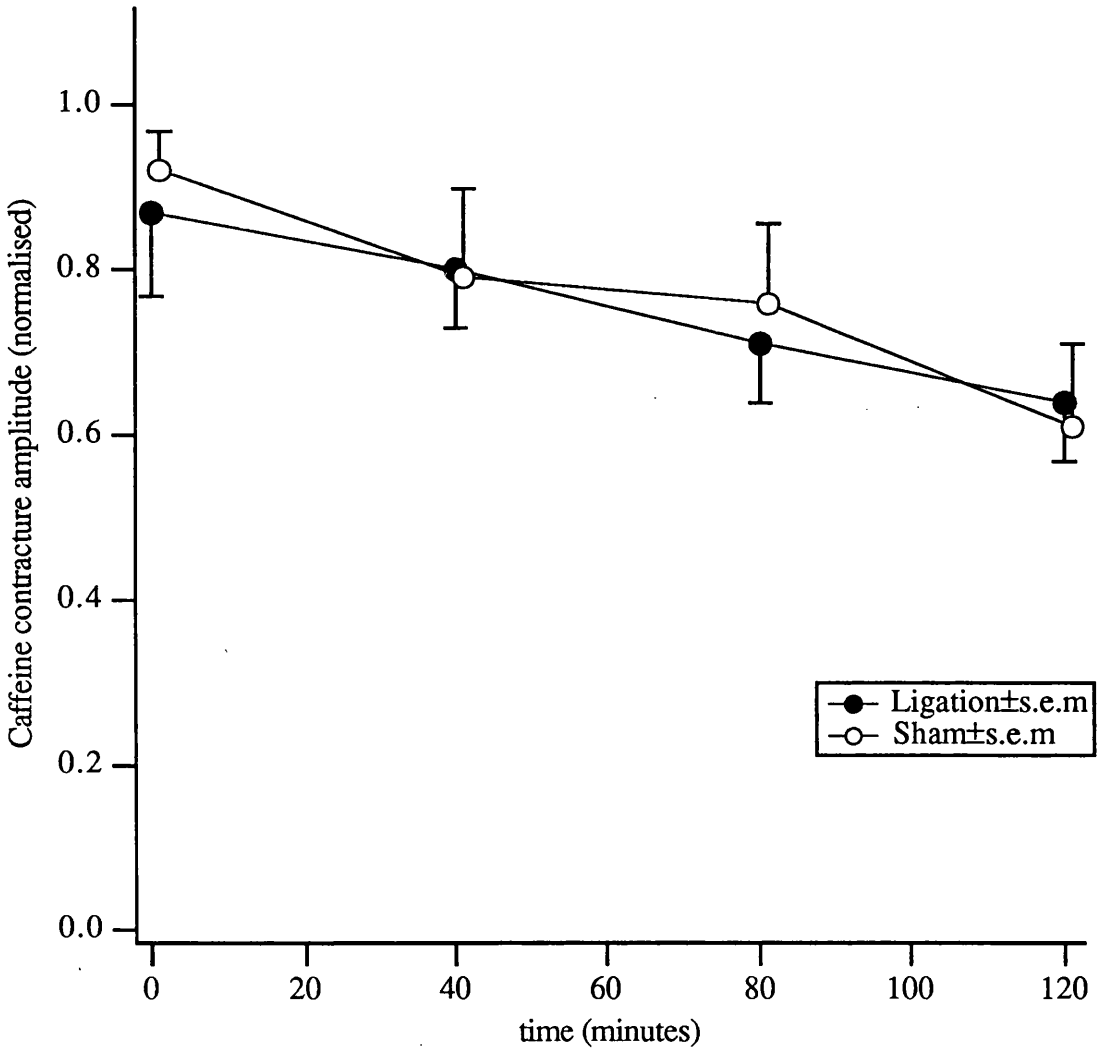


Figure 2.9 Fall-off in tension production in a saponin-treated trabeculae during a 2 hour experiment. The mean fall-off was 25% over 2 hours and was not significantly different between ligation (n=7) and sham (n=7) rabbits. This represents the fall-off in the control contractures used at intervals throughout the protocol (120s/200nM $[Ca^{2+}]$) and does not represent reduction in peak force production.

Assessing Ca^{2+}-sensitivity in Triton treated trabeculae

The Triton-treated trabeculae can be activated to produce tension by sequential stepwise increases in bathing $[Ca^{2+}]$ over the appropriate range. This approach has been widely used in pharmacological studies but has a number of disadvantages. In this laboratory, we occasionally find appreciably different force pCa curves with this protocol than with those derived from making a series of single activations (compare figure 4.1 with figure 8.4). Reasons for this difference may be due to local (*i.e.* at the middle of the preparation) increases in inorganic phosphate concentration and reductions in pH as a consequence of the preparation maintaining high levels of force for prolonged periods. In addition, prolonged activation can also accelerate the deterioration *i.e.* tension 'fall-off', of the preparation. For this reason, tension at a test pCa was achieved directly from the relaxed state in then present experiments sustained just long enough to ensure equilibration. The 'Ca^{2+}-jump', as previously described, is also useful in diminishing the rate of deterioration in such preparations.

Calculation of myofilament Ca^{2+} sensitivity using the Hill equation

The relationship between steady-state force and Ca^{2+} concentration from all otherwise satisfactory experiments is well described by the Hill equation, allowing objective definition of the position and steepness of the curve relating Ca^{2+} concentration and tension.

$$\frac{C_X}{C_{max}} = \frac{[Ca_X]^h}{EC_{50}{}^{h+}[Ca_X]^h}$$

where C_{max} is the force at a saturating Ca^{2+} concentration which is $[Ca_X]$; EC_{50} is the Ca^{2+} concentration which gives 50% of C_{max} and h is the Hill coefficient. It is generally thought that the Hill coefficient is an index of the cooperativity of Ca^{2+} binding to the regulatory protein troponin-C. However,

although skinned-cardiac muscle appears highly co-operative (Rüegg, 1986) the mechanism underlying cooperativity is poorly understood and so small changes in the Hill coefficient are difficult to interpret.

The curves obtained from the Hill equation were fitted by a least mean square fitting procedure after Levenberg and Marquat (Brown & Dennis, 1972). This yields values for $K_{1/2}$(reciprocal of the Ca^{2+} concentration producing half-maximal Ca^{2+}-activated force; log $K_{1/2}$ =pCa_{50}) and the Hill coefficient. To avoid the distortions produced by pooling experimental data, curves were fitted on an *individual* basis so that $K_{1/2}$ could be determined for each preparation. The data within the text are presented as means of Hill coefficients and pCa_{50} values calculated from the *individual* curves for each animal while the curves shown in the various figures represent the curves fitted to the mean Hill coefficient and mean pCa_{50} for sham and ligation animals.

Data handling and analysis

In isolated trabecular experiments the output of the tension transducer was displayed on a thermal array chart recorder (Astromed Dash 8), after being pre-amplified and filtered at 25Hz. The signal was simultaneously digitised using an Apple Macintosh, MacLab and the commercially available Chart software. These signals can be transferred to Macintosh graphics programmes such as Igor and MacDraw II, within which they can be scaled and annotated prior to obtaining a hard copy via an Apple Laserwriter. The experimental traces were analysed on computer screen using the MacLab software package and the data transferred to a spread sheet (Microsoft Excel) for final analysis. Curves and regressions were fitted using Igor (Wavemetrics).

Statistical analysis

Data are presented as mean±SEM unless otherwise stated. A Students two-sample, unpaired t-test was used for continuous variables if the data were normally distributed. For data that were not normally distributed, from the appearance on scatter plots, a non-parametric test was applied (Mann-Whitney test). Significance (rejection of the null hypothesis) was accepted at $P<0.05$. Correlation coefficients (Spearman's r) were used to estimate the association beteween two independent variables. Statistical significance was accepted at $P<0.05$. Data points were plotted as scattergrams and a simple regression analysis performed.

CHAPTER 3

Sarcoplasmic Reticulum Ca^{2+}-loading in Ventricular Trabeculae from Rabbits with Adriamycin-induced Cardiomyopathy

INTRODUCTION

Adriamycin is an anthracycline antibiotic used commonly for the treatment of a number of human malignancies including solid and haemopoetic cancers. However, repetitive doses exceeding a total of 550mg/m^2 are associated with significant cardiotoxic effects and can produce chronic and irreversible myocardial damage. The mechanism by which this cardiomyopathy occurs is essentially unknown despite numerous studies describing a wide range of structural and functional abnormalities.

Cellular changes can be divided into those occurring acutely and those occurring after chronic treatment. At low doses an increase in myocardial contractility, in isolated chick myocytes, has been described which is probably related to a digoxin-like effect (Kim, Akera & Brody, 1980). Myocardial contractility is depressed at higher doses in the early phase after acute administration in humans (Applefielg & Egorin, 1984). Chronic treatment is associated with structural abnormalities including myofibrillar loss, vacuolisation of the tubular network and mitochondral swelling. There are direct effects on the cell membrane which result in increased permeability brought about by electrostatic interactions with negatively charged phospholipids allowing incorporation of the drug into the lipid phase of the bilayer (Goormightigh, Chatelain, Caspers & Ruyschaert, 1980). Other membrane effects include changes in the catecholamine receptor/adenylate cyclase system (Azuma, Sperelakis, Hasegawa *et al*, 1981) and changes in the action of a variety of membrane pumps (Boucek, Olsen, Brenner *et al*, 1986).

Adriamycin is known to be a potent source of free-radicals. Under aerobic conditions adriamycin can readily donate its unpaired electron to a molecular oxygen to produce superoxide (Kalyanaraman, Perez-Reyes & Mason, 1980) which in turn can initiate the production of a number of other oxygen free-radicals. Vitamin-E deficiency has been shown to accentuate the

cardio-toxic effect of adriamycin (Singpal & Tong, 1988), thought to be related to the anti-oxidant activity of vitamin E. Thus, the generation of free-radicals is thought to be an important mechanism by which adriamycin induces cardiotoxicity (Singpal, Deally & Weinberg, 1987). Singpal *et al* also demonstrated lysosomal activation induced by adriamycin, in rats, accompanied by an increase in lipid peroxides which induce release of lysosomal enzymes. Release of these enzymes damages intracellular organelles including the sarcoplasmic reticulum and can produce Ca^{2+} overload and energy depletion within the myocyte which act in combination to depress contractile function.

Additionally, adriamycin is known to have specific effects on the sarcoplasmic reticulum. The metabolite of adriamycin, doxorubicinol is a potent inhibitor of the SR Ca^{2+} ATPase and during acute exposure can produce a marked reduction in SR Ca^{2+}-loading (Boucek, Olson, Brenner, Ogunbunm, Inui & Fleischer, 1987). However, adriamycin has also been shown to promote the open state of the ryanodine sensitive Ca^{2+} release channel on the SR rendering it more leaky with a faster rate of Ca^{2+} release (Pessah, Durie, Schiedt & Zimanyi, 1990). This would tend to predispose the cell to Ca^{2+} overload in the acute phase of drug administration. This phenomenon is readily reversible in *in-vitro* models and is brought about by an increased sensitivity of the Ca^{2+} release channel for Ca^{2+}, measured experimentally as an increased rate of occupancy of the ryanodine receptor by Ca^{2+}. However, multiple acute exposures of the SR to adriamycin sensitises the process such that even at lower doses, after repeated exposures, the 'leakiness' of the release channel becomes more marked (Pessah *et al*, 1990). These authors suggest that this might explain why cardiotoxic effects appear in humans after multiple treatments despite the use of low doses . However, long-term treatment of rabbits with adriamycin has been shown to reduce chronically the numbers of SR Ca^{2+} release channels, while the SR Ca^{2+}

uptake pump remained unaffected (Dodd, Atkinson, Olson*et al*, 1993). This reduction in ryanodine receptors could produce a net increase in the Ca^{2+}-loading ability of the SR since less leak with the same quantity of loading would result in increased total SR Ca^{2+} content.

Therefore, adriamycin-induced cardiomyopathy represents a complicated model of heart failure which is aetiologically different from the other model of heart failure studied in this thesis, namely, coronary ligation. The combination of direct adriamycin toxicity and secondary response to heart failure provide potential difficulties in interpretation. However, this represented an interesting opportunity to compare the two models.

METHODS

Animal model and assessment of heart failure

The model was produced by the protocol described in chapter 2. As indicated, this administration regime produced a significant mortality and, from 20 rabbits started, only 12 were finally studied. This reflects a low threshold for killing the animal if signs of distress developed. The adriamycin-treated (ADR) animals were assessed haemodynamically by echocardiography in all cases and seven ADR animals were assessed by invasive measurements, before being killed. This was the first group of animals to be studied my relative inexperience and the general condition of the ADR animals had initially produced a high mortality from the catheter placement procedure. It was therefore decided to perform echocardiography alone in 5 ADR animals with a clinically poor constitutional state. All saline-treated (SAL) animals had echocardiography and invasive haemodynamic assessment performed (n=7).

Isolated trabeculae experiments

The heart was excised rapidly and a trabecula isolated from the right ventricle usually situated close to the tricuspid valve annulus or occasionally at the RV apex. This was mounted for isometric tension measurement and treated with saponin for 30 minutes.

The protocol for assessing SR function is illustrated in figure 3.1. The SR, in saponin-treated trabeculae, was allowed to load [Ca^{2+}] in solution A (table 2.2) with aliquots of Ca^{2+} chloride (10mM) added to provide a range of [Ca^{2+}]. Caffeine-induced contractures were evoked by solution A (table 2) containing 10mM caffeine. Between contractures the muscle was exposed to a [Ca^{2+}] sub-threshold for tension production. During this period the SR can accumulate Ca^{2+} which is subsequently released by the next challenge with caffeine. After a standard sequence of 5 control contractures at constant [Ca^{2+}]

(200nM) and loading time (120s), the trabecula was exposed to a different loading [Ca^{2+}] for a series of 10 loading time periods ranging from 15s to 150s, arranged in a random order. After each test protocol, the standard sequence was repeated to check for any significant deterioration in the caffeine-induced contracture amplitude. The test loading [Ca^{2+}] was increased at the end of each protocol by 50nM increments from 150nM to 300nM. This range of [Ca^{2+}] was chosen to allow an assessment of the ability of the SR to load at levels similar to those in diastole and also under conditions approximating cytosolic Ca^{2+} overload. This combination of loading times and [Ca^{2+}] proved appropriate to cover the full range of SR Ca^{2+}-loading under our conditions. The amplitude of the caffeine contracture was used as a quantitative gauge of releasable Ca^{2+} content of the SR. The caffeine-induced contractures are expressed (figures 3.2 and 3.3) as a percentage of the first C_{max} measured *i.e.* immediately after Triton treatment was completed.

After the SR loading protocol was completed, the preparation was treated with Triton-x100 for 30 minutes and measurements of myofilament claim sensitivity and maximum Ca^{2+} activated force (C_{max}) were made. This protocol was followed rigidly in each experiment over a very similar time period with identical stepwise increases in loading [Ca^{2+}] for both SAL and ADR animals.

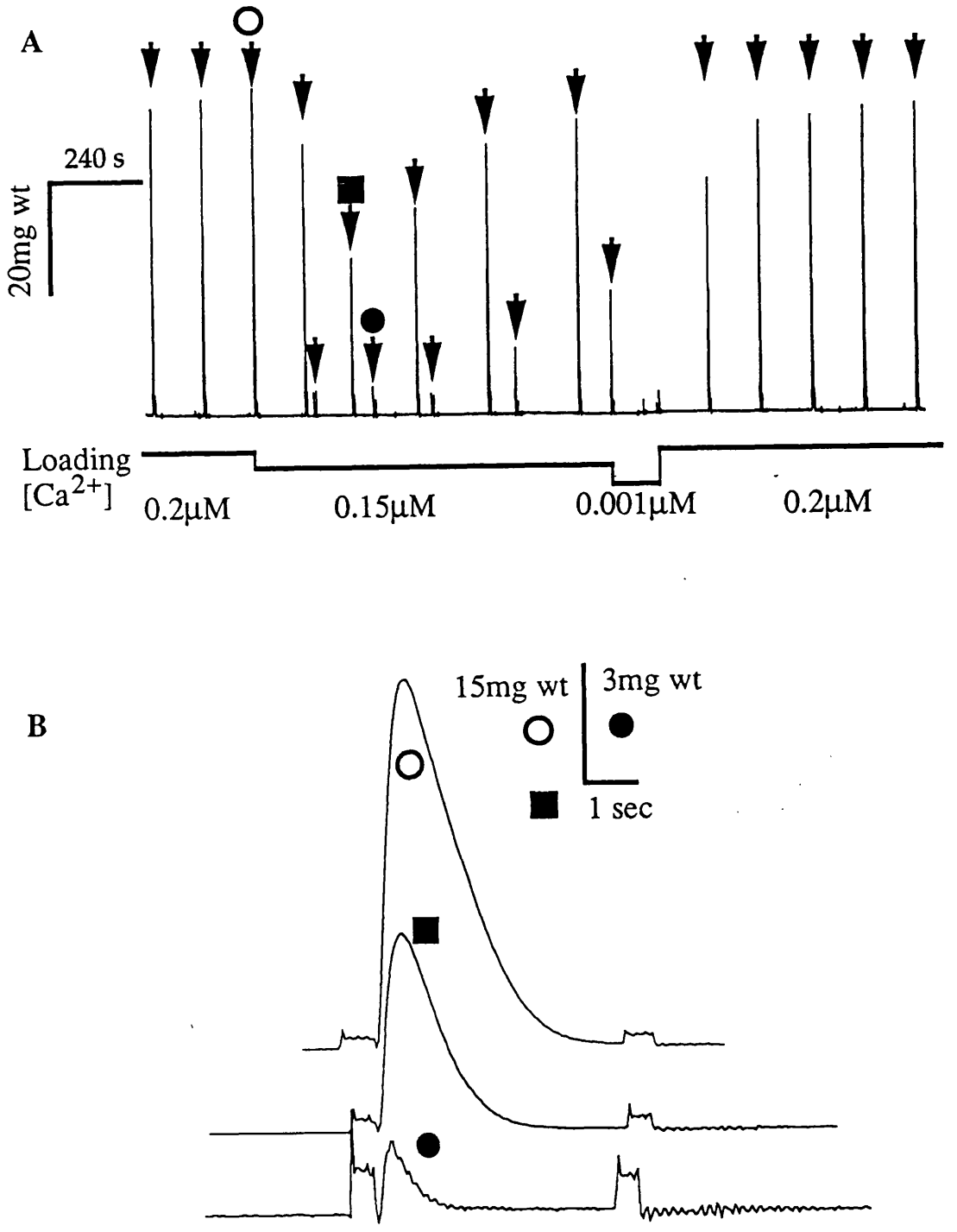


Figure 3.1 Protocol for loading the SR in saponin-treated rabbit ventricular trabeculae. Panel A shows a complete loading protocol over a period of 30 minutes. Arrowheads represent 5 s application of caffeine (10mM). A train of 3 control caffeine-induced contractures (CIC) is followed by the test loading $[Ca^{2+}]$ (150nM in this case) for various loading periods (15-150 s) arranged in random order. The preparation is then returned to the standard control conditions for a train of 5 CIC before starting another random time file at a higher loading $[Ca^{2+}]$. Panel B shows three expanded CICs from the upper panel after loading for 45 s (closed circle), 90 s (closed square), 120 s (open circle). Note how the contractures peak within 1-2 s and that the amplitude of the contracture is affected by the loading period.

RESULTS

Assessment of heart failure

Clinical features

Adriamycin animals had significantly lower body weights than the saline treated controls (ADR**3.02±0.37 vs SAL 3.68±0.25kg; P=0.001) and although they had tended to show an increase in weight after the adriamycin injections had stopped they always lagged behind the controls. 75% of the ADR animals had scrotal oedema and, although this included all of those with low LVEF (<45%), it was probably a feature of malabsorption and hypoalbuminaemia (M Pye, Personal communication). 4 ADR animals had a tachycardia at rest of >240min^{-1} and tachypnoea of >50min^{-1}. Liver weights were slightly lower in ADR animals (**69±13 vs 78±17g) and lung weights were not different (7.7±1.6 vs 7.9±0.6g).

Echocardiography

Left ventricular end-diastolic diameter was significantly greater in the ADR animals (ADR 1.63±0.11 vs SAL*1.50±0.16cm;P=0.048) although 50% of these animals had values which fell within the control range (1.2-1.6cm).

Left ventricular ejection fraction was significantly lower in the ADR animals (**54.2±9.5%) compared with SAL *(70.5±6.5%; p<0.01). However, there was considerable overlap of values (ADR range 35-70%, SAL range 60-80%).

Left ventricular pressures

Left ventricular systolic pressure was not significantly different in ADR (**93.4 ±11.8mmHg) compared with SAL *(101.5±14.1) treated animals. Left ventricular end-diastolic pressure at baseline was also not significantly different in ADR compared with SAL (**6.2±4.1 vs *5.1±3.4mmHg) with no differential response to fluid challenge between groups (**11.7±7.6 vs 11.5±7.2mmHg).

*(n=7) **(n=12)

Cardiac output

Resting cardiac output was slightly lower in ADR[**](292±71.2ml/kg/min) compared with controls[*](332±53.5ml/kg/min; P=0.1) but this did not reach statistical significance. The response of cardiac output to fluid challenge was also not significantly different between groups (31.2±10.1 vs 35±16.8; P=NS). Once again the range of values in the SAL group was smaller compared with the ADR group (SAL 303-387 *vs* ADR 211-344ml/kg/min) indicating that some ADR animals had significant LV dysfunction while others had normal haemodynamic function as assessed by this method.

Isolated trabeculae experiments

SR function

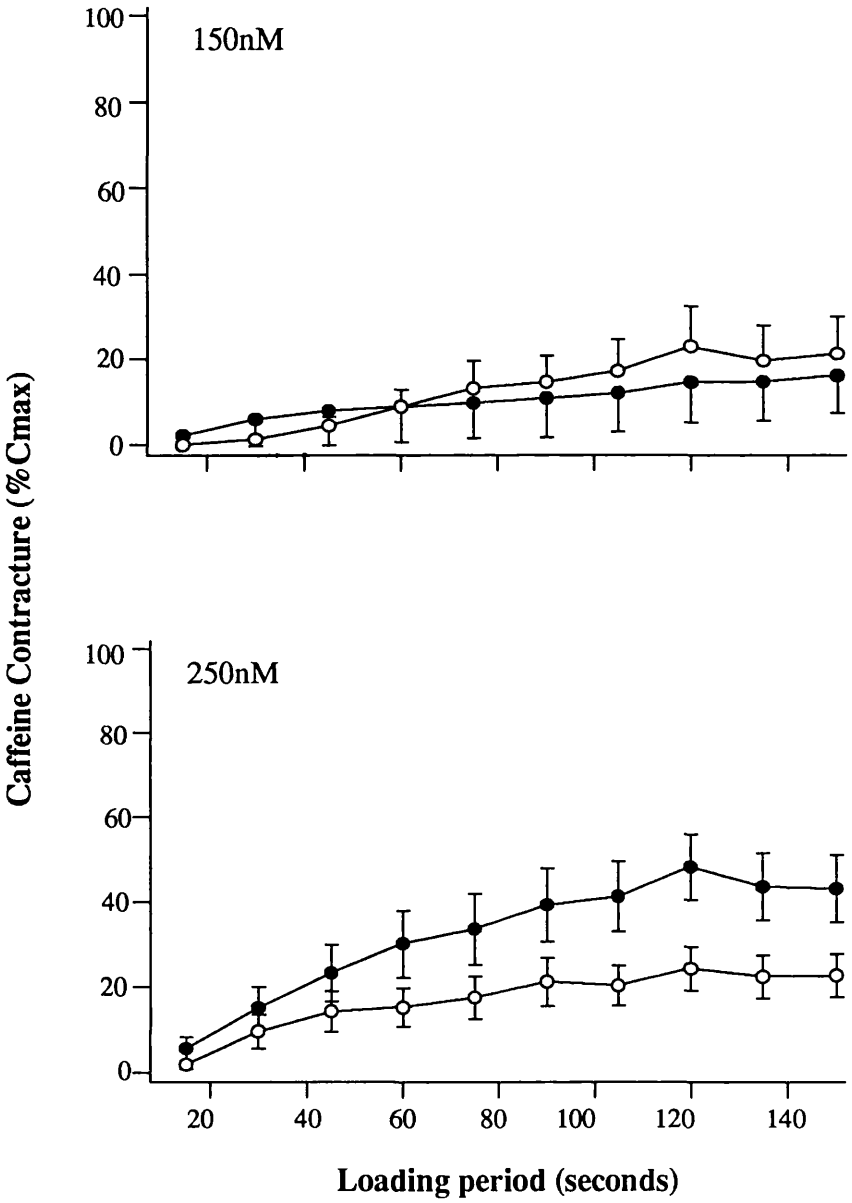
There was no significant difference in SR Ca^{2+}-loading at short or at longer loading times between ADR and SAL rabbits at lower loading [Ca^{2+}]s (150nM, 200nM). At 250nM loading [Ca^{2+}] ADR animals demonstrated enhancedCa^{2+}-loading at short and at longer loading times compared with SAL. At 300nM loading [Ca^{2+}] caffeine contractures were larger in ADR animals at longer loading times, but were not significantly different from SAL animals at shorter loading times (figure 3.2). By separating the ADR animals into two groups on the basis of left ventricular ejection fraction, animals with an EF of less than 45% (n=4) demonstrated an enhanced uptake of Ca^{2+} compared with both the SAL trabeculae and the ADR group with an EF of greater than 45% (figure 3.3). This difference between ADR animals, with and without significant LV dysfunction, was not observed at other loading [Ca^{2+}]s. At higher loading [Ca^{2+}]s and at loading times longer than 120s the amplitude of caffeine contractures tended to reduce in ADR trabeculae.

[*] (n=7) [**] (n=12)

Spontaneous tension oscillations

Enhanced SR Ca^{2+}-loading was associated with an increased tendency for spontaneous tension oscillations in trabeculae from ADR rabbits. A typical example of oscillatory behaviour in a saponin-treated trabecula is illustrated in figure 4.4 (chapter 4). These spontaneous tension transients were observed more frequently in trabeculae from ligated hearts and are dealt with in more detail in chapter 4. However, they were defined for purposes of analysis as a spontaneous increase in resting tension lasting longer than 2-3 seconds and of an amplitude greater than 3 times the baseline noise level. This pattern of spontaneous release was observed in 4 ADR trabeculae and in 2 SAL trabeculae under the SR-loading conditions examined. No formal statistical analysis of these data was possible because of low numbers in each group but the relative numbers give some information as to the occurrence in the ADR and SAL trabeculae. The $[Ca^{2+}]$•time product at which these oscillations first appeared in the loading protocol was lower in the adriamycin treated animals ($^{**}31.6\pm3.6$ vs $^{*}39.7\pm2.1$ μM.min). When these oscillations appeared there tended to be a fall in the amplitude of subsequent caffeine contractures. This feature can be clearly seen in the SR loading curve at 300nM $[Ca^{2+}]$ (figure 3.2).

$^{*}(n=7)$ $^{**}(n=12)$


150nM
250nM
100
80
60
40
20
0
20
40
60
80
100
120
140
Caffeine Contracture (%Cmax)
Loading period (seconds)

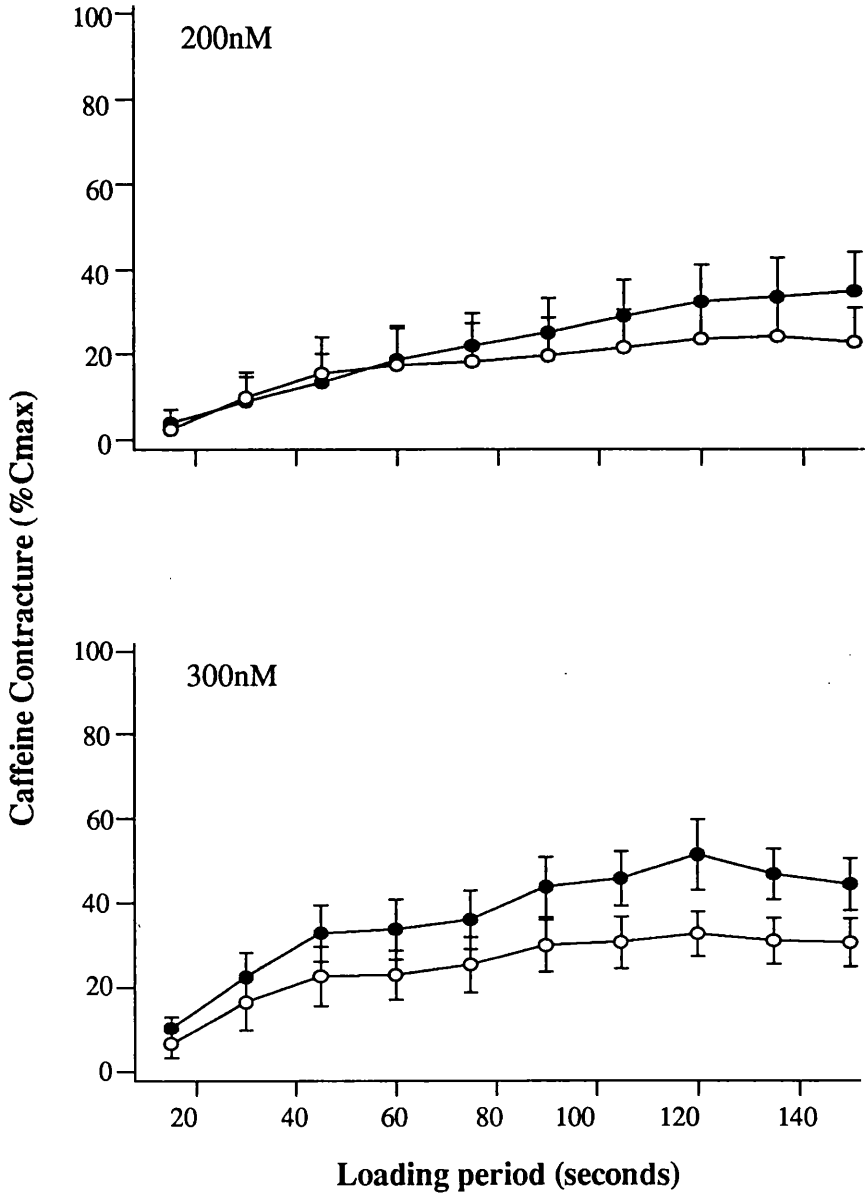


Figure 3.2 (this and facing page) SR loading curves for 4 loading $[Ca^{2+}]$s in trabeculae from adriamycin and saline treated rabbits. ADR animals (*closed circle,* n=12, mean±sem) demonstrate greater SR Ca^{2+}-loading than SAL (*open circle,* n=7) only at higher $[Ca^{2+}]$s.

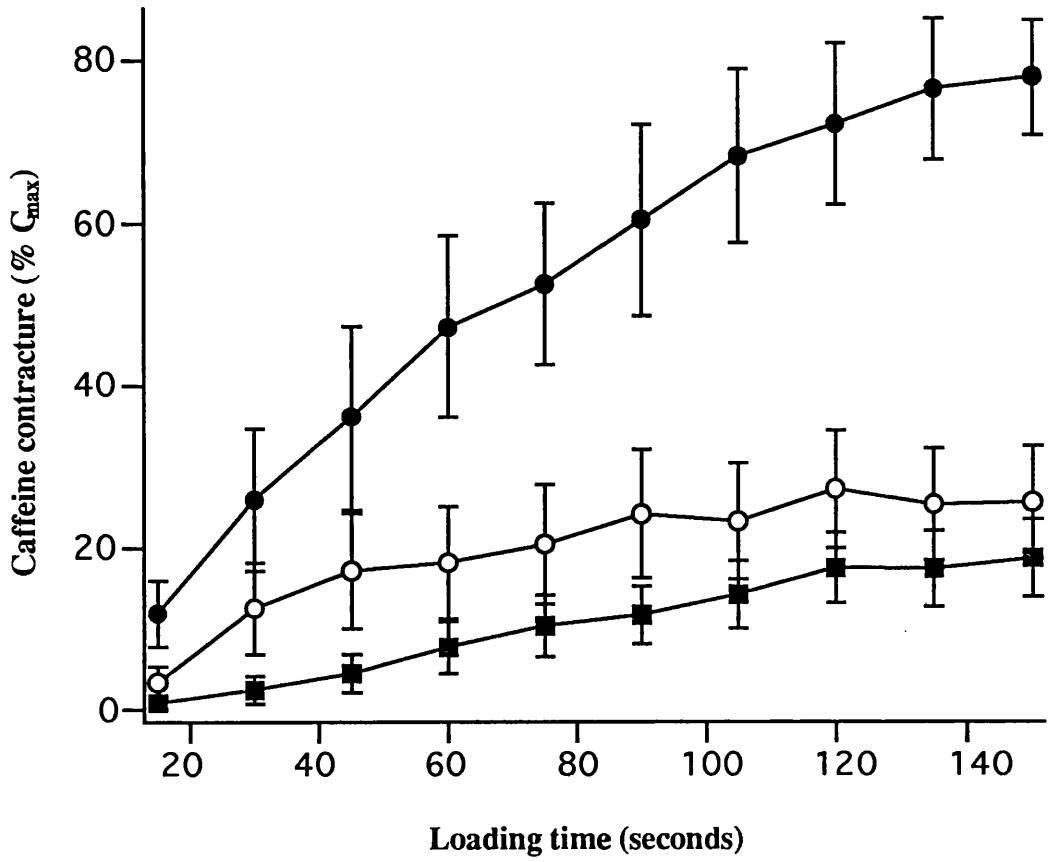


Figure 3.3 SR loading curves at 250nM loading [Ca^{2+}] for adriamycin treated rabbits separated on the basis of ejection fractions. ADR with EF<45% (*closed circles*, n=4, mean±sem) had enhanced Ca^{2+}-loading compared with ADR with EF>45% (*closed squares*, n=8) and SAL animals (*open circles*, n=7).

C_{max} and myofilament Ca^{2+} sensitivity

There was no significant difference in the cross-sectional area of trabeculae from ADR and SAL animals (0.079±0.008 vs 0.080±0.007mm^2). Maximum Ca^{2+} activated force per unit cross-sectional area was not significantly different between ADR (mean±SD, 7.42±3.0g.wt/mm^2) and SAL (6.08±2.93g.wt/mm^2; P=NS) animals. The curves representing the mean Ca^{2+}/force relationship in ADR and SAL treated animals are illustrated in figure 3.4. The [Ca^{2+}] required for half maximal activation (pCa_{50}) was not significantly different between groups (SAL 5.55±0.103 vs ADR 5.49±0.086). The mean Hill coefficient was slightly greater in ADR animals with a greater spread of values compared with controls (SAL 3.56±0.65 vs ADR 3.95±1.41).

Group with ejection fraction<45%

In view of the small number of animals in the adriamycin study it was not possible to correlate haemodynamic variables with SR function. There were four animals with an echo ejection fraction of <45% and these animals also demonstrated clinical evidence of significant adriamycin cardiotoxicity (tachycardia, oedema, tachypnoea). While this chosen ejection fraction appears arbitray the clinical impression of adriamycin toxicity was consistent with the clinical findings and therefore it is valid to analyse the SR loading characteristics of these four animals as separate group.

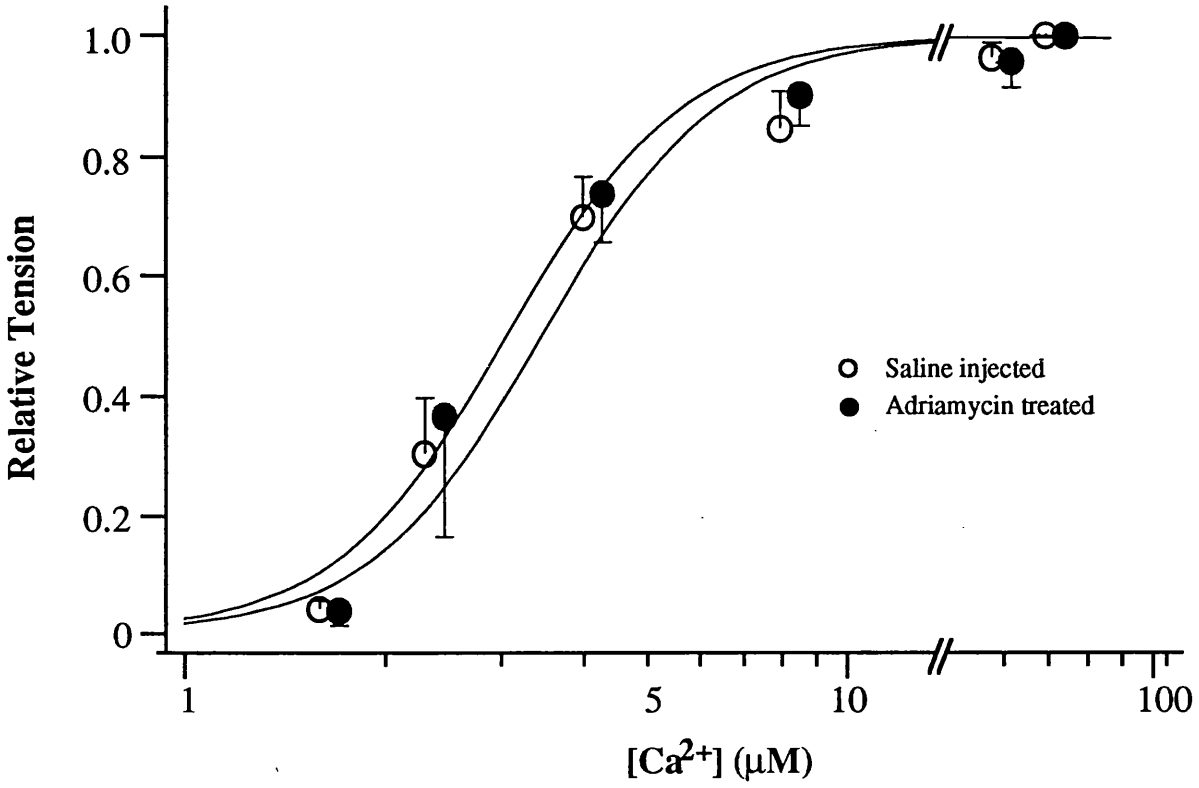


Figure 3.4 Myofilament Ca^{2+} sensitivity of adriamycin and saline treated rabbit trabeculae. ADR (*closed circles*) pCa_{50} 5.49±0.086, Hill 3.95±1 41(n=10, mean±SD);SAL (*open circles*) pCa_{50} 5.55±0.103, Hill 3.56±0.65 (n=6, mean±SD).

DISCUSSION

The severity of heart failure produced by chronic administration of adriamycin in this study was limited by non-cardiac toxic side-effects of the drug and resulted in only a small number of animals with significant LV dysfunction. Haemodynamic assessment indicated that only 4 animals in the ADR group had ejection fractions of less than 45% and a significantly greater LVEDD. However, invasive assessment of left ventricular pressures and cardiac output failed to demonstrate significant differences in the ADR animals compared with controls at baseline and following challenge with an intra-ventricular fluid load. This result is in keeping with other studies which also reported a high rate of attrition using this model (Shenasa, Calderone, Vermeulen *et al,* 1990). These animals were killed because of significant non-cardiac side-effects including weight loss, mucosal ulceration, diarrhoea and conjunctivitis and so it is possible that these were the animals most sensitive to adriamycin and therefore most likely to develop cardiomyopathy. However, 6 animals which were killed before the end of the protocol had echocardiography performed and only one had evidence of left ventricular dilatation and depressed systolic function. It is possible that they were still at an early stage of myocardial toxicity before echocardiography could detect an abnormality. Increasingly stringent guidelines for animal welfare and suffering may limit the use of this model in future.

Despite the lack of evidence for significant LV dysfunction there were differences in SR function in trabeculae isolated from ADR animals. At higher loading [Ca^{2+}] there was enhanced SR Ca^{2+} -loading revealed as larger caffeine-induced contractures in the ADR trabeculae. However, since the differences in SR loading tended to occur at longer and not shorter loading times this would suggest an increase in total loading capacity rather than an increase in the rate of SR Ca^{2+} -loading. This increased buffering capacity of

the SR for Ca^{2+} could be due to an increase in the volume of SR within the myocytes or possibly an increase in intra-SR calsequestrin. Both of these changes would bring about an increase in the Ca^{2+} buffering capacity of an isolated trabecula. Previous studies had indicated a reduction in the number of SR Ca^{2+} release channels following chronic adriamycin administration in rabbits (Dodd *et al*, 1993). In the saponin-skinned preparation the SR can load and leak Ca^{2+} in a manner similar to the physiological setting. A reduction in the number of leak channels in the presence of the same number of SR Ca^{2+} pumps might result in a net increase in the quantity of Ca^{2+} loaded by the SR during any given loading period. This hypothesis is compatible with the findings of this study: similar SR Ca^{2+}-loading at shorter loading times, implying a similar rate of uptake, but enhanced SR Ca^{2+}-uptake at longer loading times, when the SR is in a steady state, when the balance of leak and uptake becomes more important. These results are, therefore, similar to previous observations of a decrease in the number of SR Ca^{2+} release channels in rabbits treated chronically with adriamycin.

The finding of an increase in the tendency for spontaneous oscillations in the ADR trabeculae is not consistent with the notion of greater SR volume or an increase in the intra-SR Ca^{2+}-buffering protein, calsequestrin. An increase in the total SR Ca^{2+} content would provide more for release and subsequently larger caffeine contractures but would not increase the likelihood of spontaneous release. Spontaneous release of Ca^{2+} by the SR has previously been described in skinned cardiac muscle preparations after acute exposure to Adriamycin (Asayama, Yamahara, Tatsumi *et al*, 1992). These authors argue that by maintaining the SR Ca^{2+} release channel in an open state, adriamycin would increase the tendency for spontaneous release. This is a rather simplistic interpretation since it assumes that the mechanism of spontaneous release is related to the open probability of the channel which is increased in the presence of adriamycin. However, caffeine also acts at the

same ryanodine receptor on the SR and has been shown to reduce spontaneous Ca^{2+} release from the SR (Bers, 1991) probably also by increasing the open probability of the ryanodine receptor. In the chronic adriamycin model the cause of spontaneous oscillatory behaviour is likely to be Ca^{2+} over-load brought about by a reduction in Ca^{2+} leak which allows the SR to load to a greater extent. The previously observed increase in sensitivity of the release channel for Ca^{2+} (Pessah *et al*, 1990), if present in the chronically treated animal, would tend to increase Ca^{2+} leak and reduce the tendency for spontaneous Ca^{2+} release. Such differences seem to be related to the length of time of exposure to the drug.

Enhanced SR Ca^{2+} -loading was more marked in the animals with echo EF of less than 45% (figure 3.2). There are three different ways of interpreting this finding. Firstly, that these animals were the most sensitive to adriamycin and had the greatest degree of cardio-toxicity resulting in a marked reduction in the Ca^{2+} leak pathway and therefore a larger Ca^{2+} -loading capacity. The second is that these animals had significant heart failure and that this was producing a increase in SR Ca^{2+} -loading on its own. The third is that heart failure and adriamycin were acting in combination to produce a change in SR Ca^{2+} -loading. Some workers have reported that the effects of adriamycin on the SR can persist for up to 4 weeks following administration (Pessah *et al*, 1990) and so it is possible that we were observing persistent direct effects of adriamycin despite waiting for 2 weeks after the last dose.

There was no difference in the absolute force production between ADR and SAL trabeculae. This is not entirely consistent with previous work indicating that adriamycin induces myofibrillar loss (Iwasaki & Suzuki, 1991). However, Jenson (1986) also failed to demonstrate a reduction in the peak tension response in papillary muscles isolated from rats chronically treated with adriamycin. Interestingly he also demonstrated a reversal of the negative-staircase phenomenon in a number of ADR rats (perhaps as a result

of enhanced SR function). Other workers using the rabbit ADR model demonstrated a reduction in peak tension in isolated papillary muscles (Shenasa *et al*, 1990). The lack of reports in the literature on Triton treated trabeculae from animal models of adriamycin cardiomyopathy might reflect the lack of positive experimental findings. This is itself interesting since electron microscopy evidence of myofibrillar loss might be expected to produce some measurable change in myofilament function. Previous work has demonstrated a change in the myofibrillar protein, F-actin, when isolated cardiac contractile proteins are exposed acutely to adriamycin *in vitro* (Lewis, Kleinerman & Puszkin, 1982) but no such changes have been demonstrated after chronic administration.

This study demonstrated no difference in the Ca^{2+}/force relationship between ADR and SAL rabbits. Although this indicates that there has been no significant change in the response to Ca^{2+}, we can make no definitive statements of possible changes in the myofilament regulatory proteins. However, this result is important since it indicates that the myofibrils, acting as Ca^{2+} sensors, were equally as sensitive in ADR and SAL animals. The mean Hill coefficient was slightly greater in the ADR group. This can be seen in figure 3.4 as a reduced slope of the pCa/tension curve and although the Hill (h) coefficient can be used to describe the curve it is debatable whether this infers a change in the number of Ca^{2+} binding sites on troponin-C(as is implied from the Hill equation).

Conclusions

This part of the study has therefore indicated that SR Ca^{2+} -loading is increased in saponin-skinned trabeculae from rabbits with adriamycin-induced cardiomyopathy and evidence of significant LV dysfunction. As discussed, these changes may have been brought about by a direct effect of adriamycin on the SR or possibly by the LV dysfunction which resulted from adriamycin-induced myocardial damage. However, previous work on isolated SR vesicles indicated a reduction in the number of Ca^{2+} release channels and so the SR Ca^{2+} -loading assessed in these saponin-treated trabeculae might be reflecting reduced SR Ca^{2+} leak with maintained SR Ca^{2+} uptake which results in a net increase in Ca^{2+}-loading.

This concept of differences in SR leak and uptake recurs throughout this thesis and these results in ADR rabbits provide an interesting background for those obtained in the coronary ligation model of heart failure.

CHAPTER 4

Cardiac sarcoplasmic reticulum Ca^{2+}-loading in rabbits 8 weeks after coronary artery ligation

INTRODUCTION

Previous work using the rabbit coronary ligation model in this department had indicated that significant left ventricular dysfunction developed 8 weeks after coronary ligation. Few studies of Ca^{2+} handling have used the rabbit as a model of heart failure and, of those which have used the rabbit, none have studied the coronary ligation model. The rabbit is suitable since its large size allows easy instrumentation and previous work has indicated that the SR may be more like the human in contribution to myofilament Ca^{2+} activation. The initial experiments in this study were performed 8 weeks after coronary ligation. The general methods used are described elsewhere but further details of experimental protocol relating to the isolated trabeculae studies are described.

METHODS

Animal model and heart failure assessment

These methods are detailed in chapter 2. All ligation rabbits (LIG, n=17) and all sham-operated controls (SH, n=11) had echocardiography and invasive haemodynamic measurements performed.

Protocol for assessment of SR function

The methods for mounting preparations and the tension measurement techniques are detailed in chapter 2. The SR was allowed to load Ca^{2+} in solution A with aliquots of Ca^{2+} chloride (10mM) added to provide a range of $[Ca^{2+}]$. Caffeine-induced contractures were evoked by solution A (table 2) containing 10mM caffeine.

A protocol identical to that described in the adriamycin experiments (figure 3.1) was used to assess SR function in the this part of the study. Between contractures, the muscle was exposed to a $[Ca^{2+}]$ sub threshold for tension production. During this period, the SR can accumulate Ca^{2+} which is released

by the next challenge with caffeine. A range of loading periods (15-150 s) and $[Ca^{2+}]$s (150-300nM) were studied. The amplitude of the caffeine contracture was normalised to C_{max} determined in the Triton-treated state immediately after the last caffeine-contracture. This protocol was followed rigidly in all experiments.

Spontaneous tension oscillations in saponin-treated trabeculae

At higher loading $[Ca^{2+}]$ and longer loading times, the trabeculae exhibit spontaneous tension oscillations. These were defined for the purpose of this study as repeated, spontaneous tension transients (~2-3s duration) of amplitude greater than 3 times the baseline noise level. Once initiated, these oscillations occur randomly and are related to the bathing $[Ca^{2+}]$ and the length of time that the SR is exposed to this $[Ca^{2+}]$. Quantitative analysis of these tension transients is rather difficult since the number and the amplitude of oscillations can vary considerably, even in any single preparation. The MacLab software was used to calculate, by integration, the area under the tension oscillations during any given loading period. Since the baseline tension also often changed during the oscillations it was often difficult and time consuming to measure this accurately even with the software available. The critical loading conditions (loading time and loading $[Ca^{2+}]$) at which these oscillations first appeared in the protocol were recorded as an easier and more definitive method of assessing probability of oscillations. These conditions are quantitatively referred to as the $[Ca^{2+}]$•time product.

Assessment of C_{max} and myofilament Ca^{2+}-sensitivity

After assessment of SR function, each trabecula was exposed to 1% Triton-X100 for 30 min to disrupt all membranes and leave only the myofilaments functionally intact. Seven solutions with different [Ca^{2+}] were made by mixing solutions A and B in appropriate proportions. These were used to define myofilament Ca^{2+} sensitivity (expressed as the negative log of the [Ca^{2+}] required to produce 50% of maximal activation *i.e.* pCa_{50}) under conditions of strong Ca^{2+} buffering. Measurement of C_{max} at the beginning and end of the sequence was made using solution A with Ca^{2+} chloride added providing a final pCa of 4. A typical sequence of activations is illustrated in figure 4.1. The activations are arranged in random order to minimise accumulation of phosphate within the preparation and minimise fall-off in tension.

Effects of caffeine on Ca^{2+} sensitivity

The effects of caffeine on the myofilament responsiveness to Ca^{2+} was also assessed in 8 week LIG (n=5) and SH (n=5) animals. Seven solutions with different [Ca^{2+}]s were created by mixing solutions A and B in appropriate proportions. Each solution was divided into two aliquots and caffeine (10mM) was added to one. The caffeine-free solutions were used first to assess baseline myofilament Ca^{2+} sensitivity (expressed as the [Ca^{2+}] required to produce 50% of maximal activation *i.e.* pCa_{50}). Each test [Ca^{2+}] was applied after prior equilibration of the preparation in solution A. Measurement of C_{max} at the beginning and end of the sequence was made using solution A with Ca^{2+} chloride added providing a final pCa of 4. This allowed the calculation of the mean fall-off in tension throughout the protocol. The same sequence of activations was then repeated with the set of solutions containing caffeine 10mM.

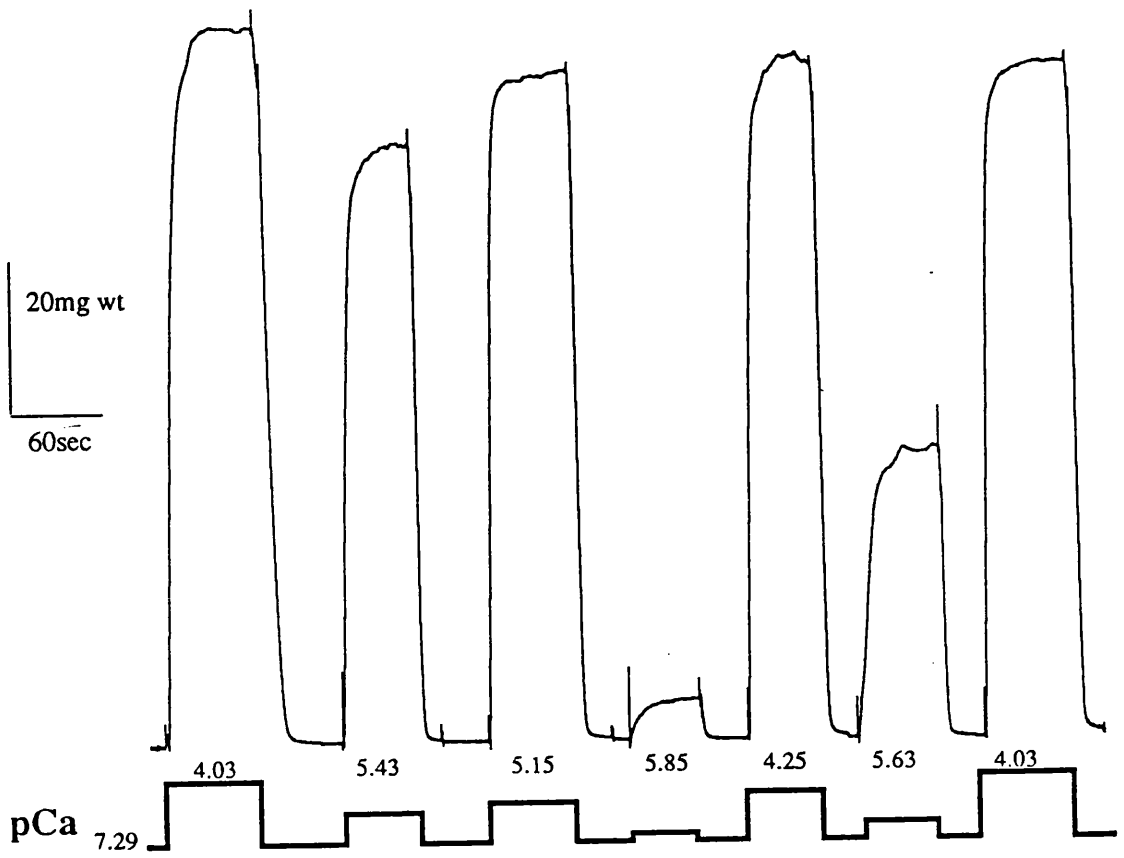


Figure 4.1 Ca^{2+}-activations in a Triton-treated ventricular trabecula used to assess Ca^{2+} sensitivity. The first and last activations are maximal activations (C_{max}), note the fall-off in tension throughout the protocol. Caffeine contractures from the SR loading protocol are normalised to the first C_{max}.

RESULTS

Haemodynamic assessment

Although coronary artery ligation using this technique produced a 25-35% infarct of the left ventricle, there was some variation in severity of left ventricular dysfunction which resulted. Full thickness infarction occurred in the majority of animals (>90%) with infarction of a large papillary muscle in 75%. Mitral regurgitation could not be detected on echocardiogram by colour or pulsed-wave doppler but there was obvious dilation of the left atrium in these animals and occasionally a suggestion of mitral valve prolapse (see figure 2.2). Results discussed below are summarised in table 3.

Clinical evidence of heart failure

47% of the ligation group (8/17) had clinical evidence of heart failure including peripheral oedema, ascites, resting tachycardia, tachypnoea and lung crepitations. 6 of these animals had ejection fractions of 40% or less on echocardiography. Further indirect evidence of left and right heart failure was provided by the significantly greater lung wet weights (0.43±0.11 vs 0.36±0.06% body weight; P=0.03) and liver wet weights (2.59±0.48 vs 2.26±0.35 % body weight; P=0.04) in the LIG. Total body weights were not significantly different between ligation and sham animals either before ligation surgery (2.98±0.21 vs 3.02±0.22kg) or at the time of haemodynamic assessment (3.75±0.46 vs 3.71±0.40kg).

Table 3 Haemodynamic indices 8 weeks after coronary ligation (mean±SD)

	Shams (n=11)	**Ligations** (n=17)	
Bodyweight *(kg)*	3.71±0.4	3.75±0.46	NS
Liver*(% body wt)*	2.26±0.35	2.59±0.48	P=0.04
Lungs*(%body wt)*	0.36±0.06	0.43±0.11	P=0.03
LVEDD *(cm)*	1.55±0.08	1.86±0.19	P=0.0001
EF *(%)*	68.3±3.9	41.9±9.5	P=0.001
LVSP *(mmHg)*	117.9±15.5	102.9±10.1	NS
LVEDP*(mmHg)*	5.8±2.7	15.1±6.9	P=0.001
CO *(ml/kg/min)*	354.3±32.9	275.2±63.1	P=0.001
ΔCO *(% increase)*	35.1±8.7	21.1±13.2	P=0.004

Echocardiography

Ejection Fraction (LVEF)

In coronary ligation animals (n=17) the mean left ventricular ejection fraction was significantly lower than sham operated (n=11) controls (mean±SD; 41.9±9.5 vs 68.3±3.9%; P=0.001). Within the controls there was little variation but in ligation animals there was a greater spread of LVEF reflected in the larger standard deviation (SD). The range of values in the control group was 65-80% with a median value of 70%. In the ligation group the range was 30-65% with a median value of 50%. 6 animals had ejection fractions of <40% with a further 6 having an EF of <50%. 2 animals had significant pericardial effusions noted on echocardiography which were later confirmed at the time of heart excision.

Left ventricular end-diastolic diameter (LVEDD)

This was an easily performed measurement in all rabbits by M-mode and was comparable in both the short and long axis. The ligation group had significantly larger LVEDD (1.86±0.19 vs 1.55±0.08cm;P=0.001) and once again the range of values was greater in the ligation group reflecting the variation in LV function. LVEDD correlated well with LVEF (r=0.81) indicating that progressive left ventricular dilatation was associated with impairment of contractile function.

Invasive measures of LV dysfunction

Heart rate

At the time of haemodynamic assessment, with catheters in-situ, resting heart rate was significantly higher in ligation animals (220.4±32.6 vs 198.1±23.2min^{-1}; P=0.04) and showed a more pronounced increase following fluid challenge (increased to 265.6±29.8 vs 220.1±45.6min^{-1}; P=0.05).

Ventricular pressures

Left ventricular systolic pressure, at rest, was slightly lower in the ligated animals (102.9±10.1 vs 117.9±15.5mmHg; P=NS) and was not significantly different after fluid challenge (119.6±12.2 vs 122.3±10.8mmHg; P=NS) compared with controls. Baseline left ventricular end-diastolic pressure was significantly higher in the ligation animals (15.1±6.9 vs 5.8±2.7mmHg; P=0.01) and demonstrated a more marked increase in the ligation animals following fluid challenge (increased to 24.9±7.8 vs 14.1±3.8mmHg: P=0.01).

Thermodilution cardiac output

Resting cardiac output was lower in ligation animals (275.2±63.1 vs 354.3±32.9ml/kg/min; P=0.01) and demonstrated a diminished response to fluid challenge (% increase (ΔCO) 21.1±13.2 vs 35.1±8.7) compared with sham operated controls. The mean peak cardiac output measured after the fluid challenge was therefore significantly reduced in the LIG group (332.75±37.9 vs 477.9±48.7ml/kg/min;P=0.01). There was a considerable spread of values within the ligation group (range 172-344ml/kg/min) with some ligated animals having a cardiac index within the normal control range. This reflected the range of left ventricular dysfunction produced. The increase in cardiac index (ΔCO) correlated well with other measures of LV dysfunction including echocardiographic ejection fraction (r=0.68; P=0.01).

Heart failure scores

Using the heart failure scoring system described in table 1, the LIG animals at 8 weeks following coronary ligation were divided into two groups (score≥5, n=7 and score≤4, n=10) with different heart failure scores (mean scores 6.5±1.11 vs 2.25±1.29).

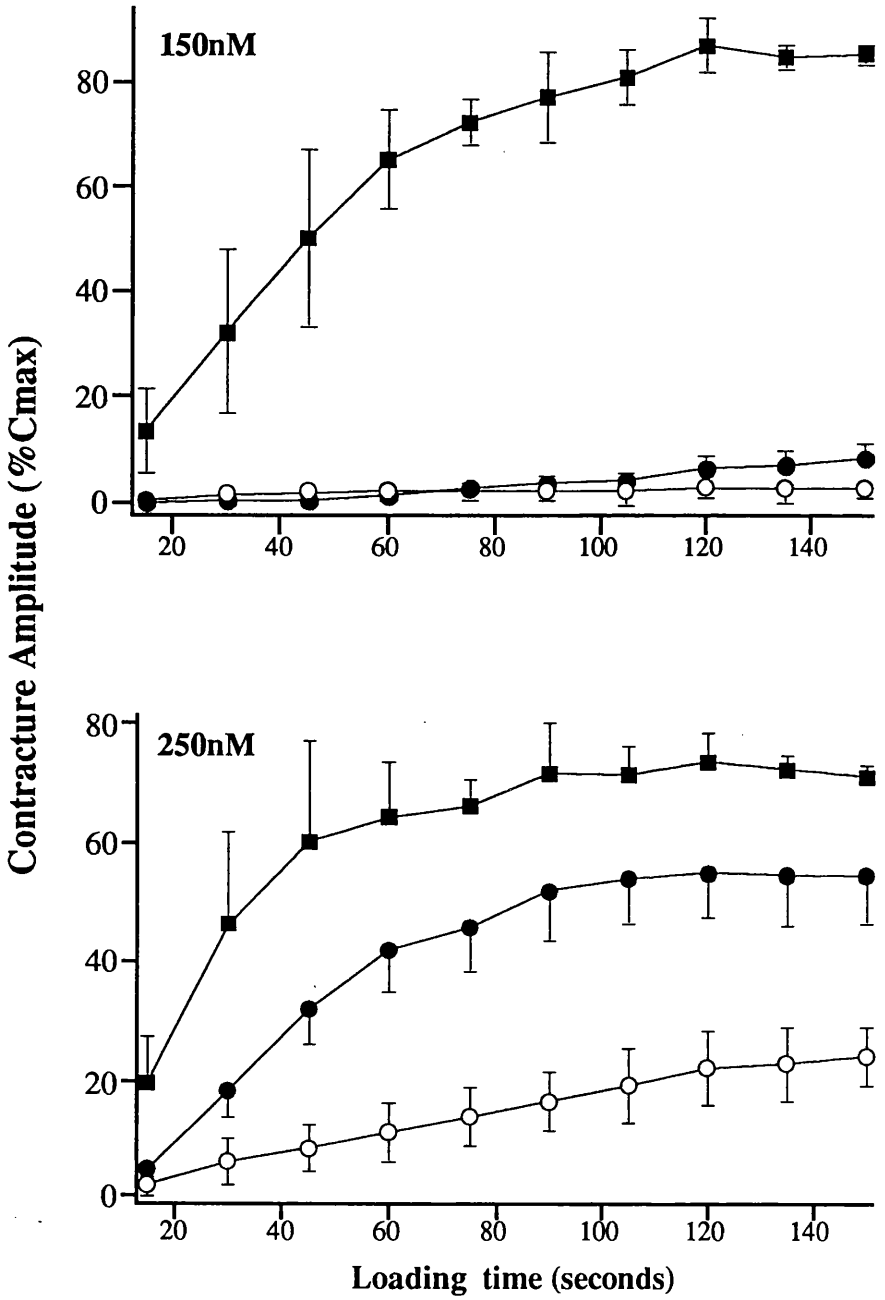
Isolated trabeculae experiments

Preparation size and shape

Trabeculae were typically elliptical in cross-section with a mean diameter of 150±56μm (mean±SD) and thickness of 85±12μm. There was no difference between the size of trabeculae obtained from ligated hearts (major diameter 148±52μm, minor diameter 78±14μm) compared with control hearts (158±46μm x 90±18μm). Preparation length when mounted at the desired sarcomere length was typically 2-3mm. The preparations were examined carefully, once mounted, to ensure they were not Purkinje fibres. Purkinje fibres are typically thinner in the rabbit than the trabeculae used here. They are also often surrounded by a sleeve of fibrous tissue which could be clearly seen on light microscopy. Purkinje fibres also tend to have a capillary plexus on the surface and such preparations were discarded. Additional evidence to support the fact that these preparations were trabeculae rather than Purkinje fibres is that the absolute force produced per unit cross sectional area was similar to that of other studies and was not significantly different between groups.

Time course of caffeine contractures

The time-course of individual caffeine-induced contractures (CIC) was not significantly different between LIG and SH animals ; mean time to peak-twitch for 5 contractures at 25-30% C_{max} was similar in a representative sample of LIG (n=6) and SH (n=6, 1.26±0.23s *vs* 1.43±0.38s, respectively, P=NS). The form of the SR Ca^{2+}-loading curves (illustrated in figure 3.1) was found to be similar using either force•time integral or peak force (amplitude) of the CIC which, again, reflects the similar nature of the time-course of CICs between LIG and SH animals.


150nM
250nM
Contracture Amplitude (%Cmax)
Loading time (seconds)
0
20
40
60
80
100
120
140

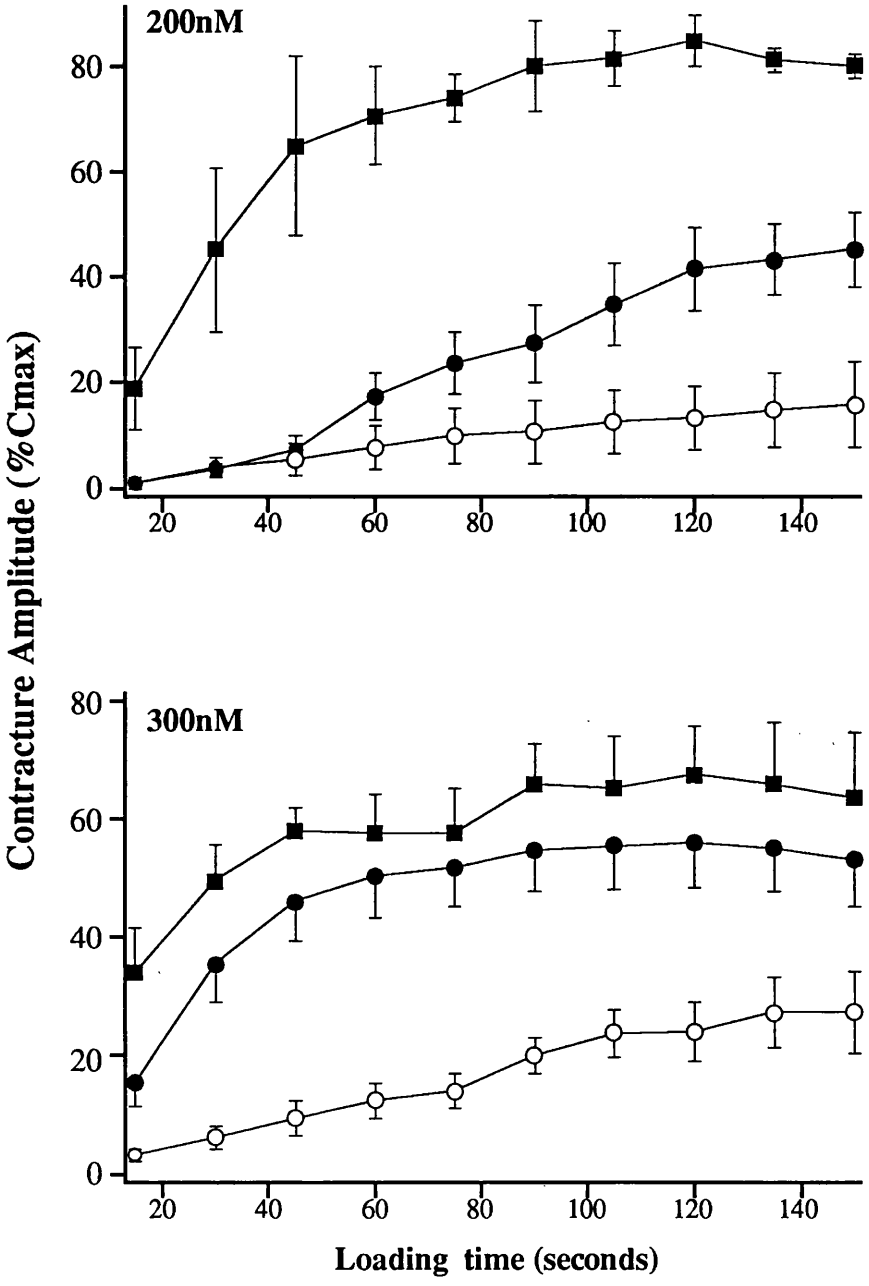
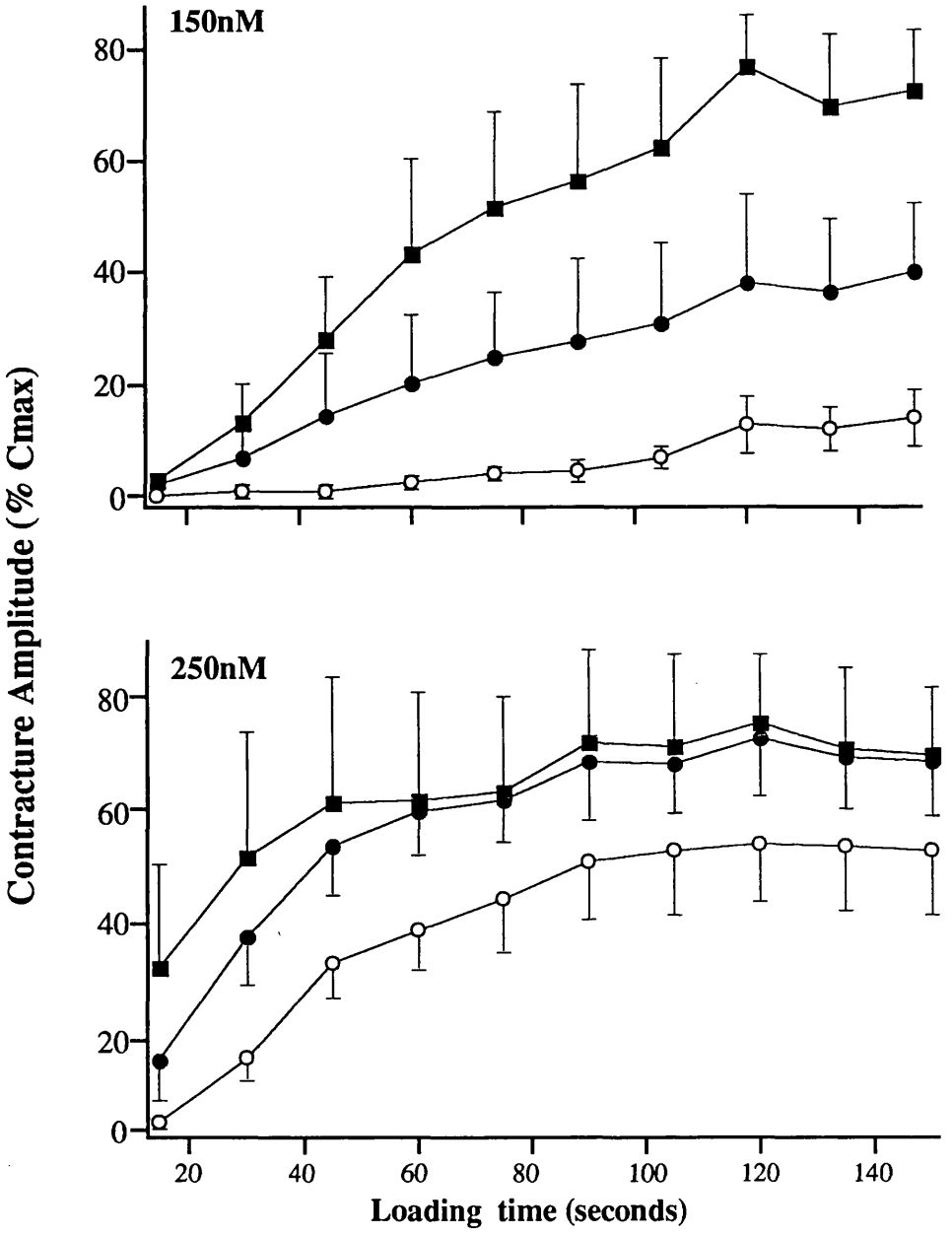


Figure 4.2 (this and facing page) SR Ca^{2+}-loading in LV trabeculae at 4 $[Ca^{2+}]$s in SH (n=5, *open circle*) and LIG animals with moderate heart failure (score≤4, n=7, *closed circle*) and more severe heart failure (score≥5, n=4, *closed squares*). Values are plotted as mean±sem.


150nM
250nM
Contracture Amplitude (% Cmax)
Loading time (seconds)
0
20
40
60
80
100
120
140

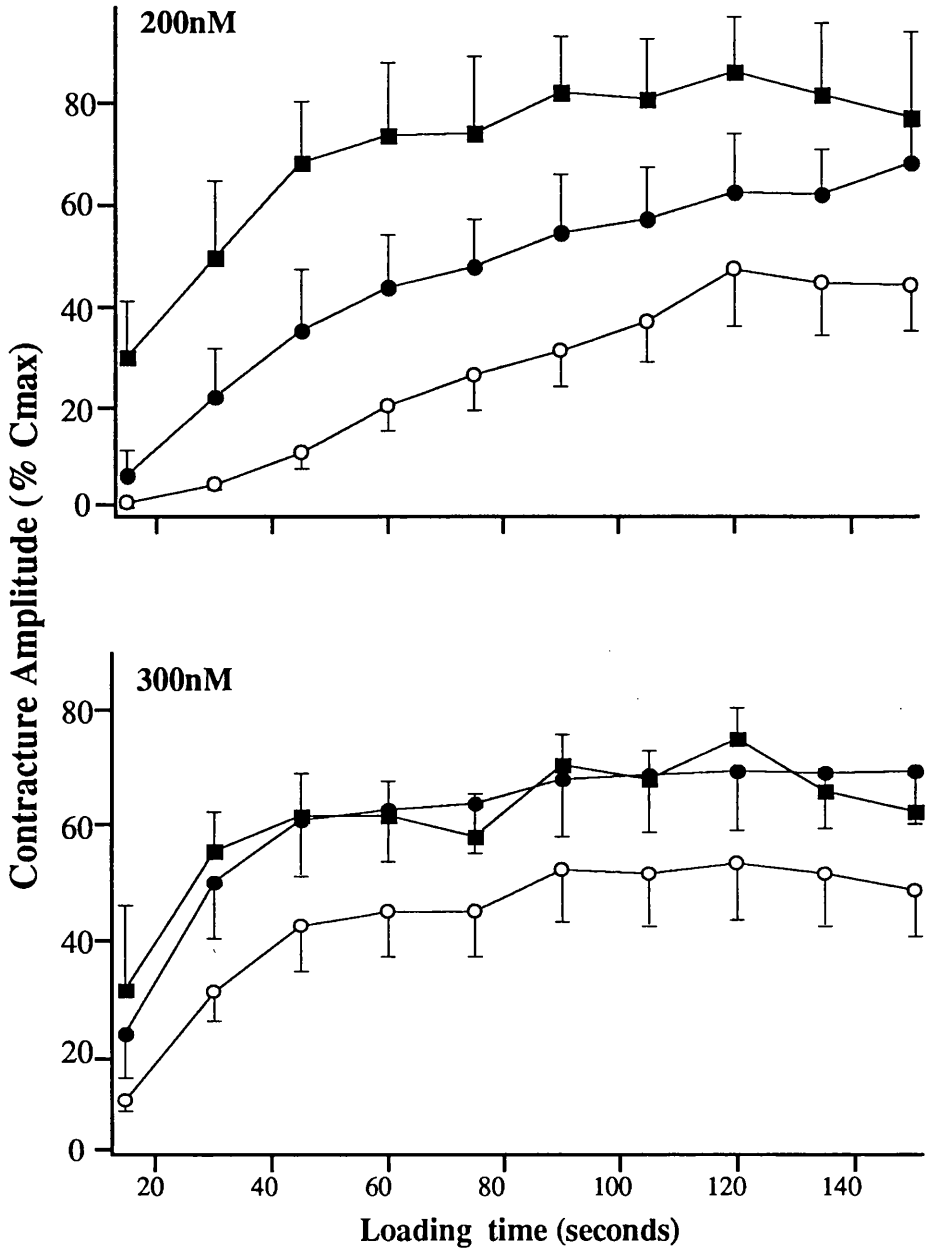


Figure 4.3 (this and facing page) SR Ca^{2+}-loading at 4 $[Ca^{2+}]$s in RV trabeculae from SH (n=8, *open circles*) and LIG animals with moderate (score≤4, n=6, *closed circles*) and more severe heart failure (score≥5, n=4, *closed squares*). Values are mean±sem.

SR function in LV trabeculae

The SR loading behaviour of LV trabeculae from 8 week sham and ligated animals is summarised in figures 4.2. At short loading periods (*e.g.* 30 seconds) LV trabeculae from animals with more severe heart failure (score≥5) produced significantly larger caffeine contractures than controls at all $[Ca^{2+}]$s studied while those animals with less heart failure (score≤4) produced contractures significantly different from controls only at higher $[Ca^{2+}]$s (250nM, 300nM). At longer loading periods (*e.g.* 120 seconds) the pattern of SR Ca^{2+} -loading in LV trabeculae was similar to that observed at short loading times except that at higher $[Ca^{2+}]$s (250nM, 300nM) the difference between the high and low scoring LIG animals became less marked but both continued to demonstrate significantly enhanced SR Ca^{2+} uptake compared with the control group. The maximum caffeine contracture obtained (relative to C_{max}) for any condition of loading time or $[Ca^{2+}]$ in LV trabeculae was significantly greater for LIG (87.9±5.0%) compared with controls (27.2±6.5%; $P<0.01$).

SR function in RV trabeculae

For RV trabeculae (figure 4.3) a similar pattern emerged but differences between high and low scoring heart failure groups were less obvious. Compared with controls, RV trabeculae from animals in both moderate (score≤4) and more severe (score≥5) heart failure groups demonstrated significantly larger caffeine contractures at low $[Ca^{2+}]$s (150nM, 200nM) and at short (30s) loading periods. At longer loading periods and at higher $[Ca^{2+}]$s (250nM, 300nM) the difference in loading patterns between the three groups became less marked. The maximum caffeine contracture obtained (relative to C_{max}) for any condition of loading time or $[Ca^{2+}]$ for RV trabeculae was significantly greater for LIG (86.0±10.5%) compared with SH (53.9±10.1; $P<0.05$).

SR function in RV *vs* LV trabeculae

For control preparations, RV trabeculae demonstrated a significantly greater peak caffeine contracture than the LV trabeculae (53.9±10.1% *vs* 27.2±6.5%; $P<0.04$). In the moderate heart failure group (score≤4), RV trabeculae were not significantly different from LV (72.7±10.3% *vs* 56.2±7.6%) and for the more severe heart failure group there was also no significant difference (RV 86.0±10.5 *vs* LV 87.9±5.0%). In control animals, RV trabeculae demonstrated increased SR loading compared with LV preparations at all loading $[Ca^{2+}]$s tested. This finding is illustrated in figure 4.4 where LV and RV preparations were obtained from the same heart for a control and a heart failure rabbit. This difference also occurred in the animals with moderate heart failure at low loading $[Ca^{2+}]$s (150nM, 200nM) but was less obvious at higher $[Ca^{2+}]$s. In those animals with a high heart failure score there was no significant difference in tension responses, at all the $[Ca^{2+}]$s tested, between RV and LV preparations.

Correlation of SR function with LV dysfunction

The enhanced Ca^{2+}-loading ability of the SR, in individual preparations, was found to correlate well with a number of indices of LV dysfunction measured by echocardiography and *in vivo* haemodynamic assessment (figure 4.5). The SR loading conditions used in this correlation are 150nM $[Ca^{2+}]$ and 120 seconds loading period. This loading condition was chosen because it represents the nominally accepted diastolic $[Ca^{2+}]$ in cardiac myocytes (Bers, 1988) and the SR is known to be in equilibrium after 2 minutes loading in saponin-skinned preparations (Orchard and Allen 1989). Using these loading conditions there was a clear spread of SR function between the three groups. The strongest correlation was found with LV ejection fraction ($r=0.79$; $P<0.01$) but there was also a relationship with LVEDD ($r=0.68$; $P<0.01$), LVEDP ($r=0.70$;

P<0.01) and resting cardiac output (r=0.54; P<0.05) suggesting that, with increasing severity of heart failure, the ability of the SR to load Ca^{2+} increases.

Spontaneous tension oscillations

In association with increased SR Ca^{2+}-loading capacity there was increased susceptibility to SR Ca^{2+}-overload revealed as spontaneous tension oscillations occurring during the loading period. A typical example of spontaneous oscillations in a ligated animal is illustrated in figure 4.6. The final caffeine contracture in the ligation animal trace (single arrow) is of lower amplitude than the preceding contracture despite a longer loading period (150s vs 60s) indicating that the SR Ca^{2+} pool available for the next activation has been depleted by spontaneous release. Oscillations occurred more commonly in LIG animals (14 of 17, 83%) than in SH (5 of 11, 45%) and tended to occur at lower [Ca^{2+}] and shorter loading times. This is summarised in figure 4.6, where the cumulative probability of oscillations is plotted against the [Ca^{2+}]•time product at which oscillations first appeared in the loading protocol for RV and LV trabeculae. In addition, RV trabeculae from the LIG group demonstrated spontaneous oscillations at lower [Ca^{2+}]•time products compared with LV trabeculae from LIG animals (26.1±2.6 *vs* 34.3±6.2 μMseconds; P<0.03) while there was no significant difference in the occurrence of spontaneous oscillations between LV and RV preparations from controls.

Myofilament force production and Ca^{2+} sensitivity

There was no significant difference in cross-sectional area (XSA) of trabeculae isolated from ligated and control animals (0.09 ± 0.002 *vs* 0.08 ± 0.003mm^2). There was also no difference in the XSA of preparations obtained from left and right ventricles (0.08±0.0025 *vs* 0.08±0.003mm^2). In the same preparations used for SR loading studies, after treatment with Triton-X100, maximum force per unit cross-sectional area was not significantly different between RV

and LV preparations in controls (RV: 5.40±2.56 *vs* LV: 5.76±3.27 g.wt./mm^2; P=0.33). In ligation animals RV trabeculae produced significantly less force per unit cross sectional area than LV trabeculae (RV 3.05±1.58 *vs* LV 5.98±3.70 g.wt/mm^2; P=0.023). This change was produced by a significant reduction in C_{max} in RV trabeculae (P=0.04) rather than a increase in LV trabecular force production.

The sensitivity of the myofilaments to Ca^{2+} was not different between the combined RV and LV trabeculae from control and LIG animals (figure 4.8). There was also no significant difference in myofilament Ca^{2+} sensitivity between RV and LV trabeculae in both groups (pCa_{50}: SH: RV 5.58±0.07 *vs* LV 5.55±0.08; LIG: RV 5.47±0.08 *vs* LV 5.48±0.09). There was no correlation between the severity of heart failure (ejection fraction) and the pCa_{50} (r=0.09).

Effects of caffeine on myofilament Ca^{2+}-responsiveness

The baseline values for Ca^{2+} sensitivity of the myofibrils were not different between the five LIG and five SH animals studied (pCa_{50} SH 5.504±0.086 vs LIG 5.455±0.141; P=0.19). The Hill values were also not significantly different at baseline (SH 2.71±0.61 vs LIG 2.18±0.89; P=0.33).

The increase in myofilament Ca^{2+}-sensitivity produced by 10mM caffeine was similar in the LIG and SH animals studied (figure 4.9). The mean Hill curves were shifted to the left equally in both groups (mean shift pCa LIG 0.16±0.07 vs SH 0.17±0.07; P=NS) with a slight but non-significant reduction in the mean Hill value in the LIG group (SH 2.39±0.57 vs LIG 1.82±0.57).

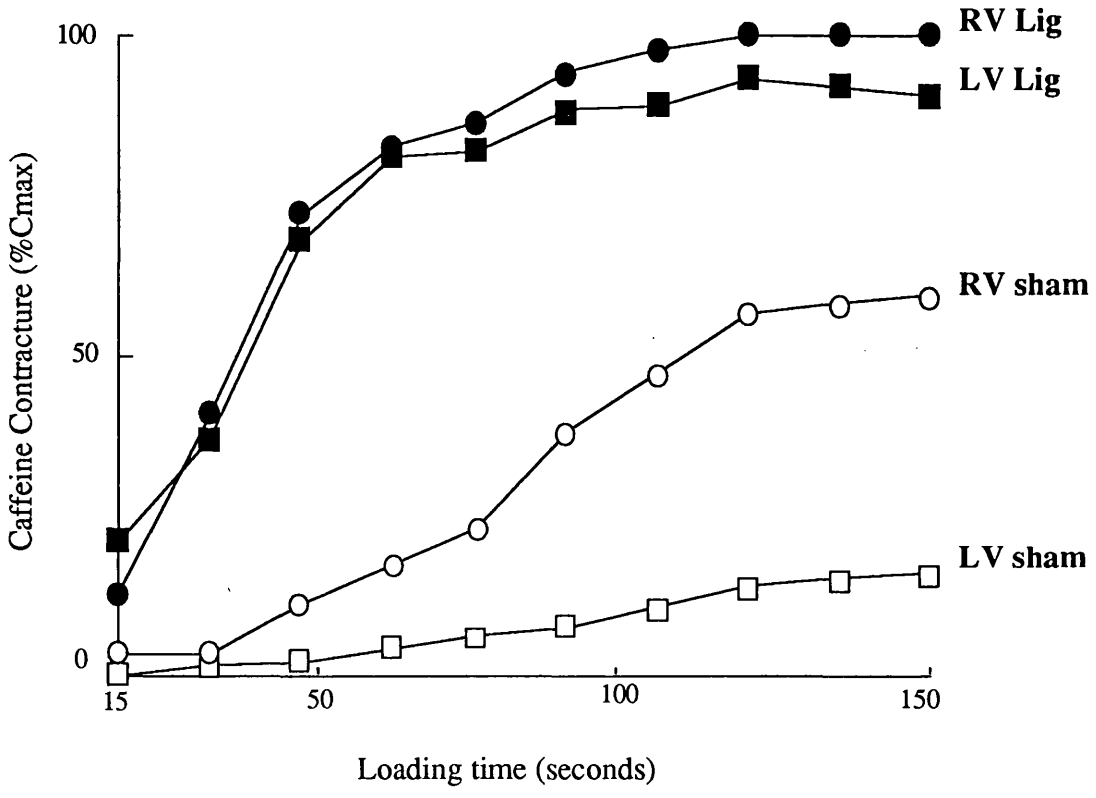


Figure 4.4 SR Ca^{2+}-loading in LV and RV trabeculae (at 200nM [Ca^{2+}]) from the same animals: comparison of SH and LIG. There is enhanced SR loading in the control RV compared with LV but this is not seen in the LIG preparation.

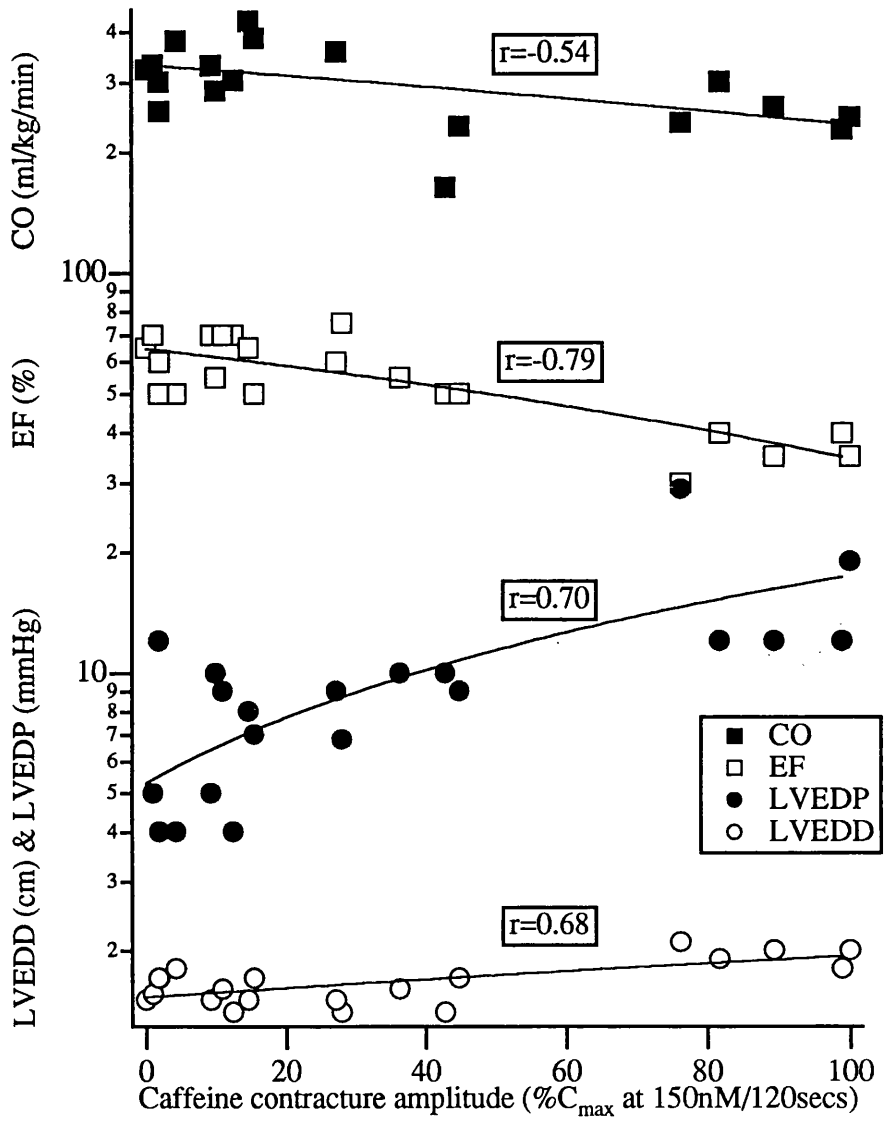


Figure 4.5 - Correlation of indices of heart failure with SR Ca^{2+}-loading. The graph plots four measures of left ventricular dysfunction against maximum caffeine contracture amplitude produced after loading the SR at 150nM $[Ca^{2+}]$ for 120seconds (expressed as a percentage of C_{max}). The curves are ***linear*** regressions with the correlation coefficient shown above each (note log ordinate). Each abscissa value represents a single animal and includes RV and LV trabeculae from LIG (n=11) and SH animals (n=9).

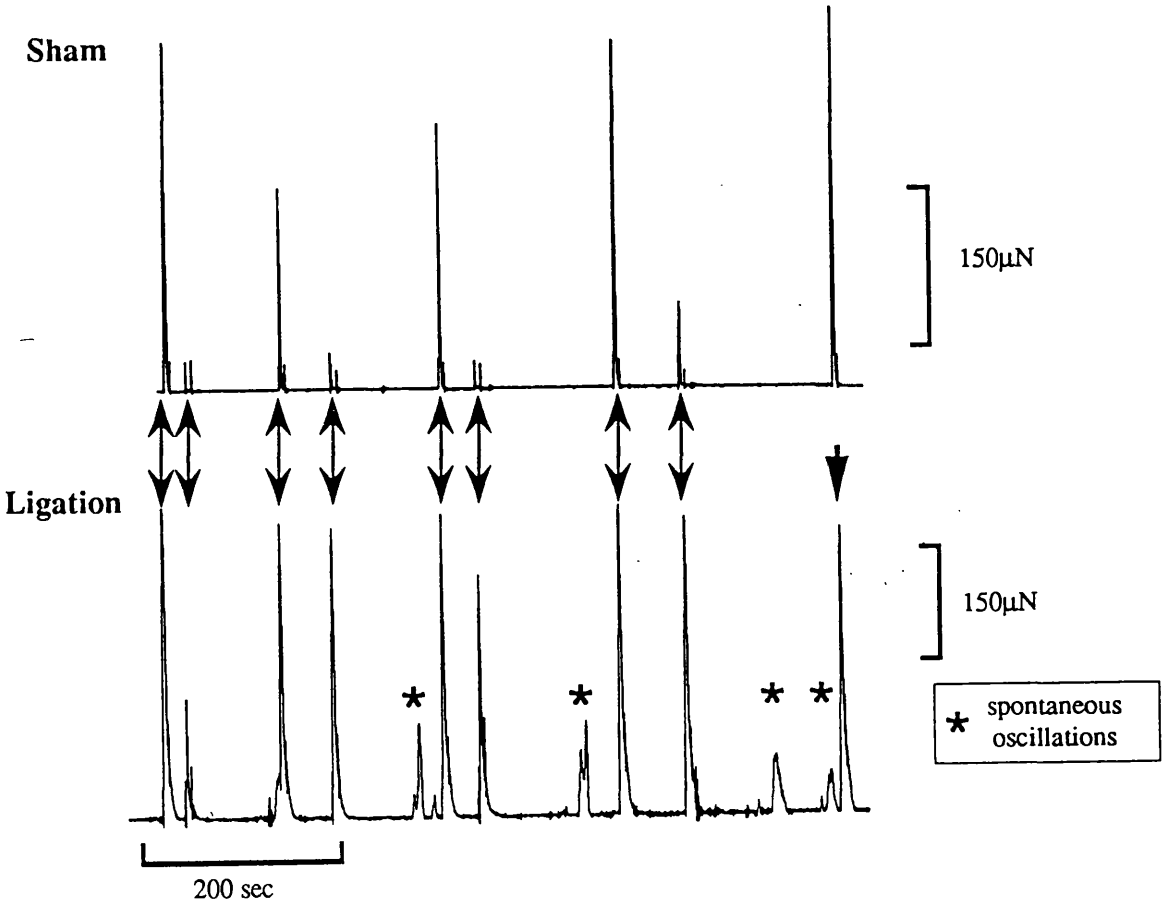


Figure 4.6 Spontaneous tension oscillations in a saponin-treated trabecula. Experimental traces showing part of an SR Ca^{2+}-loading protocol (at 150nM [Ca^{2+}]) where the applications of caffeine (10mM) are indicated by the double-headed arrows. The preparation from a ligation animal (lower trace) shows a number of spontaneous tension transients (*) indicating spontaneous Ca^{2+} release from the SR occurring during the loading period. Note how the final caffeine-contracture (single arrow) is reduced in amplitude, despite a longer loading period, as oscillations occur.

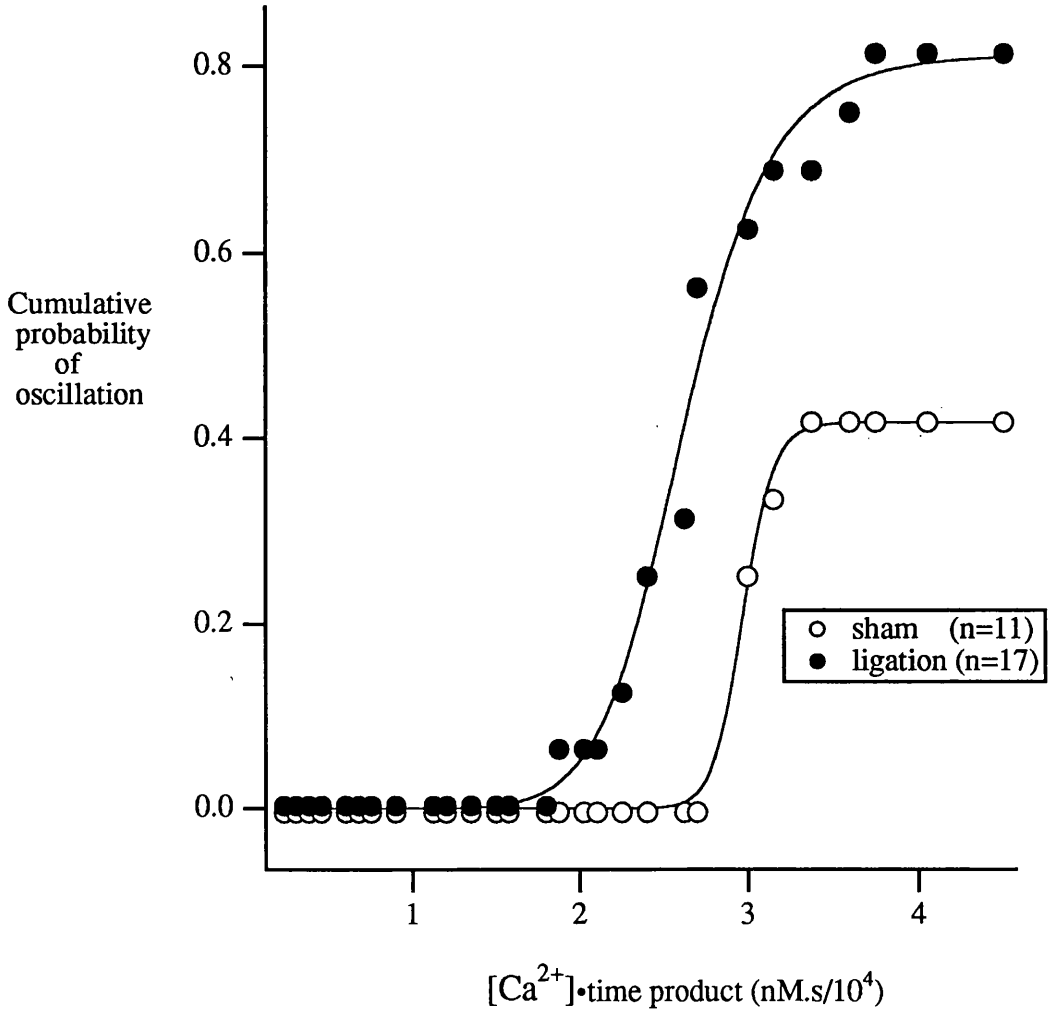


Figure 4.7 Cumulative probability of spontaneous oscillations. The percentage of trabeculae demonstrating spontaneous tension oscillations at a given $[Ca^{2+}]$•time product throughout the protocol. A greater proportion of trabeculae from LIG animals (n=17) demonstrated this spontaneous activity compared with controls (n=11) and were more likely to oscillate at lower $[Ca^{2+}]$•time products.

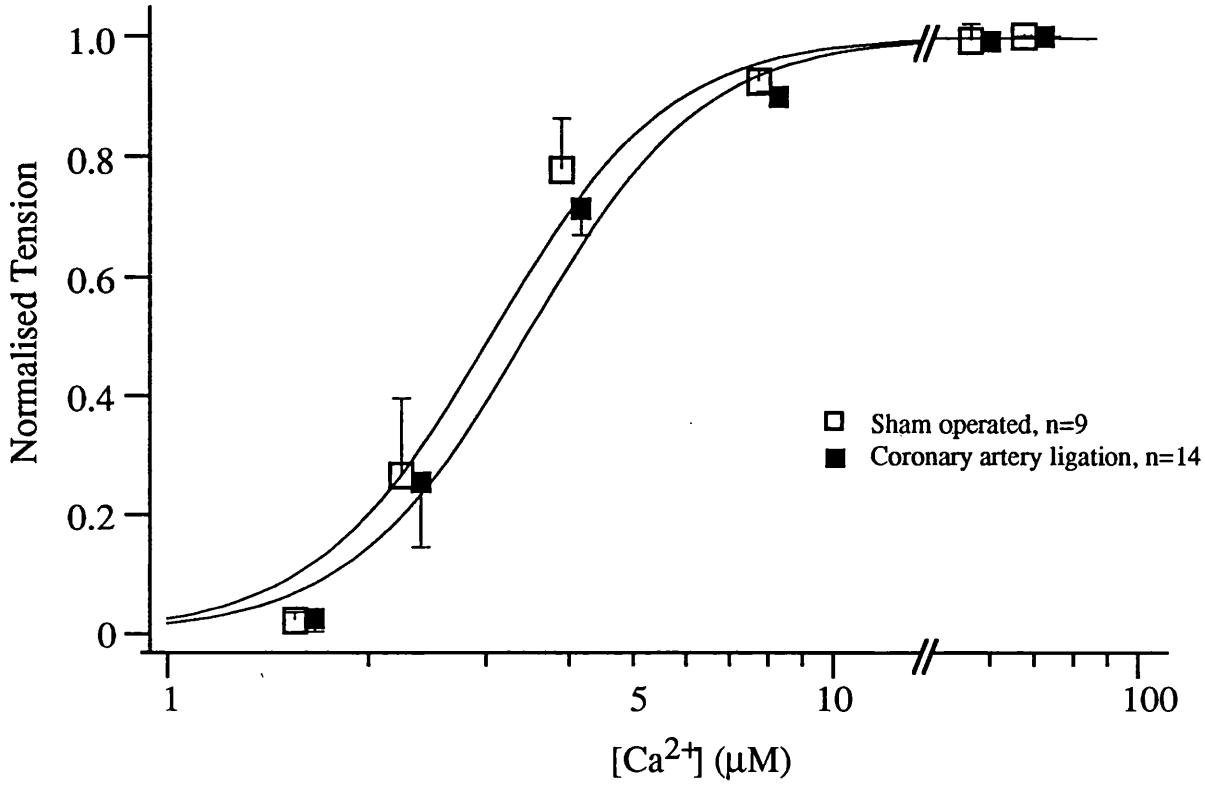


Figure 4.8 Combined Ca^{2+} sensitivity curves of 8 week SH (n=7) and LIG (n=14) animals. Values are mean±SD.

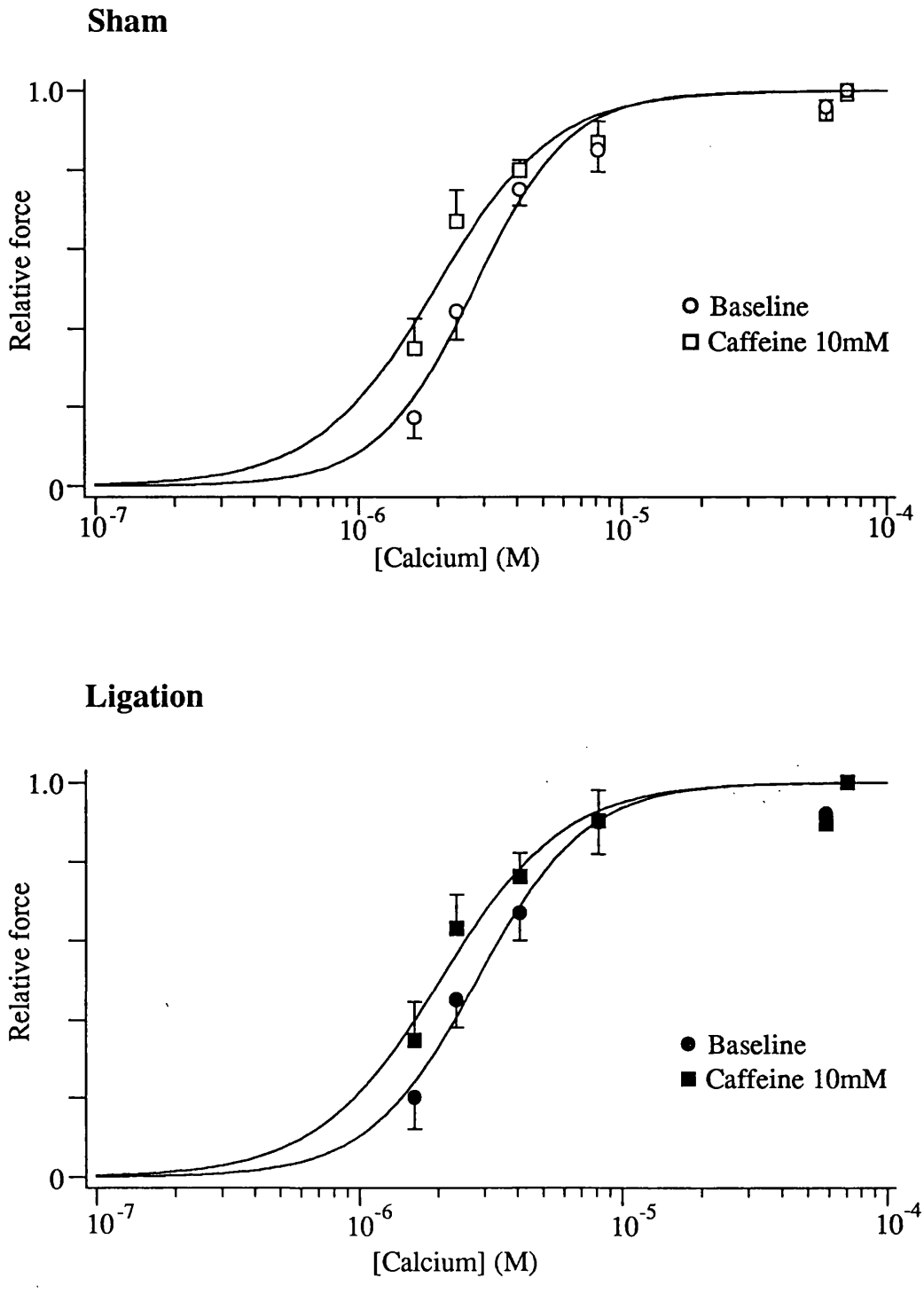


Figure 4.9 Effects of caffeine (10mM) on Ca^{2+} sensitivity of Triton-treated LIG (n=5) and SH (n=5) trabeculae.

DISCUSSION

This study has demonstrated an increased Ca^{2+} -loading capacity of sarcoplasmic reticulum in saponin-treated isolated cardiac trabeculae from rabbits 8 weeks following left coronary artery ligation. Haemodynamic performance, assessed in the conscious animal by echocardiography, LV pressures and thermodilution cardiac output measurement (table 3) demonstrated clear differences between the controls and the ligated animals indicative of significant left ventricular dysfunction in the ligation group. This assessment of heart failure in each animal allowed changes in the SR function to be correlated with the severity of left ventricular dysfunction. Heart failure was found to correlate strongly with the SR Ca^{2+}-loading capacity (figure 4). In those animals with a myocardial infarction but with little or no heart failure, SR Ca^{2+} uptake was similar to controls in the LV but was enhanced in RV trabeculae (figures 2 and 3). Clear differences between SR Ca^{2+}-loading of LV and RV trabeculae present in the controls at lower loading $[Ca^{2+}]$ were less obvious at higher $[Ca^{2+}]$. With more severe heart failure RV and LV trabeculae demonstrated a differential enhancement of SR Ca^{2+} uptake such that these differences between LV and RV were not observed.

SR function in heart failure

Animal studies

The indications of enhanced Ca^{2+} uptake in heart failure reported in this study contrast with a number of studies where SR Ca^{2+}-uptake was reported to be reduced or unchanged (Lamers & Stinis, 1979, Movsesian, Bristow & Krall, 1989, Limas, Olivari, Goldenberg, Levine, Benditt & Simon, 1987). A depression of SR Ca^{2+}-uptake has been reported in a number of studies using animal models of heart failure including the cardiomyopathic Syrian hamster (Nagano, Takeda & Dhalla, 1994) the spontaneously hypertensive rat

(Bing, Brooks, Conrad, Sens, Perreault & Morgan, 1991) and pacing-induced heart failure in dogs (Perreault, Shannon, Komamura, Vatner & Morgan, 1992). Idiopathic cardiomyopathy in dogs (Dobermans) appears to reduce the SR Ca^{2+}-uptake and also reduces the SR Ca^{2+}-release channel resulting in a possible net increase in SR Ca^{2+}-content (Crory, McCutcheon, O'Grady, Pang, Geiger & O'Brien, 1993). Enhanced SR Ca^{2+} uptake has been reported in mild cardiac hypertrophy produced by constriction of the descending aorta in dogs (Limas, Spier & Kahlon, 1980). Surprisingly, few studies of SR function have used the rabbit as a heart failure model. The rabbit SR is less extensive than that of the rat (Fabiato, 1982) and might, therefore, be more like the human (Bers, 1991). Ito *et al* (Ito, Suko & Chidsey, 1974) using a volume overload model in rabbits, demonstrated no difference in SR Ca^{2+}-uptake in the early stages of hypertrophy with a reduction as heart failure occurred.

Myocyte hypertrophy was not measured directly in our rabbit model but LIG hearts were significantly heavier and additionally the haemodynamic data suggest significant left ventricular dysfunction. Therefore it seems likely that we had produced significant hypertrophy. Chronic pressure overload hypertrophy of either the left or right ventricles by aortic or pulmonary banding has been extensively studied as a method of inducing heart failure. However, chronic pressure overload sufficient to provoke heart failure may represent a different patho-physiological process from that produced by infarction.

Undoubtedly, some of the observed differences between studies are related to species and model while the technique used to measure SR Ca^{2+}-uptake may also give rise to variable results. Assessment of Ca^{2+} uptake in isolated SR vesicles is expressed per mg protein which, if different in heart failure, could give rise to errors in such measurements. Additionally, Ca^{2+}-uptake is oxalate-supported in isolated SR experiments, allowing only a measurement of Ca^{2+}-uptake and not the total or net loading capacity. In the present study,

the caffeine-induced contracture, admittedly, represents an indirect method of assessing the SR Ca^{2+} content. However, in the saponin-skinned preparation, the SR is anatomically undisturbed and can load and leak Ca^{2+} in a similar way to that occurring in the intact cell.

SR function in human heart failure

The bulk of evidence suggests that in human heart failure SR Ca^{2+}-loading is reduced (Hassenfauss, Mulieri, Leavitt, Allen, Haeberle & Alpert, 1991, Gwathmey *et al*, 1987, Mercadier, Lompre, Duc *et al.*, 1990, Feldman *et al*, 1987). Studies using heat measurements to assess the ATP consumption by the SR have indicated a reduced activity of the SR Ca^{2+}-ATPase pump (Hassenfauss *et al*, 1990) While one study using isolated SR preparations demonstrated no significant change in SR Ca^{2+}-uptake (Limas *et al*, 1987), similar preparations in the hands of other workers have demonstrated reduced rates of Ca^{2+} uptake (Movsesian *et al*, 1989) Brillantes *et al* (Brillantes, Allen, Takahashi, Izumo & Marks, 1992) reported that mRNA for the SR Ca^{2+} release channel was significantly reduced in ischaemic cardiomyopathy but not in idiopathic dilated cardiomyopathy, while the SR Ca-ATPase mRNA was reduced in both. This result is similar to that found in dog heart failure where changes in the balance of Ca^{2+} release and uptake mechanisms could produce a net increase in SR Ca^{2+}-loading in the intact myocyte. Other factors which might give rise to variable results in such studies on human tissue from end-stage heart failure patients include the use of antiarrhythmic and immuno-suppressive drugs immediately prior to organ removal. These drugs are known to affect Ca^{2+} regulation in myocytes (Watson, Balke & Gold, 1993, Banijamali, ter Keurs, Paul & ter Keurs, 1993). Additionally, human control tissue used in these studies is often from donor hearts deemed unsuitable for transplantation because of excessive inotropic support and frequently is from patients with cranial and intra-cranial pathology which can produce profound effects on myocardial function prior

to organ removal. Furthermore, while most studies have used myocardial preparations from patients with end-stage heart failure SR function may be different in mild to moderate heart failure or earlier in the process of development of contractile dysfunction. This rabbit model of heart failure at 8 weeks following coronary ligation may represent such a compensated state which could also be present in human heart failure but is not observed in end-stage disease.

Left vs Right ventricle SR Ca^{2+}-loading

Differences of Ca^{2+} transport between isolated SR vesicles from RV and LV have previously been reported by Afzal *et al* (1992) suggesting that the RV had diminished rates of SR Ca^{2+} uptake compared to the LV. As heart failure developed SR Ca^{2+}-loading reduced in the LV but increased in the RV. These authors argue that this could reflect the different haemodynamic stress to which the ventricles are exposed. This is different from my finding, where the RV Ca^{2+}-loading was greater than LV in controls but both showed enhancement as failure ensued such that there was no significant difference between chambers in animals with more pronounced heart failure. The finding of an increase in the SR Ca^{2+}-loading in the RV preparations prior to changes in the LV in the moderate heart failure group (score$\leq$4) suggests that circulating factors such as catecholamines or angiotensin/renin products may be acting on the RV during moderate LV dysfunction since at this stage it is unlikely that significant right heart failure has occurred. Alternatively, the RV SR responds more rapidly or is more sensitive to subtle changes in LV performance, perhaps related to alterations in wall stress in the LV.

Spontaneous Ca^{2+}-release

The finding of increased probability of spontaneous release of Ca^{2+} from the SR in this model represents a possible arrhythmogenic mechanism in heart failure. Tension oscillations, as a result of spontaneous Ca^{2+}-release, occurred

during the protocol and were not present before the experiment commenced. Each preparation was treated in an identical manner for each experiment and was exposed to a standard set of solutions with stepwise increases in $[Ca^{2+}]$. Therefore, the probability of Ca^{2+} overload developing in these experiments must be related to some difference in the intrinsic properties of the SR in LIG animals. Possible mechanisms underlying this phenomenon might be related to a change in the balance of SR Ca^{2+} influx and efflux or an increase in the sensitivity of the Ca^{2+}-induced Ca^{2+}-release mechanism in LIG animals. This phenomenon of spontaneous oscillatory activity has previously been described in Langendorff perfused hearts from cardiomyopathic hamsters by demonstration of increased laser-light scattering in response to catecholamine challenge (Hano O, Lakatta E, 1991). Capogrossi *et al* (Capogrossi, Houser, Bahinski & Lakatta, 1987) have shown that large, synchronous spontaneous releases of Ca^{2+} from the SR can produce localised depolarisations sufficient to produce an action potential. This has therefore been proposed as an arrhythmogenic mechanism in heart failure since failing hearts appear more likely to exhibit spontaneous oscillations both under standard conditions, as in this study, or with catecholamine stress as in the study by Lakatta *et al*, 1991. A spontaneous action potential would therefore represent triggered spontaneous activity which, provided with re-entry substrate, could give rise to a sustained arrhythmia.

Spontaneous release of Ca^{2+} from the SR may also contribute to contractile dysfunction in heart failure. Spontaneous release during a diastolic period depletes the SR store and would therefore reduce the amount of Ca^{2+} available for a subsequent activation. This point is illustrated in figure 6 where the final contracture (single arrow) in the LIG preparation trace shows a fall in amplitude despite a longer loading period than the preceding contracture because the SR has spontaneously released some of its store

immediately prior to the 'caffeine activation'. This phenomenon could not only reduce systolic contractile force but would also tend to increase diastolic tension producing slower relaxation and a rise in resting tension *i.e.* a so called negative lusitropic effect.

Possible mechanisms for enhanced SR Ca^{2+}-loading capacity

SR Ca-ATPase rate was not measured in this study but a reduced SR Ca^{2+}-ATPase rate in the rabbit ligation model could still be consistent with an enhancement of *net* Ca^{2+}-loading. This apparent paradox can be explained by considering that the net uptake of Ca^{2+} by the SR during any given loading period is a function of the relative magnitudes of Ca^{2+}-influx and Ca^{2+}-efflux. Reduction in the leak of Ca^{2+}, via the so-called SR Ca^{2+} leak channel, during the loading period could significantly increase the total or net Ca^{2+} accumulated by the SR. In dogs with heart failure, one study indicated a 25% reduction in SR Ca^{2+}-ATPase activity but a 75% reduction in the Ca^{2+} release channel (Crory *et al*, 1993). Such a change could produce a net increase in the Ca^{2+}-loading ability of the SR. A reduction in the number or a change in function of SR Ca^{2+} release channels are mechanisms which could explain enhanced SR Ca^{2+}-loading in this model.

Enhanced SR Ca^{2+}-loading could also result from an increased Ca^{2+} buffering capacity of the SR in heart failure, due, either to an increase in intra-SR calsequestrin or an increase in the total volume of SR. Time did not permit a quantitative electron microscopic study of the SR in these preparations which would be required to answer the latter question. Previous studies of the calsequestrin gene have not suggested enhanced activity in heart failure (Masahi, Alpert, McLennan, Barton & Periasamy, 1993). Furthermore, an increase in buffering capacity, alone, would reduce and not increase the probability of Ca^{2+}-overload as demonstrated in this study by the prevalence of spontaneous oscillations. Enhanced Ca^{2+}-loading at shorter loading periods (i.e. <60s, figure 4.2) does suggest that the SR is, in fact, loading faster

in LIG preparations. However, a faster rate of SR Ca^{2+} pumping could not fully explain the current results since, under these conditions, once the SR was filled to capacity, the Ca^{2+}-pump would be inhibited by the SR intraluminal [Ca^{2+}] and Ca^{2+}-uptake would cease. Therefore, both an increase in the total buffering capacity and an increase in SR Ca^{2+}-uptake rates must play a role in the enhanced SR-loading capacity observed in these preparations.

Myofilament force production

The time-course of the caffeine-induced contractures was not significantly different between LIG and controls. This suggests that the rate of SR-Ca^{2+} release and the subsequent activation of the myofilaments was not significantly different in LIG preparations since the rate of diffusion of caffeine in these preparations is known to be rapid (Wendt & Stephenson, 1983) and can be assumed to be similar in both groups. However, since caffeine sensitises the myofilaments to Ca^{2+} and diffuses into the preparation rapidly then even during a twitch there will be an increase in myofilament Ca^{2+} sensitivity. Subsequently if a greater increase in myofilament Ca^{2+} sensitivity occurred in LIG animals compared with controls this could produce an apparent enhancement of caffeine induced contractures in the ligated group. Differential enhancement of myofilament Ca^{2+} sensitivity has been described in a rat model of hypertrophy by Baudet *et al* (1990). However, I have clearly shown that the change in responsiveness to Ca^{2+} produced by caffeine was not different in SH and LIG animals (figure 4.9).

Maximum Ca^{2+} activated force per unit XSA was not different between LV trabeculae from ligated and control animals but was significantly lower in the LIG RV trabeculae. This might partly explain the apparent enhanced Ca^{2+} uptake in the RV at moderate levels of heart failure since the same SR function with a reduced C_{max} would result in an apparent increase in Ca^{2+} uptake because the data are normalised to C_{max}. The absolute force produced

was similar to that described in a number of studies (Maughan, Low, Litten, Brayden & Alpert, 1970, Perreault *et al*, 1990). This result is important since it suggests that there was no untoward fall-off in myofilament force production throughout the experiment (C_{max} was first assessed at the end of the SR testing protocol). In addition the preparation was assessed by control contractures at standard intervals through the protocol (see figure 2.9) and no significant fall-off in force production was observed (approximately 25% fall-off over 2 hours). Preparation size was carefully measured in this study using light microscopy to measure the width and depth of the trabecula while mounted at the sarcomere length at which tension measurements were made. This method may also have improved the accuracy of the calculation of force per unit area. The finding of a reduction in maximum isometric force development in the RV in LIG animals is in contrast to Perreault *et al* (1990) where an increase in force development was reported in RV trabeculae from rats with heart failure. Reduced force production, found in this study, could be related to an increase in myocardial interstitial collagen which occurs in response to heart failure but these findings would require more significant fibrosis in the RV compared with LV since maximum force production was unchanged in LV trabeculae from failing hearts.

Ca^{2+} sensitivity of the myofilaments was not significantly different between RV and LV trabeculae or between sham and LIG animals even for those assessed to have the most severe heart failure. For the purpose of this study this was an important finding for a number of reasons. Firstly, tension was used as an index of Ca^{2+} release and any underlying difference in myofilament response to Ca^{2+} could have been interpreted as a change in SR Ca^{2+}-loading. Secondly, tension oscillations were taken to indicate spontaneous release of Ca^{2+} from the SR sufficient to cause activation of the myofilaments. This approach cannot detect sub-threshold releases of Ca^{2+} from the SR but since Ca^{2+}-sensitivity of the myofilaments was the same in

both ligation and control animals then the [Ca^{2+}] at which activation of the myofilaments occurred would be comparable.

Contractile dysfunction and enhanced SR function

Enhanced SR Ca^{2+}-loading in heart failure contrasts with a number of animal and human studies and gives rise to the question as to what mechanism could produce contractile dysfunction in the intact LIG heart in the presence of this enhanced SR function. Metabolic factors, such as inorganic phosphate (P_i) and pH, could give rise to contractile abnormalities in the intact cell, SR Ca^{2+} overload (as demonstrated in this study) or changes in beta-receptor responses could each contribute to contractile failure in the presence of enhanced SR function. The apparent enhanced SR Ca^{2+}-loading in saponin-treated preparations could therefore represent a physiological adaptation by which the SR compensates for reduced levels of intracellular phosphorylation (resulting from chronic beta-receptor down regulation) by increasing Ca^{2+}-loading capacity. Under our experimental conditions (saturating ATP, creatine phosphate and controlled pH) the 'compensated' SR pump could then load Ca^{2+} to a greater extent than control tissue. In the intact myocyte, however, in the presence of low levels of cAMP[4], raised P_i and lower pH, Ca^{2+} transients may be of similar amplitude (Vahl, Bonz, Timek & Hagl, 1994, Bing *et al*, 1991) to those in control tissue.

Conclusion

In summary, therefore, isolated RV and LV trabeculae from rabbits with LV dysfunction 8 weeks after coronary ligation demonstrated an increase in sarcoplasmic reticulum Ca^{2+}-loading capacity. At this stage in the study there were a number of questions which had arisen concerning the possible reasons for this rather paradoxical and previously unreported finding:

1. Is the SR Ca^{2+} release channel different in the LIG animals ?

2. Is there a difference in the phosphorylation state of phospholamban on the SR ?

3.Was this an earlier stage of heart failure than had previously been studied where the SR was in a compensated state ? Does SR function change later in the development of heart failure ?

The following chapters describe a series of experiments which set out to examine these questions.

CHAPTER 5

Sarcoplasmic reticulum Ca^{2+}-loading in rabbits 15 weeks after coronary artery ligation

INTRODUCTION

The reasons for allowing a separate group of animals to proceed to 15 weeks after coronary ligation were mainly that the results from the 8 week animals were in direct contrast to other studies of SR function in heart failure. It was possible that I had chosen a window during the development of heart failure where there was compensation of SR function and that later, either as heart failure became more severe or with increasing duration of heart failure, then SR function would change further. One previous study in dogs with pressure-induced left ventricular hypertrophy had indicated enhanced SR function during mild cardiac hypertrophy (Limas *et al,* 1980). Therefore, although I had demonstrated significant LV dysfunction at 8 weeks after ligation there could be additional factors operating later in the course of heart failure which could change SR function. Few studies have addressed the question of linear changes in SR function in heart failure.

METHODS

Animal model and assessment of heart failure

Coronary ligation was performed as in chapter 2 and the animals allowed to develop heart failure over the following 15 weeks (LIG n=12, SH n=7). Echocardiography was performed 2 days before invasive haemodynamic assessment. A left ventricular catheter and a femoral thermodilution catheter were inserted under general anaesthesia as with 8 week animals. Measurements of haemodynamic function were made in the conscious animal and were repeated after a fluid challenge.

Isolated trabeculae experiments

In all of the 15 week animals only RV trabeculae were isolated for assessment of SR function. Unfortunately, no data were obtained from LV trabeculae due to difficulties in obtaining suitable preparations and also due to the length and technical nature of experiments at this stage in the study

which only allowed one experiment per day to be performed. The SR loading protocol was shortened to include only 5 loading times ranging from 30-150 seconds (instead of 10 ranging from 15-150 as in the 8 week animals). However, the protocol was once again followed stringently for LIG and Sh animals with Triton treatment at the end of the SR protocol and assessment of C_{max} and myofilament Ca^{2+} sensitivity at the end of the experiment.

RESULTS

Assessment of heart failure

Clinical features

4 of 12 animals had clinical signs of heart failure in this group including scrotal oedema and tachypnoea compared with no animals in the SH group. These features had, however, appeared within 8 weeks of ligation and did not progress significantly during the following 7-8 weeks. There were no sudden deaths in 15 week SH or LIG animals. The mean total body weight of LIG animals was not significantly different from SH animals (3.80±0.24 vs 3.64±0.51kg). Mean lung wet weight was significantly greater in the LIG group (14.4±2.3 vs 12.8±1.3g; P=0.048) mean liver wet weight was not significantly different between groups (99.3±15.8 vs 94.8±14.7g). After excision of the hearts the size and extent of infarction was examined macroscopically. The overall size of infarction was, on average, similar to that in the 8-week animals, often involving a large papillary muscle. However, it was noted that the organisation of the infarct appeared more complete with a more clearly defined margin between infarcted and healthy tissue compared with the 8 week infarcts. In addition, the infarct site appeared thinner walled and almost aneurysmal in a few cases.

Echocardiography

Left ventricular ejection fraction was significantly lower in LIG animals (51.3±7.6 vs 67.2±4.1%; P=0.001). Left ventricular end-diastolic diameter was significantly greater in LIG animals (2.0±0.26 vs 1.63±0.11cm; P=0.002).

Cardiac output and left ventricular pressures

Mean left ventricular systolic pressure at baseline was not significantly different between LIG and SH animals (141.3±6.6 vs 145.1±7.4mmHg) and was not significantly different after fluid challenge (140.7±3.4 vs 148.6±8.8mmHg). Mean left ventricular end-diastolic pressure was significantly greater in LIG animals at baseline (15.5±4.9 vs 9.3±2.6mmHg; P=0.001) and also increased to a greater extent after the standard fluid challenge (26.4±6.21 vs 18.0±4.4mmHg; P=0.002).

Mean baseline cardiac output was significantly lower in LIG compared with controls (260±45 vs 320±23ml/kg/min;P=0.001). The peak cardiac output in response to a fluid challenge was also significantly lower in the LIG animals compared with SH (343±69 vs 465±22ml/kg/min;P=0.01). This represents a significantly reduced increase in cardiac output in response to the standard fluid challenge of 15ml/kg (30.7±13.3 vs 45.8±8.8% increase; P=0.03).

Heart failure scoring

Using the same scoring system employed to assess the 8 week rabbits there were only 3 rabbits with scores≥5 at 15 weeks. The remaining 9 animals had scores≤4 and therefore fell into the category of moderate heart failure using the criteria laid down in chapter 4. The general impression was that although these rabbits had significant LV dysfunction compared with the sham animals they had less heart failure than the group produced for the 8 week study.

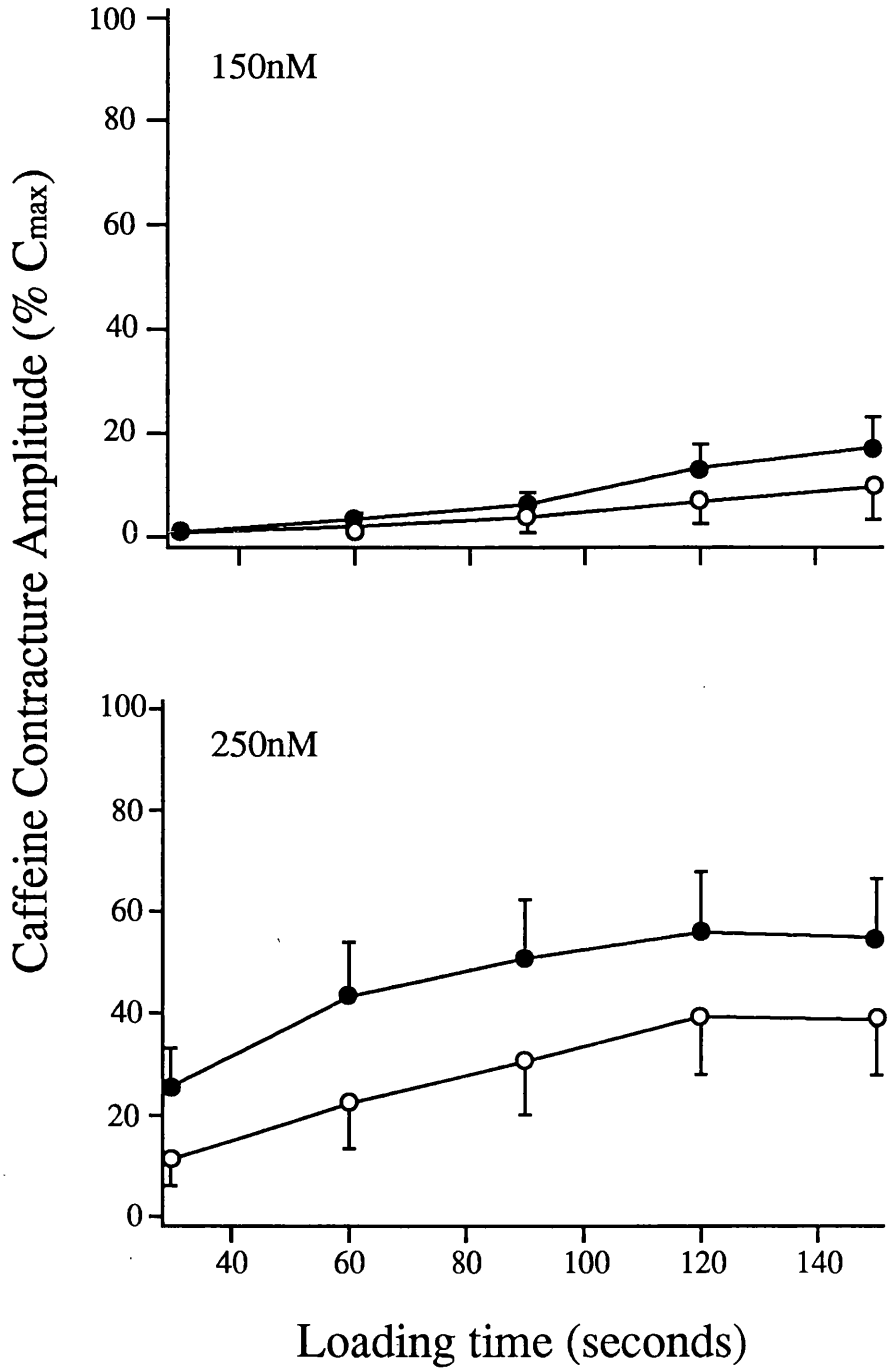
Isolated trabeculae experiments

SR function

The mean data for SR loading curves in 15 week animals are summarised in figure 5.1. These curves represent all data and are not separated into groups with differing severity of heart failure. There was a tendency for the LIG animals to produce larger caffeine contractures than the SH group but this was not significantly different at any of the loading conditions of time or $[Ca^{2+}]$ studied. At low loading $[Ca^{2+}]$s (150nM,200nM) the loading curves for both groups were very similar but at higher loading $[Ca^{2+}]$s the LIG animals produced slightly larger caffeine contractures. Attempting to separate the LIG animals into moderate and more severe heart failure groups was not possible since of the 3 with score≥5, 2 produced peak caffeine contractures which were 100% of C_{max} while the other produced only 10% of C_{max} . Therefore, two behaved very similarly to the animals at 8 weeks while one produced contractures which were even smaller than the controls. For the remaining 9 animals with scores≤4 the mean SR loading curves were virtually identical to SH SR loading curves with no significant differences at any of the $[Ca^{2+}]$ or times studied. The mean peak caffeine contracture obtained for any condition of $[Ca^{2+}]$ or time was slightly larger for the LIG group as a whole compared with SH animals (59.5±12.6 vs 45.4±12.8% C_{max}) but this was not significantly different. For those animals in the LIG group with a heart failure score≤4 the mean peak caffeine contracture was not significantly different from SH animals (46.1±11.8 vs 45.4±12.9% C_{max}).

Correlations of SR function with heart failure

There were no significant correlations of SR function (at a number of different loading conditions) with various indices of LV dysfunction including: EF; r=0.19, LVEDD; r=0.24, resting CO; r=0.11.


150nM
250nM
Caffeine Contracture Amplitude (% Cmax)
Loading time (seconds)
100
80
60
40
20
0
40
60
80
100
120
140

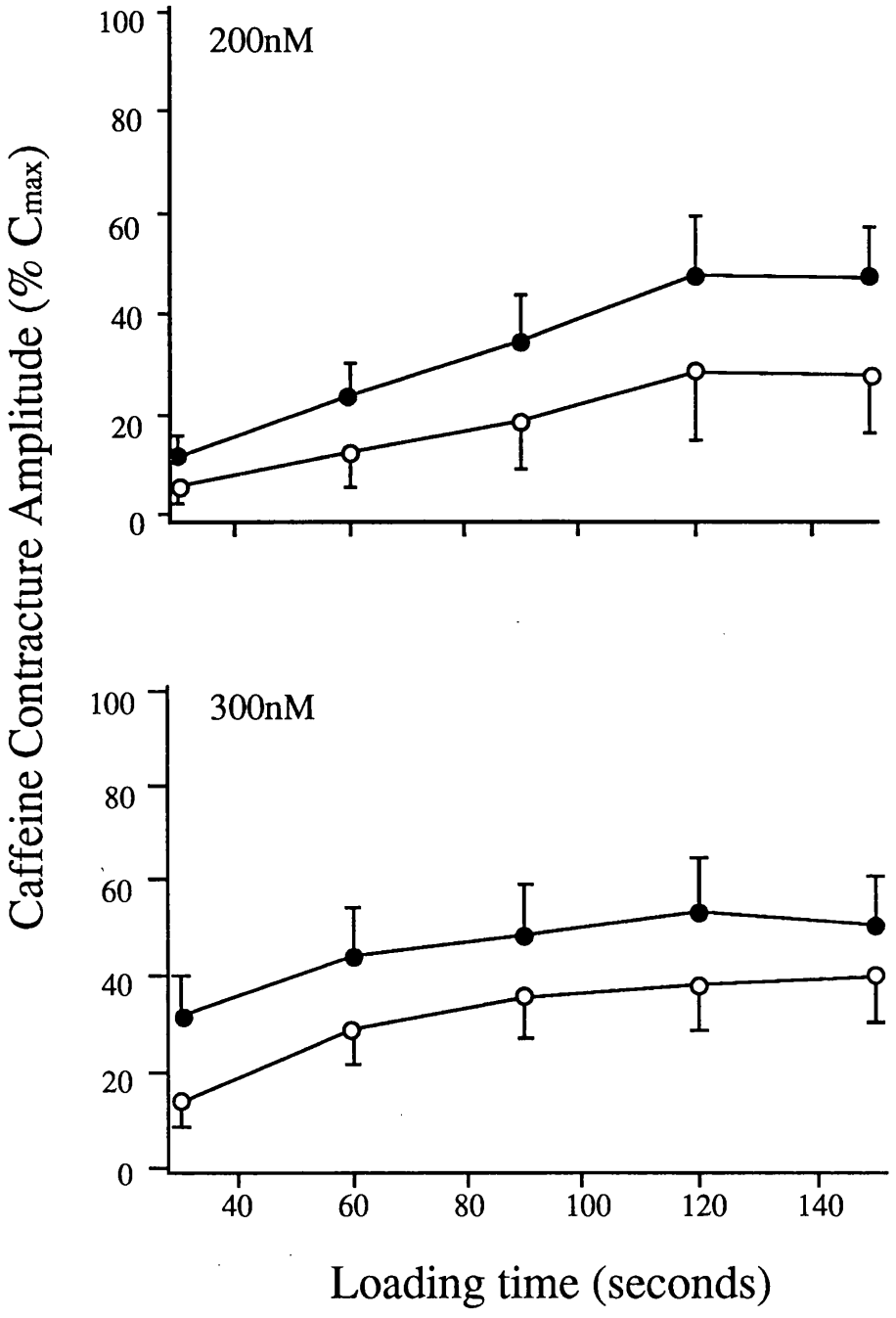


Figure 5.1 (this and facing page) SR Ca^{2+}-loading in RV trabeculae from 15 week ligation rabbits. Values are mean±sem. 15SH (n=7) animals are indicated by the open circle and 15LIG (n=12) by the closed circle.

Spontaneous Oscillations

There was no significant difference in the incidence of spontaneous tension oscillations between ligated and control rabbits. 3 of 7 SH and 4 of 12 LIG animals demonstrated oscillations during the standard loading protocol. Oscillations tended to occur at longer loading times and higher $[Ca^{2+}]$ in both groups compared with the oscillations seen in the 8 week study with [Ca]•time products that were not significantly different between groups (SH 37.6±3.1 *vs* LIG 38.7±4.7 μMseconds).

C_{max} and myofilament Ca^{2+} sensitivity

All trabeculae were obtained from the right ventricle. The mean cross-sectional area of trabeculae was not significantly different between groups (0.011±0.007 vs 0.0085±0.002mm^2). The maximum force produced per unit cross sectional area was slightly greater in the SH animals (LIG 5.6±4.3 vs SH 8.54±3.86g.wt/mm^2; P=0.31) but this did not reach statistical significance. There was also no difference in the Ca^{2+} force relation between groups (pCa_{50} LIG 5.56±0.09 vs SH 5.76±0.10).

DISCUSSION

Comparison of severity of LV dysfunction at 15 weeks and 8 weeks

A comparison of haemodynamic data obtained from the 15 week (15-LIG) and 8 week (8-LIG) coronary ligation groups are summarised in table 4. The overall impression is of significant LV dysfunction present in the 15-LIG group compared with the 15 week shams but the severity of heart failure was no worse than in the 8-LIG group. The mean left ventricular end-diastolic diameter was slightly greater in the 15-LIG animals but the ejection fractions were also slightly higher. The baseline cardiac output was slightly lower but there was a very similar response to fluid challenge.

There are a number of possible ways of interpreting these findings:

i) A smaller size of myocardial infarction in the 15-LIGs and we were observing the progression of LV dysfunction after a longer time period.

ii) there was a similar degree of initial myocardial damage in the 8 and 15-LIG groups but there was no further progression of LV dysfunction after 8 weeks.

iii) A further possibility is selection bias *i.e.* sick rabbits with more severe heart failure were removed from the protocol at 8 weeks or died before 15 weeks. Since no animals died between 8 and 15 weeks and since all 15 week animals completed the protocol, then, this latter suggestion is unlikely.

The absence of more severe LV dysfunction at 15 weeks appeared disappointing since it was hoped that a longer period after ligation would allow study of a more advanced degree of cardiac failure. However, since a similar degree of LV dysfunction was seen after 8 and 15 weeks then differences in SR function could be interpreted as occurring secondary to the 'duration of heart failure' and not necessarily due to more severe heart failure.

Table 4- comparison of LV dysfunction in 15 and 8 week ligations

	8-LIG	15-LIG	
Ejection fraction (%)	41.9±9.5	51.3±7.6	P=0.1
LVEDD (cm)	1.86±0.19	2.0±0.26	P=0.09
LVEDP (mmHg)	15.1±6.9	15.5±4.9	P=0.71
baseline CO (ml/kg/min)	275.2±63.1	260.2±45.3	P=0.53
Peak CO (ml/kg/min)	332.75±37.9	343.8±69.1	P=0.68
Body weight (kg)	3.75±0.46	3.80±0.24	P=0.9

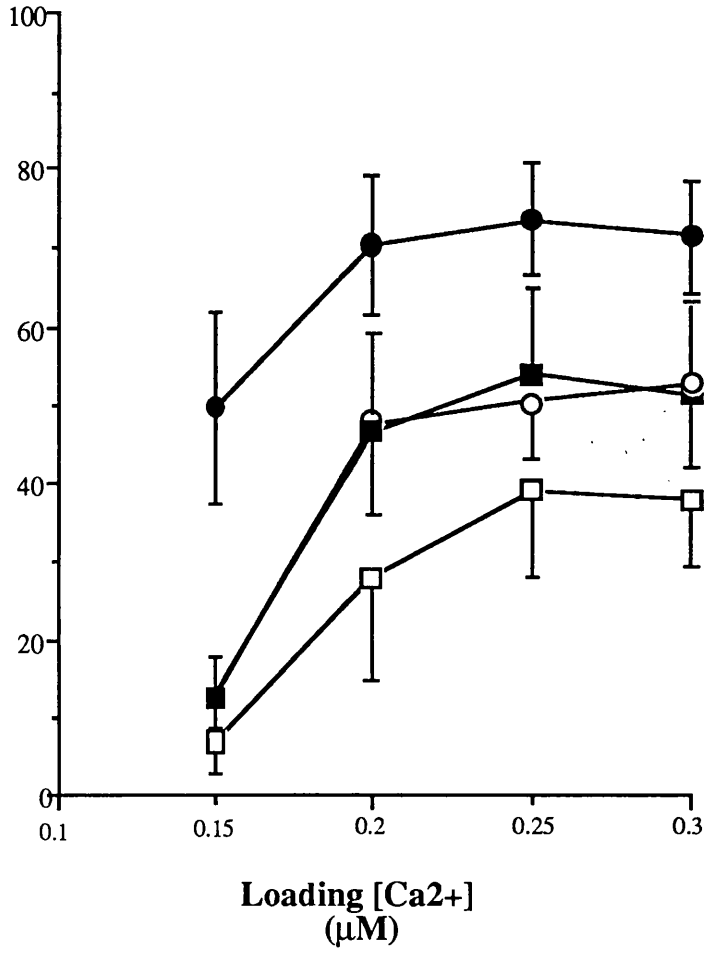


Figure 5.2 SR Ca^{2+}-loading (120 second loading period) in 8 and 15 week LIG and SH rabbits (8 week SH (*open circle*, n=8) and LIG (*closed circle*, n=9) and 15 week SH (*open square*, n=7) and LIG (*closed square*, n=12), values are mean±sem).

Comparison of SR function at 8 and 15 weeks

There were no clear differences in SR function between the ligations and controls at 15 weeks following coronary ligation. However, the LIG group tended to demonstrate enhanced SR function, although not statistically different, at all of the $[Ca^{2+}]$s examined. The SR Ca^{2+}-loading at 120s loading periods are summarised for both 8 and 15 week animals in figure 5.2. Compared with RV trabeculae from 8 week-LIG animals SR Ca^{2+}-loading returned towards control levels 15 weeks after ligation. This apparent secondary reduction in SR function at 15 weeks, compared with 8 weeks post-ligation, was most marked at low loading $[Ca^{2+}]$ (150nM) where 8-LIG animals produced caffeine contractures which were significantly larger than the 15-LIG group (49.9±12.1 vs 13.1±4.6%C_{max}; P=0.001) but there was also a significant difference at higher loading $[Ca^{2+}]$ (250nM, 73.8±7.0 vs 54.2±4.2%C_{max}; P=0.048). The sham-operated controls at 15 weeks also showed a tendency to have lower SR function compared with the 8 weeks controls but this did not reach statistical significance at any of the conditions of $[Ca^{2+}]$ or loading time studied.

These results suggest that the ability of the SR to load Ca^{2+} becomes depressed from an enhanced level at 8 weeks after coronary ligation to levels which are not different from non-heart failure controls at 15 weeks. The various methods of assessing LV dysfunction indicated significant decompensation in the 15-LIG group but this did not represent more severe heart failure than that observed in the 8-LIG group. This would indicate that further depression of SR function at 15 weeks was related to duration of heart failure and not progression of LV dysfunction.

Previous studies have suggested that the process of ageing alone may have effects on myocardial contractility (Harding, Jones, O'Gara, del Monte, Vescovo & Poole-Wilson, 1992, Bristow *et al*, 1982). In SH hearts, there was a

slight but non-significant reduction in SR function at 15 weeks compared with the 8 weeks. The reduction in SR function in 15-LIG compared with 8-LIG, without the development of more severe LV dysfunction, could, therefore, represent the process of ageing which may occur more rapidly in heart failure animals. The changes occurring at a cellular level in heart failure might represent an accelerated form of presbycardia.

There was no difference in the incidence of spontaneous tension oscillations in the 15-LIG group compared with controls which is in contrast to the findings at 8 weeks. This result suggests that spontaneous release of Ca^{2+} from the SR is dependent on the intra-SR [Ca^{2+}] and not on a change in the sensitivity of the Ca^{2+} release channel to cytosolic [Ca^{2+}], since in the 8-LIG group the SR was loading to a greater extent the intra-luminal [Ca^{2+}] reaches a higher level which predisposes the SR to spontaneous release. An alternative theory is that the SR is qualitatively different and that the Ca^{2+} release channel is more sensitive to cytosolic [Ca^{2+}] which produces spontaneous releases of Ca^{2+} by way of the mechanism of Ca^{2+}-induced Ca^{2+} release. However, more recent work using isolated SR Ca^{2+} release channels incorporated into liposomes has indicated that the open probability of the channel is related to the luminal SR [Ca^{2+}] (Tripathy & Meissner, 1994). This is in keeping with the result found in this study where the degree of loading of Ca^{2+} by the SR predicts the probability of spontaneous release. In the intact heart this would mean that, at 8 weeks, enhanced SR Ca^{2+}-loading would tend to over-fill the intracellular Ca^{2+} stores and so predispose to spontaneous release. This could then act in an arrhythmogenic way by activating the transient inward current and give rise to triggered activity. However, at 15 weeks the SR Ca^{2+}-loading has returned to control values and therefore the risk of this as an arrhythmogenic mechanism would be diminished. Extrapolating this (with permission) to the clinical setting suggests that the underlying mechanisms for arrhythmic death in heart

failure may change during the course of development of chronic left ventricular dysfunction. In the early stages, triggered activity may play a significant part, while later, the development of fibrosis and scarring may allow re-entry to play a more important role.

Myofilament force production and Ca^{2+} sensitivity

Maximum force production per unit cross sectional area was lower in RV trabeculae from 15-LIG animals compared with the respective controls although this did not reach statistical significance (15-SH-8.54±3.86.vs 15-LIG 5.6±4.3g.wt/mm^2; P=0.31). This result is similar to the 8-LIG group where RV trabeculae from heart failure animals produced significantly less force than control trabeculae (8-SH-5.40±2.56 vs 8-LIG 3.05±1.58; P=0.043). The apparent reduction in maximum force between the sham animals at 8 and 15 weeks does not reach statistical significance but does suggest some loss of force production with age at the level of the myofilaments. However, previous workers have reported no difference in maximum amplitude of contraction with increasing age in isolated myocytes from patients with heart failure although changes in β-receptor responses have been noted with age (Harding SE *et al*, 1992, Fan & Banerjee, 1985.).

The Ca^{2+} force relationship was not different in 15-LIG rabbits compared with respective controls. There was also no significant difference from the 8 week animals. This would suggest that no significant change in the expression of the myofilament regulatory proteins has occurred.

Conclusions

The results from rabbits allowed to develop LV dysfunction over a longer time period suggest that SR function returns to values similar to control animals at 15 weeks. The enhanced SR Ca^{2+}-loading ability observed at 8 weeks following coronary ligation may therefore represent a period of compensation for the initial myocardial insult. With increasing duration of

heart failure, the SR loading ability returns to baseline levels. The question which remains to be answered is whether, after more prolonged duration of LV dysfunction, SR function would decrease below control levels, as has been reported in a number of other studies. This could certainly be studied in this model but the cost of keeping animals for lengthy intervals becomes prohibitive. All of the animals used for both groups (8 and 15 weeks) in this study were of a very similar age and weight when entered onto the experimental protocol. One further possibility, which would represent an interesting future study, would be to examine the effect of age at the time of infarction on the severity and rate of development of LV dysfunction following a myocardial infarction.

CHAPTER 6

Studies on the sarcoplasmic reticulum Ca^{2+}-release mechanism in the rabbit coronary artery ligation model of heart failure

INTRODUCTION

The SR Ca^{2+} release channel has been identified as a 30 S tetrameric complex protein comprising M_r 565,000 polypeptides as determined by cDNA cloning and sequencing (Otsu, Willard, Khanna *et al*, 1990).The plant alkaloid, ryanodine, binds specifically to this Ca^{2+} channel and not to the voltage-sensitive Ca^{2+} channel of the sarcolemma. Single channel recordings have indicated that purified ryanodine receptor conducts Ca^{2+} and monovalent cations. It is activated by Ca^{2+}, ATP and caffeine, inhibited by Mg^{2+} and ruthenium red and modulated by ryanodine (Lai & Meissner, 1989). During Ca^{2+} release there is charge-compensation across the SR membrane by cations from the cytosol. Candidates for this charge-compensating current include Mg^{2+}, K^+ and the Ca^{2+} channel itself (Lindsay & Williams, 1991). Ca^{2+} influx during the action potential "triggers" Ca^{2+} release from the SR by Ca^{2+}-induced Ca^{2+} release (Fabiato, 1983).

A number of studies have reported a reduction in the number of SR-Ca^{2+} release channels in heart failure. However, the main method of assessing this is by analysis of m-RNA (Brillantes *et al*, 1992, Arai *et al*, 1993) for the Ca^{2+} release channel or by measurement of [H^3]-ryanodine binding (Crory, McCutcheon, O'Grady, Pang, Geiger & O'Brien, 1993). These measurements provide information about the number of channels but do not represent a measure of functional activity. The functional activity of the SR Ca^{2+} release channel is extremely difficult to assess under conditions which are close to physiological. Fabiato (1983) demonstrated that the mechanism for Ca^{2+} release in cardiac muscle cells was by the process of Ca^{2+}-induced Ca^{2+} release (CICR). That is, Ca^{2+} enters the cell during the action potential and reaches a sufficiently high concentration, sufficiently quickly in the region of the cytosolic surface of the SR and activated the ryanodine receptor causing release of Ca^{2+}. This was previously demonstrated in single, mechanically

skinned myocytes and requires that cytosolic [Ca^{2+}] rises very rapidly. Some workers have reproduced CICR by using caged Ca^{2+} compounds which release their bound Ca^{2+} when illuminated with certain wavelengths of light (Kentish, Barsotti, Lea *et al*, 1990). CICR is difficult to produce in multi-cellular, chemically-skinned preparations because of problems related to diffusion. Placing the preparation into a high [Ca^{2+}] causes a large increase in the cytosolic [Ca^{2+}] which should be able to induce CICR. However, because diffusion in large preparations is slow relative to that in single cells the rate of rise of [Ca^{2+}] is insufficient to produce CICR. Some workers have been able to isolate SR Ca^{2+} release channels into lipid bilayers and study their activity under modelled conditions. There was no difference found in the way the channel from failing myocardium behaved (Holmberg & Williams, 1992). However, these conditions are far removed from the physiological setting and interpretation of this negative result must be made bearing this in mind.

Aims of the chapter

The aim of this part of the study was to examine possible differences in Ca^{2+} release mechanisms in multi-cellular preparations in a functional way. This was examined by:

1. Assessing the response of Ca^{2+} release mechanism to various concentrations of caffeine under a standard set of SR-loading conditions.
2. Examining the sensitivity of the SR Ca^{2+} release channel to ryanodine.
3. Assessing SR leak of Ca^{2+} by constructing SR Ca^{2+} 'unloading' curves.
4. Assessing Ca^{2+}-induced Ca^{2+} release in failing and control hearts.

METHODS

Haemodynamic assessment

Coronary ligation rabbits (LIG) were produced in the way described in chapter 2. Sham-operated rabbits (SH) were used as controls. All data presented relate to tissue derived from animals 8 weeks after ligation. Animals were assessed by echocardiography and invasive haemodynamic measures prior to experiments on isolated trabeculae. The LIG rabbits had significantly lower ejection fractions (43.2±6.9 vs 68.3±5.8%; P=0.01) and lower peak cardiac output following a fluid challenge (309.6±39.8 vs 515.7±87.9ml/kg/min; P=0.002) than SH.

Response of SR Ca^{2+} release to various [caffeine]

In a separate set of experiments using LIG (n=7) and SH (n=5) animals the effect of various concentrations of caffeine ([Caff]) on SR Ca^{2+} release was assessed in saponin-skinned trabeculae. A train of 5 caffeine-induced contractures was elicited under a standard set of SR loading conditions (120 seconds, 200nM [Ca^{2+}]) at each of 7 caffeine concentrations: 0.1-50mM applied for 5 seconds. The various [Caff]s were applied in a random order and the protocol was followed strictly in each experiment. A dose-response curve was constructed where the mean amplitude of the final 3 contractures (at each applied [Caff]) was normalised to the peak contracture obtained during this protocol. K_{caff} was calculated as a measure of the sensitivity of the calcium release mechanism to caffeine and represents the concentration of caffeine required to release 50% of the total SR Ca-content loaded under the standard set of conditions applied.

Effects of ryanodine on SR Ca^{2+} release

Ryanodine acts specifically on the SR Ca^{2+} release channel. At low concentrations (10^{-7}M) it binds to the channel causing it to remain in the open state, thus making the SR more leaky. At higher concentrations

ryanodine is believed to block the Ca^{2+} release channel. One other feature of ryanodine is that its effect on SR calcium release is dependent on the activity of the release channels *i.e.* the more active the channels, the more become blocked. Ryanodine has been used in a number of previous studies to examine the contribution of the SR to contraction in both skeletal and cardiac muscle (Bers, 1991). Ryanodine has previously been shown to block oscillatory behaviour of the SR at low concentrations probably by rendering the SR more leaky and preventing the accumulation of a high intra-SR $[Ca^{2+}]$.

The aim of this protocol was to determine possible differences in the sensitivity of the SR Ca^{2+} release channel to ryanodine. Because of the use-dependent nature of the drugs action and the differing actions at low and high concentrations it was not possible to perform a simple dose-response curve. An alternative way of examining its action was to use the same concentration of the drug (10^{-7}M) and assess its effects over time. To do this, saponin-treated trabeculae were placed in a solution B containing 1μM added Ca^{2+} (oscillating solution) until there were clear tension oscillations observed (see figure 6.2). Ryanodine (10^{-7}M) was added to the bath and the time taken for the oscillations to cease was recorded. Once the oscillations had stopped completely, caffeine was applied and the amplitude of the resulting contracture measured. This protocol was performed only once since the effects of ryanodine are use-dependent and although they are partially reversible it was not possible to assess whether the preparation had returned to a comparable baseline state.

Ca^{2+}-leak from the SR

As previously discussed, the net loading capacity of the SR is dependent on the quantity loaded minus the quantity leaked during any given time period. During the SR loading protocols described previously, in chapter 3, the trabecula is placed in a relaxing solution with a low free $[Ca^{2+}]$. The SR

therefore begins the loading protocol with (nominally) zero Ca^{2+} content. Once ATP and Ca^{2+} are provided, the pump becomes active and loads the SR. The conditions are such that they favour Ca^{2+}-uptake rather than Ca^{2+}-leak. However, in this experiment the SR was, firstly, loaded with Ca^{2+} under standard conditions and was then placed in solution B with no added Ca^{2+}, i.e. under conditions which favoured leak rather than uptake. The trabecula was placed in solution B for various time periods ranging from 15-360 seconds. Caffeine was applied for 5s at the end of each 'leak-period' and the amplitude of the resulting contracture measured. At the end of this protocol the net-uptake of Ca^{2+} in the leak solution (solution B with no added Ca^{2+}) was assessed by attempting to evoke a caffeine-contracture after 120s loading period in solution B. In all cases no response was observed.

Ca^{2+}-induced Ca^{2+} release (CICR)

CICR is difficult to produce in multi-cellular preparations for the reasons discussed earlier in this chapter. The reasons for this are mainly related diffusion which is relatively slow in large multi-cellular preparations but in a few cases, where thinner trabeculae (<100μm) were obtained, this was attempted. The preparation was placed in solution B containing 200nM added Ca^{2+} for 2 minutes to allow the SR to load Ca^{2+} and was then rapidly switched to solution B containing $[Ca^{2+}]$s ranging from 0.6-1.5 μm.

RESULTS

Caffeine dose-response curve

For the standard set of loading conditions studied, the relationship between applied [Caffeine] and Ca^{2+} release (assessed by contracture amplitude) was similar in sham and LIG animals (Figure 6.1, LIG K_{caff} 1.80±0.16mM *vs* SH K_{caff} 1.98±0.19mM). The contracture amplitude peaked at 10mM in both groups although there was a tendency for a slight fall-off in tension in the LIG animals at higher [Caff]. There was no significant difference in the response of SR Ca^{2+} release to low concentrations of caffeine.

Effects of ryanodine

Ryanodine produced cessation of spontaneous oscillations in all preparations studied (Figure 6.2). During continuous oscillations resting tension rises and the observed oscillations are superimposed on this increased resting tension. Addition of ryanodine initially caused a decrease in the frequency of oscillations and subsequently a fall in resting tension. Oscillatory behaviour becomes infrequent and finally stops altogether. At this point, it is clear from the trace shown (figure 6.3) that the SR still contains a significant quantity of releasable Ca^{2+}, since the application of caffeine produces a large contracture. The time taken for oscillations to stop and resting tension to return to baseline was significantly shorter in the ligations compared with shams (136.2±35.4 vs 385.1±94.7 seconds; P=0.001). The size of the post-ryanodine caffeine-contracture was slightly greater in the ligation group (38.4±8.1 vs 26.9±7.3 $\%C_{max}$; P=0.2).

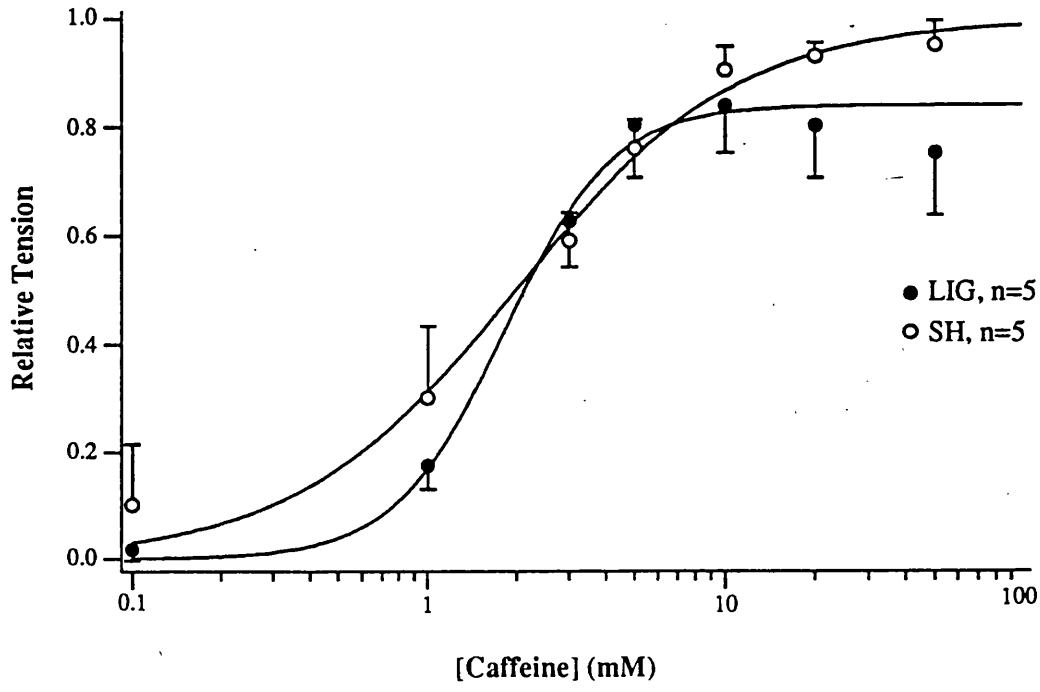


Figure 6.1 Effect of different caffeine concentrations on the Caffeine-induced contracture in saponin-treated SH and LIG rabbit trabeculae.

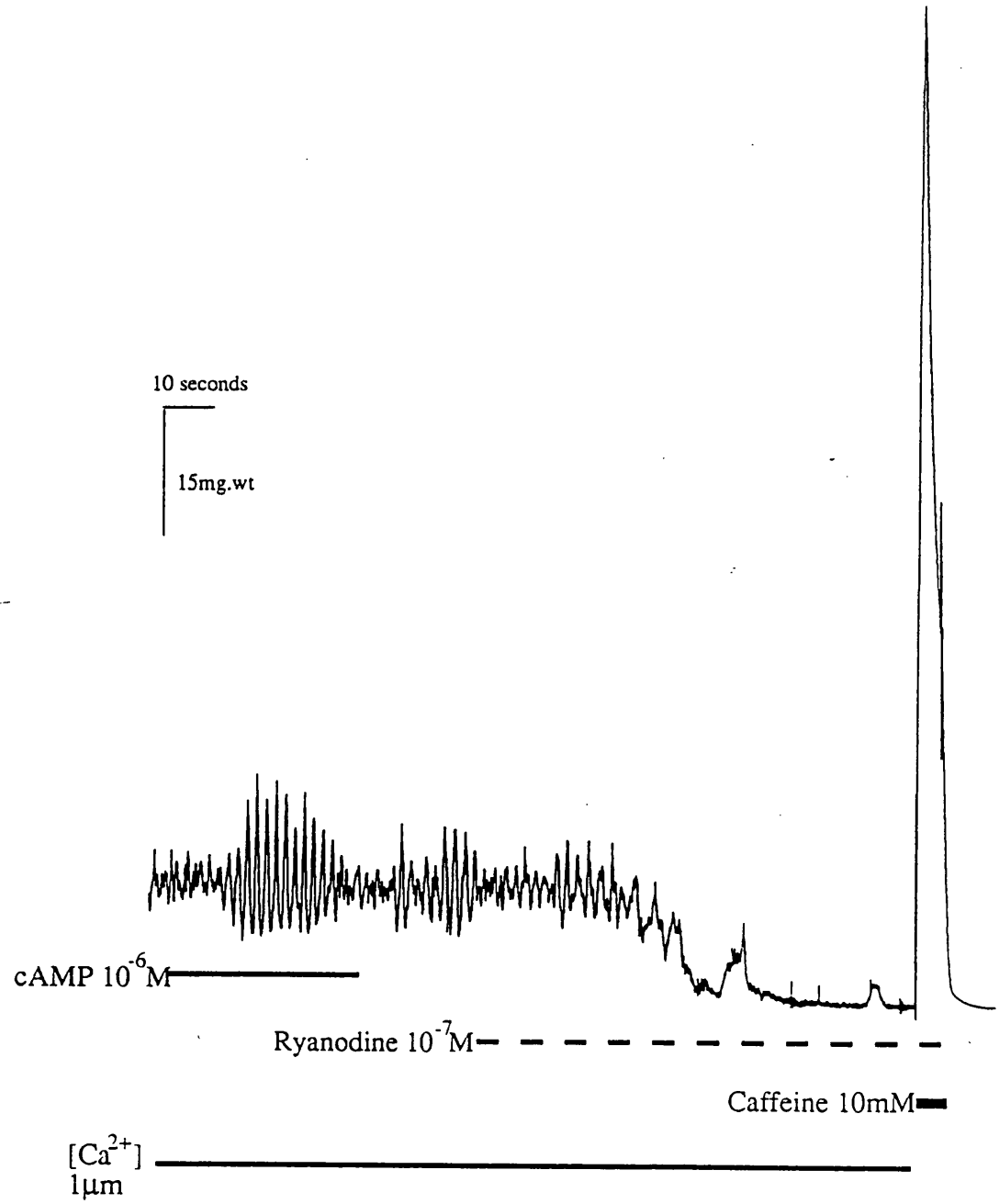


Figure 6.2 Effect of cAMP and ryanodine on tension oscillations in a saponin-treated ventricular Trabecula. The preparation is allowed to oscillate in a solution containing high [Ca^{2+}]. cAMP (10^{-6}M) is added and produces a marked increase in the frequency and amplitude of oscillations. Ryanodine is added later and produces a gradual reduction in the frequency of oscillations and eventually resting tension falls to baseline levels and oscillations cease totally. The large caffeine-induced contracture, evoked at this stage, indicates that although the oscillations have stopped a significant quantity of Ca^{2+} remains within the SR. In addition, ryanodine clearly has not completely blocked the Ca^{2+} release channel.

SR Ca^{2+} leak

The leak curves for both LIG and SH animals are shown in figure 6.3. Contractures fell rapidly during the first minute to 49.1±8.1% of control contractures in the ligation group and to 33.9±8.3% in the shams. After 150s leak time period the ligations had fallen to 38.1±7.3% of controls and shams to 22.2±7.7% of control values. At longer leak periods (6 minutes) the same pattern emerged but although the mean caffeine contractures were slightly greater in the ligation group this failed to reach statistical significance. Ca^{2+}-loading, assessed in the leak solution (solution B with no added Ca^{2+}), was zero after 120s in all cases for both LIG and SH animals studied.

Ca^{2+}-induced Ca^{2+} release

As expected, there were no consistent results produced in attempting to induce CICR in a total of 6 different preparations from 3 LIG and 3 SH animals. However, in one SH preparation there was a definite release of SR Ca^{2+} produced by the high bathing [Ca^{2+}] (figure 6.4). There was no release produced until 0.8μM [Ca^{2+}] with a larger release produced at 1μM [Ca^{2+}]. This was clearly not produced by mechanical perturbation since the bath-change at 0.6μM [Ca^{2+}] failed to induce release. The twitches appeared more brief and were of lower amplitude than the caffeine-induced contractures. In other preparations studied in this way, when a contracture was produced, there was a delay of approximately 1-2s before tension rose. This was interpreted as sufficient time for the SR to start loading in the solution containing the high [Ca^{2+}] and the resulting contracture could equally well be interpreted as a large spontaneous release of Ca^{2+} rather than true CICR.

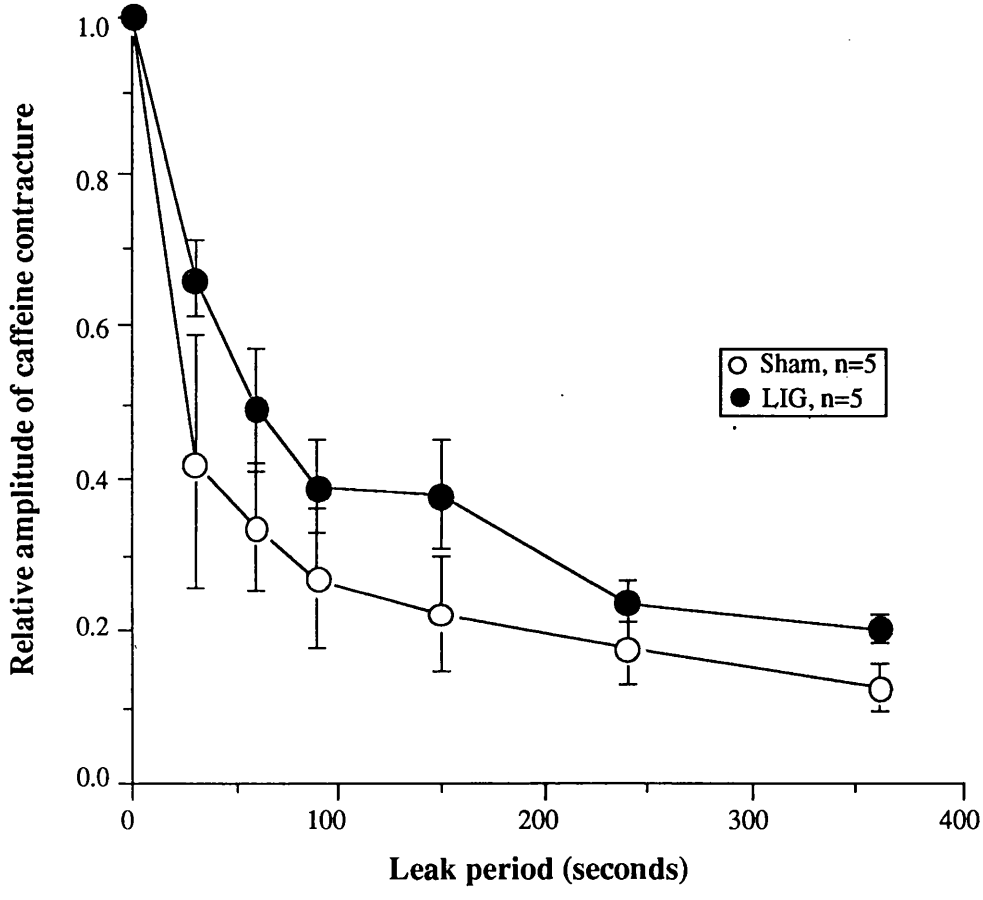


Figure 6.3 Leak of Ca^{2+} from the SR in saponin-treated trabeculae from LIG and SH rabbits.

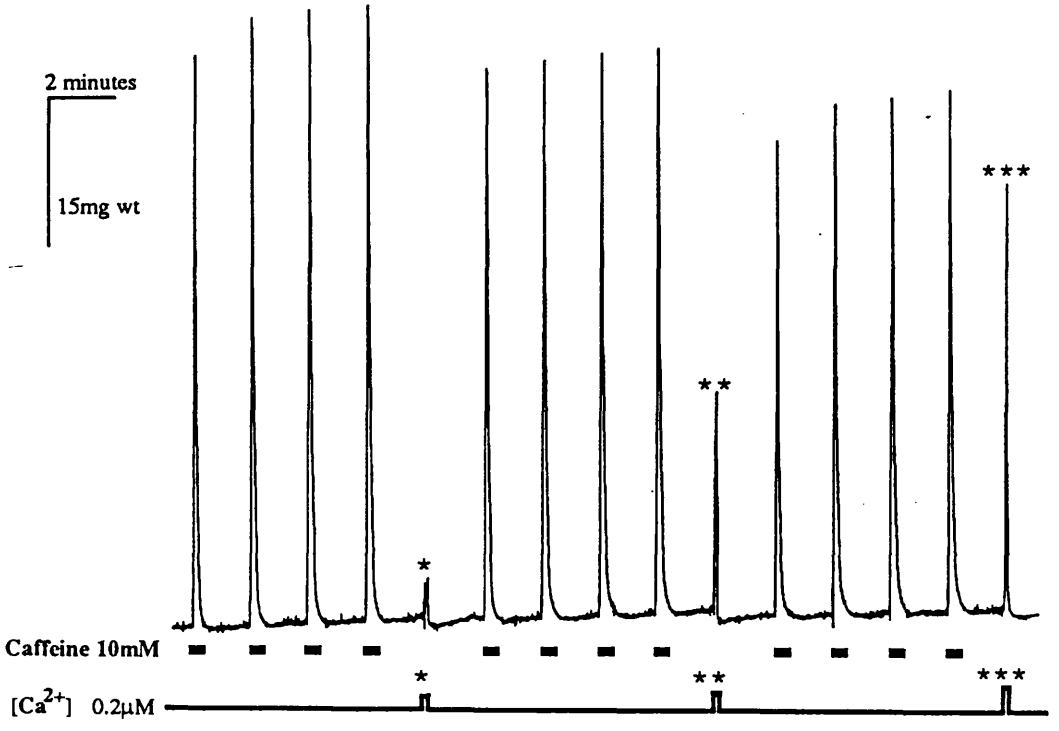


Figure 6.4 Ca^{2+}-induced Ca^{2+} release (CICR) in a saponin-treated ventricular trabecula. A train of four caffeine-induced contractures (0.2μM $[Ca^{2+}]$/ 2 minute loading period) is followed by a similar loading period then a 2.5 second exposure to a * 0.6μM, ** 0.8μM, *** 1μM [Ca] (no caffeine) solution. There is no CICR at 0.6μM (*) but there is a release at 0.8μM and 1μM $[Ca^{2+}]$ which is attributable to CICR. Note how the CICR contractures are much briefer than the caffeine contractures.

DISCUSSION

A reduction in the number of SR Ca^{2+} release channels has previously been reported in heart failure (Crory *et al*, 1993, Brillantes *et al*, 1992). However, these studies provided no evidence for a functional change in the SR as a result of a reduced number of channels. It is possible to speculate that a reduced number of channels will produce a slower rate of release of Ca^{2+} from the SR and will also reduce the amount of Ca^{2+} leak.

Caffeine-induced Ca^{2+} release

There was no significant difference in the response of the SR in failing hearts to various concentrations of caffeine compared with controls in this study. This might suggest that the response of the SR to caffeine does not alter in heart failure and therefore that there has been no significant qualitative change in the function of the caffeine-sensitive SR Ca^{2+} release channel. It is an important finding in interpreting the results from chapters 4 and 5, since it indicates that the net Ca^{2+} release in response to caffeine was similar in both LIG and SH animals. Additionally, it demonstrates that 10mM (the concentration used in all experiments testing SR function) was sufficient to induce maximal release of calcium from the SR with no further increase in tension responses in both LIG and SH animals at higher [Caff]s.

In ligation animals mean tension responses tended to decrease at caffeine concentrations greater than 20mM. This was not a distortion produced by averaging the data and can be seen as a clear effect in a number of individual LIG animals. Caffeine has previously been shown to depress maximum C_{max} at high concentrations (Miller, Lamont & O'Dowd, 1993) and this might explain why this was more obvious in the ligation animals. That is, since these values are normalised to C_{max}, and LIG trabeculae tended to produce contractures closer to C_{max}, then the effect of high concentrations of caffeine on force production was more pronounced.

Effect of ryanodine on SR function

Ryanodine was found to halt oscillations more quickly in trabeculae from LIG animals. Since the effect of ryanodine is greater when channels are more active then this could be interpreted as a higher level of channel activity during oscillations in LIG trabeculae. Alternatively, it could suggest that the calcium content was lower in the LIG trabeculae during the oscillations and so were stopped by locking-open, proportionally, fewer channels. However, this is not consistent with the findings that SR Ca-content was greater in LIG trabeculae. A further possible explanation is that the Ca-release channel is more sensitive to ryanodine in LIG trabeculae, although, there is no evidence for this in isolated SR-release channel studies (Holmberg & Williams, 1992). It is, therefore, difficult to resolve these experimental findings with the hypothesis that the number of Ca-release channels is reduced in LIG trabeculae. However, if the process of halting oscillations by ryanodine was a stochastic process. then the effect of locking-open a few channels would be more likely to halt oscillations completely than in control trabeculae where the effect would be lost in background noise. Therefore, although these results are do not directly indicate a reduction in the number of calcium release channels in failing trabeculae they are consistent with this hypothesis. It is clear that despite the cessation of oscillations the SR still contains a large quantity of releasable Ca^{2+} and the Ca^{2+} release channels are not completely blocked since we know that caffeine can induce release. This would suggest that oscillations can be stopped by a quantity of ryanodine, sufficient to switch-off those channels involved in spontaneous release, but not enough to cause the SR to leak all of its Ca^{2+} content. The difference in the size of the post-ryanodine contracture between LIG and SH trabeculae may simply be the result of greater leak of Ca^{2+} in the SH preparations during the period ryanodine was acting which was longer in the SH trabeculae. Alternatively, it

may reflect a greater SR Ca^{2+} content in the LIG trabeculae as suggested by the Ca^{2+}-loading experiments.

Leak of Ca^{2+} from the SR

There was some evidence that the rate of leak in LIG trabeculae was slightly less than in controls (figure 6.3) although this failed to reach statistical significance. However, the LIG leak curve does remain consistently above that of the SH animals and perhaps with such a small difference we simply require to increase the number studied to make this significant. The protocol used to assess the leak of Ca^{2+} in these preparations was crude, in many ways, but still managed to reveal some evidence that the rate of leak of Ca^{2+} was different in heart failure animals. The conditions used to assess this were identical to the loading solutions used in earlier experiments but the bathing [Ca^{2+}] was extremely low (~20nM) such that the gradient for Ca^{2+} movement was from the lumen of the SR into the bathing medium. The SR could still take up Ca^{2+} but at a rate that could not counteract the rate of leak. This resulted in the net movement of Ca^{2+} from the SR into the bathing medium. An alternative method to assess this would have been to allow the SR to load Ca^{2+}, then to remove ATP from the bathing solution thus halting the action of the Ca^{2+} pump altogether: this condition would also have favoured Ca^{2+} leak. In fact, this was not attempted but judging from the rate of leak in the presence of ATP, the rate of leak in its absence may have been far too rapid to assess using this protocol.

The net loading of Ca^{2+} in the leak solution was zero in all cases. This indicates that for the conditions provided which favoured leak from the SR, the curves for both LIG and SH would finally reach zero leakage was allowed for a sufficient time. However, there may have been very small releases of Ca^{2+} from the SR which were subthreshold for myofilament activation. These experiments require to be repeated to measure leak at even shorter time periods using Ca^{2+} indicators to measure Ca^{2+} directly.

Ca^{2+}-induced Ca^{2+} release

Ca^{2+}-induced Ca^{2+} release was difficult to obtain in these saponin-treated trabeculae. In the course of studying 6 separate trabeculae from different animals we were convinced of observing true CICR in only one (result shown, figure 6.4). The conditions required to observe this phenomenon need to be strictly controlled and so this result is not surprising. The contractures which did result in the example shown were of shorter duration and of lower amplitude than the caffeine contractures. Presumably, this difference is a consequence of the properties of caffeine which sensitises the myofilaments, increasing peak force attained and slowing the rate of relaxation. In addition, in the CICR contractures, the absence of caffeine might allow the SR to contribute more significantly to the fall in $[Ca^{2+}]$ thus increasing the rate of relaxation and making the contracture more brief. There are certainly reasons for attempting further experiments using this method although the preparation itself may well not be suited to assessment of CICR.

Conclusions

These data provide indirect evidence for a possible reduction in the number of Ca^{2+} release channels in failing ventricular trabeculae. A reduction in the number of Ca^{2+} release channels could provide a mechanism by which the SR might load more Ca^{2+} and consequently contain more for release by caffeine. Such a change could give rise to the enhanced SR Ca^{2+}-loading ability observed in this study in ligation animals at 8 weeks.

CHAPTER 7

Sarcoplasmic reticulum Ca^{2+}-loading in a rabbit model of adreno-receptor down regulation

INTRODUCTION

There are a number of ways in which the heart can respond to demands for increased blood flow and oxygen delivery. EH Starling (1918) first described the relationship between force and end-diastolic volume in isolated working hearts. This intrinsic inotropic mechanism is thought to result mainly from the myofilament length/tension relationship which allows a greater force production at cell lengths which favour a greater overlap of sarcomeres. However, additional changes in myofilament Ca^{2+} sensitivity (Hibbert & Jewell, 1982) and changes in stretch activated membrane channels (Gamble, Taylor & Kenno, 1992) may also play a role in increasing the force of contraction of cardiac muscle. There are additional factors which increase the force of contraction in the heart in the whole animal. The heart is under direct influence of the autonomic nervous system. Sympathetic nerve endings in the ventricular wall can release noradrenaline locally bringing about an increase in the force of contraction. Circulating noradrenaline and adrenaline, released from the adrenal gland, will also produce both an inotropic and chronotropic response.

Beta-receptor stimulation activates adenylate cyclase which brings about an increase in the formation of intracellular cyclic adenosine mono-phosphate (cAMP). This activates a cAMP-dependent protein kinase which phosphorylates a number of intracellular sites involved in the regulation of Ca^{2+}. These include the voltage dependent Ca^{2+} channels on the cell membrane, the regulatory protein phospholamban on the SR and troponin-C on the myofilaments. This results in a greater influx of Ca^{2+} during the action potential, increased rate of Ca^{2+} sequestration by the SR and a decrease in the sensitivity of the myofilaments for Ca^{2+}. The net result is more force and faster relaxation, an ideal physiological combination for producing an increase in cardiac output and dealing with an increase in venous return.

Heart failure is accompanied by a widespread activation of the neurohormonal axis. Chronic activation of the sympathetic nervous system brings about a decrease in the number of beta-receptors on the cardiac myocyte and results in a diminished response to catecholamines (Bristow, Kantrowitz, Minobe *et al*, 1982). The heart, therefore, loses an important inotropic mechanism which normally allows it to respond to increased demands. This could be interpreted as a physiological protective mechanism since it limits the inotropic response and hence the increased oxygen demands which follow catecholamine stimulation. However, a number of studies treating heart failure patients with beta-blockers have succeeded in demonstrating significant improvements in myocardial function and symptoms accompanied by a return of beta-receptor response (Heilbrunn, Shaw, Bristol *et al*, 1989).

Since beta-receptor stimulation produces an increase in SR Ca^{2+} uptake it is possible that the observed enhancement of SR Ca^{2+} uptake in the rabbits with heart failure (at 8 weeks) in this study is the result of a change in the phosphorylation state of the SR. Down-regulation of β-receptors, however, would be expected to reduce the level of intracellular cAMP, reduce intracellular phosphorylation and so reduce the activity of the SR Ca^{2+} pump. Such a change could explain the diminished Ca^{2+} uptake observed in failing human tissue but would not explain the enhanced Ca^{2+} uptake observed in the rabbit coronary ligation model after 8 weeks. However, if the rabbit model did *not* have beta-receptor down regulation but *did* have high levels of circulating catecholamines then increased beta-stimulation at the time of removal of the heart might result in a more phosphorylated SR Ca^{2+} pump and an enhanced Ca^{2+}-loading ability. For this hypothesis to be correct, the phosphorylated state, resulting from catecholamine stimulation, would require to persist after removal of the heart and after the subsequent saponin-skinning procedure. To examine this hypothesis, we used a previously

validated model of adreno-receptor down regulation in the rabbit (Jones *et al,* 1990). The experimental protocol for assessing possible down-regulation of catecholamine response allowed us to examine the effect of acute administration of isoprenaline immediately prior to removal of the heart in saline infused control animals. This protocol allowed us to assess the effects of diminished beta-receptor stimulation in the chronic-isoprenaline infused animal and also the possible persistence of the acute effects of isoprenaline in control hearts on SR and myofilament function .

METHODS

Animal model

The rabbit model of adrenoreceptor down-regulation was produced using the method described in chapter 2 (after Jones *et al*, 1990). Isoprenaline filled mini-osmotic pumps (Iso-OP, n=7) were implanted for a period of 7 days. Saline-filled mini-osmotic pumps were implanted for a period of seven days as controls (Sal-OP, n=5). On the sixth day, under general anaesthesia, a thermodilution catheter was placed in the descending aorta and a fluid filled 4F catheter placed in the LV via the right carotid artery. The animal was placed in a protective jacket and allowed to recover from the anaesthetic overnight.

Haemodynamic assessment

Haemodynamic assessment was performed in the conscious animal on the morning after implantation of catheters. The protocol followed was designed to minimise the general distress to the animal and so hopefully reduce the background level of sympathetic activation secondary to handling and moving around. The animal was placed in a quiet, isolated room and allowed to settle for a period of 15-20 minutes. The LV pressure catheter was then connected to the Gould pressure transducer and the thermodilution catheter connected to the cardiac output computer. The initial LV pressure trace was observed for a further 10-15 minutes until the trace was stable with no ectopic activity. A baseline assessment of left ventricular systolic pressure (LVSP), end-diastolic pressure (LVEDP) and heart rate (HR) was made. The heart rate was measured by simply counting the peaks on the pressure trace over a period of one minute. Mean baseline cardiac output was calculated from five 1ml injections of cold dextrose (19-22°C). The peak heart rate response to increasing doses of isoprenaline (1-5μg), given as bolus injections into the LV, was measured. The heart rate was allowed to recover to baseline

levels (within 10%) between injections of isoprenaline (generally ~10 minutes). Immediately after the final bolus injection of 5μg of isoprenaline, 5 injections of 1ml cold dextrose were used to calculate the mean cardiac output after isoprenaline. The Sal-OP animals were assessed in an identical way with incremental doses of isoprenaline given to assess heart rate response and a similar bolus of 5μg of isoprenaline given within 10 minutes of the animal being killed. A lethal dose of Euthatal was given and the heart rapidly excised and placed in Ca^{2+} free ringer with 2,3-butanedione monoxime (30mM) added. A free-running trabecula was dissected from the right ventricle and mounted for isometric tension measurement. The preparation was treated with saponin and exposed to a series of solutions at various [Ca^{2+}] for various loading periods. At the end of the SR loading protocol, the preparation was treated with Triton-X100 for 30 minutes and measurements of myofilament Ca^{2+} sensitivity and maximum force production made.

RESULTS

Clinical features

Iso-OP animals appeared more anxious and agitated consistent with elevated circulating catecholamines. There were no wound infections in either group and no problems with erosion of the mini-osmotic pump through the skin or the wound. However, two animals in the Iso-OP group died suddenly with no obvious preceding clinical problems. Post-mortem examination revealed no abnormality and the pumps contained the appropriate contents of isoprenaline (assessed by comparing the weight of the pump before implantation and after death). The most likely cause of death in these animals was thought to be an arrhythmia although this could not be confirmed. At the time of excision of the heart, 2 of the isoprenaline animals were noted to have significant pericardial effusions. Although this could have resulted simply from placement of the LV catheter it is not a feature seen in any of the other rabbits except the coronary ligation animals with more severe heart failure . The reason for this is unclear but could be related to discrete areas of myocardial necrosis which are known to result from large doses of intravenous catecholamines (Rona, Chapel, Balazs and Godfrey, 1959).

Haemodynamic assessment

The haemodynamic results obtained at baseline and after isoprenaline are summarised in table 5. Total body weight was not significantly different between groups at the time of haemodynamic assessment (3.13±0.15 vs 3.18±0.15 kg: P=0.62). Baseline LVSP was significantly higher in Iso-OP animals compared with Sal-OP (163.2±9.1 vs 140.5±5.4mmHg; P=0.01). The response of LVSP to the final bolus of isoprenaline was diminished in the Iso-OP group (3.1±2.4 vs 14.2±5.6% increase; P=0.01). Baseline LVEDP was not significantly different between groups (7.5±1.9 vs 8.5±1.5mmHg; P=0.52).

There was no significant difference in LVEDP after the final bolus of isoprenaline between groups (6.8±1.3 vs 7.0±1.6mmHg). Baseline heart rate was significantly greater in Iso-OP compared with Sal-OP (259.6±41.3 vs 212.3±21.3 min^{-1}; P=0.01). The heart rate response to various bolus injections of isoprenaline, in increasing doses, was less marked in the Iso-OP compared with Sal-OP animals (figure 7.1). In addition to the difference at baseline, there was a significantly lower peak heart rate response to the maximum bolus of isoprenaline (5μg) in the Iso-OP animals (336.7±8.1 vs 368.6±6.3min^{-1} ; P=0.001). The dose-response curve was shifted to the right in keeping with a down-regulated response to isoprenaline in the Iso-OP group. The mean heart rate immediately before sacrifice was similar in both groups but was significantly higher than mean resting heart rate in both groups indicating persistent chronotropic stimulation (Iso-OP 296.2±11.1 & Sal-OP 311.8±8.3min^{-1}). Baseline cardiac output was higher in the Iso-OP animals (413.8±40.1 vs 306.2±15.6ml/kg/min; P=0.01) and was also significantly higher following intra-ventricular isoprenaline (506.5±55.9 vs 386.1±12.6ml/kg/min; P=0.01). However, the relative increase in cardiac output following the final bolus of isoprenaline was slightly lower in the Isoprenaline treated animals (22.9±6.7 vs 27.2±4.3%).

Table 5 Haemodynamic results in isoprenaline and saline infused rabbits. Values given are those at baseline and after the final bolus injection of intra-cardiac isoprenaline (mean±sem) for both saline infused (Sal-OP, n=5) and isoprenaline infused (Iso-OP, n=7) rabbits.

	Iso-OP	**Sal-OP**	
LVSP(mmHg)			
Baseline	163.2±9.1	140.5±5.4	P=0.01
Post-isoprenaline	168.3±8.6	159.9±10.2	P=0.21
LVEDP(mmHg)			
Baseline	7.5±1.9	8.5±1.5	P=0.52
Post-isoprenaline	6.8±1.3	7.0±1.6	P=0.48
Heart Rate(min^{-1})			
Baseline	259.6±41.3	212.3±21.3	P=0.01
Post-isoprenaline	336.7±8.1	368.6±6.3	P=0.001
CO (ml/kg/min)			
Baseline	413.8±40.1	306.2±15.6	P=0.01
Post-isoprenaline	506.5±55.9	386.1±12.6	P=0.01

Isolated trabeculae experiments

SR Ca^{2+}-loading

There was no significant difference in the size of RV trabeculae taken from Sal-OP or Iso-OP animals (major diameters 146±32 vs 155±36μm; minor diameters 75±26 vs 81±27μm). The SR loading curves for all conditions of time and loading [Ca^{2+}] are presented in figure 7.2. There was no significant difference in the ability of the SR to load Ca^{2+} between Iso-OP and Sal-OP groups. This relationship held at a range of loading periods and at all the [Ca^{2+}] studied. The peak caffeine contracture obtained for any of the loading conditions studied was not different between groups (Iso-OP 34.3±10.6 vs Sal-OP 32.6±6.2% C_{max}; P=0.65). Comparing the saline controls used in this part of the study (Sal-OP) with saline controls from the adriamycin study reveals no difference in the SR loading curves for any of the conditions of Ca^{2+}-loading. There was also no difference between the Sal-OP and the Sham-operated controls in the coronary ligation study. The SR loading curves for all three of these control groups are plotted together in figure 7.3 for comparison. There was no difference in the incidence of spontaneous oscillations between Sal-OP and Iso-OP. 2 control animals and 3 isoprenaline treated animals demonstrated oscillations but all occurred at high loading [Ca^{2+}] and long loading periods.

Myofilament force production and Ca^{2+} sensitivity

There was no significant difference in the maximum force produced per unit cross sectional area in Triton-skinned trabeculae from Iso-OP and Sal-OP animals (4.76±3.55 vs 5.21±3.88gwt/mm^2). The myofilament responsiveness to Ca^{2+}, expressed as the Ca^{2+} required for 50% full activation (pCa_{50}), was slightly lower in the Iso-OP animals compared with Sal-OP controls (pCa_{50} 5.51±0.07 vs 5.61±0.05; P=0.053). This difference only just fails to reach statistical significance. The mean Hill fit curves for saline and isoprenaline

osmotic pumps are plotted in Figure 7.4. This shift to the right of the Ca^{2+}-tension relationship in the Iso-OP animals represents a reduction in myofibrillar Ca^{2+} responsiveness.

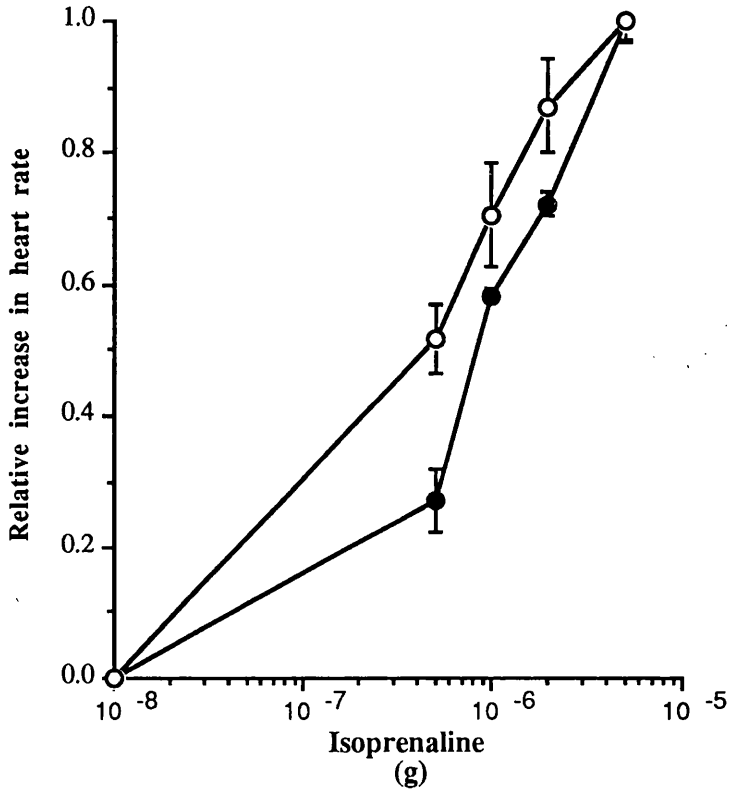
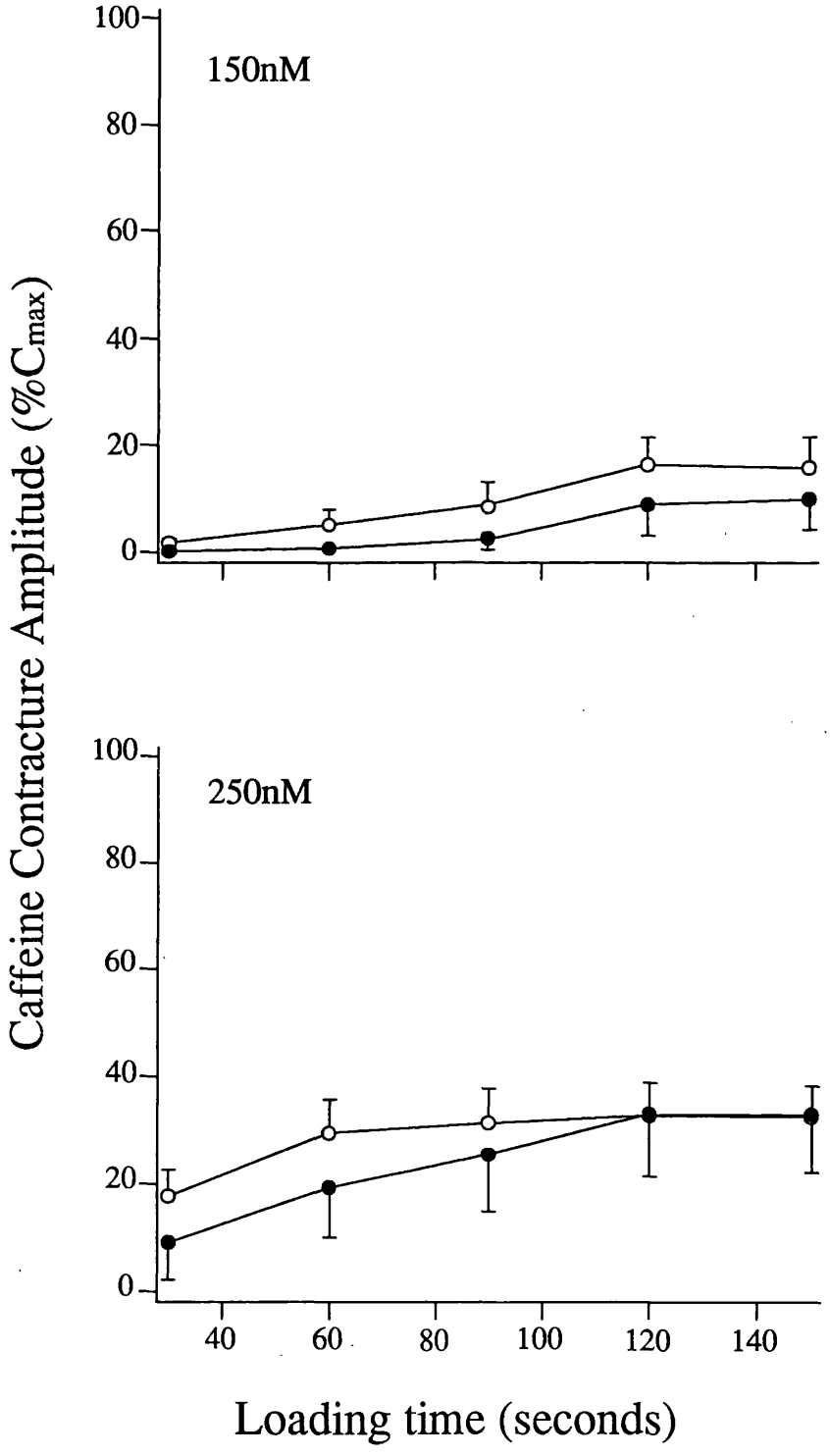


Figure 7.1 Heart rate response to intra-cardiac boluses of isoprenaline in saline (*open circle,* n=7) and isoprenaline (*closed circle,* n=5) infused rabbits.


150nM
250nM
100
80
60
40
20
0
40
60
80
100
120
140
Caffeine Contracture Amplitude (%Cmax)
Loading time (seconds)

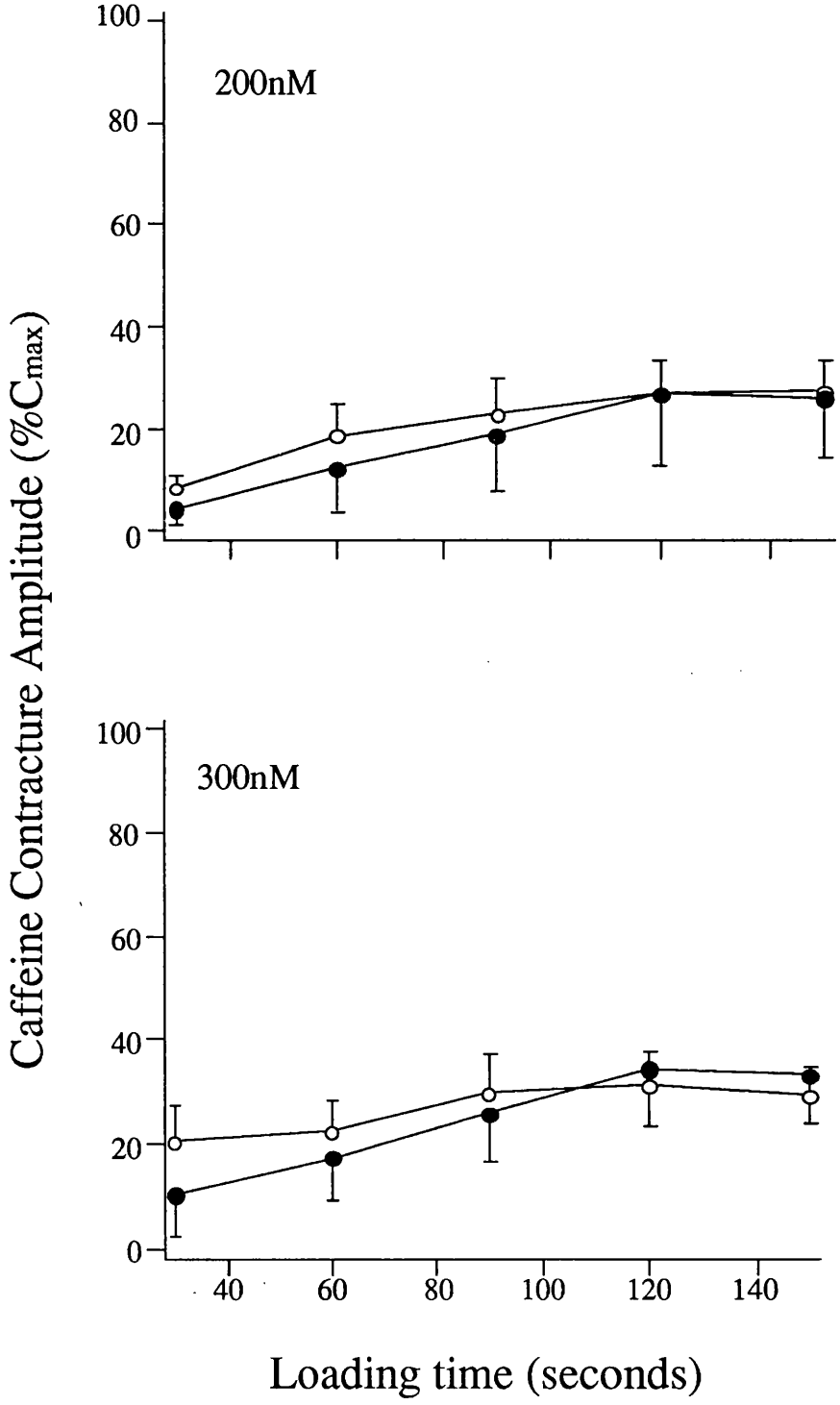


Figure 7.2 (This and facing page) SR Ca^{2+}-loading in saponin-treated RV trabeculae from isoprenaline-infused (Iso-OP, closed *circle*, n=7) and saline-infused (Sal-OP, *open circle*, n=5) rabbits.

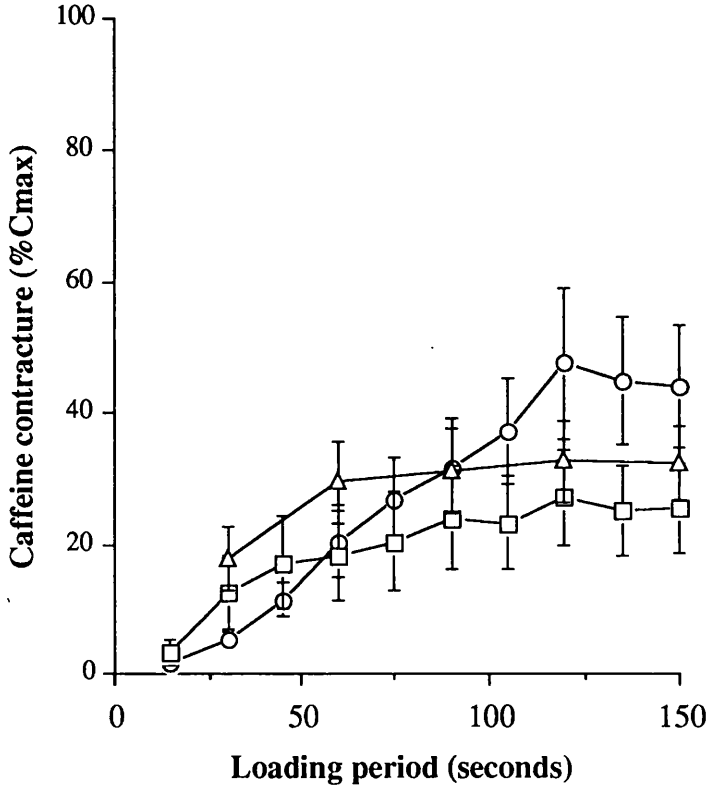


Figure 7.3 Comparison of SR Ca^{2+}-loading (200nM $[Ca^{2+}]$) in RV trabeculae from all three rabbit control groups from this thesis: saline (adriamycin study, *open square*, n=7), Sham-operated ligation (*open circle*, n=10) and saline (isoprenaline study, *open triangle*, n=5).

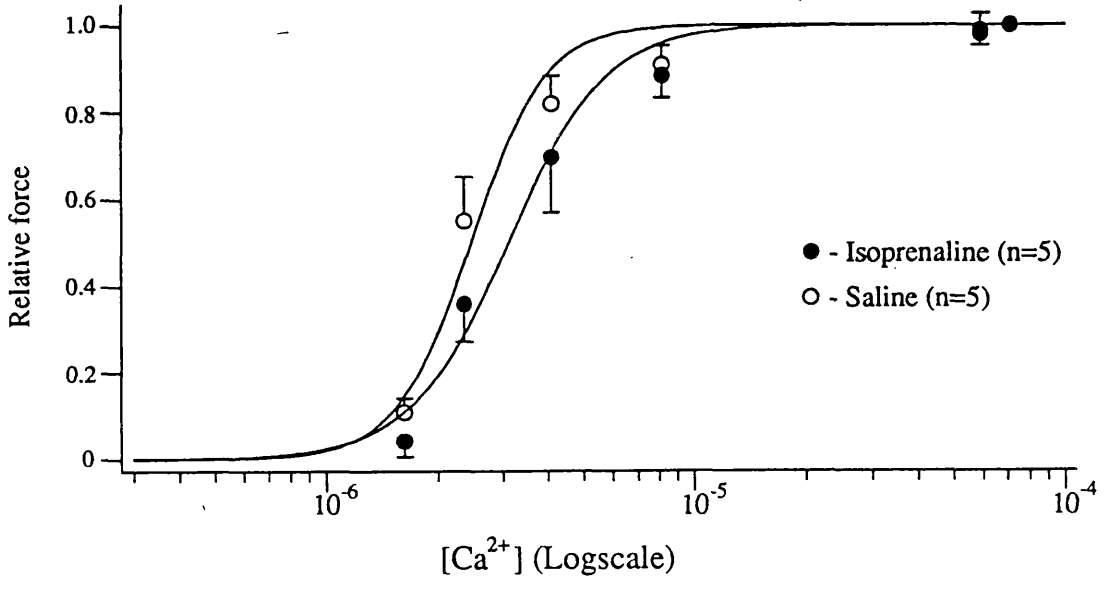


Figure 7.4 Ca^{2+} sensitivity curves of ventricular trabeculae from saline (Sal-OP, *open circle,* n=5) and isoprenaline (Iso-OP, *closed circle,* n=5) infused rabbits.

DISCUSSION

These results indicate that the ability of rabbit cardiac SR to load Ca^{2+} is not affected by chronic infusion of isoprenaline for a period of 7 days. Since it was possible to demonstrate a diminished response of these hearts to isoprenaline, then this degree of adreno-receptor down-regulation does not appear to change the Ca^{2+}-loading ability of the SR. Acute administration of isoprenaline, within 10 minutes of death, did not appear to increase the SR Ca^{2+}-loading ability in the Sal-OP controls compared with Iso-OP animals, saline controls in the adriamycin study or sham-operated controls in the ligation study (figure 7.3). There is evidence indicating a raised heart rate immediately before sacrifice which suggests that there was persistent catecholamine stimulation at this time. This suggests that the previously demonstrated effects of isoprenaline on SR function (Endoh & Blinks, 1988) do not persist after the animal is killed and do not persist after the myocardial preparation is treated with saponin. Maximum force production in Triton-skinned trabeculae was not significantly different between controls and chronically infused animals. However, Ca^{2+} responsiveness of the myofilaments was reduced by chronic treatment with isoprenaline.

Evidence for adreno-receptor down-regulation in this model

In view of this lack of difference in SR Ca^{2+}-loading between the two groups, the important question which arises is whether adreno-receptor down regulation had occurred in the hearts of the chronically infused rabbits. Baseline values for cardiac output, LVSP and heart rate were significantly increased in the Iso-OP group indicating a persistent response to the isoprenaline from the mini-osmotic pump. However, there is clear evidence suggesting a differing heart rate response to intra-cardiac isoprenaline in the chronically infused animals (figure 7.1). The rise in LVSP after a bolus of 5μg of intra-cardiac isoprenaline was diminished in the Iso-OP animals compared

with controls. In addition, although *peak* cardiac output following a bolus injection of isoprenaline was greater, the *relative* increase was diminished in the Iso-OP animals. These features, in combination, would tend to suggest a diminished adrenergic response and imply adreno-receptor down-regulation in the chronically infused animal.

β-receptor down-regulation: possible changes in SR Ca^{2+}-loading

There was no difference in the Ca^{2+}-loading ability of cardiac SR from rabbits chronically infused with isoprenaline compared with controls. This indicates that β-receptor down-regulation does not influence the ability of the SR to load Ca^{2+} and so the enhanced SR Ca^{2+}-loading ability in ligation rabbits after 8 weeks could not have been a consequence of β-receptor down-regulation (secondary to heart failure) and must be due to additional factors operating in the ligated rabbit heart.

Reduced β-receptor response is associated with reduced levels of intracellular cAMP (Feldman *et al*, 1987) which results in reduced levels of phosphorylation of phospholamban. This would tend to reduce the SR Ca-ATPase rate and reduce the loading ability of the SR. When these factors are considered, the hypothesis that this mechanism could be responsible for enhanced SR Ca^{2+}-loading appears flawed. However, extending this argument further, one could suggest that as a consequence of chronically low levels of cAMP, there is a compensatory increase in the factors downstream from cAMP which, when provided with a controlled intra-cellular environment (such as in the skinned-fibre), produce enhanced SR Ca^{2+}-loading. There is some evidence that the mechanism of inotropic stimulation, down-stream from the β-receptor, remains intact, since previous work has shown that the tension response to forskolin and cAMP is similar in failing and control human papillary muscles (Bohm, Beuckelmenn, Brown *et al*, 1988). However, there is no evidence for a compensatory increase in response to these type of agents in heart failure and

so this suggestion, of a compensatory increase in down-stream mechanisms, is unlikely to explain enhanced SR Ca^{2+} uptake in our rabbit heart failure model.

Adrenergic stimulation - effects on SR Ca^{2+}-loading

An alternative suggestion is that the 8 week-ligation animals did not have β-receptor down-regulation but did have increased circulating levels of catecholamines as a result of heart failure. This persistent catecholamine stimulation could produce chronic phosphorylation of the SR Ca^{2+} pump and result in enhanced Ca^{2+}-loading. This hypothesis is, however, strongly dependent on phospholamban remaining phosphorylated from the time the animal was killed until after the trabeculae were mounted and treated with saponin. Previous work has demonstrated that pre-treatment of single isolated cardiac myocytes with α and β-agonists, before chemical skinning, could produce phosphorylation of the contractile proteins, measured as changes in Ca^{2+} sensitivity, which persisted after chemical skinning treatment with *Triton* (Puceat, Clement, Lechene, Pelosin, Clapier & Vassort, 1990). This implies that, at least, the chemical skinning procedure does not necessarily cause dephosphorylation of intra-cellular sites but dephosphorylation could still occur in the period between death and the chemical skinning procedure in the rabbits in our study. Indeed, Puceat *et al* (1990) demonstrated that incubation of skinned single cardiac myocytes with alkaline phosphatase could reverse the phosphorylation state induced by adrenergic stimulation. This raises the question of whether phosphatase enzymes remain active within the cytosol and reverse the phosphorylated state of phospholamban during the period between death and measurement of SR Ca^{2+} uptake. It is possible that these enzymes may still be active even in the saponin-treated trabeculae since a number of other enzyme systems, including the mitochondria, are known to remain functionally intact in saponin-treated trabeculae (Miller & Steele, 1990, Steele & Miller, 1992). This

might explain why there was no measurable difference in the SR Ca^{2+}-loading between control animals given boluses of isoprenaline and the saline controls from the adriamycin study. That is, despite phosphorylation of the SR Ca^{2+} pump immediately before death, induced by isoprenaline, phosphatase enzymes remain active and dephosphorylate the Ca^{2+} pump before measurement of SR Ca^{2+}-loading is made. A second possibility is that all three control groups described in figure 7.3 have persistent phosphorylation of the SR Ca^{2+} pump which is present in all control trabeculae at the time of SR Ca^{2+} uptake assessment. This could result from high circulating levels of catecholamines in all animals prior to death as a result of the stress associated with handling. This might produce saturation of all intra-cellular phosphorylation sites such that further doses of isoprenaline would have no effect. One way to test this hypothesis would be to treat the preparations with protein phosphatase after the skinning procedure which would dephosphorylate any phosphorylated sites on phospholamban (Kranias, 1985). β-blockers, given to control animals for a period of time prior to sacrifice might also be expected to reduce stimulation by endogenous circulating catecholamines, reduce levels of intra-cellular cAMP and so produce a reduction in SR Ca^{2+}-loading compared with animals not given β-blocker.

Adrenergic stimulation - effects on the myofilaments

There was no difference in maximum force production in Triton-skinned fibres from Iso-OP and controls. This result is similar to other published reports of a maintained response to Ca^{2+} in cardiac myocytes despite evidence of β-receptor down regulation (Jones *et al*, 1990, Bohm *et al*, 1988). At the level of the myofilaments there is no mechanism by which *maximum* force production may be increased via adrenergic stimulation (Solaro, 1993). However, myofibrillar responsiveness to Ca^{2+} can be altered by catecholamines in a number of ways. Phosphorylation of the amino-terminal

region of troponin-I at Ser 20 increases following stimulation of the heart with catecholamines (Solaro, Moir & Perry, 1976). This results in an alteration in the Ca^{2+} binding properties of troponin-C and inhibits the ability of Ca^{2+}-troponin-C to release the thin filament from the troponin-tropomyosin complex. The net result is a reduction in the responsiveness of the myofibrillar proteins to Ca^{2+} and a rightward shift in the Ca^{2+}-tension relationship (Herzig, Kohler, Pfitzer, Ruegg & Wolffle, 1981). In my study, there was a slight rightward shift in the Ca^{2+}-tension relationship in Iso-OP animals suggestive of a reduction in myofilament responsiveness to Ca^{2+}. This could indicate a greater level of phosphorylation of sites on troponin-I in the chronically infused animals. However, there was no significant difference in the values obtained for myofilament Ca^{2+} responsiveness, represented as pCa_{50}, between the Sal-OP animals given isoprenaline immediately prior to sacrifice and the other control groups used in this thesis (e.g. RV Sham-ligation: 5.58±0.07, P=NS, RV saline-injected ADR controls 5.55±0.103; P=NS). This suggests that there was no significant phosphorylation of troponin-I produced by acute administration of isoprenaline. Alternatively, phosphorylation of troponin-I had occurred in all of the control groups and therefore no measurable difference in myofilament Ca^{2+} responsiveness could be revealed between groups.

In conclusion, therefore, there was no apparent change in the Ca^{2+}-loading ability of cardiac sarcoplasmic reticulum induced by chronic infusion of isoprenaline in rabbits. The phosphorylation state of the phospholamban in these preparations was not known and although there was some slight evidence for a myofibrillar phosphorylation in the chronically-infused rabbits, suggested by a decrease in the myofibrillar Ca^{2+} responsiveness, we could not be sure of the level of phosphorylation within these preparations after the skinning procedure was completed. If the phosphorylated state did persist, then one would expect that the control animals would be most

sensitive to catecholamines (i.e. having no adreno-receptor down regulation) and would demonstrate evidence of intra-cellular phosphorylation (enhanced SR Ca^{2+} uptake, reduced myofibrillar responsiveness to Ca^{2+}) while the chronically treated animals (and the heart failure animals) would not show evidence of intra-cellular phosphorylation. The possibility of persistent baseline phosphorylation of the SR Ca^{2+} pump has not been examined as a possible explanation of the difference in SR Ca^{2+} uptake observed in many previous studies of failing and normal myocardium.

CHAPTER 8

Sarcoplasmic Reticulum Ca^{2+}-loading in Ventricular Trabeculae from Patients with Heart Failure

INTRODUCTION

There are a number of mechanisms responsible for contractile abnormalities in heart failure including myocardial cell-slippage (Weber & Brilla, 1991), abnormal myocardial architecture, peri-cellular fibrosis, β-receptor down-regulation (Bristow, 1993) and abnormalities of myocyte Ca^{2+} regulation. Previous studies examining Ca^{2+} transients in failing human cardiac muscle fibres suggested that the rate of uptake of Ca^{2+} by the SR was reduced (Gwathmey *et al,* 1987). This was supported by evidence from a number of studies using isolated SR vesicles which demonstrated a reduced rate of uptake of radio-labelled $^{45}Ca^{2+}$ in heart failure (Movsesian, Bristow & Krall, 1989). Further evidence for a reduced activity of the SR Ca^{2+}-ATPase was obtained by demonstrating a reduced levels of m-RNA coding for this protein in failing human myocardium (Brillantes *et al,* 1991). While this evidence is fairly convincing there are a number of limitations to these studies.

The Ca^{2+} transients reported by Gwathmey *et al* (1989) suggest two phases to the Ca^{2+} transient in failing human myocardium. These are: (1). L1, which represents Ca^{2+} release from the SR (2) L2 - sarcolemmal Ca^{2+} influx via the voltage dependent Ca^{2+} channels. The authors propose that the amplitude of L2 is more pronounced in failing myocardium as a result of reduced re-uptake of Ca^{2+} by the SR . There are a number of problems with this result . Firstly, the method of loading aequorin into the cells requires high concentrations of EGTA which can damage a significant portion of the preparation. Preparations which demonstrated a greater than 30% fall in peak twitch during the aequorin loading procedure were discarded. This creates a rather biased selection procedure and additionally indicates that as much as 30% of the preparation was damaged in the chemical loading procedure in preparations which were deemed *acceptable.* Secondly, it is interesting to

note, that since the widespread use of more sensitive and ratiometric Ca^{2+} indicators in recent years, there have been no further reports of this type of biphasic Ca^{2+} transient associated with heart failure. A number of studies have reported a slower rate of fall of the Ca^{2+} transient in heart failure tissue but the amplitude of the transients have often been similar in control and failing hearts (Perreault, Gonzalez, Litwin, Sun, Armstrong & Morgan, 1993, Gwathmey & Morgan, 1985, Bing *et al*, 1991).

Measurements of Ca^{2+} uptake in isolated vesicles represents an interesting and rather simple way of assessing SR function. However, the vesicles are typically incubated over long periods with oxalate anions which act in a similar way to the endogenous protein, calsequestrin. That is, once Ca^{2+} is taken-up into the SR, calsequestrin binds to it and effectively reduces the free-$[Ca^{2+}]$ within the SR lumen. The SR Ca^{2+} pump is therefore not inhibited by high intra-SR $[Ca^{2+}]$, the gradient for Ca^{2+} across the SR membrane is maintained and Ca^{2+} uptake continues. There is, however, a finite quantity of calsequestrin within the SR and once the buffering capacity is exceeded then free Ca^{2+} will increase and inhibit the Ca^{2+}-pump. Oxalate, on the other hand, can pass freely into the SR where it forms an insoluble precipitate with the pumped Ca^{2+} ions providing an infinite Ca^{2+} accumulating capacity. The SR Ca^{2+}-pump is therefore never inhibited by intra-luminal $[Ca^{2+}]$ and continues to work at very high rates. Therefore, although the rate of Ca^{2+} uptake can be measured, there is no outward leak of Ca^{2+} as would occur physiologically. Furthermore, the uptake of Ca^{2+}, in isolated vesicles, is expressed per unit of protein (of SR vesicles) and so changes in the level of protein as a consequence of heart failure will confuse the interpretation of the calculated result.

Measurement of the m-RNA for the SR Ca-ATPase provides information on the turnover of this protein within the myocyte. It is assumed that a lower level of m-RNA represents a reduced expression of the protein. While this

may be a valid assumption it may not be representative of how the SR functions within the cell. One study which attempted to relate molecular biology with function demonstrated that the levels of m-RNA for SR Ca-ATPase could be negatively correlated with m-RNA for atrial naturetic peptide (Arai, Alpert, McLennan *et al*, 1993).

There have been no previous studies which have specifically examined SR function in saponin-permeabilised ventricular trabeculae in human heart failure. In this study, human RV trabeculae were obtained from patients undergoing cardiac transplantation. The right ventricle is more trabeculated than the left and provided free-running preparations of appropriate length and diameter for the experimental set-up. Additionally, the left ventricle tended to contain large infarcted areas of myocardium with widespread diffuse areas of fibrosis and so suitable preparations were extremely difficult to find with consistency.

METHODS

All tissue was obtained with written consent from patients undergoing elective orthotopic cardiac transplantation. These patients were assessed by coronary angiography and cardiac catheterisation 3±1.3 months before transplantation (range 1-5 months). Measurements of right and left sided cardiac pressures were done using a fluid filled system and were calculated from a paper trace obtained from an ink-jet pen recorder (Siemens Mingograph 7). Left ventricular ejection fraction was measured by radio nuclide blood pool imaging.

Transplantation was routinely performed during the hours of 2300 and 0500 and so all experiments were done in the early hours of the morning immediately after excision of the heart. The heart underwent in-situ cold cardioplegic arrest and, after excision, was plunged in cold cardioplegia solution (4-6°C, modified St Thomas' solution). The RV was opened along the length of the interventricular septum from the tricuspid valve ring towards the apex. A number of free-running trabeculae were excised from the free wall of the RV and were placed in cold Ringer containing 2,3-butanedione monoxime (30mM). These were immediately transported to the laboratory and a suitable preparation, from those dissected, was chosen under the dissection microscope. This was mounted for isometric tension measurement and placed in relaxing solution (solution B) containing saponin (50μg/ml) for 30 minutes. The trabecula was stretched to a length which provided a sarcomere length of 2.1-2.2μm and the preparation diameter (major and minor) was measured under light microscopy. The experimental protocol described in chapter 3 (figure 3.1) was followed. Caffeine contractures were evoked at a variety of $[Ca^{2+}]$ (150-400nM) and loading times (15-150s). At the end of the protocol assessing SR function, the trabecula was treated with Triton-X100 to remove all cell membranes leaving

only the myofilaments intact. C_{max} was measured in all preparations and the Ca^{2+}/force relation was assessed in 8.

Human control tissue

Various sources were pursued, including fast-track post mortem, to try to obtain fresh human tissue which could act as a control. Several experiments were attempted using this tissue but even when removed within 1 hour of death there was obvious rigor and no experimental data were obtained. On one occasion, tissue was near to being obtained from a donor heart turned down for transplantation on the basis of single vessel coronary disease but the relatives finally refused permission for experimental use.

RESULTS

Clinical details

Experimental data were successfully obtained on eighteen patients. Details relating to clinical diagnosis, drug therapy and severity of heart failure are described in table 6. The majority had an underlying diagnosis of ischaemic heart disease. However, eight patients were transplanted with a principle diagnosis of heart failure with New York Heart Association (NYHA) class IV symptoms, six were transplanted on the basis of left ventricular dysfunction but with an additional diagnosis of inoperable coronary artery disease and a further four patients had a diagnosis of idiopathic dilated cardiomyopathy (IDCM). Five had a history of previous coronary artery by-pass surgery. Five patients had a history of essential hypertension controlled on drug therapy and one had type IIa hyperlipidaemia.

All patients were taking Frusemide, ACE inhibitors and nine were anticoagulated with warfarin. Two patients were taking beta-blockers. One patient was taking amiodarone therapy at the time of transplantation and one was taking the lipid-lowering agent, bezafibrate. Six were taking digoxin and three were taking Ca^{2+} antagonists.

Examining the haemodynamic data, it is clear that there is a range of left ventricular dysfunction ranging from severe to moderate. Unfortunately, not all data was obtained for some of the measures of LV function such as cardiac output (CO), cardiac index (CI), right atrial pressure (RA) and maximum oxygen consumption. The data are complete for the measures of pulmonary capillary wedge pressure (PCWP), mean pulmonary artery pressure (MPA) and left ventricular ejection fraction (LVEF). For the purpose of some aspects of data analysis the IHD patients were separated into two groups on the basis of ejection fraction and pulmonary capillary wedge pressure. Those with more marked haemodynamic evidence of heart failure (LVEF of <19%,

PCWP>20mmHg) were defined as severe left ventricular dysfunction while those with less haemodynamic impairment (LVEF>20%, PCWP<20mmHg). were defined as having moderately impaired LV function. The patients with IDCM also had evidence of severe left ventricular dysfunction and, haemodynamically, are equivalent to the IHD group with severe LV impairment (all 4 patients had LVEF<19%, PCWP>20mmHg).

Table 6 - Patient Data										
Age	DIAGNOSIS	RA	MPA	PCWP	LVEF	CO	CI	urea	Creat	DRUGS
54	IDCM	10	30	20	14			7.2	121	Capto, Frus, Dig,Warf
40	IDCM	11	37	25	16	3.6		5.1	106	Frus,Dig,Capto,Amio,Warf,Hydral
39	IDCM	12	36	22	15	2.9		6.9	125	Frus,Dig, Enal,Warf, Nit
20	IDCM	6	32	22	11		2.1	8.6	114	Capto, Frus, Warf
43	IHD,CABG,MVR	11	35	32	8	2.4		9.9	95	Capto,Dig,Frus,Asp
49	IHD,CABG		30	22	11		1.7	7.2	102	Capto, Frus, Warf, Nit
45	IHD	13	33	25	8	3.8	2.7	10.2	103	Enal, Dig, Bur, Asp, Beza
44	IHD	5	28	22	7	4.3		4.3	93	Capto,Bumet,Ismo,Ranit,Warf
58	IHD,VF		35	30	8.7	2.1		7.7	114	Burinex,Enal,Warf,GTN
57	IHD		35	24	10.5	4.3	2.1	9.4	129	Enal,Dig,Frus, Nif
46	IHD,CABG		22	20	11	4	1.9	5.6	113	Enal,Frus,Temaz, Nicard
39	IHD	9	38	29	7.8			2.5	74	Capto, Frus, Amil Asp
42	IHD,VT/VF	8	21	17	21			3.6	89	Capto, Frus, Asp, Nit
54	IHD	9	19	10	28			2.3	82	Ranit,Dilt,Aten,Warf,GTN
53	IHD	1	20	2	22.5			6.8	111	Capto,Frus,Asp,Warf
47	IHD,CABG		23	8	22	3.8		11.1	134	Enal,Bendro,Dig,Allop,Frus
55	IHD	4	25	16	20	4.8	2.6	9.2	113	Enal, Metop, Frus, Asp
42	IHD, CABG		16	5	30		2.1	6.3	114	Capto,Frus,Asp,Warf

Isolated trabeculae experiments

SR function

Trabeculae used for experiments were typically elliptical in cross-section with mean major and minor diameters of 213.5±58.4μm and 85.9±25.5μm (mean±SD) respectively. Figure 8.1 illustrates the amplitude of caffeine contractures produced at various [Ca^{2+}] at 120 second loading periods for 15 patients studied. There was a wide range of SR loading abilities as assessed by caffeine contracture amplitude. The patients with ischaemic heart disease (IHD) were separated into two groups on the basis of left ventricular function as described above. There were six patients with reasonably well preserved ventricular function and eight with more severe heart failure. Patients with a diagnosis of IDCM (n=4) are also presented as a separate group.

Figure 8.2 illustrates the mean caffeine contractures produced after 30,60,90 and 150 seconds loading periods at various [Ca^{2+}]s for the 3 groups of patients. There was some separation in the SR loading abilities of the two IHD groups, with the moderate heart failure patients demonstrating a tendency for larger caffeine contractures, reflecting a greater SR loading ability. At 60 seconds loading period, the moderate LV dysfunction group demonstrated significantly larger caffeine contractures at lower [Ca^{2+}] and had produced contractures at 200nM loading [Ca^{2+}] while the more severe heart failure group had failed to produce contractures (0% vs 7.3±4.1% C_{max}; P=0.011). Differences between the three groups was most marked at 250nM loading [Ca^{2+}] at 60 seconds and was most pronounced at 150 seconds loading period (severe HF group 32.8±9.0 vs moderate HF group 65.2±11.3% C_{max}, P=0.016). However, at higher loading [Ca^{2+}], at all loading periods, there was no significant difference in the amplitude of caffeine contractures between the two IHD groups and between those and the DCM patients. The moderate HF group demonstrated a marked fall in caffeine contractures at [Ca^{2+}] above

250nM which was not an effect produced by averaging but is indeed present in a significant number of individual trabeculae (see figure 8.1). This pattern was seen in preparations exhibiting spontaneous oscillations at higher $[Ca^{2+}]$s.

Trabeculae from patients with IDCM demonstrated significantly smaller mean caffeine contractures than the moderate and more severe IHD groups at a range of $[Ca^{2+}]$s up to 300nM but at higher $[Ca^{2+}]$ there was no significant difference between any of the three groups. The shape of the loading curves was also different in that the DCM group did not show a plateau phase until $[Ca^{2+}]$ was greater than 350nM at 150 seconds loading. This is consistent with the observation that none of the DCM preparations demonstrated spontaneous oscillations at any of the conditions of $[Ca^{2+}]$ or time tested.

Using the range of SR loading abilities at 250nM $[Ca^{2+}]$ and 150 seconds loading it was possible to correlate clinical indices of heart failure and SR loading capacity. This loading condition of $[Ca^{2+}]$ and time was chosen because it showed maximum spread of data allowing a possible relationship between SR function and severity of heart failure to be established. A number of these relationships are illustrated in figure 8.3. There was a relationship between the ability of the SR to load Ca^{2+} and the severity of heart failure for a number of indices of LV dysfunction. The strongest relationship was with pulmonary capillary wedge pressure (PCWP, $r = -0.68$, $P<0.01$) but there was also a relation with mean pulmonary artery pressure (MPA, $r = -0.56$, $P<0.04$) and left ventricular ejection fraction (LVEF, $r = 0.59$, $P<0.05$).

Spontaneous oscillations

Spontaneous oscillatory behaviour was observed less often in trabeculae from failing human hearts than in those from failing rabbit hearts. The Ca^{2+}•time product at which oscillations first appeared in the loading protocol

tended to be greater i.e. longer loading periods and higher [Ca^{2+}] ([Ca^{2+}]•time product - 54.3±3.6 μMseconds), than in failing rabbit hearts (26.1±2.6 μM seconds). The [Ca^{2+}]•time product at which oscillations were first observed was slightly lower in the moderate heart failure group (48.2±3.8 vs 57.2±3.1 μM seconds). None of the four trabeculae from patients with IDCM demonstrated spontaneous oscillations. The first appearance of these oscillations in the SR loading protocol was not correlated with severity of LV dysfunction (PCWP - r = 0.09, P=NS).

Myofilament force production and Ca^{2+} sensitivity

Ca^{2+} sensitivity of the myofilaments was assessed in 8 preparations from patients with a range of clinical LV impairment. A typical experimental trace of a cumulative Ca^{2+} activation, used to assess the Ca^{2+}/force relationship in a human trabecula, is shown in figure 8.4. Hill equation curves were plotted to the data for seven of the patients studied (figure 8.5). There was a range of values for pCa_{50} (5.05-5.79) with a mean value of 5.45±0.19 for all 8 patients. The Hill coefficients for these patients ranged from 0.41-2.66 (mean 1.63±0.45). There was no significant relationship between the myofilament responsiveness to Ca^{2+} (pCa_{50}) or the maximum Ca^{2+}-activated force and the severity of heart failure (figure 8.6b).

Maximum force produced per unit cross-sectional area was not significantly different between the three heart failure groups. For the more severe heart failure group, the mean peak force production was slightly lower than the moderate heart failure group (2.92±2.15 vs 5.05±2.60gwt/mm^2; P=0.12). There was no significant difference between the IHD patients, as a single group, and the patients with IDCM (IHD 3.98±2.38 vs DCM 3.78±0.71gwt/mm^2; P=0.3) There was no correlation between the severity of heart failure as assessed by a number of clinical parameters and the maximum force developed by the Triton skinned preparation (Figure 8.6a; mean PAP, r = -0.19, LVEF, r = 0.18).

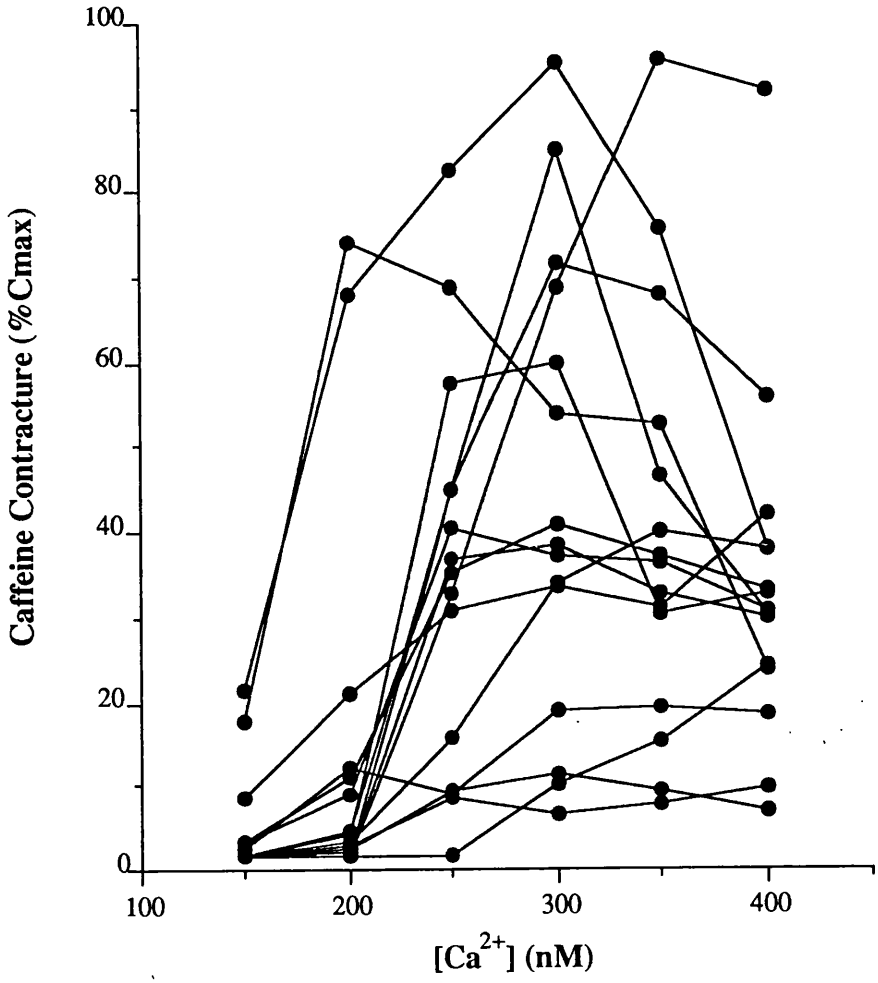
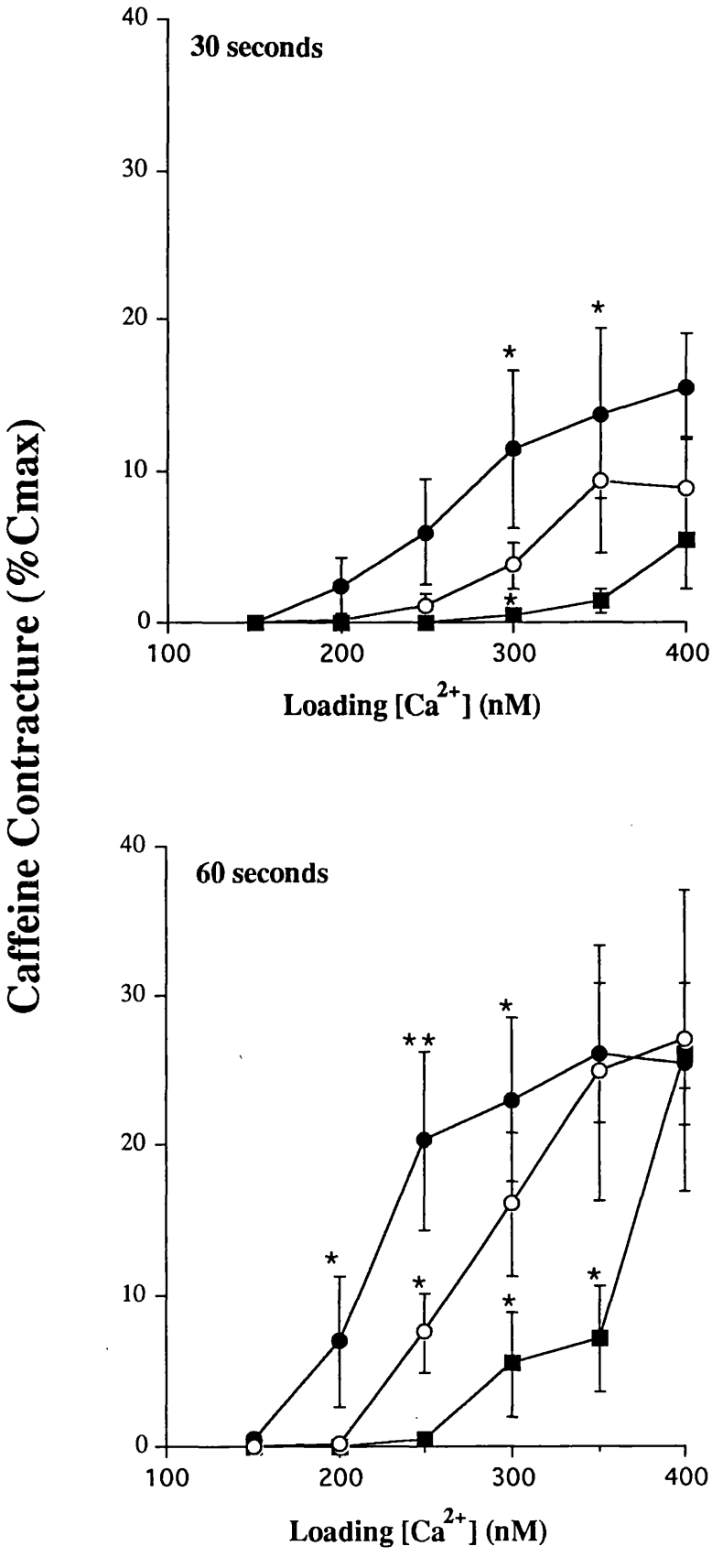


Figure 8.1 SR Ca^{2+}-loading (120 seconds) in human saponin-treated ventricular trabeculae.


30 seconds
60 seconds
Caffeine Contracture (%Cmax)
Loading [Ca2+] (nM)
Loading [Ca2+] (nM)
0
10
20
30
40
100
200
300
400

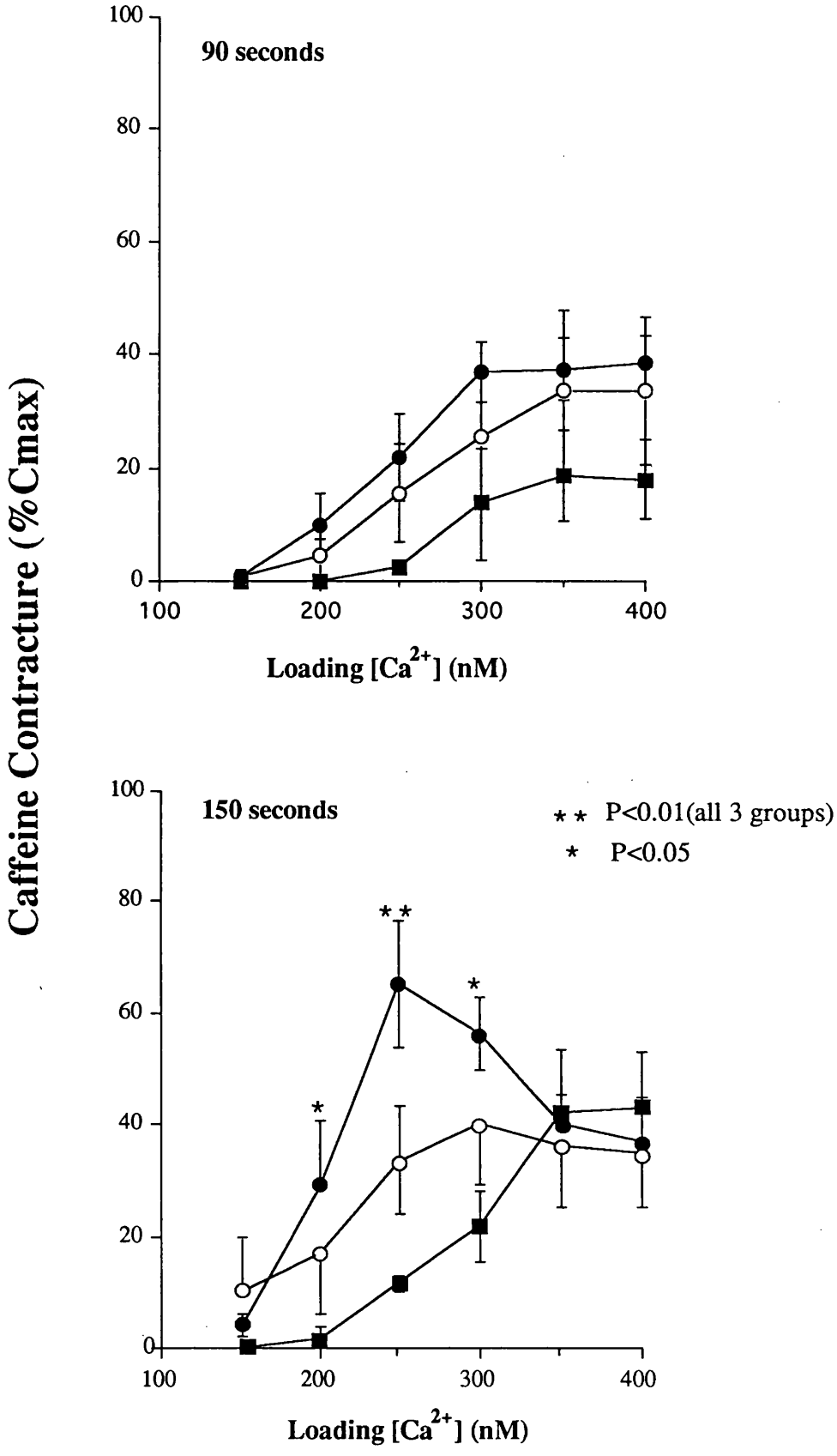
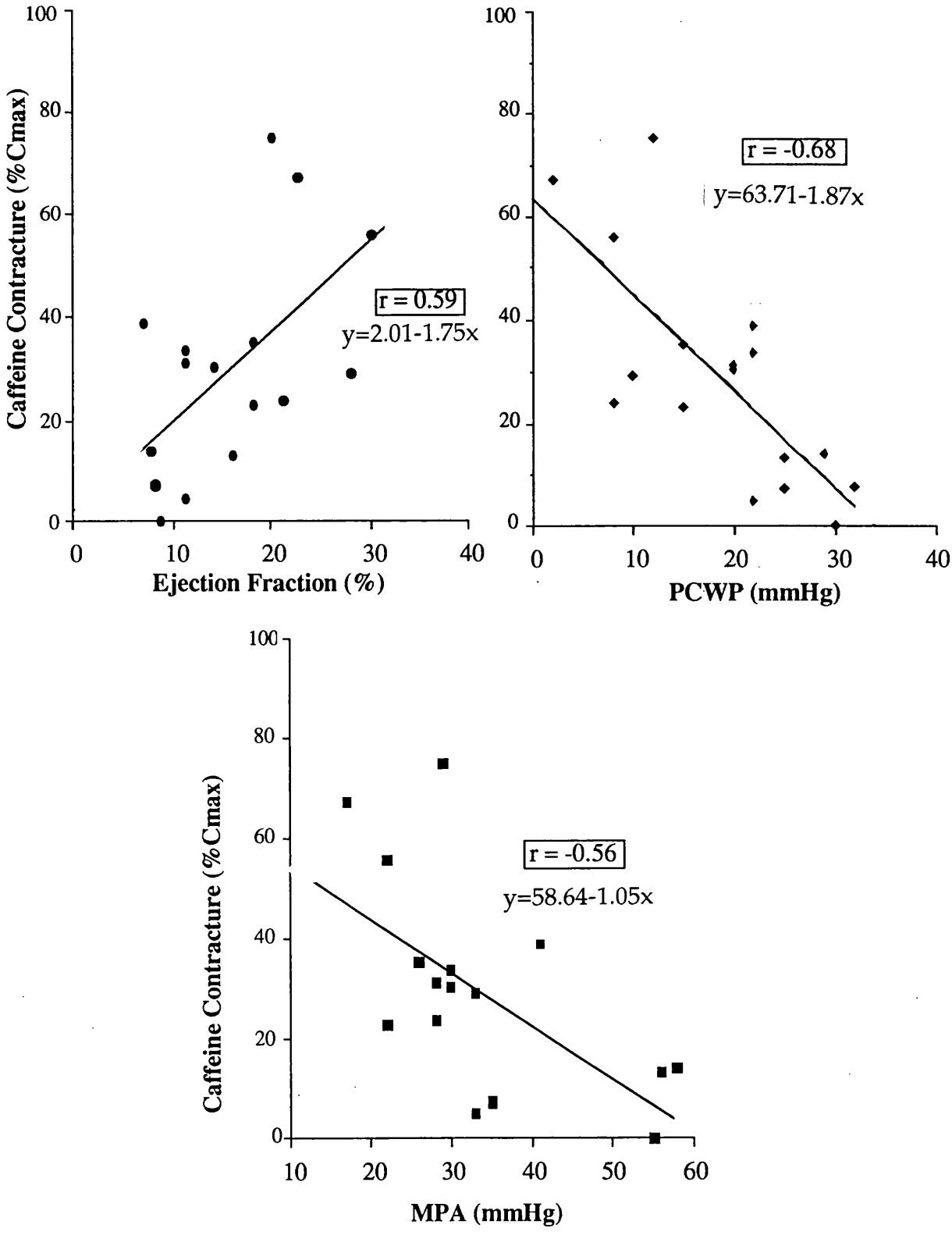


Figure 8.2 (and facing page) SR Ca^{2+}-loading at 30-150 seconds in human trabeculae from patients with severe (*open circle*, n=8) and moderate (*closed circle*, n=6) LV dysfunction, and dilated cardiomyopathy (*closed square*, n=4).

Figure 8.3 Various correlations of severity of heart failure with SR Ca^{2+}-loading ability (120s/250nM Ca^{2+}).



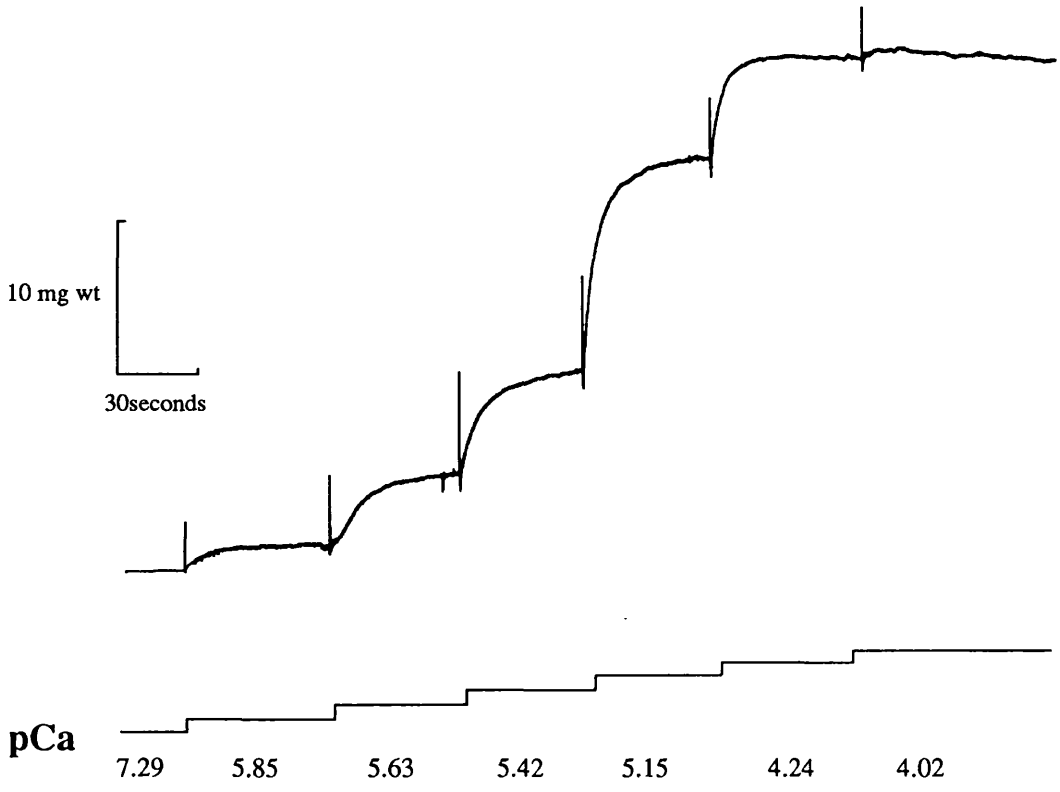


Figure 8.4 Experimental trace of a cumulative Ca^{2+}-activation curve in a human ventricular trabecula from a patient with heart failure.

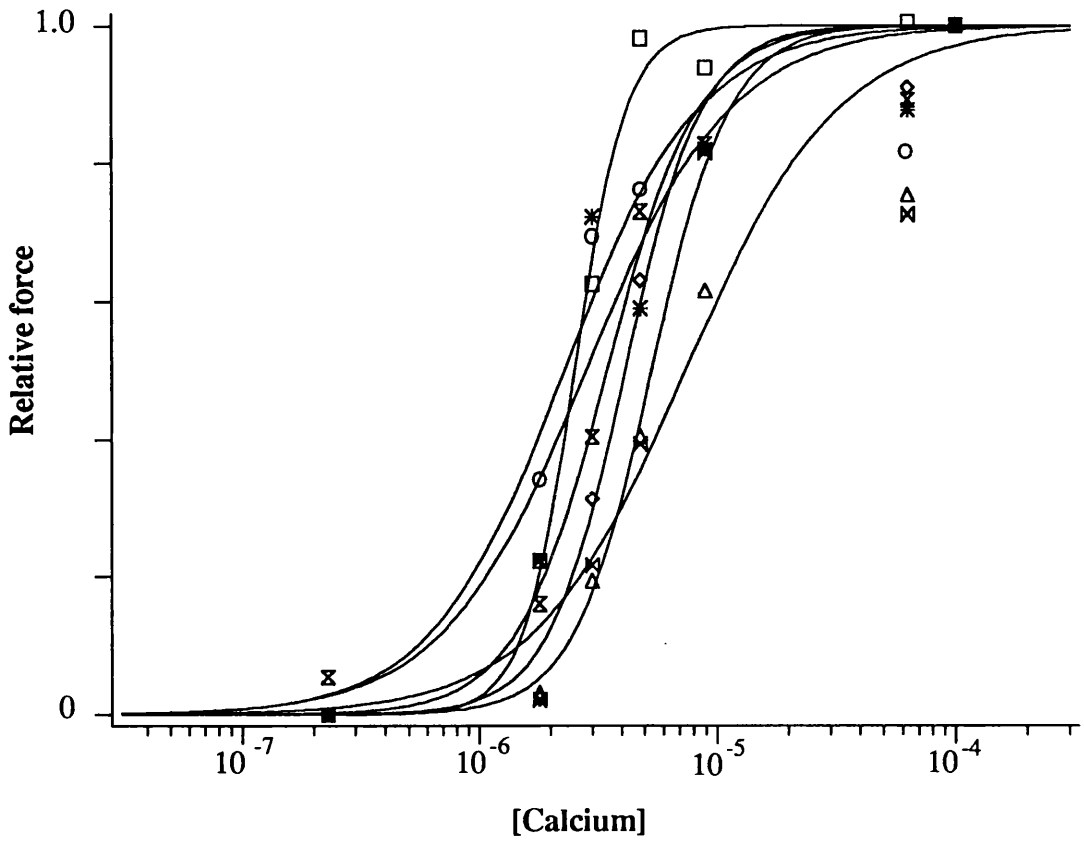


Figure 8.5 Ca^{2+} sensitivity curves of Triton-treated human ventricular trabeculae.

Figure 8.6a Correlation of maximum Ca^{2+}-activated force (C_{max}) of human trabeculae with severity of heart failure.

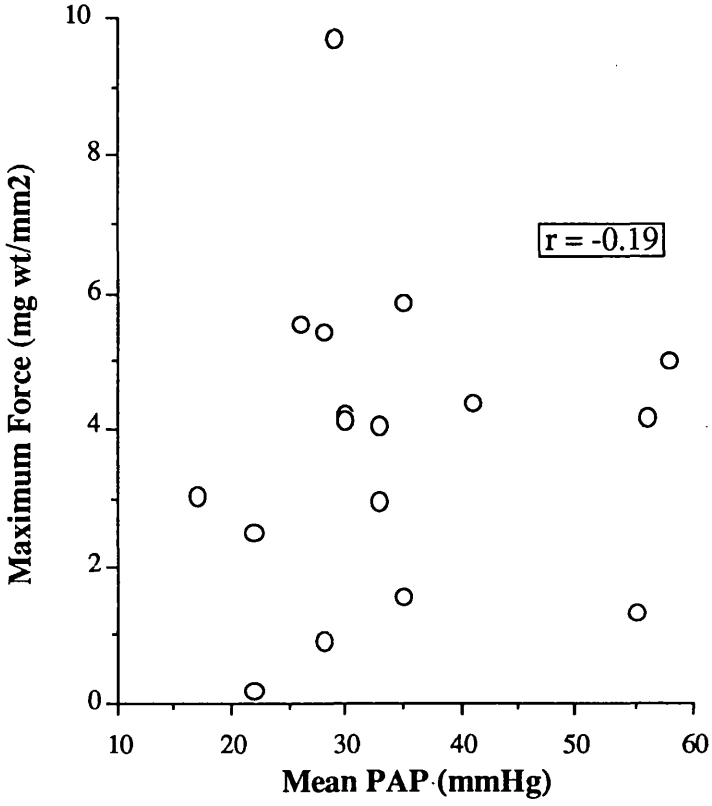
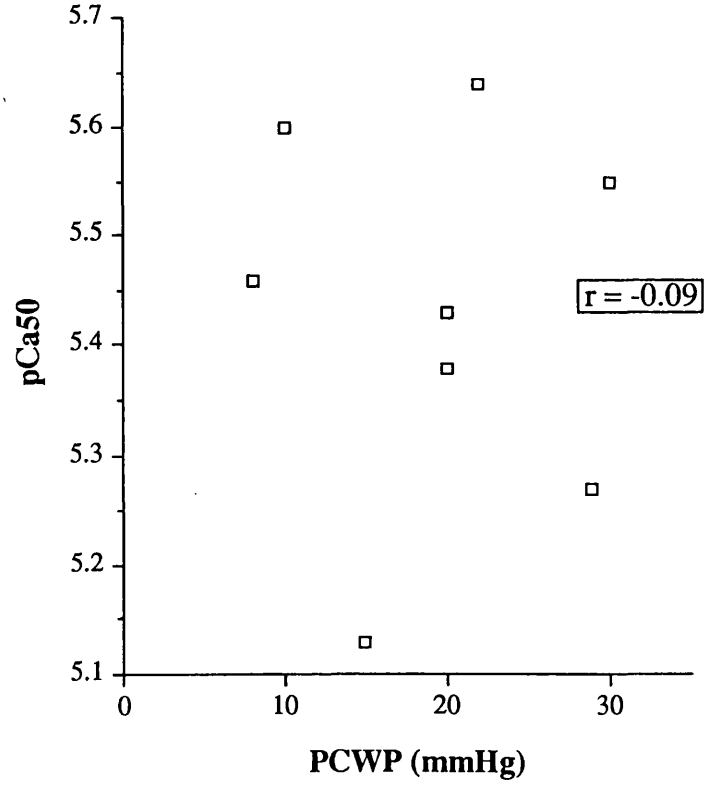


Figure 8.6b Correlation of Ca^{2+} sensitivity (pCa) of human trabeculae with severity of heart failure



DISCUSSION

The results from this study suggest, that with increasing severity of heart failure, the ability of the SR to load Ca^{2+} diminishes. SR Ca^{2+}-loading, reflected as the amplitude of the caffeine-induced contracture, was reduced in a sub-group of patients with more severe LV dysfunction compared with a group of patients with moderate LV dysfunction. Patients with idiopathic dilated cardiomyopathy demonstrated SR Ca^{2+}-loading capacity which was even lower than the IHD group with the most severe LV dysfunction. These IDCM patients did have severe LV impairment and the markedly reduced SR Ca^{2+}-loading ability may therefore indicate a more severe form of heart failure rather than a separate and distinct group. Previous work examining m-RNA indicated possible differences in the expression of a number of SR proteins between ischaemic and idiopathic dilated cardiomyopathy (Brillantes *et al*, 1992).

Correlation of SR function with severity of heart failure

SR loading capacity correlated with a number of clinical indices of left ventricular function. The strongest relationship was with pulmonary capillary wedge pressure which is an indirect measure of diastolic function of the left ventricle. However, the measures of SR function used *right ventricular* trabeculae, one must speculate that the same influences on relaxation in the LV were also active in the RV. This is unlikely to be the case, although, widespread RV and LV fibrosis in heart failure can produce diastolic dysfunction in both chambers. Ideally, it would have been more appropriate to obtain some measure of right ventricular diastolic function in order to attempt to relate SR function to RV relaxation. Right atrial pressure was obtained in some patients but was often not recorded. The relationship observed here between PCWP and SR Ca^{2+}-loading is therefore more likely to be due to an overall reflection of heart failure and left ventricular

performance. There was also a relationship with ejection fraction which is more a reflection of systolic function of the LV. Once again, there were some data available for RV ejection fraction but not in a sufficient number of patients to allow correlations. The relationship with mean pulmonary artery pressure probably also reflects the severity of left ventricular dysfunction although it may also reflect RV hypertrophy secondary to pressure and volume overload.

Clinical and haemodynamic data

The patient data of left ventricular function were acquired in the clinical setting during assessment of the patients suitability for transplantation. Clearly, there is a considerable possibility of error and variation in these measurements especially since treatment of heart failure can change the values obtained significantly. However, this can be interpreted as being of added value since they were obtained, in the clinical setting, in the routine clinical assessment of patients, with no bias towards the SR experiments finally performed. The measurements were made several months before the hearts were finally excised and the condition of some of the patients had deteriorated in the period immediately before the transplantation. This could certainly have altered the values for some of the measures of LV dysfunction. No attempt was made to correlate patient symptoms with SR function since most of the patients were in NYHA III-IV which is often a primary reason for inclusion on the transplant program.

Control human tissue

Without normal human tissue for comparison it is not possible to make direct statements as to the nature of the changes in SR function in heart failure. However, there are reasons why comparison of SR function within the same group of patients is more valid than attempting to use non-heart failure human controls. Normal human control tissue is now very difficult

to obtain and is often taken from patients that are brain-dead following cranial injury. Raised intra-cranial pressure is known to produce profound effects on cardiac performance, both at a clinical and cellular level (McLeod, Dwyer, Meyer, Richardson, Cruikshank & Bartlett, 1982). Additionally, brain-dead patients are often treated with large doses of inotropic drugs to maintain circulation in the terminal stages of their illness. Indeed potential donor-hearts are often rejected for transplantation on the basis that they are over-stimulated with inotropic drugs. It is these hearts which become available for experimental analysis as 'normal control tissue'. Some studies have used control tissue from post-mortem specimens (Hassenfauss *et al*, 1992). Unless these were obtained very rapidly the effect of warm-ischaemia is marked, in our experience, and will have a profound effect on contractile performance at a cellular level.

Patient drug therapy at the time of removal of the heart may have additional effects on cellular function. The recipient heart tissue may have been exposed to diuretics, ACE inhibitors, Ca^{2+} antagonists, beta-blockers and anti-arrhythmic drugs. Amiodarone, for example, has been shown to have effects on SR Ca^{2+} uptake (Watson, Balke & Gold, 1993). Clearly, control tissue is not exposed to this pharmacological cocktail. Most of the patients in this study were also given the immunosuppressant drug, cyclosporin, intravenously before going to theatre for transplantation. Cyclosporin is known to have effects on Ca^{2+} regulation within cardiac myocytes and so might alter SR function (Banijamali, ter Keurs, Paul & ter Keurs, 1993). Therefore, the tissue used for this study was from patients on a comparable baseline long-term drug regimen, treated in the same way in the period immediately before the heart was excised and all tissue was removed rapidly from the heart during cold-ischaemia. For these reasons a comparison of SR function between patients with heart failure undergoing transplantation is a more valid and controlled study.

Myofilament force production and Ca^{2+} sensitivity

Maximum force production by the myofilaments was not different between groups and did not correlate with severity of heart failure. This is similar to other reports that the myofilaments are intrinsically able to produce a similar quantity of force in heart failure. The Ca^{2+} sensitivity of myofilaments was not significantly different from that reported in other studies (Morano *et al*, 1988, Schwinger *et al*, 1994) and was not correlated with severity of heart failure. This indicates that measured force would accurately reflect Ca^{2+} in a similar way in all patients, regardless of the severity of heart failure. This is an important finding for this study since we did not measure Ca^{2+} directly. This result indicates that the differences observed in SR loading were due to changes in the quantity of released Ca^{2+} and not due to changes in response of the myofilaments to similar quantities of Ca^{2+}. This result also suggests that the co-operativity of the regulatory proteins is essentially unchanged even in severe heart failure. Changes in the regulatory proteins have been reported in human heart failure (Solaro *et al*, 1993). Changes in troponin-I have been demonstrated but how these changes influence function is less clear. There is no evidence for a significant change in myosin types in human heart failure as occurs in heart failure seen in small mammals.

Spontaneous oscillations

Spontaneous oscillations were observed in human trabeculae but these tended to occur at higher loading [Ca^{2+}] and longer loading times than had been observed in the rabbit model. There was no evidence of spontaneous SR Ca^{2+} release in any of the patients with IDCM. These patients had the lowest SR loading capacity and therefore this would suggest that the appearance of oscillations is more likely in SR which loads to a greater extent. There is some evidence that the probability of oscillations is related to the absolute

[Ca^{2+}] within the SR (Tripathy and Meissner, 1994). This suggests that the final intra-SR [Ca^{2+}] reached during the various loading conditions was lower in the IDCM trabeculae than in those trabeculae which demonstrated spontaneous oscillations. This implies that there was not only a reduction in the total quantity of releasable Ca^{2+} but that the final luminal SR [Ca^{2+}] was lower in those patients with IDCM and with more severe heart failure. This lower intra-SR [Ca^{2+}] would result from a reduced SR Ca^{2+}-pump activity and not simply from a reduction in the volume of SR. However, a change in the intra-SR Ca^{2+} buffering capacity might bring about a similar experimental result. For example, an increased level of calsequestrin within the SR of IDCM patients could reduce the probability of oscillations. However, studies of the calsequestrin gene have failed to demonstrate significant changes in expression of this protein in heart failure (Takahashi, Allen, Lacro *et al*, 1992). The conclusion from this must be that the rate of Ca^{2+} pumping is reduced, and therefore, that there is indeed a qualitative change in SR function and not simply a change in the quantity of SR.

Further support for this argument is revealed by examining the SR loading patterns of the three heart failure groups (figures 8.2). All three curves reach the similar maximum point at 400nM loading [Ca^{2+}] for each of the loading periods shown. This suggests that the total volume of SR is similar in each group but clearly the patients with more severe heart failure require higher loading [Ca^{2+}] to achieve this maximum. This also suggests that the SR volume is the similar but that the rate of Ca^{2+} uptake is different.

Skinned *vs* Intact preparations

The sarcolemma of preparations used in this study was permeabilised with saponin and therefore the contribution to developed force from Ca^{2+} influx via the voltage -gated Ca^{2+} channels could not be assessed. While a number of studies on 'sarcolemma-intact' cardiac muscle preparations have indicated a reduced rate of Ca^{2+} re-uptake, the amplitude of the Ca^{2+} transient has been

reported as unchanged in human heart failure (Gwathmey *et al*, 1987, Vahl *et al*, 1994). The Ca^{2+} transient in such tissue is made up of Ca^{2+} entry via the sarcolemmal voltage dependent channels and Ca^{2+} release from the SR. Since the bulk of evidence suggests that the SR Ca^{2+} content is reduced in heart failure then for the transient to be of the same size there must be compensation by increased influx across the sarcolemma. This theory has not been supported by other evidence suggesting a reduction in the m-RNA coding for L-type Ca^{2+} channels in heart failure (Takahashi *et al*, 1992, Brillantes *et al*, 1992). This discrepancy remains rather difficult to explain. However, reduced re-uptake of Ca^{2+} in the presence of increased sarcolemmal influx does provide conditions for cytosolic Ca^{2+} overload which is recognised as a possible mechanism for inducing spontaneous electrical activity by activation of the transient inward current. Thus, in addition to the effects on contractile function a reduced rate of re-uptake of Ca^{2+} will predispose the heart to arrhythmogenic conditions.

Consequences of reduced SR Ca^{2+}-loading ability

Reduced SR Ca^{2+} uptake will contribute to diastolic dysfunction by reducing the rate of relaxation of the myocyte. The diminished rate of uptake will also tend to reduce the Ca^{2+} content of the SR and therefore reduce the quantity of Ca^{2+} available for subsequent activation. During conditions of adrenergic drive the increased influx of Ca^{2+} across the sarcolemma may overwhelm the SR buffering capacity. This would produce Ca^{2+} overload and could generate arrhythmogenic activity.

Conclusions

These results indicate that with increasing severity of heart failure the ability of the SR to load Ca^{2+} becomes diminished. The data support the hypothesis that total SR volume is not reduced but that the rate of Ca^{2+} uptake is diminished. SR function was reduced to the greatest extent in patients with

dilated cardiomyopathy. Whether this represents a specific response to the cause of the cardiomyopathy or simply reflects more severe heart failure is not clear. Such a reduction in SR function would tend to predispose the myocyte to Ca^{2+} overload and delay relaxation.

CHAPTER 9

Effects of cyclic Adenosine Monophosphate (cAMP) on Sarcoplasmic Reticulum Ca^{2+}-loading in Ventricular Trabeculae from Rabbits and Humans with Heart Failure

INTRODUCTION

Adrenergic stimulation of cardiac tissue produces an increase in the intra-cellular second messenger, cyclic adenosine-monophosphate (cAMP). Adenylate cyclase is activated by way of a stimulatory GTP-dependent protein. ATP is converted to cAMP which activates protein kinase C and which, in turn, phosphorylates a number of intra-cellular sites including the Ca^{2+} channel on the sarcolemma, phospholamban on the sarcoplasmic reticulum and sites on the myofibrillar proteins. This results in an increased force of contraction and an increased rate of relaxation *i.e.* a positive inotropic and lusitropic effect. Therapeutically, this physiological mechanism is of considerable potential benefit for patients with heart failure. However, adrenoreceptor down regulation, resulting from chronic sympathetic activation, limits its effect in improving cardiac performance.

cAMP metabolism

cAMP is hydrolysed by the enzyme phosphodiesterase to the inactive 5'-AMP. Drugs which directly inhibit this enzyme, effectively, by-pass the adrenoreceptor increasing levels of cAMP by inhibiting its breakdown. Phosphodiesterase inhibitors, such as milrinone and enoximone, have proved successful in improving cardiac output and various haemodynamic indices (Colucci, Wright & Braunwauld, 1986) but have been associated with increased mortality with more long-term use in the treatment of heart failure (Packer *et al,* 1991). The reasons for this increased mortality are unclear but are thought to be related to an increased incidence of arrhythmias (Holmes *et al,* 1985) secondary to Ca^{2+} overload. The relative increase in levels of cAMP induced by PDE inhibitors is dependent on the level of background substrate available (Feldman *et al,* 1987) and on the background activity of the enzyme. The levels of PDE enzyme were found to be similar in isolated SR vesicles from failing and normal human myocardium

(Movsesian, Smith, Krall, Bristow & Manganiello, 1991) although the measurement in such myocardial extracts should not be interpreted as representative of the cytosol in the intact cell. Attempting to assess the functional activity of PDE enzymes in intact tissue is extremely difficult since many PDE inhibitors have direct effects on a number of cellular systems including the SR and myofilaments in addition to the effects produced indirectly via cAMP.

cAMP in heart failure

Pervious studies have demonstrated that the reponse of isolated cardiac myocytes to cAMP is abnormal in heart failure (Harding *et al*, 1994). These authors also reported that a diminished response to cAMP was also present in a guinea-pig model of β-receptor down-regulation. This suggests that adrenoreceptor changes in the sarcolemma have effects, downstream, on the mechanisms which control the reponse to cAMP. At what point downstream does this abnormality of response to cAMP exist? In isolated SR vesicles from rabbits with pressure overload hypertrophy Lamers and Stinis (Lamer & Stinis, 1979) demonstrated a reduced baseline SR Ca^{2+} uptake and this failed to reach control levels in the hypertrophied hearts after incubation with cAMP and protein kinase. However the relative increase was similar in controls and hypertrophied animals suggesting that the sensitivity of the cAMP mechanism remained intact. This result, however, does indicate that the abnormality lies further downstream than protein kinase C. Movsesian (1992) used a phospholamban stimulating antibody on isolated SR vesicles from failing human heart and could find no difference in the response of SR Ca^{2+} uptake. This result suggests, therefore, that the abnormality of response to cAMP lies between protein kinase C and phospholamban. However, it should be noted that, in this study by Movsesian, the baseline levels of Ca^{2+} uptake by SR vesicles was not significantly different between failing and control myocardium.

There are, therefore, conflicting reports on the response of failing myocardium to cAMP and the direct effects on Ca^{2+} uptake in 'in-situ SR' has not previously been directly evaluated in failing myocardial tissue.

Aim of this chapter

This chapter examines the direct effects of cyclic-adenosine monophosphate on SR Ca^{2+}-loading in saponin-treated ventricular trabeculae. The results obtained have general implications for SR Ca^{2+} regulation in heart failure but are also an interesting extension of the results obtained in chapter 3 (Ligation 8 weeks). The hypothesis follows from chapter 7 in which the possible difference in the phosphorylation-state of phospholamban was discussed. In 8 week LIG animals, the scope for further increase in SR Ca^{2+}-loading might be reduced compared with SH animals. Therefore, cAMP may increase SR Ca^{2+}-loading to a greater extent in the Sham-operated animals compared with LIG animals. By comparing the cAMP response of SR Ca^{2+}-loading in isoprenaline infused rabbits with the response in heart failure animals this will also allow an assessment of the effects of adrenoreceptor down-regulation alone. These results are compared with the SR response of failing human saponin-treated trabeculae to cAMP under identical loading conditions. The results of this part of the study are presented as a separate chapter because they represent a comparison of the two rabbit models and also of these models with the human failing trabeculae.

METHODS

cAMP: effects on SR Ca^{2+}-loading

The effect of cAMP on SR Ca^{2+}-loading was studied in rabbit and human right ventricular trabeculae : coronary ligation rabbits(LIG) with heart failure and sham-operated controls (SH); rabbits chronically infused with isoprenaline by mini-osmotic pump (Iso-OP) and saline infused controls (Sal-OP); human hearts explanted at the time of cardiac transplantation. Haemodynamic assessment was undertaken in the way described in chapter 2 for all rabbits studied. The patient details related to the human preparations are presented in table 6, chapter 8.

The trabeculae were prepared and mounted as previously described. Following treatment with saponin for 30 minutes, the preparation was placed in solution B with 200nM [Ca^{2+}] to allow the SR to load Ca^{2+} for 120 seconds. Caffeine (10mM for 5 seconds) was used to effect release of Ca^{2+} from the SR and evoke a contracture. A train of four contractures was produced at baseline and at various [cAMP]s ranging from 10^{-7} to 10^{-5} M. The mean of the final three contractures at each [cAMP] was calculated.

Ca^{2+} measurements

In two experiments, one using a trabecula from sham-operated animal and one using a ligated rabbit, Ca^{2+} was measured using the fluorescent indicator Fura-2 at the same time as measuring tension. The experimental set-up is described in chapter 2. The effect of cAMP on Ca^{2+}-loading was observed using a similar protocol to that used in the experiments where tension was measured alone.

cAMP: effects on the myofilaments

At the end of the SR protocol the preparation was treated with Triton to disrupt all cell membranes. C_{max} was assessed and the effect of 10^{-5} cAMP on

myofibrillar responsiveness to Ca^{2+} was also assessed in a small number of rabbit preparations.

Experimental note: cAMP stock solution

The sodium salt of cAMP was used, dissolved in solution B to make a stock which was frozen in 1ml aliquots and defrosted for use in each experiment. Quantities of this solution were added to the bath to obtain the desired concentration. Vigorous agitation with an electric stirrer ensured a rapid diffusion of the cAMP throughout the bath chamber . However, some of the observed delay in response to cAMP may be related to diffusion throughout the bath and into the preparation. The problem of lipid solubility of different types of cAMP, experienced by workers using intact preparations (Harding *et al*, 1994), does not apply in skinned preparations since the holes in the sarcolemma allow free access to ions and small molecules including cAMP.

RESULTS

Effects of cAMP on SR Ca^{2+}-loading

A typical response of cAMP on the caffeine-evoked contracture is illustrated in a rabbit and a human preparation in figure 9.1 (upper and middle panel). The amplitude of the caffeine contracture increased significantly after a period of approximately 2 minutes in both species. This delay in response was a consistent finding in all preparations which responded with the increase always occurring at least one cycle of loading and caffeine release after the addition of cAMP. There were a number of additional features of note. Firstly, the response to cAMP was all or nothing and did not follow a dose response relationship in any of the trabeculae studied. Secondly, this response was not observed in all preparations studied (figure 9.1, lower panel). A response to cAMP was defined as an increase in the CIC of >25% occurring at any of the [cAMP]s tested (figure 9.2). Using this definition, 90% (9 of 10) of the human trabeculae studied produced a response. However, only 20% (1 of 5) of the Sal-OP animals responded compared with 86% (6 of 7) of the Iso-OP animals but a similar number of preparations responded in the SH (5 of 8) and LIG groups (7 of 11). The third feature, which is not shown, was that after the initial increase in amplitude of the CIC with cAMP there was often a gradual fall in the size of the CIC over a period of 4-5 cycles of Ca^{2+}-loading and release. The possible mechanisms for this third observation are discussed.

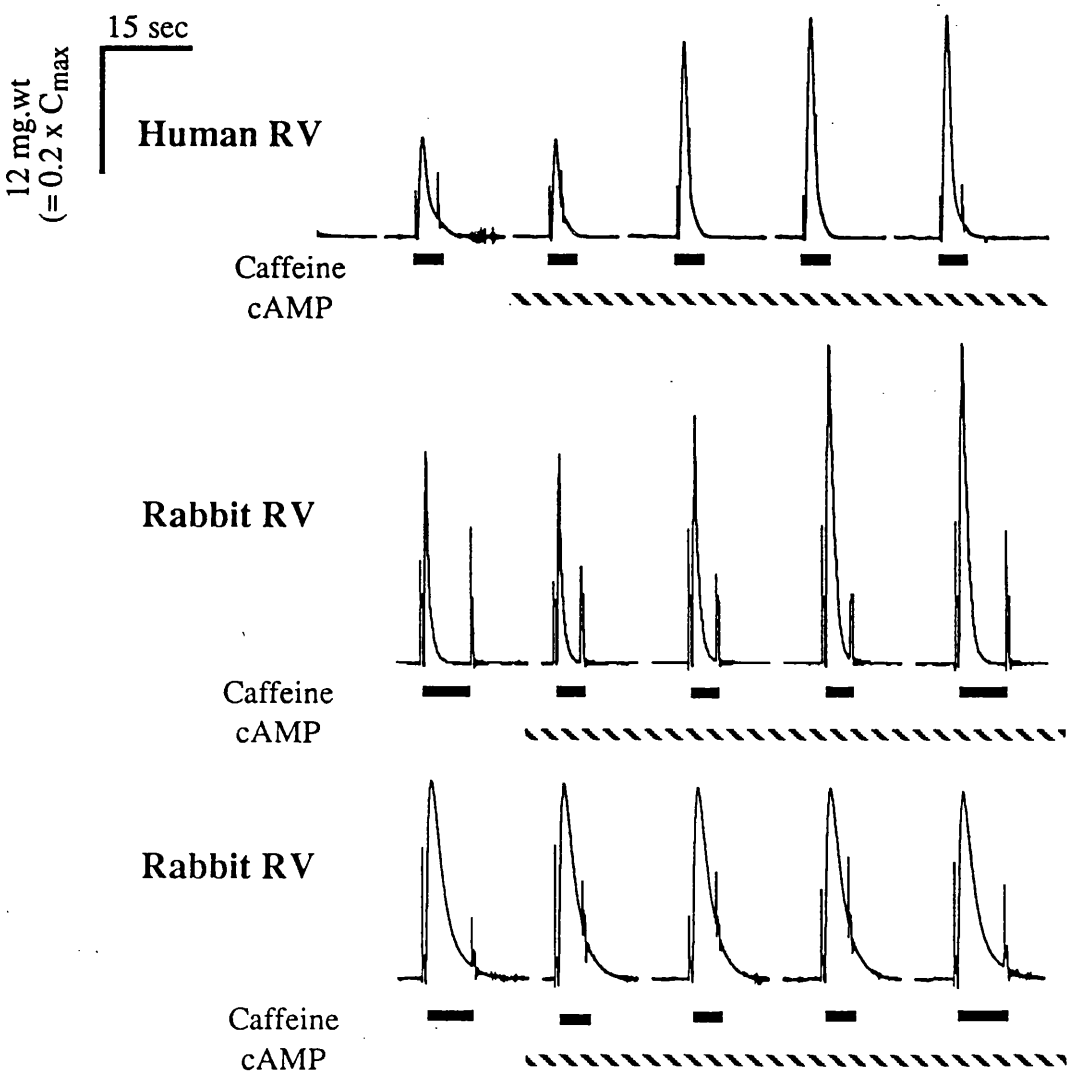
There are a number of ways in which the actual magnitude of response to cAMP may be illustrated. The first is to express the mean increase in the amplitude of the CIC as a percentage of the baseline level (figure 9.3). The second is to express the increase as a proportion of the maximum force produced by the trabecula (C_{max}) (figure 9.4). Using the former, the human trabeculae demonstrated a marked increase in the amplitude of the CIC of

approximately x4.5 fold (364.5±102.8% increase above baseline). The LIG animals demonstrated a slightly greater increase than the SH controls (170.7±60.4 vs 126.9±31.9% increase; P=0.58) but both are significantly less than the human (P=0.02,P=0.03). The Iso-OP animals demonstrated a significantly greater response than the Sal-OP animals (96.4±18.1 vs 33.4±7.6% increase, P=0.038). However, the SH group are not significantly different from the Iso-OP animals. When these responses are normalised to C_{max} the magnitude of the response to cAMP in terms of absolute force becomes more apparent. The human trabeculae produced a relatively large increase in SR Ca^{2+}-loading which was equivalent to a 0.2-fold increase (20.2±4.7%). LIG rabbits produced a similar increase in absolute force compared with the respective controls (LIG 8.3±2.86 vs SH 10.6±3.22 % C_{max}; P=0.55) while the isoprenaline treated animals produced a greater increase in force compared with controls (15.1±5.4 vs 2.2±1.1 % C_{max}, P=0.02). Although the Iso-OP animals appear to produce a greater increase in SR loading than the LIG or SH animals the difference did not reach statistical significance (P=0.48, P=0.30).

The baseline caffeine contractures, before application of cAMP, were not significantly different between groups (LIG 8.1±4.0, SH 12.4±6.0, Human 11.5±4.6, Iso-OP 14.5±3.4, Sal-OP 19.9±4.2 % C_{max}). The scope for further increase in the caffeine contracture, with respect to C_{max}, was therefore roughly similar between groups (approximately 80-90% C_{max}).

Trabeculae which demonstrated no significant response to cAMP, by the definition used above (<25% relative increase in CIC), tended to have larger caffeine contractures at baseline than the responders (27.4±7.4 vs 13.3±4.4; P=0.04) but this was clearly not close enough to C_{max} to limit the scope for increase. The relative increase in amplitude of the CIC in the 'non-responders' group was significantly lower than any of the responding groups (10.3±3.9 %) and represented only a very small proportion of C_{max} (2.3±1.8 %).

Figure 9.1 Experimental trace of the response of human and rabbit saponin-treated trabeculae to cAMP. The SR is loaded in 200nM [Ca^{2+}] for 120 seconds, the subsequent challenge with caffeine induces a Ca^{2+} release and a contracture results. The increase in amplitude of the caffeine contracture is due to enhanced SR Ca^{2+}-loading as a result of phosphorylation of phospholamban. The lower trace is from a SH rabbit which failed to demonstrate a significant response to cAMP.



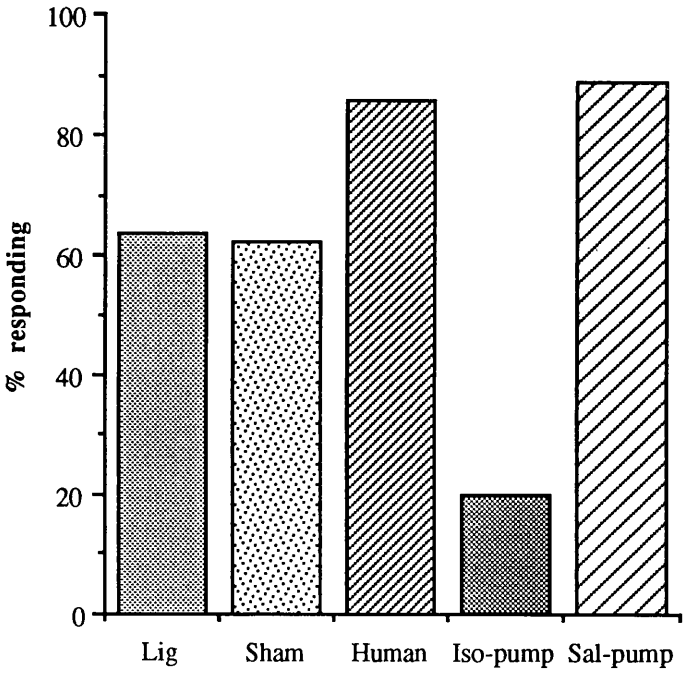


Figure 9.2 Proportion of trabeculae from human (n=10) and rabbit models (LIG n=11, SH n=8, Sal-OP n=5, Iso-OP n=7) demonstrating a response to cAMP (>25% increase in baseline caffeine contracture).

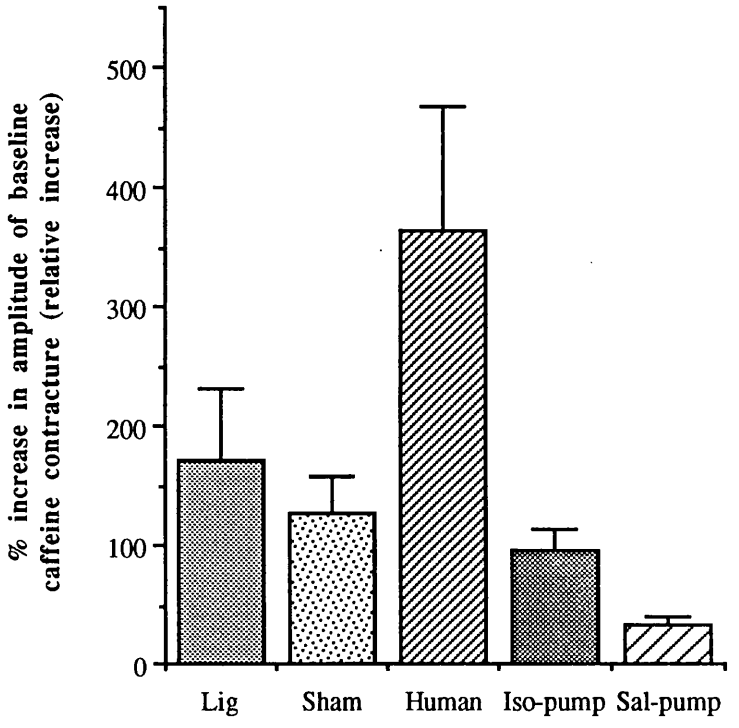


Figure 9.3 Effect of cAMP on the amplitude of the caffeine contracture relative to baseline caffeine contracture (numbers are as in figure 9.2).

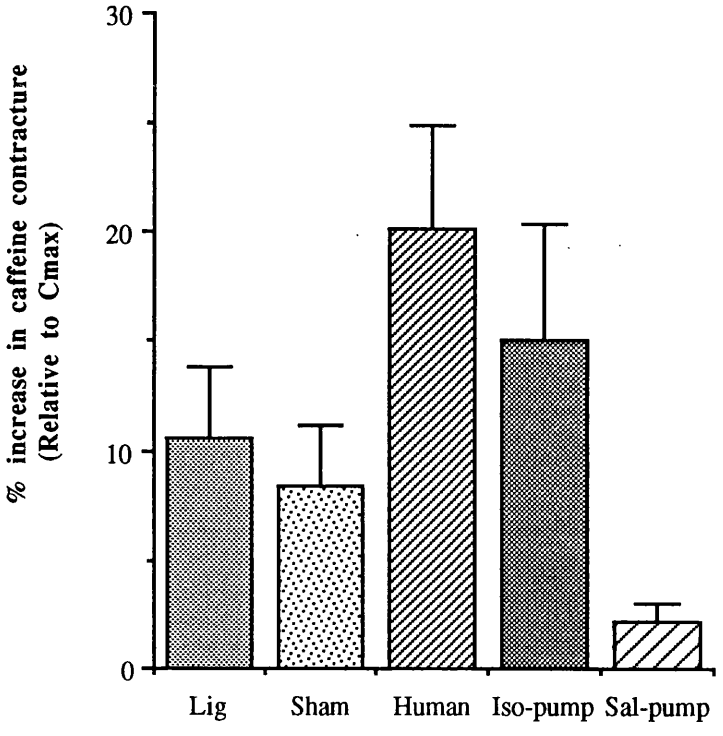


Figure 9.4 Effect of cAMP on the amplitude of the caffeine contracture relative to C_{max} (numbers are as in figure 9.2).

Ca^{2+} measurements

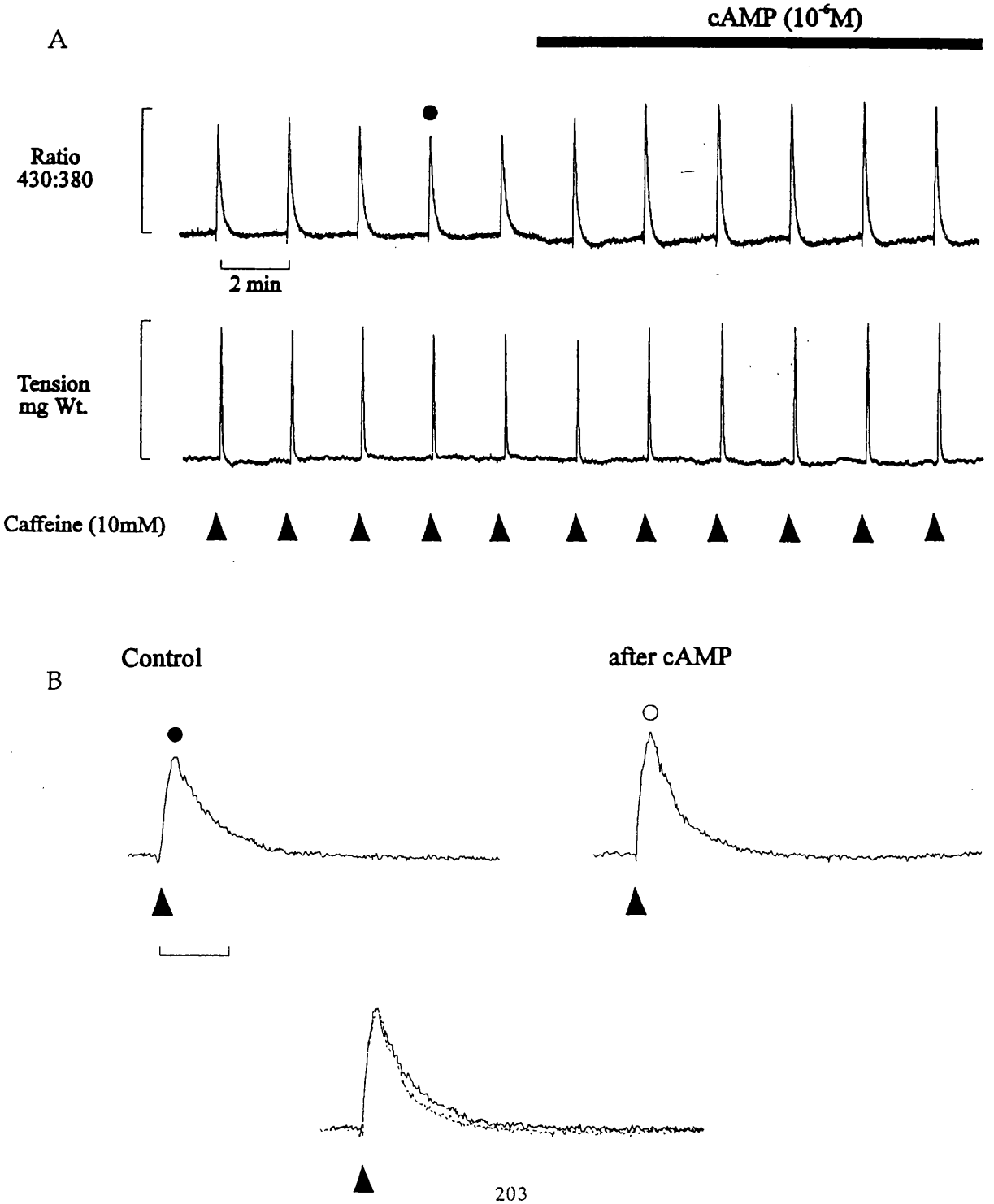
The LIG rabbit in which SR Ca^{2+}-loading was measured using Fura-2 demonstrated a modest increase in the amplitude of the Ca^{2+} transient (figure 9.5, panel A) following application of 10^{-6} cAMP (24.1±2.6% increase in peak [Ca^{2+}]) associated with a small increase in the tension transient (8.8±3.6%). The SH trabeculae failed to demonstrate an increase in either the Ca^{2+} or tension transient in response to cAMP.

In addition to the increase in the amplitude of the Ca^{2+} transient, there was a slight increase in the rate of fall of the Ca^{2+} transient in the LIG rabbit studied with Fura-2 (figure 9.5, panel B). This increased rate of decline corresponded with the increased peak light transient observed in this rabbit. In addition the overshoot in the Ca^{2+} trace normally observed immediately after the Ca^{2+} transient was more marked after cAMP was applied suggesting that the rate of Ca^{2+} uptake was significantly increased. The SH rabbit Trabecula failed to show an increase in the peak Ca^{2+} transient and demonstrated no change in the rate of decline of the Ca^{2+} transient

cAMP : effect on myofilaments

The time to peak-twitch (TPT) of the CIC was not significantly different between the rabbit groups at baseline (LIG 1.8±0.8s, SH 1.9±1.0s, Iso-OP 1.7±0.9s, Sal-OP 2.1±0.7s; P=NS). However, the human trabeculae demonstrated significantly longer TPT than all of the rabbit groups (3.2±1.6s; P=0.048). Following treatment with cAMP there was no significant change in TPT in any of the rabbit groups and no significant change in the human trabeculae (3.8±3.1s). The direct effects of cAMP was tested on a number of Triton-treated preparations. There was no effect produced on myofilament force production, at sub-maximal activations (result not shown), in any of the rabbit models tested.

Figure 9.5 Simultaneous Ca^{2+} and tension measurements in a saponin-treated ventricular Trabecula from a rabbit with heart failure (LIG). The arrow heads represent the brief application of caffeine. Panel A demonstrates the response of caffeine-induced Ca^{2+} transient (upper trace) and tension (lower trace) to cAMP (10^{-6}M, thick bar). There is a 15-20% increase in the amplitude of the Ca^{2+} transient over a period of 2-3 cycles of loading and release. There is only a small increase in the amplitude of the caffeine induced contracture. Panel B demonstrates the Ca^{2+} transient before cAMP (closed circle) and after the addition of cAMP (open circle) to the bathing solution. The traces are normalised and super-imposed in the lower part of panel B to demonstrate the more rapid decline in the Ca^{2+} transient following application of cAMP.



DISCUSSION

The response to cAMP in saponin-treated trabeculae tended to occur as an all-or-nothing increase in the amplitude of the caffeine contracture rather than as a dose-response relationship. In addition, the effect of cAMP was only very slowly reversible and so once an effect was observed it was not possible to return to a true baseline state. For practical reasons the concentration of cAMP was increased by a factor of five each time but it is possible that this was not tuned finely enough to reveal a dose-response relationship. The apparent step-like response to cAMP may have been due to a very steep dose-response relationship between cAMP and phospholamban phosphorylation. Reviewing the literature, few workers observing the effect of cAMP on SR Ca^{2+} uptake have reported their results in the form of a dose-response relationship. Indeed, previous work in this laboratory, using rat ventricular trabeculae, also demonstrated a similar step-like response to cAMP (DS Steele, PhD thesis, 1990). It is possible that a threshold concentration is required to activate protein kinase which, once activated, causes phosphorylation of all sites on the SR such that further increases in [cAMP] are ineffective.

A response to cAMP was not observed in all trabeculae (figure 9.1, lower panel). I have represented this as the proportion of trabeculae responding by a >25% increase in amplitude of the baseline caffeine contracture (Figure 9.2). Although this seems an arbitrary cut-off, it reduces the effect of averaging for a number of caffeine contractures and does, in fact, reflect the actual increase, or lack of increase, in caffeine contractures observed during the experiment. It is assumed that those trabeculae failing to show a response in the tension transient failed to increase the amount of Ca^{2+} loaded by the SR. This may not be the case since an increase in the Ca^{2+} transient in a rabbit trabecula with only a very small increase in the size of the caffeine contracture was

observed. This finding might be explained by a simultaneous increase in SR Ca^{2+}-loading and a reduction in myofilament Ca^{2+} responsiveness. Therefore, an alternative way of interpreting an absent response to cAMP is that the more dominant effect was a reduction in myofilament Ca^{2+} sensitivity with a smaller increase in Ca-uptake while those which did respond produced a more marked increase in SR Ca^{2+} uptake and little change in Ca-sensitivity. However, in another experiment where Ca^{2+} and tension were measured simultaneously, cAMP had no effect on tension or Ca^{2+} even at high concentrations (result not shown) indicating a complete failure to respond. One can only conclude, therefore, that since Ca^{2+} was not measured directly in the majority of the experiments, some of those trabeculae, but not all, which apparently failed to respond may have produced an increase in SR Ca^{2+} uptake which could not be detected by measuring tension alone.

The relative increase in the caffeine contracture produced by cAMP was greatest in the failing human trabeculae studied (figure 9.3). However, there is no human control tissue with which to compare this result. Therefore, although it represents an interesting comparison with the rabbits, it would not be appropriate to make definitive statements concerning the responsiveness of failing human myocardium to cAMP. The LIG animals demonstrated a slightly greater response to cAMP than the SH but this failed to reach statistical significance. The Iso-OP animals did show a significantly greater response to cAMP than the respective controls although the response was no greater than in the LIG or SH animals. When this increase is expressed in terms of proportion of C_{max} then the human and Iso-OP rabbits demonstrate the greatest increase in force production with the LIG animals only slightly greater than the SH controls. This finding suggests that the size of response to cAMP might reflect the degree of β-adrenergic receptor down-regulation - the human hearts followed by the Iso-OP rabbits then LIG rabbits.

The responsiveness to cAMP might, therefore, be a reflection of the severity of heart failure and the degree of resulting β-adrenergic receptor down-regulation. One possible reason why the Sal-OP rabbits are less responsive than the SH group could be that they were given boluses of isoprenaline immediately before sacrifice. This may have caused phosphorylation of all intracellular sites and, because the process is slowly reversible, this state persisted such that no further effect could be produced *in-vitro*. The LIG and Iso-OP animals however, because of β-receptor-down regulation, had sites available for phosphorylation which resulted in increased SR Ca^{2+}-uptake and an increase in amplitude of the caffeine contracture.

cAMP in heart failure

These data suggest that the response of SR Ca^{2+}-loading to cAMP is, at least, maintained in heart failure which is similar to other reports in isolated SR vesicles (Movsesian, 1992, Lamars, 1977). This maintained response is somewhat different from other reports in isolated failing myocytes (Harding *et al*, 1994). Clearly, the nature of the preparations in these studies are different and this may explain the differences observed in functional response to cAMP. The results from this study and the studies on isolated SR vesicles take no account of changes in sarcolemmal Ca^{2+} flux in response to cAMP. Therefore, these apparently conflicting reports can be interpreted together to suggest that if the response to cAMP is diminished in intact failing myocytes then the SR might not be the limiting factor.

The mechanism responsible for the diminished response to cAMP, observed by Harding *et al*, might not due to diminished Ca^{2+} uptake by the SR and could be the result of reduced influx of Ca^{2+} via voltage dependent Ca^{2+} channels. Such reduced influx might delay the process of Ca^{2+}-induced Ca^{2+} release and reduce the Ca^{2+} available for myofilament activation compared with control tissue. There is evidence supporting a reduction in the number of dihydropyridine-sensitive Ca^{2+} channels in heart failure (Takahashi,

Allen, Lacro *et al*, 1992) and this has often been proposed as a mechanism for contractile dysfunction in the failing heart. The hypothesis suggested is, therefore, that cAMP is able to increase SR Ca^{2+} uptake in the failing heart but the sarcolemma is unable to increase the influx of Ca^{2+} to support a significant increase in contractile function. There is, however, evidence which indicates that the response to isoprenaline in (non-failing) isolated myocytes is mainly mediated via an increase in Ca^{2+} uptake by the SR (Sham, Jones & Morad, 1991). This was demonstrated by using phospholamban stimulating antibody to stimulate SR Ca^{2+} uptake resulting in an increase in contraction amplitude with the subsequent addition of isoprenaline failing to increase contraction further. The authors interpret this finding as a marked SR-dependence of inotropic response to isoprenaline. Since we found the SR to have a normal or enhanced response to cAMP in heart failure then it seems unlikely, therefore, that a simple explanation of reduced Ca^{2+} influx via voltage-gated channels could be responsible for the depressed response in the intact single myocyte reported by Harding *et al.* (1994)

Time-course of response to cAMP

Previous studies have made no reference to a time-delay in the response of SR Ca^{2+}-loading to cAMP. In this study, the increase in SR Ca^{2+} uptake often occurred within 2-4 minutes of adding the drug to the solution. Admittedly, some of this delay was related to diffusion from the solution into the preparation but some of the delay could have involved activation of endogenous protein kinase and subsequent phosphorylation of phospholamban. It is possible that in 'sarcolemma-intact' preparations the increase in Ca^{2+} influx via the sarcolemmal Ca^{2+} channel, mediated by an increase in intra-cellular cAMP, occurs at a slower rate than the increase in SR Ca^{2+} uptake. This would result in a faster rate of relaxation at a time when there had been no increase in absolute force production. Such an uncoupling of Ca^{2+} regulation has not been previously reported although Harding *et al*

(1994) commented only on maximum amplitude of contraction, not on rate of relaxation and also did not comment on the time delay in the response to cAMP. However, if the long-term toxicity of drugs which increase cAMP is related to Ca^{2+} overload, as has been suggested, then perhaps a slower process of increased $[Ca^{2+}]_i$ occurs via the sarcolemmal Ca^{2+} channel over a period of days, or even weeks, which eventually overcomes the SRs' ability for Ca^{2+} re-uptake resulting in Ca^{2+} overload and generation of arrhythmias.

cAMP and the myofilaments

The apparent fall-off in the tension transient with cAMP which follows the initial increase could have been due to a number of factors. Firstly, a straight-forward decrease in sensitivity of the myofibrils for Ca^{2+} may occur, thus, despite an increased Ca^{2+}-loading by the SR, the tension reponse to this increased quantity of releasable Ca^{2+} was diminished. The direct measurements of Ca^{2+} provide some evidence to support this suggestion since we observed that despite an increase in Ca^{2+}-loading of the SR the caffeine contracture failed to increase significantly which must represent a decrease in myofilament Ca^{2+} sensitivity. The second suggestion is that oscillatory Ca^{2+} release from the SR increases as a result of cAMP (see figure 6.2, increased oscillations following cAMP). This would tend to deplete the SR pool of Ca^{2+} and reduce the size of the caffeine contracture. These oscillations might not be observed on the tension trace because of a simultaneous reduction in myofilament Ca^{2+} sensitivity induced by cAMP.

There was no effect of cAMP on the Triton skinned fibre. This is not surprising since enzymes, including protein kinase C, are lost from the cytosol as a result of this treatment . However, these preparations had already been treated with cAMP and therefore myofilaments may already have been phosphorylated. Addition of further cAMP would therefore have had no effect on Ca^{2+} sensitivity. There was some indication that the myofilaments did show a decrease in Ca^{2+} responsiveness (figure 7.4).. These preparations

had all been treated with cAMP in the saponin treated state and so this shift in response to Ca^{2+} may have been related to persistent phosphorylation of troponin-I.

Conclusions

The ability to manipulate intracellular levels of cAMP represents a potentially important mechanism for the treatment of heart failure. Early studies of PDE inhibitors were encouraging with reports of significant improvements in both haemodynamic indices and patient symptoms. This study suggests that the response of the SR to cAMP is maintained in heart failure and is possibly even enhanced. Therefore if the increased mortality associated with long-term use of PDE inhibitors is related to Ca^{2+} overload, then the mechanism which brings this about must be related to increased influx of Ca^{2+} across the sarcolemma or a secondary reduction in the SR uptake of Ca^{2+} with chronic exposure to the drug. Alternatively, the initial improvements in heart failure produced by PDE inhibitors could be associated with a reduction in sympathetic stimulation which in turn replenishes the β-receptor population. Thereafter, combined PDE inhibition and a return of β-receptor sensitivity might then cause a marked increase in the level of intracellular cAMP which would result in Ca^{2+} overload.

CHAPTER 10

General discussion

GENERAL DISCUSSION

A number of previous studies have examined SR function in animal models of heart failure and virtually all have suggested reduced cardiac SR Ca^{2+}-loading ability although there have been a few where enhanced SR function was observed such as in mild pressure overload hypertrophy (Limas *et al*, 1980) and after training (Greenen, White & Lapman, 1987). The results presented in this thesis from the rabbit models are therefore at odds with the weight of evidence in the literature. Before discussing each chapter in turn, therefore, it would be appropriate to discuss the validity of the technique used for assessment of SR Ca^{2+} content.

The caffeine-contracture as an indicator of SR Ca^{2+}-content

Tension was used as a 'bio-indicator' of SR Ca^{2+} content. That is, it was assumed that when the SR contained more Ca^{2+} for release this resulted in a larger contracture following application of caffeine. The question which arises is whether the caffeine-induced contracture was accurately reflecting SR Ca^{2+} content?

Firstly, we demonstrated that the Ca^{2+}/force relation was not significantly different between LIG and SH trabeculae, assessed in Triton-treated preparations, under steady state conditions. To exclude the possibility that caffeine was having a differential effect on LIG and SH myofilaments during caffeine-induced contractures the effects of 10mM caffeine on the myofilaments was examined. This demonstrated that the shift in Ca^{2+} sensitivity produced by caffeine was similar in LIG and SH rabbits (figure 4.9). These experiments indicate that the enhanced SR Ca^{2+}-loading observed in LIG trabeculae was not the result of increased responsiveness of the myofilaments to Ca^{2+}.

Secondly, the release of Ca^{2+} by caffeine was examined in LIG and SH rabbit trabeculae. These experiments showed that 10mM caffeine released all

'caffeine-releasable' Ca^{2+} from the SR and this effect had saturated in both ligation and control rabbits at the concentration used in the SR experiments i.e. 10mM (figure 6.1). Thus, one may conclude that caffeine was having a similar effect on Ca^{2+} release in the two groups. However, it is theoretically possible that in control rabbits, maximal concentrations of caffeine (even 50mM) released proportionally less of the total SR Ca^{2+} content compared with LIG rabbits. This would result in an apparent increase in SR loading in LIG trabeculae. The SR in control trabeculae might load Ca^{2+} to the same extent and at the same rate as LIG trabeculae but might only release *e.g.* 50%, of that loaded when challenged with caffeine compared with *e.g.* 100% in the case of LIG preparations. Previous work has indicated that caffeine might not release all Ca^{2+} from the SR (Fabiato, 1985, Smith, McAinsh & Steele, 1994). However, the saponin-treated preparations used in this study were 'plunged' into caffeine causing a rapid increase in the [caffeine] within the preparation. Under these conditions, the release of Ca^{2+} by caffeine may, itself, generate Ca^{2+} release by the mechanism of Ca^{2+}-induced Ca^{2+} release (Fabiato, 1985) and, therefore, it is likely that all Ca^{2+} was released from the SR in these preparations.

In separate experiments, Ca^{2+} and tension were measured simultaneously in saponin-treated trabeculae from a ligation and a sham-operated rabbit, using the Ca^{2+} indicator Fura-2. These experiments indicated that the SR Ca^{2+}-loading curve, represented by the tension transient, followed a similar pattern to that of the Ca^{2+} transient in both the LIG and SH rabbit (figure 10.1). Although the numbers are small, this implies that the measurements of tension alone, in the majority of experiments performed in this thesis, was indeed reflecting Ca^{2+} release from the SR under the various conditions, of $[Ca^{2+}]$ and loading-time, applied.

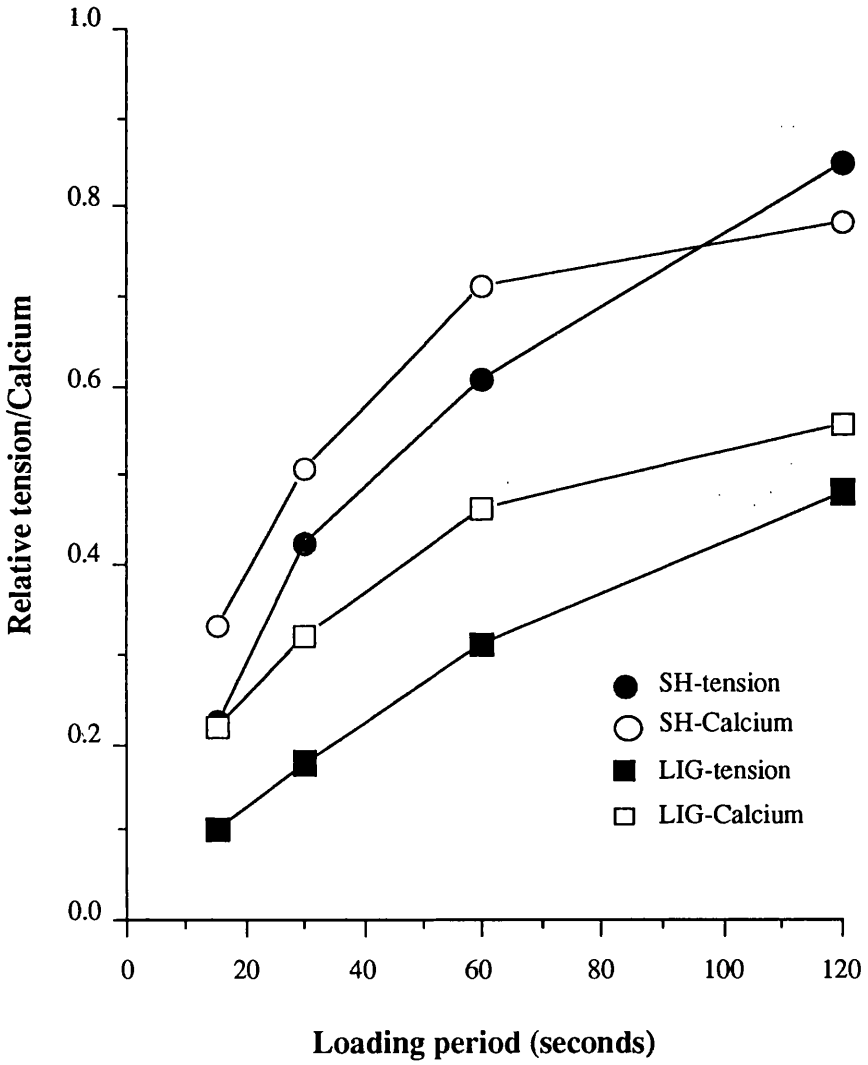


Figure 10.1 Comparison of the tension and Ca^{2+} transients to assess SR Ca^{2+}-loading in a ligation and sham trabecula (loading [Ca^{2+}] 200nM). The amplitude of the sham Ca^{2+} signal (*open circle*) and tension signal (*closed circle*) show a parallel response to increasing loading periods. A similar pattern is seen in the ligation Ca^{2+} (*open square*) and tension signal (*closed square*). These values for LIG and Sham rabbits are normalised to their own maxima and comparison between the amplitude of signals is not possible (hence why the LIG appear lower than SH).

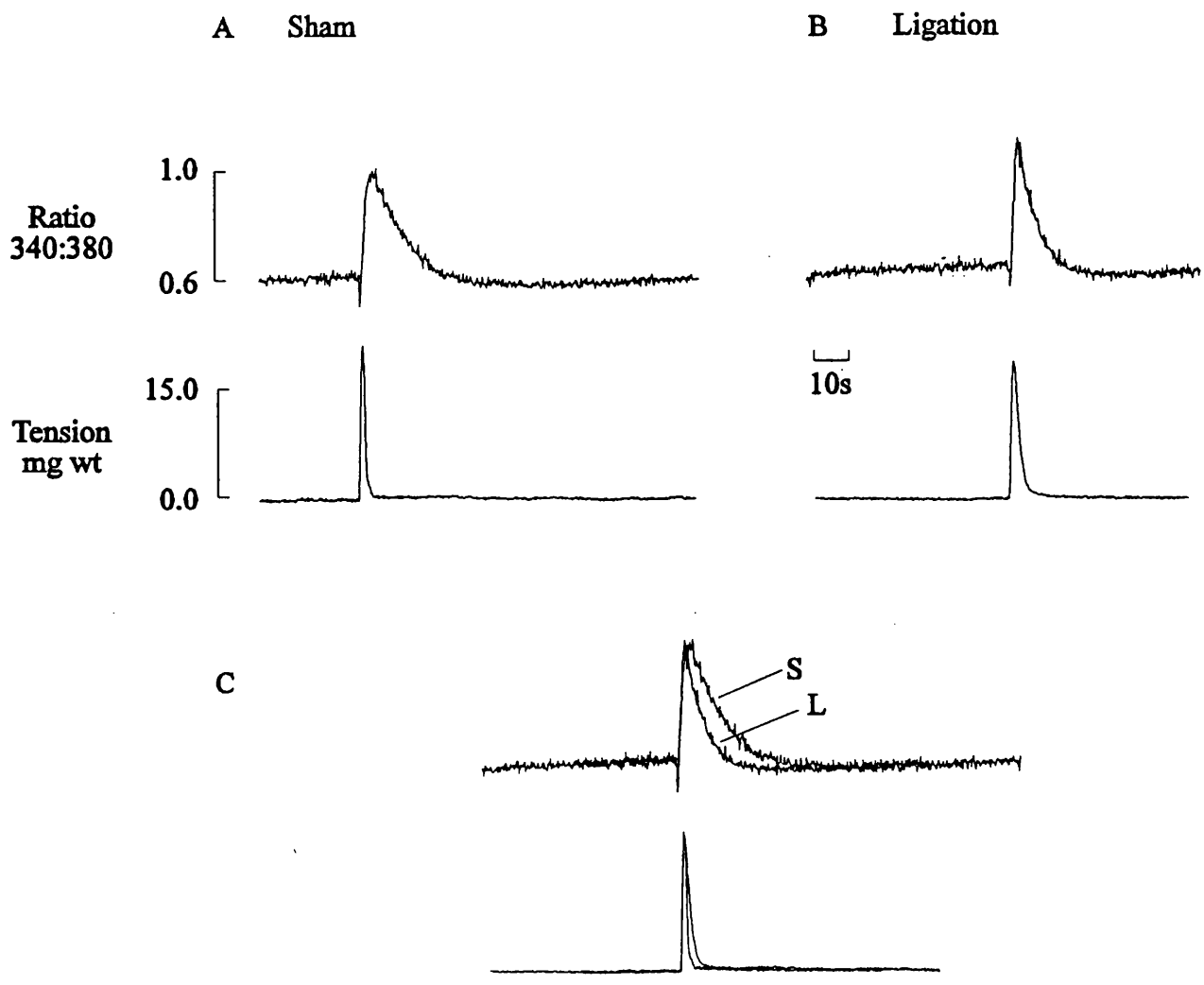


Figure 10.2 Representative Ca^{2+} (upper noisy trace) and tension transients from a Sham (Panel A) and ligation (Panel B) trabecula. The trabeculae used for these experiments were of similar size (~200μm diameter). The Ca^{2+} transient from the sham is of a similar amplitude to the ligation but has a slower rate of decay. The tension transient is, however, more brief in the sham. The Ca^{2+} and tension transients are super-imposed in panel C demonstrating the more rapid decay phase in the LIG trabecula.

A further interesting observation in these experiments was that the rate of fall of the Ca^{2+} transient was more rapid in the LIG Trabecula (figure 10.2) suggesting that there was a faster rate of Ca^{2+} re-uptake by the SR. This falling phase of the Ca^{2+} transient is dependent on diffusion characteristics of the individual preparation but can be affected by the rate of Ca^{2+} uptake by the SR (as demonstrated by the effects of cAMP, figure 9.5). This provides further evidence to support the finding of enhanced SR Ca^{2+} uptake in LIG rabbit hearts.

Taken together, this evidence suggests that the caffeine contracture was a valid indicator of SR Ca^{2+} content in saponin-treated trabeculae in this study.

Adriamycin-induced cardiomyopathy

There was evidence that the SR Ca^{2+}-loading ability was greater in those animals with evidence of significant myocardial toxicity secondary to adriamycin administration. Previous studies have suggested that the long-term effects of adriamycin may be the result of block of the Ca^{2+} release channel of the SR thus rendering it less leaky (Dodd *et al,* 1993) while others had suggested increased leak from the SR (Pessah *et al,* 1990). The results from the present study suggest either that the SR Ca^{2+} pump is more active or Ca^{2+}-leak is reduced after chronic adriamycin treatment. Since adriamycin is known to inhibit membrane pumps (Boucek *et al,* 1987) then it is more likely that the enhanced Ca^{2+}-loading resulted from a reduction in Ca^{2+}-leak.

This finding may be considered further by examining the relative uptake and leak of Ca^{2+} from the SR under steady state conditions.

The rate of uptake of Ca^{2+} by the SR can be described by the following equation:

$$\frac{d[Ca^{2+}]_i}{dt} = \frac{[Ca^{2+}]_o V}{K_o + [Ca^{2+}]_o} - \frac{[Ca^{2+}]_i V}{K_i + [Ca^{2+}]_i} - D([Ca^{2+}]_i - [Ca^{2+}]_o)$$

(Hara & Kasai, 1977)

The first part of the equation (on the right) represents the rate of Ca^{2+} uptake by the SR Ca^{2+} pump and is dependent on the Ca^{2+} concentration outside the SR ($[Ca^{2+}]_o$), the second represents the efflux from the SR by active transport (dependent on SR internal Ca^{2+} concentration ($[Ca^{2+}]_i$)) and the third portion represents the passive flux of Ca^{2+} across the SR membrane, as defined by Fick's first law, where D is a diffusion constant. It is clear from this simple equation that a reduction in efflux of Ca^{2+} from the SR (second part) would result in an increase in the rate of uptake. Similarly, a reduction in both the first and second parts might result in no net change in the measured rate of uptake.

This principle of altering SR Ca^{2+}-loading ability by changing the balance of uptake and release provides a more interesting interpretation of changes in SR function rather than simply proposing a change in the rate of the SR Ca^{2+}-ATPase pump.

Coronary ligation model of heart failure

This proved to be an extremely reliable and reproducible model of left ventricular dysfunction in the rabbit. The spectrum of heart failure which results is beneficial in that it allows correlation of measurements made in the whole animal with those made at a cellular level over a range which spans severely impaired to essentially normal left ventricular function (but with an infarct present). In this way, ligation animals provide a complete group since the most appropriate 'control' is a rabbit with an infarct but no significant LV dysfunction.

8 weeks following ligation there was evidence of significant LV dysfunction which correlated with the ability of the SR to load Ca^{2+}. In a group of animals with the most consistent evidence of the heart failure, there was a marked increase in SR Ca^{2+}-loading (in both RV and LV) compared with a group with less severe heart failure which, in turn, also demonstrated a greater SR Ca-loading than controls. At 15 weeks, the ability of the SR to load Ca^{2+} had returned to baseline levels, in RV trabeculae, with no significant difference from control hearts despite evidence of a similar degree of left ventricular dysfunction to that found in 8 week ligations. This suggests that there may be changes in SR Ca-loading which are not related to deterioration of left ventricular function but which occur with increasing duration of heart failure. Similar changes were reported by Afzal *et al* (1992) in a rat infarct model where SR Ca^{2+}-uptake increased at 8 weeks then fell below control values 15 weeks after infarction. This period of increased uptake, early in the development of heart failure, could represent a period of compensation which is followed by a gradual fall in SR Ca^{2+}-uptake. In my study, at 15 weeks, it is possible that SR Ca^{2+}-uptake had not yet fallen below control values but if allowed to continue, beyond this time, a further fall might be observed.

Human SR function in heart failure

The human preparations studied represented a spectrum of clinical disease from very severe to moderate LV dysfunction. The average duration of disease was approximately 3 years although the range was much larger (0.8-7 years). In these patients there was a clear trend for SR Ca^{2+}-loading ability to be negatively correlated with severity of heart failure (or put alternatively -positively correlated with LV function). Indeed, some of the patients with moderate LV function demonstrated caffeine contractures close to C_{max} which represents a greater SR Ca^{2+} content than has previously been estimated in the human (Bers, 1991). This could be interpreted as enhanced

SR Ca^{2+}-loading during moderate LV impairment in the human similar to that observed in the 8 week ligation rabbits. The human trabecular numbers are small but the results in chapter 8 support the idea that the SR may respond to hypertrophy in a biphasic way - a period of enhanced function followed by a fall below control values as heart failure becomes more severe.

Adreno-receptor down-regulation

There was no evidence to suggest that chronic infusion of isoprenaline produced a significant change in SR function in the rabbit. However, although there was some evidence for down-regulation, there was clearly a higher resting heart rate and cardiac output in the isoprenaline treated rabbits consistent with a retained response to chronically high circulating catecholamines. Chronic phosphorylation of phospholamban (for 7 days) produced no measurable change in SR Ca^{2+}-loading. It is possible that phosphorylation, present in the heart before the animal was killed, did not persist beyond saponin-treatment as a result of the action of endogenous phosphatase enzymes. This did, however, raise the question as to whether the baseline state of phosphorylation of SR-phospholamban could influence the Ca^{2+}-loading ability as measured in the saponin-treated fibre. Saline-infused rabbits (control mini-osmotic pumps), given boluses of isoprenaline within 15 minutes of being killed, failed to demonstrate an increased rate of SR Ca^{2+}-loading compared with other control animals not given isoprenaline immediately before death (figure 7.3). There are three possible reasons for this finding: (i) dephosphorylation by endogenous phosphatases occurs rapidly before skinning or (ii) dephosphorylation occurs during saponin treatment or (iii) all the controls were equally phosphorylated as a result of high endogenous levels of catecholamines immediately prior to excision of the heart.

Variation in the level of SR-phosphorylation is important since it may account, at least in part, for the reduced SR Ca^{2+}-loading observed in severe

human heart failure where levels of phospholamban have been reportedly reduced (Arai, Alpert, MacLennan, Barton & Periasamy, 1993). There is certainly a need to repeat these experiments both in the rabbit and in the human under conditions where intracellular phosphorylation is minimised. This could be done in the rabbit by administering β-blockers intra-venously immediately before excision of the heart, placing β-blocker in the Ringer solution used for dissection or by bathing the saponin-treated trabecula in a solution containing a protein phosphatase enzyme before assessing Ca^{2+}-loading.

Response to cAMP

The hypothesis being tested in this chapter was that the SR Ca^{2+}-pump was more phosphorylated in LIG rabbits and would respond less to cAMP than controls. Ligation and sham rabbits demonstrated a similar response to cAMP with the LIG rabbits showing a tendency for a slightly greater response. The isoprenaline treated rabbits also demonstrated an increased response to cAMP compared with controls. The greatest increase in SR Ca^{2+}-loading in response to cAMP was observed in human preparations. These results indicate that the preparations in which a reduced level of phospholamban was expected demonstrated the greatest sensitivity to cAMP. The reason for this rather paradoxical result is unclear but there are a number of possibilities. Firstly, the levels of phosphodiesterase enzyme may be reduced in those failing hearts therefore reducing the breakdown of exogenously applied cAMP. Secondly, the levels of protein kinase may be different and thirdly the activity of phosphatase enzymes may also be different. The ideal experiment (which is planned) would be to, firstly, treat the preparation with phosphatase enzyme then apply phospholamban stimulating antibody and observe the increase in SR Ca^{2+}-loading. This removes the problem of intermediary metabolism of cAMP and places the SR from failing and non-

failing tissue in a similar baseline state before phosphorylating phospholamban by exogenous means.

Changes in SR function during the progression of heart failure

The combined results of this study of SR Ca^{2+} regulation in rabbits and humans with heart failure suggest that there may be a biphasic response of the SR to progressive left ventricular dysfunction. Early after a myocardial infarction there is a period of compensation where SR Ca^{2+}-uptake is enhanced. As heart failure becomes more severe or prolonged there is a reduction in Ca-loading ability which eventually reduces below control levels.

There are a number of ways in which these changes might occur but one hypothetical scheme is described in figure 10.3. This scheme takes account of the findings of this thesis with enhanced uptake at 8 weeks, normal (or not different from controls at 15 weeks) and diminished Ca^{2+}-loading in humans with a longer history of heart failure. Since the rate of Ca^{2+} uptake by the SR is the result of the net Ca^{2+} loaded minus the net Ca^{2+} leaked then, by implication, the relative proportion of Ca^{2+} pumps and Ca^{2+} release channels will determine the quantity of Ca^{2+} loaded by the SR during any given loading period (*i.e.* Net Ca^{2+}-loaded = Ca^{2+}-uptake - Ca^{2+}-leaked). Since very few studies have indicated that the rate of the SR Ca^{2+} pump is increased in heart failure this model assumes that there is, initially, no change and then a gradual fall in the rate of Ca^{2+} uptake (open circle). For the total or net Ca^{2+} loaded to be increased there would require to be a more rapid fall in the number of Ca^{2+} release channels (open squares). This combination of changes would result in an initial rise in net SR Ca^{2+}-loading (closed circles), a fall back to baseline values and then a reduction as both Ca^{2+}-pump and leak rates reduced further. The curve representing the net Ca^{2+} loaded is simply the arithmetic sum of the other two curves.

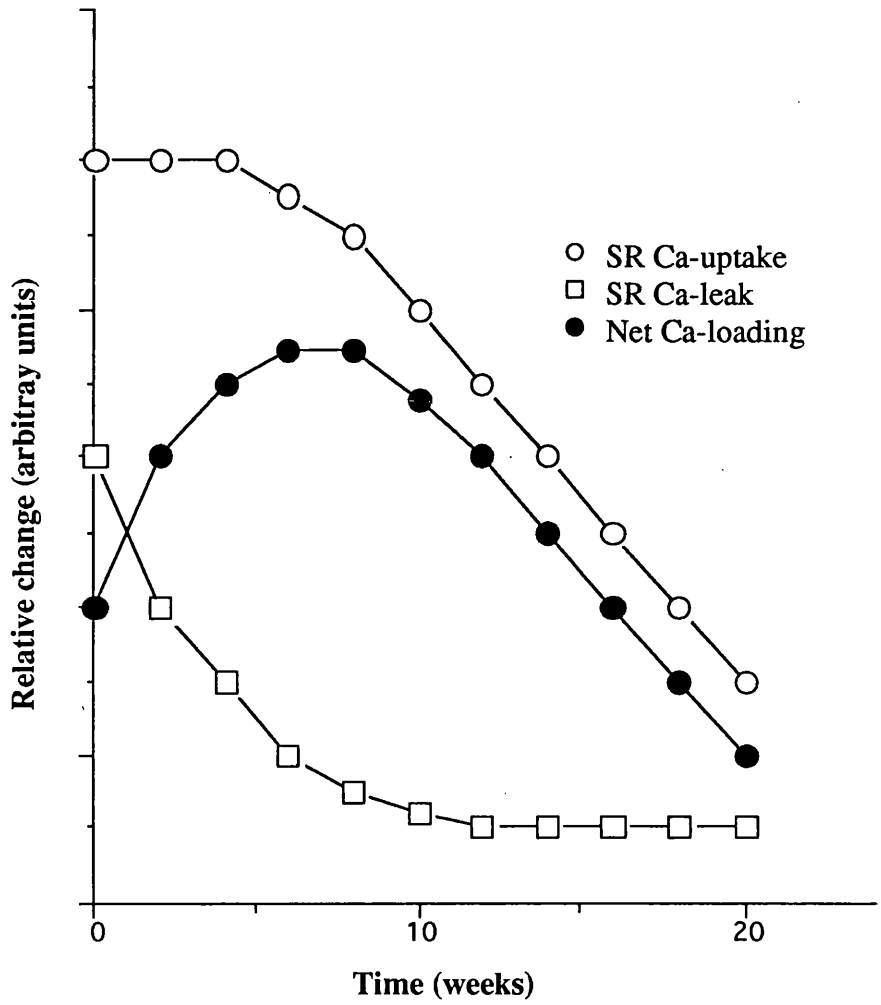


Figure 10.3 Hypothetical scheme for changes in SR Ca^{2+}-loading, with time, during the development of heart failure.

This biphasic change in SR Ca^{2+}-loading would tend to predispose the myocyte to Ca^{2+} overload in the early and late stages of heart failure but by different mechanisms. In the early stages of heart failure while net SR Ca^{2+}-loading was increased his would tend to over-fill the SR resulting in spontaneous Ca^{2+} release as demonstrated in the ligation rabbits. These spontaneous releases of Ca^{2+} could generate triggered activity and arrhythmias. In the later stages of heart failure reduced net SR Ca^{2+}-loading might predispose the myocyte to cytosolic Ca^{2+} overload which could also generate arrhythmogenic activity. The overall picture is one of disturbed Ca^{2+} regulation within the cell which could act to alter contractile function and predispose to arrhythmogenesis.

There are several qualifications to this model which deserve consideration. The relative contributions of SR Ca^{2+}-uptake and leak described in the equation (page 228) apply to steady state conditions and the majority of models of Ca^{2+} efflux and influx have been based on such steady state conditions in isolated SR vesicles (Feher & Briggs, 1983). There is no evidence to indicate that this condition applies in the physiological setting where time periods involved are short, processes occur more rapidly and where the SR may never reach a state of equilibrium during the cardiac cycle. However, although this is true, the hypothesis is valid in that it goes some way to explain the experimental findings of this thesis since at longer loading periods (100-150s), in saponin-treated trabeculae, these conditions of steady state are relevant. The second important qualification has already been discussed in some detail. It is assumed that the phosphorylation state of phospholamban is similar in failing and non-failing myocardium to allow claims that the relative pump and leak rates are intrinsically altered in failing myocardium. As was revealed in the experiments using cAMP this may be a false assumption and requires further investigation.

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