Studies Of Sarcoplasmic Reticulum Function In Rabbit And Human Left Ventricular Dysfunction.

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Abstract.

Heart Failure is a common clinical problem with high morbidity and mortality. While there are several pathological changes in cardiac failure, abnormalities of myocardial contraction at the cellular level are well documented. Myocyte Ca^{2+} handling which underlies excitation-contraction coupling is abnormal in heart failure and contributes to mechanical dysfunction. Changes in intracellular Ca^{2+} during contraction and relaxation are mediated by both sarcolemmal Ca^{2+} transport and sarcoplasmic reticulum (SR) Ca^{2+} release and re-uptake. Abnormalities of both sarcolemmal Ca^{2+} transport, and SR function have been described in heart failure and thus the pathophysiological mechanisms of abnormal Ca^{2+} handling and subsequent myocardial contraction need to be clarified.

In this thesis, abnormalities of Ca^{2+} handling at the level of the whole myocyte are characterised in a coronary artery ligation model of heart failure in rabbit. Following this, a novel preparation for the specific study of SR function in permeabilised single, myocytes was developed. SR Ca^{2+} content was significantly reduced in myocytes from failing hearts compared to shams. Spontaneous SR Ca^{2+} release occurred less frequently in failure myocytes and with reduced amplitude than in myocytes from sham hearts. SR Ca^{2+} uptake was then directly measured in suspensions of single, permeabilised myocytes from failing rabbit hearts. SERCA 2A mediated SR Ca^{2+} uptake was significantly slower in failure cells than sham cells, and this difference was maintained in the presence of ruthenium red, a blocker of the SR Ca^{2+} release channel. Inhibitors of specific aspects of SR function were then used to model the changes in SR function seen in heart failure. Inhibition of SR Ca^{2+} uptake with thapsigargin in healthy permeabilised myocytes most closely modelled the heart

failure phenotype. Inhibition of SR Ca^{2-} release at the ryanodine receptor with tetracaine, and stimulation of SR Ca^{2+} leak with ionomycin did not accurately model the changes in SR function seen in heart failure.

The experimental techniques described in this thesis were then applied to human myocytes dissociated from small ventricular biopsies to ascertain whether similar protocols could be used in future studies to investigate SR function in human heart failure.

Chapter 1: Introduction.

1.1 Heart Failure - definition and clinical problem.

Heart failure is a syndrome which can be defined in clinical terms as a condition which develops as a result of cardiac disease or damage and produces recognised symptoms of fatigue, poor exercise tolerance, breathlessness and fluid retention secondary to complex circulatory and neurohormonal responses (Cowie *et al.* 1997). It can also be defined as a state when cardiac output is insufficient to meet the requirements of metabolising tissues (Braunwald & Grossman, 1992). Heart Failure is a common clinical problem with high morbidity and mortality. Left ventricular dysfunction (LVD) is present in around 3% of all adults, and is symptomatic in around half of these (McDonagh *et al.* 1997). The prevalence of LVD rises to 5-6% in the elderly population (Clarke *et al.* 1995). The resources spent on treating heart failure account for 1-2% of the total healthcare budget in this country (McMurray & Davie, 1996). Mortality is high in patients with heart failure: even with optimal therapy patients with moderate to severe heart failure (NYHA class III-IV) have between 16% (Stevenson *et al.* 1995)and 46% (The SOLVD investigators, 1991) one year mortality rates.

The aetiology of cardiac failure is complex since the syndrome is the end result of many disease processes. In the Framingham population hypertensive heart disease was the most frequent underlying aetiology of heart failure (Kannel & Belanger, 1991). Coronary artery disease has been a more common underlying pathology in more recent studies (McDonagh *et al.* 1997), but hypertension and coronary artery disease together underlie the vast majority of cases of heart failure in the developed world (Cowie *et al.* 1997). Rheumatic valvular heart disease accounts for fewer

cases than previously, but remains common in the developing world (Kannel *et al.* 1994). There are also infective, nutritional, toxicological, endocrine and idiopathic aetiologies, the incidences of which vary geographically and between series.

Previous research has shown that there are several pathological changes in cardiac failure including myocyte loss, interstitial fibrosis and neurohormonal activation (for review see (Figueredo & Camacho, 1994; Davies *et al.* 1996; Carter & Rubin, 1994; Gwathmey & Ingwall, 1995)), but the mechanical consequences of cellular contractile dysfunction are central to the pathophysiology of left ventricular dysfunction.

1.2 Evidence for contractile and lusitropic dysfunction at cellular level.

Evidence for contractile dysfunction in myocytes comes from both single cell and multi-cellular studies. Mulieri (Mulieri *et al.* 1992) examined the isometric tension development of left ventricular myocardial strips from patients with end stage idiopathic dilated cardiomyopathy. In control tissue from non-failing hearts there was a positive relationship between stimulation frequency and developed twitch tension at frequencies of up to 3Hz. This has previously been termed the positive force frequency relationship and was initially described over 100 years ago (Bowditch, 1871) and has been confirmed in many mammalian species (Buckley *et al.* 1972). In Mulieri's experiments, the positive force frequency relationship seen in control tissue was absent in muscle strips from failing hearts, and peak tension development was reduced by 80% at high frequency of stimulation compared to control tissue. Peak rate of tension development was also reduced by 75% in tissue from failing hearts. Peak tension development and rate of tension development have also been found to be reduced in other human muscle human experiments (Hasenfuss *et al.* 1992).

These finding were extended in another study looking at the force-frequency relationship in papillary muscle strips from patients with both moderate (New York Heart Association (NYHA) class II-III) heart failure and severe (NYHA IV) heart failure. In moderate heart failure the force-frequency relationship was flattened at low stimulation rates (0.5-2Hz) and negative at higher frequencies (2.5-3Hz), whereas in severe heart failure the relationship was negative throughout the stimulus range (Schmidt *et al.* 1994). This data are shown in fig. 1.1. Schwinger (Schwinger *et al.* 1992; Schwinger *et al.* 1993) found that the negative force-frequency relationship in papillary muscle strips from patients with terminal heart failure could be only partially reversed with β-adrenergic stimulation, although full reversal was seen with a combination of ouabain (a cardiac glycoside) and BDF 9148 (a Na^+ channel activator). There was no difference in developed tension at basal stimulation between papillary muscle strips from failing and non-failing hearts, but when force of contraction was raised approximately three-fold using inotropic stimulation, both rate of developed tension and rate of relaxation increased significantly more in normal than failing hearts. Evidence of contractile dysfunction in trabecular preparations also comes from a canine model of pacing-induced heart failure (Perreault *et al.* 1992). Here isometric tension development in control tissue rose with increases in extracellular [Ca^{2+}], but this relationship was blunted in tissue from heart failure animals. Taken together this evidence suggests that systolic and diastolic contractile abnormalities in multicellular preparations, while present at baseline in some studies are most marked at high stimulation frequencies and during other forms of physiological inotropism. This reflects the *in vivo* finding that cardiac function in

heart failure is most compromised during exercise or inotropic stimulation (Mulieri *et al.* 1992).

There are a number of theoretical problems associated with the study of myocardial physiology in multicellular preparations, however. In particular, it has been suggested that core hypoxia in multicellular preparations may affect results (Atar *et al.* 1995). In addition diffusion time within preparations may be limiting for either nutrients or other compounds. All of these considerations raise the possibility that there may be differences in function of myocardial tissue within regions of the preparation. For this reason, abnormalities of contraction and relaxation at the level of the single myocyte have been studied in both human heart failure and in animal models of heart failure. Most of these studies were performed by Harding's group in London. In early studies there was no apparent difference in contraction amplitude or velocities of contraction and relaxation between cells from patients with terminal heart failure and control subjects (Harding *et al.* 1991), and maximum contraction amplitude in high [Ca^{2+}] media was also unchanged, although responses to catecholamine stimulation were reduced (Harding *et al.* 1992a). However in later studies performed at 37°C using physiological stimulation rates there was a clear blunting in the positive force frequency relationship in cells from failing hearts compared to control cells. Time to peak contraction, and time to 50% and 90% relaxation were all significantly prolonged (Davies *et al.* 1995). Other studies have shown that atrial (Harding *et al.* 1990) and ventricular (Harding *et al.* 1992b) myocytes from failing human hearts have reduced contractile responses to isoprenaline, forskolin (which acts distal to β-adrenoceptors to stimulate adenyl cyclase) and dibutyryl cAMP (a membrane permeant analogue of cAMP) compared

to control cells. In a pacing-induced canine model of heart failure, a rate dependent decrease in contraction amplitude has also been seen in single ventricular myocytes, although abnormalities of relaxation were not seen (Perreault *et al.* 1992).
Left ventricular hypertrophy, which often accompanies heart failure in remodelled myocardium, is also associated with abnormalities of myocyte contraction and relaxation. In a further study from Harding's group (Del Monte *et al.* 1995), cells from patients with left ventricular hypertrophy associated with heart failure showed prolonged contraction and relaxation times in comparison to cells from failing hearts without hypertrophy.

Despite some contradiction between studies, this evidence from multicellular and single myocyte preparations shows that myocyte dysfunction does occur in heart failure, and that there is interaction between this dysfunction and other pathophysiological changes seen in heart failure, for example neurohormonal activation and left ventricular hypertrophy. Amplitude of contraction may be normal or reduced at low stimulus frequencies, but contractile dysfunction is increasingly apparent at higher frequencies. Contraction and relaxation times may also be slower, and these changes are more significant in hearts with ventricular hypertrophy. Myocytes from diseased hearts do not respond normally to physiological β-adrenergic inotropic stimulation or to post-receptor activation of β-adrenergic pathways.

1.3 Evidence that abnormal calcium homeostasis contributes to myocyte dysfunction in intact preparations.

Gwathmey was the first to note abnormal Ca^{2+} homeostasis in heart failure (Gwathmey *et al.* 1987). Using aequorin, a bioluminescent Ca^{2+} indicator in trabeculae from hearts explanted from patients with terminal heart failure, it was found that in tissue from myopathic hearts, the Ca^{2+} transient was markedly prolonged in comparison with control tissue, and exhibited two distinct phases (shown in figure 1.2). This was seen in tissue from patients with both dilated cardiomyopathy and hypertrophic cardiomyopathy, but was more marked in hypertrophic tissue. This correlated with slowed relaxation when tension was measured simultaneously. Prolongation of Ca^{2+} transients could not be totally blocked with either verapamil or ryanodine, suggesting that there were abnormalities of both sarcolemmal Ca^{2+} transport and SR function. Prolongation of Ca^{2+} transients would be expected to cause primarily diastolic myocardial dysfunction, however at physiological stimulation rates, fusion of Ca^{2+} transients occurred which was associated with increased diastolic tone, and reduced systolic contractile force. Later experiments (Gwathmey *et al.* 1990) confirmed that end-diastolic $[Ca^{2+}]$ was raised during steady state contraction in myopathic tissue. There was no observed difference in peak systolic $[Ca^{2+}]$, despite the fact that frequency-related force potentiation was attenuated in myopathic tissue. Pharmacological manipulations which increased cytosolic $[Ca^{2+}]$ potentiated these diastolic abnormalities, whereas drugs which lowered $[Ca^{2+}]$ ameliorated them (Gwathmey *et al.* 1991).

In contrast, Pieske (Pieske *et al.* 1995) correlated reduced peak $[Ca^{2+}]$ levels with a negative force-frequency relationship in failing human trabeculae. In a pacing-

induced canine model, Perrault (Perreault *et al.* 1992) found a diastolic prolongation in Ca^{2+} transients, and a reduction in peak tension development, but peak and resting $[Ca^{2+}]$ levels were normal.

Studies in trabecular preparations in human heart failure and animal models therefore show a consistent finding of impaired diastolic Ca^{2+} handling, but there is conflicting evidence on a reduction in peak systolic Ca^{2+} availability.

Beuckelmann (Beuckelmann *et al.* 1995; Beuckelmann *et al.* 1992) studied Ca^{2+} transients in isolated ventricular myocytes from patients with terminal ischaemic and dilated cardiomyopathy. Diastolic decay of Ca^{2+} transients was significantly prolonged and peak Ca^{2+} levels were significantly depressed in heart failure. Resting cytosolic $[Ca^{2+}]$ was significantly raised. Smaller Ca^{2+} transients have also been seen in isolated myocytes from an infarction model of cardiac failure in the rat (Zhang *et al.* 1995), but predominantly diastolic changes were seen in a hypertensive model. Capasso (Capasso *et al.* 1993) also found increased resting $[Ca^{2+}]$, diastolic prolongation and reduced systolic amplitude in myocytes from a post-infarction rat model. Peak $[Ca^{2+}]$ has also been found to be reduced in Ca^{2+} transients in isolated myocytes from cardiomyopathic hamsters (Kruger *et al.* 1994).

Clear evidence of altered systolic and diastolic Ca^{2+} handling is therefore seen in isolated myocyte studies. Whether differences in findings in systolic Ca^{2+} handling from trabeculae and single cell studies represent differences in experimental preparation or in pathophysiology is unclear, however, since the nature of the single cell preparation has an effect on Ca^{2+} handling. Altering load in cardiac tissue has been shown to qualitatively alter Ca^{2+} transients (Vahl *et al.* 1994). Cell specialisation within the ventricular wall is also known to affect electrophysiological

properties, which would in turn affect Ca^{2+} handling (Drouin *et al.* 1995). The weight of evidence, however, supports abnormalities in both systolic and diastolic Ca^{2+} handling.

1.4 Evidence that abnormal SR function contributes to altered Ca^{2+} handling in heart failure.

Gwathmey (Gwathmey *et al.* 1987) suggested that abnormal Ca^{2+} transients reflected both abnormal sarcolemmal Ca^{2+} transport and SR dysfunction. There is conflicting evidence of abnormal sarcolemmal Ca^{2+} transport in heart failure. Several studies have found reduced density of the L-type Ca^{2+} current in animal models of cardiac hypertrophy and failure (Ming *et al.* 1994; Ouadid *et al.* 1995; De Brito Santos *et al.* 1995). In contrast no reductions in Ca^{2+} current density have been found in myocytes isolated from failing human hearts (Mewes & Ravens, 1994; Beuckelmann *et al.* 1992). Both increased and reduced function of the Na^{+} / Ca^{2+} exchanger have been suggested in heart failure. Studer (Studer *et al.* 1994) found increased expression of Na^{+} / Ca^{2+} exchanger mRNA in patients with heart failure and coronary artery disease, suggesting that upregulation of the exchanger may be a compensatory change in response to raised diastolic [Ca^{2+}]. Flesch (Flesch *et al.* 1996) also found raised levels of exchanger mRNA, and found that Na^{+} channel activator BDF 9148 was a more potent inotrope in patients with heart failure and suggested that the Na^{+} / Ca^{2+} exchanger may be a possible modulator of contractility in heart failure. Enhanced Na^{+} / Ca^{2+} exchange activity has also been reported in cardiomyopathic hamsters (Hatem *et al.* 1994). In contrast Dixon (Dixon *et al.* 1992) found reduced activity of the Na^{+} / Ca^{2+} exchanger in sarcolemmal preparations from rats with post

infarct cardiac failure, and Na^+ / Ca^{2+} exchange activity is also reduced in hypertrophied guinea pig hearts (Naqvi & MacLeod, 1994).

Conflicting evidence of altered sarcolemmal Ca^{2+} transport in heart failure may reflect species differences in the relative contribution of sarcolemmal Ca^{2+} currents and SR Ca^{2+} release to the Ca^{2+} transient (Tada & Kadoma, 1997). Evidence of abnormal SR function comes from a number of sources.

1.4.1 Altered SR protein and gene expression.

A reduction in protein levels of the SR Ca^{2+} uptake protein SERCA 2A would be expected to cause slowed diastolic re-uptake of Ca^{2+}, consistent with findings of prolonged Ca^{2+} transients in heart failure, and would also contribute to lower SR Ca^{2+} content which would cause reduced systolic Ca^{2+} release. There is general agreement that mRNA for SERCA 2A is reduced in cardiac failure and hypertrophy (see (Arai *et al.* 1994) for review). SERCA 2A mRNA has been found to be reduced in human heart failure (Schwinger *et al.* 1995; Arai *et al.* 1993; Mercadier *et al.* 1990) and in animal models of heart failure (Matsui *et al.* 1995; Feldman *et al.* 1993; Boluyt *et al.* 1994). There is also broad agreement that levels of the SERCA 2A protein itself are reduced in animal models of heart failure and hypertrophy (Stein *et al.* 1996; Matsui *et al.* 1995; Kiss *et al.* 1995), although some studies have found levels to be unchanged (Cory *et al.* 1994). Changes in levels of SERCA 2A in human heart failure remain controversial however. Schwinger (Schwinger *et al.* 1995) found unchanged levels of SERCA 2A in left ventricular tissue from patients with dilated cardiomyopathy. Similar findings have been reported by Movsesian (Movsesian *et al.* 1994). In contrast Hasenfuss (Hasenfuss *et al.* 1994) found reduced levels of SERCA 2A, which correlated with reduced twitch potentiation at high frequencies in

muscle strips from the same patients. Meyer (Meyer *et al.* 1995) also found reduced levels in human heart failure. The reason for different findings in these studies has not been explained. Some investigators have found a wide variation in SERCA 2A expression within the heart failure patient population (Lehnart *et al.* 1998) and therefore small numbers of patients in these studies may make real differences in protein levels difficult to prove due to inadequate statistical power. Given the reduction of mRNA expression for SERCA 2A, the expected finding would be that protein levels are also reduced, however this assumes that rates of protein degradation are equivalent in failing and non-failing hearts, which remains to be confirmed (Movsesian *et al.* 1994).

Alterations in gene and protein expression for the SERCA 2A regulatory protein phospholamban have also been studied. Since phospholamban is an inhibitory regulator of SERCA 2A, decreases in protein levels would have a dis-inhibitory effect on SR Ca^{2+} uptake, antagonising the effects of reduced SERCA 2A in heart failure. In general reduced levels of phospholamban mRNA have been found, both in animal models (Rockman *et al.* 1994; Matsui *et al.* 1995) and in human studies (Arai *et al.* 1993; Schwinger *et al.* 1995).Measurements of protein levels are less uniform, however, with studies showing decreased levels (Meyer *et al.* 1995; Kiss *et al.* 1995) or unchanged levels (Bohm *et al.* 1994; Movsesian *et al.* 1994; Schwinger *et al.* 1995). In those studies showing a reduction in phospholamban, the degree of reduction was less than that of SERCA 2A, implying that while a reduction in phospholamban would have a dis-inhibitory effect on SERCA 2A, the net effect of changes in SERCA 2A and phospholamban protein expression would be a reduction in SERCA 2A mediated SR Ca^{2+} uptake. This has been confirmed in the coronary

artery ligation model of heart failure in the rabbit where both phospholamban and SERCA 2A protein expression were found to be reduced, and SR Ca^{2+} uptake in vesicle experiments was reduced(Currie & Smith, 1999).

Fewer studies have examined levels of mRNA and protein for the SR Ca^{2+} release channel (the ryanodine receptor). Arai (Arai *et al.* 1993) found reduced mRNA and Brillantes (Brillantes *et al.* 1992) found reduced cDNA levels in patients with ischaemic but not dilated cardiomyopathy, and Go (Go *et al.* 1995) found reduced mRNA and ryanodine binding in human failing hearts, but others have found protein levels to be unchanged (Meyer *et al.* 1995; Movsesian *et al.* 1994). There are also conflicting reports on functional changes of the ryanodine receptor in heart failure. Nimer (Nimer *et al.* 1995) found differential effects of ryanodine (an inhibitor of Ca^{2+} release through the ryanodine receptor) in SR microsomes from failing and non-failing hearts. In contrast the properties of single ryanodine receptors under voltage clamp conditions have been found to be unchanged in heart failure (Holmberg & Williams, 1989).

There is general agreement that levels of protein and mRNA levels for the SR Ca^{2+} storage protein calsequestrin are unchanged in heart failure (Arai *et al.* 1993; Meyer *et al.* 1995; Movsesian *et al.* 1994).

In conclusion there is good evidence that mRNA for SERCA 2A is reduced in human and animal heart failure and that protein levels are lower in animal studies. Changes in SERCA 2A expression in human heart failure remain controversial. Reductions in mRNA and protein expression of the ryanodine receptor and phospholamban have been reported in some studies, but found to be unchanged in others.

1.4.2 Altered phosphorylation of phospholamban.

The inhibitory effect of phospholamban on SERCA 2A can be modulated by phosphorylation. Phosphorylation at the serine 16 and threonine 17 sites of the phospholamban monomer reduces phospholamban mediated inhibition of SERCA 2A (Cornwell *et al.* 1991; Colyer & Wang, 1991). Currie (Currie & Smith, 1999) studied the phosphorylation status of phospholamban in a rabbit model of heart failure and found increased levels of phosphorylation, and increased intrinsic kinase activity in tissue from heart failure animals, suggesting that phosphorylation of phospholamban may be an adaptive response to reduced SERCA 2A mediated SR Ca^{2+} uptake in failure. Bartel (Bartel *et al.* 1996) found reduced cAMP dependant phospholamban phosphorylation of trabeculae from failing human hearts, although phosphorylation capacity could be normalised by the addition of dibutyryl cAMP.

1.4.3 SR vesicle studies.

Ca^{2+} uptake in SR vesicles has been used to study SR function in heart failure. Movsesian (Movsesian *et al.* 1989) measured Ca^{2+} uptake in purified SR vesicles from patients with end-stage idiopathic dilated cardiomyopathy. Maximal Ca^{2+} uptake (V_{max}), affinity constant ($K_{0.5}$) and Hill coefficient were unchanged in failing tissue. Ohkusa (Ohkusa *et al.* 1997) found increased rates of Ca^{2+} uptake in SR vesicles in the early stages of development of pressure-induced left ventricular development in rats. Indices of left ventricular function were increased throughout the study period, but in later stages of development of ventricular hypertrophy rates of SR Ca^{2+} uptake returned to normal. Other studies have found reduced Ca^{2+} uptake in heart failure. Schwinger (Schwinger *et al.* 1995) found uptake rates decreased to 50% of control levels and reduced SERCA 2A activity in human dilated

cardiomyopathy, despite finding unchanged protein levels of SERCA 2A. Hasenfuss (Hasenfuss *et al.* 1994) correlated reduced Ca^{2+} uptake in homogenised human myocardium with reduced levels of SERCA 2A in failing hearts. Ca^{2+} uptake was reduced by 50-75% in ischaemic and dilated cardiomyopathy in a further study using homogenised human myocardium (O'Brien & Gwathmey, 1995). SERCA 2A activity and SR Ca^{2+} uptake is also reduced in left (but not right) ventricle in the post-infarction rat model (Afzal & Dhalla, 1992), and pacing-induced canine model (Cory *et al.* 1993).

1.4.4 Measures of SR Ca^{2+} content in heart failure.

Reductions in protein levels or activities of SERCA 2A would be expected to reduce steady state SR Ca^{2+} content. This has been measured in several ways. Denvir (Denvir *et al.* 1995) examined the amplitude of caffeine-induced contraction as a measure of SR Ca^{2+} content in right ventricular trabeculae from failing human hearts. Contraction amplitude was reduced in patients with severe heart failure compared to moderate failure. Using the same techniques, SR function was found to be enhanced in a rabbit model of heart failure (Denvir *et al.* 1996). Reduced caffeine-induced Ca^{2+} release have been found in isolated myocytes from failing human hearts (Beuckelmann *et al.* 1994), and SR Ca^{2+} content was reduced in heart failure when measured using electron probe micro-analysis (Darvish & Moravec, 1994).

1.4.5 Genetic manipulation of SR function.

Over-expression of SERCA-2A in rat myocytes by adenoviral transfer has been shown to increase Ca^{2+}-ATPase activity, shorten the Ca^{2+} transient and increase and shorten cellular contraction (Hajjar *et al.* 1997a), suggesting that cellular changes seen in heart failure may be remediable if normal levels of SERCA 2A were present.

Similar findings have been reported in transgenic mice over-expressing SERCA 2A (Ile *et al.* 1997). Over-expression of phospholamban in rats by adenoviral transfection mimics the changes seen in heart failure, prolonging the Ca^{2+} transient and slowing relaxation (Hajjar *et al.* 1997b), and these effects can be prevented by maintaining relative levels of phospholamban and SECRA 2A by concomitant gene transfer of SERCA 2A.

There are several potentially valuable endpoints of these experiments of genetic manipulation of SR proteins. While treatment of human heart failure by adenoviral gene transfer remains a distant possibility, genetic manipulation allows hypotheses of causes of SR dysfunction in heart failure to be tested. This type of study can be used in animal models to predict whether potential pharmacological modulators of specific aspects of SR function will have beneficial effects without the harmful side effects seen with so many other treatments of heart failure.

1.5 Overview of experimental work.

As reviewed, there is a substantial amount of evidence that abnormal Ca^{2} handling contributes to cellular contractile dysfunction in heart failure. Much of this work, however, relates to studies of intact tissue, where it is difficult to differentiate between dysfunction of sarcolemmal Ca^{2+} transport and SR function. The majority of studies of SR function in heart failure relate to mRNA and protein expression, or to studies of SR function in isolated SR vesicles. The purpose of the work reported here was to study SR function in an animal model of heart failure in isolation from changes in sarcolemma function, but still in working myocardial tissue. The preparation chosen for these studies was the permeabilised myocyte.

Chapter 2 of this thesis describes preliminary studies in intact myocytes from a coronary artery ligation rabbit model of heart failure, to establish the presence of abnormal Ca^{2+} handling at the whole cell level. Ca^{2+} transients were recorded from electrically stimulated single myocytes at frequencies of 0.1-3Hz. The measurements taken from these transients were amplitude, duration, and time to peak. These were compared in cells from failing and sham hearts.

Chapter 3 explains the development of the permeabilised single myocyte preparation, which allowed the study of SR function in isolation from sarcolemmal Ca^{2+} transport. SR Ca^{2+} content was measured in myocytes from failing and sham hearts by application of caffeine. Spontaneous SR Ca^{2+} release was stimulated in these cells by increasing cytosolic Ca^{2+} concentration, and the amplitude and frequency of spontaneous release compared in failing and sham cells.

Chapter 4 describes measurements of SR Ca^{2+} uptake in suspensions of oxalate-loaded, permeabilised myocytes. These experiments were performed in the absence and presence of ruthenium red, a blocker of Ca^{2+} release at the ryanodine receptor. These experiments were undertaken to explore whether SR Ca^{2+} content was reduced in heart failure due to lower SERCA 2A activity.

In chapter 5, pharmacological manipulation of SR function is described, in an attempt to model the abnormal SR function described in earlier chapters.

Finally, chapter 6 describes the application of these experimental methods to the study of SR function in human myocytes, dissociated from small ventricular biopsies taken at the time of cardiac surgery.

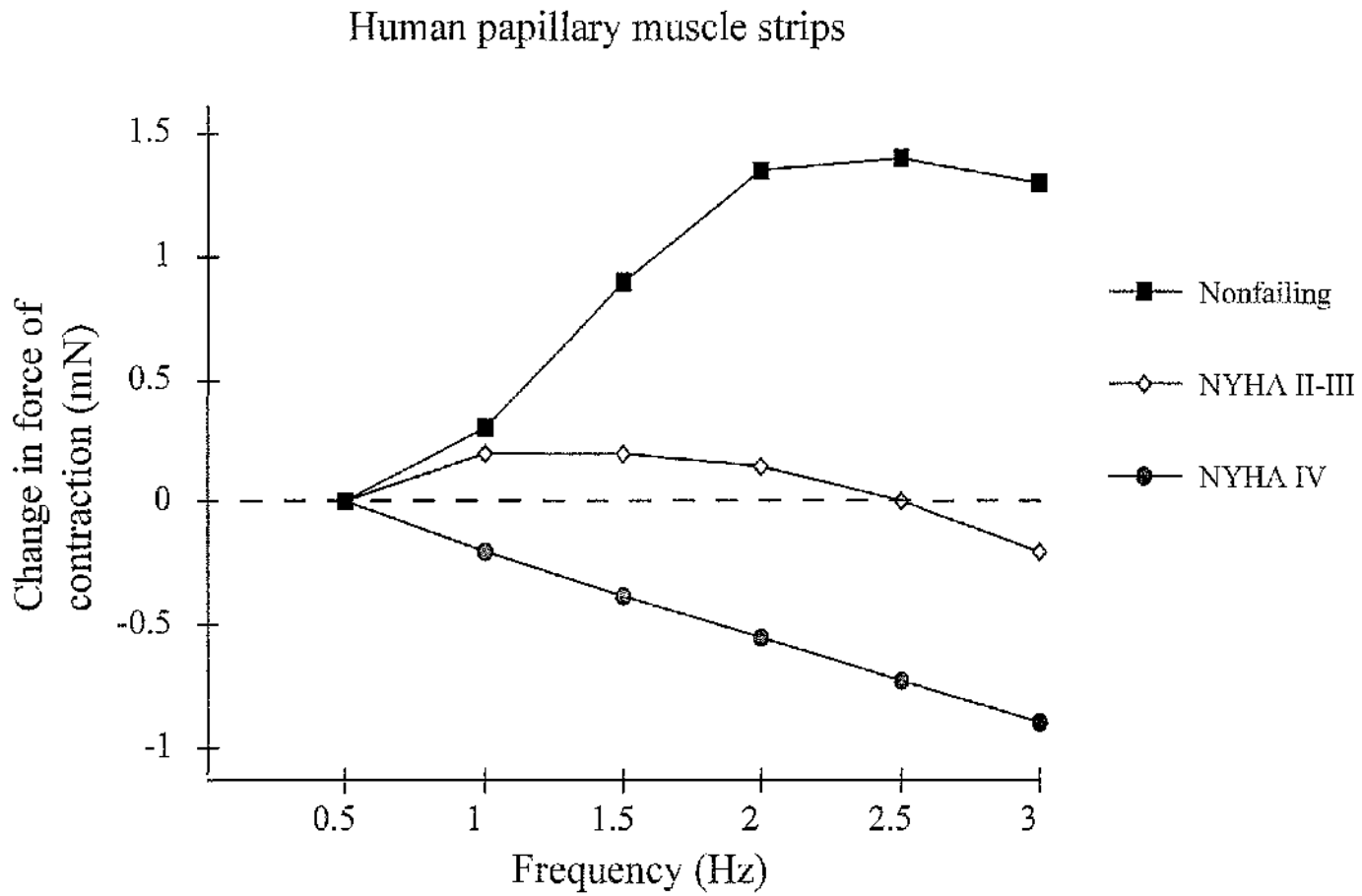

Fig. 1.1 Change in isometric force of contraction with increasing frequency of stimulation in human papillary muscle strips from non-failing, moderately failing and terminally failing hearts. (Redrawn from Schmidt et al 1994).

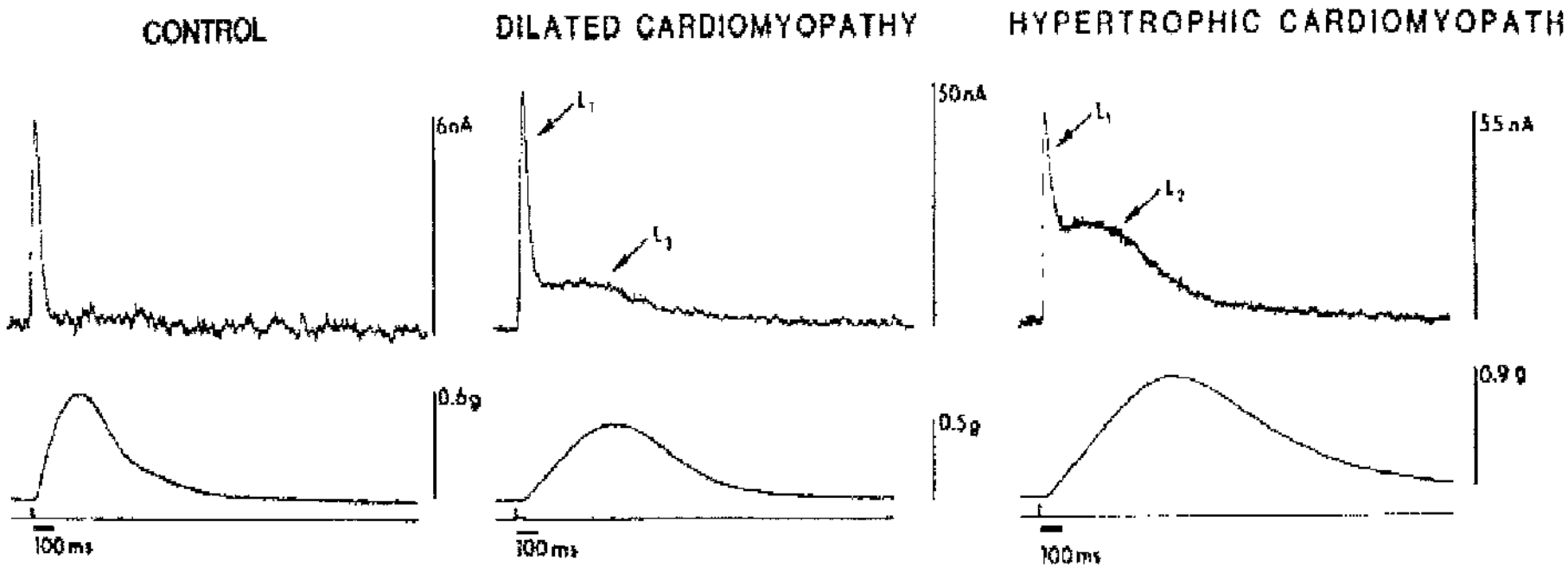

FIGURE 1. *Representative light tracing* (upper noisy trace) *and tension tracing* (middle trace) *in control and myopathic muscles;* lower trace *is stimulus artifact. Light expressed in nanoamperes (nA) of anode current; tension in grams (g). The cross-sectional areas of the muscles are (in mm^2) control, 1.3; dilated cardiomyopathy, 0.5; hypertrophic cardiomyopathy, 0.5.*

Fig. 1.2 Original tracings from Gwathmey's experiments showing prolongation of the Ca^{2+} transient in heart failure. (From Gwathmey et al 1987)

Chapter 2. Studies in intact myocytes.

2.1 Methods.

2.1.1 Rabbit model of left ventricular dysfunction.

A coronary artery ligation model of left ventricular dysfunction was used in these studies as described by Pye et al(Pye *et al.* 1996). Experiments were undertaken in adult New Zealand white rabbits of either sex weighing initially 2.5 to 3 kg. Surgery was performed by technicians in the animal house at Glasgow Royal Infirmary. General anaesthesia was induced using Hypnorm and maintained with Halothane and Nitrous Oxide/Oxygen. Following induction a left thoracotomy was performed and the large circumflex branch of the left coronary artery was identified and occluded approximately midway between the left atrial appendage and the cardiac apex. This procedure gives rise to a large homogeneous infarct in view of the minimal collateral circulation in the rabbit. Intravenous Quinidine was administered to minimise perioperative ventricular fibrillation. Post operative analgesia was provided and prophylactic antibiotics were administered. Animals were monitored for signs of distress and were withdrawn from the experimental protocol in cases of weight loss greater than 20% of baseline, evidence of overt infection or severe distress. Sham operated animals were prepared in an identical manner, but the coronary artery was not tied. Following surgery, animals were allowed to recover for 8 weeks prior to being sacrificed for in vitro experimentation.

Prior to sacrifice all animals underwent echocardiographic examination to assess the degree of LVD produced by the experimental infarct. This was undertaken using a 5 MHz focused paediatric transducer under light sedation with Hypnorm. M-mode measurements of ventricular end diastolic diameter was made and area ejection

fraction calculated from 2-D measurements of end systolic and end diastolic dimensions in the short axis (Pye *et al.* 1996).

2.1.2 Cell isolation.

Rabbits were injected via a marginal ear vein with 500U of heparin and a terminal overdose of sodium pentabarbitone. Following this the heart was quickly excised and mounted onto a Langendorff perfusion column. The heart was retrogradely perfused at 37° C via the aorta with Ca^{2+} free base Krebs solution buffered with 0.1mM Ethylene bis[oxyethylenenitrilo] tetraacetic acid (EGTA). After 5 minutes the perfusion solution was changed to base Krebs solution containing 80μM $CaCl_2$ and 1.5mg ml^{-1} collagenase (type 1, Worthington Chemicals) and 0.14mg ml^{-1} protease (type XIV, Sigma). The heart was perfused with this for 10-20 minutes to allow initial digestion of connective tissue, judged by a softening of ventricular tissue. The atria and right ventricle were dissected from the heart and discarded. The left ventricle was then removed from the aortic cannula and dissected into epicardial, mid-myocardial and endocardial layers. These layers were cut into small chunks and incubated at 37°C in further enzyme solution containing 1% bovine serum albumen (BSA, fraction V, Sigma) for 5 minutes before being filtered through a 250μm nylon mesh. Undigested material was then incubated for a further 5 minutes, while the cell suspension was centrifuged at 900rpm for 1 min, and the resulting cell pellet re-suspended in Krebs solution containing 1mM $CaCl_2$ and 1.5% BSA. When the cells has settled they were finally stored in Medium 199 (Gibco) with 5mM taurine, 5mM creatine and 0.2% BSA until use. The timing of incubation with digestive enzymes were altered from time to time as properties of different batches of enzymes varied.

2.1.3 Experimental Procedure.

Changes in cytosolic [Ca^{2+}] during the cell cycle were measured using the fluorescent Ca^{2+} indicator Fura-2 (Molecular Probes)(Kobayashi *et al.* 1989; Roe *et al.* 1990; Timmerman & Ashley, 1986). Cells were loaded with 5μM Fura-2 AM for 20 minutes at 37°, before being transferred to a perfusion chamber mounted on an inverted Nikon Diaphot microscope. The chamber was perfused with 5ml min^{-1} of rat Ringer solution at 37° C. Single myocytes were stimulated at 0.1-3Hz using a 0.5mm silver-silver chloride electrode system, with one electrode placed near the cell, and a second electrode serving as a reference electrode at the side of the bath. Square wave 20ms pulses were delivered to the electrodes at 80-90V using a Digitimer DS2 stimulator. Fluorescence was stimulated 340nm and 380nm from a Xenon arc light source and a spinning wheel system (Cairn Research Systems) at 60Hz. Evoked fluorescence at wavelengths above 500nm was collected using a 500nm interference filter and passed through a dichroic mirror to a photomultiplier. Measured fluorescence was restricted to the area of the microscope field containing the cell by the use of a variable rectangular window on the side port of the microscope The output voltage from the photomultiplier was measured by a spectrophotometer (Cairn Research Systems). A schematic diagram of the experimental apparatus is shown in fig. 2.1. A ratiometric measurement of intracellular [Ca^{2+}] was calculated from fluorescence excited at 340 and 380nm. Data from these two wavelengths and their ratio was output to a chart recorder (Gould Instruments Ltd) and to videotape, and simultaneously digitised using the program WCP (John Dempster (1993-4) and stored on hard disc. Ca^{2+} transients from cells stimulated for 2 minutes at frequencies 0.1, 0.3, 1, 2 and 3Hz were recorded, with a standard protocol.

2.1.4 Data Analysis.

Data were analysed off-line. Ca^{2+} transients from two minute periods of stimulation were averaged for the different frequencies using the program WCP. Using this program the timecourses of the transients were analysed. Two measurements of timecourse were taken from each averaged Ca^{2+} transient. Time from the initial upstroke to half peak $[Ca^{2+}]$ (T_{50}) was used as a measurement of the rate of release of Ca^{2+}. D50% was calculated as a measure of the duration of the Ca^{2+} transient, largely reflecting the time taken for Ca^{2+} re-uptake by the SR and extrusion across the sarcolemma during the diastolic phase of the contraction cycle. D50% was measured as the time taken from reaching 50% of the maximum amplitude of Ca^{2+} release to reaching the same level during the relaxation / re-uptake phase. Measurement of T_{50} and D_{50} is shown in fig. 2.2 From the chart recorder records of experiments, the mean peak and trough ratios were measured after calibration of the chart recorder. These values represented peak systolic and resting (diastolic) intracellular $[Ca^{2+}]$.

Cells which did not follow all stimulation frequencies were considered to be unhealthy, and were not included in the analysis. Data collected for each cell were myocyte length and width, time to peak Ca^{2+} release (T_{50}), D50%, peak Fura 2 ratio and trough Fura 2 ratio. Mean data (± SEM) for all cells from failing and sham cells was compared using unpaired Student t tests. Statistical significance was taken as a probability of <0.05.

2.2 Results.

2.2.1 Left ventricular function.

Mean left ventricular ejection fraction was 68.5 ± 2.7% in sham operated animals. LVEF was significantly reduced in experimental animals at 43.2 ± 1.9% ($p<0.001$).

2.2.2 Ventricular remodelling.

Since all hearts were enzymatically digested to obtain single myocytes in this study, it was not possible to measure total ventricular mass in experimental and sham hearts. Thus it was not possible to assess global remodelling changes. In a published study from this laboratory, dry weights of both the left and right ventricle have been found to be increased by 22.7 ± 0.5% and 50.0 ± 1.6% respectively 8 weeks post infarction in this rabbit model (Ng *et al.* 1998).

Remodelling changes at the cellular level were investigated in the present study by measuring myocyte dimensions. Endocardial myocytes from failing hearts were significantly longer than those from sham hearts (125.2 μm ± 2.9 μm, n=52 cells compared to 109.1 μm ± 2.6 μm, n = 32 cells, $p < 0.001$), but were of equivalent width (26.8 μm ± 1.6 compared to 25.0 μm ± 1.2 μm, p = n.s.). In contrast epicardial myocytes from failing hearts were both longer and wider than sham cells (117.5 μm ± 4.5 μm, n=34 cells, vs 105.0 μm ± 3.3 μm, n= 30 cells, $p < 0.05$, and 27.4 μm ± 2.4 μm vs 21.6 μm ± 1.2 μm, $p < 0.05$).

2.2.3 Rate of Ca^{2+} release.

T_{50} was measured in 52 endocardial and 34 epicardial cells at 0.1- 3 Hz from 8 experimental hearts, and in 33 endocardial and 30 epicardial cells at the same frequencies from 6 sham operated hearts. Mean values for these cells are shown in table 2.1 and fig. 2.3 and 2.4. T_{50} was significantly prolonged in endocardial cells at

frequencies 0.1- 2 Hz compared to control cells. There was a trend towards a frequency dependent decrease in T_{50} from 0.1 - 2 Hz in experimental endocardial cells. T_{50} was significantly lower at 1 and 2 Hz compared to 0.1 Hz ($p<0.05$). This trend was not seen in control cells. There was no difference in T_{50} values between control and experimental epicardial cells. No frequency-dependant reduction in T_{50} was seen in epicardial myocytes.

	Expt.					Sham				
(a) Endocardial cells										
	0.1 Hz	0.3 Hz	1 Hz	2 Hz	3Hz	0.1 Hz	0.3 Hz	1 Hz	2 Hz	3Hz
Mean	67.33	62.67	58.33	57.94	58.53	54.88	54.94	55.09	55.27	57.42
SEM	4.13	2.62	0.96	0.78	0.9	1.03	0.72	0.73	0.71	0.97
p	<0.05	<0.05	<0.05	<0.05	>0.05					
(b) Epicardial cells										
Mean	54.45	55.81	54.77	54.94	56.32	56.1	55.79	55.17	55	56.36
SEM	0.62	0.73	0.64	0.53	0.63	0.99	0.75	0.56	0.63	0.73
p	>0.05	>0.05	>0.05	>0.05	>0.05					

Table 2.1: Mean T_{50} values for endocardial and epicardial myocytes. All times are in ms. Probabilities are compared to sham cells at the same frequency.

2.2.4 Duration of Ca^{2+} transients.

D_{50} was measured in 56 endocardial and 33 epicardial cells at 0.1- 3 Hz from 8 experimental hearts, and in 38 endocardial and 30 epicardial cells at the same frequencies from 6 sham operated hearts. Mean values for these cells are shown in table 2.2 and fig. 2.5 and 2.6. Ca^{2+} transients were prolonged at all frequencies in endocardial cells compared to shams. Prolongation was statistically significant at

frequencies 0.1 - 2 Hz. Ca^{2+} transients were prolonged in epicardial myocytes only at higher frequencies (1-3 Hz), and statistical significance was only reached at 3 Hz. In all cells there was a marked frequency-dependant shortening of transients. In myocytes from heart failure animals, Ca^{2+} transients were longer in endocardial cells than epicardial cells at 0.1 and 0.3 Hz ($p<0.05$), but there were no differences at higher stimulation rates. There were no significant differences in transient length between endocardial and epicardial cells in sham operated animals.

In all cells there was a marked reduction in Ca^{2+} transient duration with increasing frequency of stimulation.

	Expt.					Sham				
(a) Endocardial cells										
	0.1 Hz	0.3 Hz	1 Hz	2 Hz	3Hz	0.1 Hz	0.3 Hz	1 Hz	2 Hz	3Hz
Mean	533.5	563.2	364.8	229	172.6	402.2	431.7	299.4	211.4	167.7
SEM	38.9	34	15.8	4.6	2	47.8	40.6	14.9	5.3	3.7
p	<0.05	<0.01	<0.01	<0.01	>0.05					
(b) Epicardial cells										
Mean	411.7	458.6	348	225.6	172.5	440.7	439.5	326.5	213.5	163.7
SEM	45.2	38	20.7	5.9	2.3	41	31.7	14.3	4.7	2.8
p	>0.05	>0.05	>0.05	>0.05	<0.05					

Table 2.2 Mean D_{50} values for endocardial and epicardial myocytes. All times are in ms. Probabilities are compared to sham cells at the same frequency.

2.2.5 Peak systolic Fura-2 ratios.

Peak systolic Fura 2 ratios were measured in 54 endocardial and 31 epicardial cells at 0.1- 3 Hz from 10 experimental hearts, and in 34 endocardial and 31

epicardial cells at the same frequencies from 6 sham operated hearts. Mean values for these cells are shown in table 2.3 and fig. 2.7 and 2.8.

Peak Ca^{2+} release was strongly related to frequency in both endocardial and epicardial myocytes from sham animals. Increasing frequency from 0.1 to 0.3, 1, 2 and 3 Hz, caused increases in peak Fura 2 ratios of 3.5%, 24.1%, 58.6% and 92.4% respectively in endocardial cells, and of 4.0%, 17.2%, 37.5% and 58.0% in epicardial cells. This is analogous to the positive force-frequency relationship seen in cellular contraction. Peak Fura-2 ratio was higher in endocardial cells at all frequencies studied, this difference reached statistical significance at higher frequencies (1-3 Hz).

The frequency related increase in peak Ca^{2+} release was blunted in both endocardial and epicardial myocytes from heart failure animals. Peak Fura-2 ratios were significantly lower at all frequencies in endocardial myocytes from failure hearts compared to shams. Ratios were also lower in epicardial cells, but statistical significance was only reached at 3 Hz. When frequency - associated increases in Fura 2 ratios were normalised to peak level at 0.1 Hz, increases with higher stimulation rates were still blunted at 8.8% (p=ns vs control), 19.0% (p=ns), 36.22% ($p<0.05$) and 49% (p=0.01) in endocardial cells and 6.7%, 21.2%, 40.0% and 54.5% in epicardial cells (all ns vs control). In cells from failing hearts, endocardial Fura 2 ratios were also higher than those from epicardial cells, but these differences were not significant.

	Expt.					Sham				
(a) Endocardial cells										
	0.1 Hz	0.3 Hz	1 Hz	2 Hz	3Hz	0.1 Hz	0.3 Hz	1 Hz	2 Hz	3Hz
Mean	1.02	1.07	1.18	1.36	1.48	1.43	1.47	1.74	2.13	2.5
SEM	0.06	0.06	0.07	0.09	0.1	0.13	0.13	0.15	0.18	0.21
p	<0.001	<0.001	<0.001	<0.001	<0.001					
(b) Epicardial cells										
Mean	0.94	0.99	1.09	1.24	1.35	1.15	1.18	1.33	1.57	1.78
SEM	0.08	0.08	0.08	0.09	0.09	0.12	0.12	0.13	0.17	0.19
p	>0.05	>0.05	>0.05	>0.05	<0.05					

Table 2.3 Mean Peak Fura 2 ratios for endocardial and epicardial myocytes. Probabilities are compared to sham cells at the same frequency.

2.2.6 End diastolic Fura 2 ratios.

End diastolic Fura 2 ratios were measured in 54 endocardial and 31 epicardial cells at 0.1- 3 Hz from 10 experimental hearts, and in 34 endocardial and 31 epicardial cells at the same frequencies from 6 sham operated hearts. Mean values for these cells are shown in table 2.4 and fig. 2.7 and 2.8.

End diastolic Fura 2 ratios rose with increased frequency in both endocardial and epicardial myocytes from sham hearts. Normalised increases from 0.1 to 0.3, 1, 2, and 3Hz were 4%, 15%, 38% and 58% in endocardial cells and 4%, 14%, 28% and 49% in epicardial cells. There were similar increases in cells from failing hearts of 4%, 15%, 30% and 45% in endocardial myocytes and 5%, 16%, 30% and 48% in epicardial myocytes. Comparing end diastolic results between failure and sham cells

showed no difference in epicardial cells, and endocardial cells at low frequencies, but levels were significantly higher in endocardial sham cells at 2 and 3 Hz.

	Expt.					Sham				
(a) Endocardial cells										
	0.1 Hz	0.3 Hz	1 Hz	2 Hz	3Hz	0.1 Hz	0.3 Hz	1 Hz	2 Hz	3Hz
Mean	0.67	0.7	0.77	0.87	0.97	0.72	0.75	0.83	0.99	1.14
SEM	0.03	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.05
p	>0.05	>0.05	>0.05	<0.05	<0.01					
(b) Epicardial cells										
Mean	0.63	0.66	0.73	0.82	0.93	0.72	0.75	0.82	0.92	1.07
SEM	0.06	0.06	0.06	0.06	0.06	0.04	0.05	0.05	0.05	0.06
p	>0.05	>0.05	>0.05	>0.05	>0.05					

Table 2.4 Mean end diastolic Fura 2 ratios for endocardial and epicardial myocytes. Probabilities are compared to sham cells at the same frequency.

2.2.7 Amplitude of Ca^{2+} transients.

Given the fact that there are differences in both systolic and diastolic Fura 2 ratios between cells from failing and sham hearts, it is important to establish whether the amplitude of Ca^{2+} transients differs in failure and sham cells. Table 2.5 shows these amplitudes calculated by subtracting diastolic from systolic Fura 2 ratios in individual cells. All cells show a positive amplitude-frequency relationship, but this is blunted in cells from failure hearts. Amplitude is significantly reduced in failure endocardial myocytes compared to sham cells. The reduction in amplitude seen in epicardial myocytes is only significant at 3 Hz. In sham cells the amplitude of Ca^{2+}

transients was significantly greater in endocardial than epicardial cells ($p<0.05$ at all frequencies). There was no significant difference in amplitude between Ca^{2+} transients from endocardial and epicardial regions in cells from failing hearts.

	Expt.					Sham				
(a) Endocardial cells										
	0.1Hz	0.3Hz	1 Hz	2 Hz	3Hz	0.1Hz	0.3Hz	1 Hz	2 Hz	3Hz
Mean	0.36	0.39	0.42	0.49	0.54	0.72	0.74	0.93	1.16	1.37
SEM	0.03	0.03	0.04	0.06	0.08	0.11	0.11	0.14	0.17	0.21
p	<0.001	<0.001	<0.001	<0.001	<0.001					
(b) Epicardial cells										
Mean	0.31	0.33	0.36	0.42	0.42	0.42	0.41	0.5	0.63	0.69
SEM	0.03	0.04	0.04	0.04	0.04	0.09	0.08	0.1	0.13	0.15
p	>0.05	>0.05	>0.05	>0.05	<0.05					

Table 2.5 Mean amplitudes of Ca^{2+} transients from endocardial and epicardial myocytes. Probabilities are compared to sham cells at the same frequency.

2.3 Discussion.

Background studies in intact myocytes thus confirm abnormal Ca^{2+} handling in this model of heart failure. Previous studies linking abnormal Ca^{2+} handling to both contractile and lusitropic dysfunction in heart failure suggest that the altered Ca^{2+} handling seen in these cells contributes to the impairment of ventricular function documented in vivo.

2.3.1 Systolic dysfunction.

Previous studies have shown reduced peak tension development and reduced rate of tension development in failing myocardium. This has been shown in both muscle strip studies (Hasenfuss *et al.* 1992) and single cell studies (Davies *et al.* 1995). Data from intact cells in the present study support these findings. Tension development and rate of tension development depend upon the increase in cytosolic Ca^{2+} concentration produced from sarcolemmal transport and sarcoplasmic reticulum release and the rate at which these processes occur at the onset of systole. In this study peak $[Ca^{2+}]$ and rate of rise of $[Ca^{2+}]$ were reduced at all frequencies in endocardial cells and at higher frequencies in epicardial cells. (A caveat in acceptance of the T_{50} data is that measurements are approaching the limit of the systems temporal capacity, given that the spinning wheel allowed sampling at 60Hz.) Similarly several studies have shown a blunting of the positive force frequency relationship in heart failure (Schwinger *et al.* 1993; Schmidt *et al.* 1994). This is reflected in the current study in the blunting of the increased Ca^{2+} release as frequency increases, notably in endocardial cells, but also in epicardial cells at high frequencies.

Dimensions of all cells were measured to asses whether peak Ca^{2+} release was a simple function of cell size. Clearly a reduction in systolic Ca^{2} release in cells from heart failure animals can not be explained in this way since heart failure cells were significantly larger from both endocardial and epicardial regions from heart failure animals. In both failure and sham animals there was no relationship between cell size and peak Ca^{2+}. This is shown for endocardial cells in fig. 2.9.

The possible pathophysiological processes underlying the abnormalities of systolic Ca^{2+} release include both abnormal sarcolemmal and SR Ca^{2+} handling. At the sarcolemmal level, the most important effector of Ca^{2+} influx is the L-type Ca^{2+} channel. Several studies have found reduced density of the L-type Ca^{2+} current in animal models of cardiac hypertrophy and failure (Ming *et al.* 1994; Ouadid *et al.* 1995; De Brito Santos *et al.* 1995) although no reductions in Ca^{2+} current density have been found in myocytes isolated from failing human hearts (Mewes & Ravens, 1994; Beuckelmann *et al.* 1992). Since sarcolemmal Ca^{2+} entry contributes a smaller fraction of the systolic increase in cytosolic Ca^{2+} than SR release (Tada & Kadoma, 1997), it would seem unlikely, however, that a reduction in the L-type Ca^{2+} current alone would cause the decreases seen in systolic Ca^{2+} release. Since Ca^{2+} entry through the L-type channel is the initiating step in the release of a larger SR store of Ca^{2+} it is possible that a reduction in this Ca^{2+} current could cause the observed slowed release of Ca^{2+}. A further explanation for this slowed release of Ca^{2+} could be a reduction in the efficiency of the link between sarcolemmal entry and Ca^{2+}-induced Ca^{2+} release. This could be explained by an increase in the spatial separation of the L-type channel and the ryanodine receptor, or by a detuning of the gain of the signal transduction process (Cannell *et al.* 1997).

A reduction in the expression of SERCA 2A as described in some previous studies (Currie & Smith, 1999; Hasenfuss *et al.* 1994; Kiss *et al.* 1995) could contribute to reduced SR Ca^{2+} release secondary to reduced SR Ca^{2+} stores as measured by Darvish (Darvish & Moravec, 1994). A reduction in levels of the ryanodine receptor (Brillantes *et al.* 1992) could also explain both the reduction in Ca^{2+} release and slowed rate of release.

Any of the above mechanisms causing reduced systolic Ca^{2+} release would contribute to the blunted relationship of Ca^{2+} release with frequency. Phosphorylation of phospholamban is also thought to contribute to the force-frequency relationship (Colyer & Wang, 1991). Increased phosphorylation removes the inhibitory regulation of phospholamban on SERCA 2A, thus increasing diastolic Ca^{2+} uptake and SR Ca^{2+} content. This mechanism may be less effective in heart failure, if basal phospholamban phosphorylation is increased (Currie & Smith, 1996). This hypothesis is consistent with previous findings that the positive inotropic action of isoprenaline and cAMP analogues is reduced in failing myocardium (Harding *et al.* 1992b).

2.3.2 Diastolic dysfunction.

The predominant finding from these experiments in intact myocytes, is that there is slowed diastolic re-uptake of Ca^{2+} as indicated by prolonged Ca^{2+} transients in myocytes from failing myocardium. As with findings of systolic dysfunction, this is more marked in endocardial myocytes than in epicardial myocytes. During diastole, Ca^{2+} is removed from the cytosol by re-uptake into the SR, effected by SERCA 2A, and extrusion across the sarcolemma by the sarcolemmal Ca^{2+} - ATPase and the Na^{+}-Ca^{2+} exchanger. As with Ca^{2+} release, SR re-uptake processes are quantitatively more important than sarcolemmal extrusion processes in mammalian myocardium (Tada & Kadoma, 1997).

On an individual cell basis, there was no relationship between cell area and duration of Ca^{2+} transients. This shows that slowing of diastolic Ca^{2+} re-uptake is not a simple function of increase in cell size during the remodelling process. This is shown for endocardial cells in fig 2.10.

As discussed in the introduction, the strongest evidence from previous studies is that the cause of abnormal Ca^{2+} handling in heart failure is abnormal function of or expression of SERCA 2A, and evidence for abnormal sarcolemmal extrusion of Ca^{2+} is unconvincing. Reduced SERCA 2A expression or function therefore seems the most likely explanation for the prolongation of Ca^{2+} transients seen in this rabbit model of heart failure. Since a reduction in SERCA 2A activity can also explain the findings of abnormal systolic Ca^{2+} handling, this is a possible unifying hypothesis for the changes seen in these cells.

The difficulty in accepting this hypothesis, however, is the finding that diastolic $[Ca^{2+}]$ is not raised in failing cells in this study, even at high stimulation rates. Raised diastolic $[Ca^{2+}]$ has been found in some previous studies (Gwathmey *et al.* 1987; Beuckelmann *et al.* 1992), though others have found normal diastolic levels (Perreault *et al.* 1992). If the sole or prime defect in Ca^{2+} handling in heart failure were reduced activity in SERCA 2A, causing reduced diastolic Ca^{2+} re-uptake, and a secondary reduction in SR Ca^{2+} content, an expected finding would be that diastolic Ca^{2+} levels were raised, and this would be particularly true at high stimulation frequencies. There are several possible explanations for this discrepancy.

(i) Up-regulation of the Na^{+} - Ca^{2+} exchanger in heart failure may be a compensatory mechanism. Some previous studies have found increased activity (Hatem *et al.* 1994) and increased mRNA for the exchanger (Flesch *et al.* 1996; Studer *et al.* 1994). In this scenario, reduced SR Ca^{2+} uptake would be compensated for by increased sarcolemmal extrusion, and diastolic $[Ca^{2+}]$ would not rise. In order to avoid SR Ca^{2+} depletion or overload, there must be overall Ca^{2+} balance during repeated contraction - relaxation cycles. This implies that if proportionately more

Ca^{2+} is extruded across the sarcolemma during diastole, unless an equivalent amount of increased Ca^{2+} enters the cell during systole, there will be progressive SR Ca^{2+} depletion and final contractile failure. There is no evidence for increased L-type Ca^{2+} current in heart failure however. It is possible that the compensatory mechanism of increased extrusion of Ca^{2+} through the exchanger is utilised during short periods of increased contraction frequency, incurring an effective Ca^{2+} debt which is restored when contraction rates return to normal, but there is no evidence to support this hypothesis. In these experiments 2 min stimulation protocols were used at all frequencies of stimulation. During this period cells appeared to be in steady state, with no reduction in amplitude or increase in diastolic [Ca^{2+}] towards the end of the stimulation period.

(ii) Reduction in the activity of SERCA 2A prolonged Ca^{2+} transients, but there was sufficient activity to attain normal cytosolic [Ca^{2+}] by end-diastole. Those studies which have found increased diastolic [Ca^{2+}] have been performed in end stage heart failure, in hearts explanted at the time of cardiac transplantation (Gwathmey *et al.* 1987; Beuckelmann *et al.* 1992). The post-infarct model of heart failure in the rabbit is a less severe model of heart failure, indicated by a more modest reduction in ejection fraction (around 70% to 40%), and low mortality in rabbits which survive the peri-operative period (Pye *et al.* 1996). If myocardial function is depressed less than in other studies, it is possible that there is a concomitantly smaller reduction in SERCA 2A activity, and this smaller reduction may have the observed effect of only prolonging Ca^{2+} transients, and not affecting end-diastolic [Ca^{2+}]. This hypothesis is reasonable, but if correct means that although diastolic SR Ca^{2+} accumulation would be slowed, there would be no effect on total SR Ca^{2+} accumulation in diastole, and

reduced SERCA 2A activity alone would not explain the reduction in systolic Ca^{2+} release.

(iii) The stimulation frequency may have been too low to cause cytosolic Ca^{2+} accumulation in diastole. The experimental protocol encompassed a wide range of stimulation frequencies, from 0.1 Hz to 3Hz. However, 3Hz, while physiological in the rabbit, does not represent peak heart rate, and it is possible that higher stimulation frequencies would have further limited Ca^{2+} re-uptake / extrusion and caused raised diastolic $[Ca^{2+}]$. Again the difficulty with this hypothesis is that a partial reduction in SERCA 2A activity at relatively low stimulus rates, causing slowed Ca^{2+} transients but not raised end-diastolic $[Ca^{2+}]$ would not cause reduced SR Ca^{2+} content, and again could not therefore account for the systolic dysfunction seen.

(iv) SR Ca^{2+} content / storage facility is chronically reduced in failing hearts. This hypothesis suggests that chronically reduced activity of SERCA 2A reduces the SR pool of Ca^{2+} available for release, and the amount of Ca^{2+} cycled through the SR during contraction and relaxation is permanently reduced. In this new steady state, reduced SERCA 2A activity causes prolonged Ca^{2+} transients, but since total systolic Ca^{2+} released is smaller, slower re-uptake does not cause end-diastolic $[Ca^{2+}]$ to be raised. Previous findings that levels of calsequestrin are unaltered in heart failure argues against this hypothesis(Meyer *et al.* 1995; Movsesian *et al.* 1994).

In summary, the finding that end-diastolic $[Ca^{2+}]$ is not raised, despite impaired diastolic Ca^{2+} handling indicates that reduced activity of SERCA 2A is insufficient to explain both the diastolic and systolic dysfunction in Ca^{2+} handling seen in these cells, and that other alterations in sarcolemmal and / or SR Ca^{2+} handling must also be present.

The effects of these cellular abnormalities of diastolic Ca^{2+} handling would be to cause lusitropic dysfunction at the whole heart level. In vivo, diastolic dysfunction would cause decreased ventricular compliance and slowed diastolic relaxation. A secondary effect of diastolic dysfunction would be to reduce ventricular filling, and therefore cardiac output.

2.3.3 Correlation of observed changes with left ventricular function.

If the changes observed in Ca^{2+} handling in cells from failing hearts reflect pathophysiological processes, and are not simply epiphenomena, it should be possible to correlate these changes with indices of left ventricular function. Fig. 2.11 shows the relationship between ejection fraction and Ca^{2+} transient duration in endocardial cells stimulated at 1Hz. Fig. 2.12 shows the relationship between peak Ca^{2+} release and ejection fraction in endocardial cells stimulated at 3Hz. In both cases there were weak ($R= -0.42$ and $R= 0.38$ respectively) but statistically significant correlations ($p< 0.001$ for both).

This poor correlation between ejection fraction and cellular measurements may reflect inherent difficulties in measuring ejection fraction using echocardiography, where even the intra-observer error may be as high as 6% in the rabbit (Pye *et al.* 1996). Other possible explanations include cell heterogeneity within both experimental and sham populations, since some mid-myocardial (M) cells (which have different electrophysiological properties, and therefore possibly different Ca^{2+} handling) may have been inadvertently sampled when endocardial and epicardial layers were separated during the dissociation procedure. Following infarction it is possible that remodelling and cell death changes in the ventricular wall change the relative thickness of the sub-endocardial, sub-epicardial and mid-myocardial layers,

thus M cell contamination may contribute different numbers of cells in experiments from sham and failure hearts. Finally it is difficult to exclude differential cell isolation from any single cell experiment. Since there is appreciable cell death during the isolation procedure, it is possible that a population of relatively healthy cells are isolated from failing hearts, thus diluting the extent of cellular changes in the population available for study.

2.3.4 Differences between endocardial and epicardial myocytes.

Clear differences are evident between the abnormalities of Ca^{2+} transients seen in endocardial and epicardial myocytes from failing hearts. In general changes are more marked in endocardial cells, and tend to be present at all stimulation frequencies. In epicardial cells, abnormalities are only present at higher stimulation frequencies. These patterns are similar in both Ca^{2+} transient duration measurements, levels of systolic Ca^{2+} release and Ca^{2+} transient amplitude, suggesting that there are real differences in abnormalities of Ca^{2+} handling in heart failure in myocytes from different sites in the ventricular wall.

Previous studies in this model have confirmed that there is left ventricular remodelling post-infarction, with an increase in left ventricular mass (Ng *et al.* 1998). The finding in this study that endocardial myocytes were longer but not wider in failure cells than sham cells, and that epicardial myocytes were both longer and wider suggests that the remodelling process is not uniform throughout the ventricular wall. This may reflect different adaptive mechanisms in endocardial and epicardial regions, or adaptation to different stresses or degrees of stress. In the normal heart tissue systolic pressure is not uniform throughout the thickness of the ventricle. There is an increasing pressure gradient from the epicardial surface to the

endocardium (Wong & Rautaharju, 1968; Kirk & Honig, 1964). This is reflected in higher systolic and diastolic cytosolic [Ca^{2+}] in endocardial than epicardial regions (Figueredo *et al.* 1993).It is thus possible that during ventricular remodelling and dilatation, increasing wall stress may affect Ca^{2+} handling in endocardial cells to a greater extent or at an earlier stage in the remodelling process in endocardial than epicardial cells. It has also been shown that there is reduced sub-endocardial coronary blood flow reserve in the presence of left ventricular hypertrophy (Hittinger *et al.* 1995). This may be a further reason why maladaptive responses in ventricular remodelling might be more evident in endocardial than epicardial cells.

2.4 Summary.

Experiments in intact myocytes have therefore confirmed abnormal Ca^{2+} handling in this model of left ventricular dysfunction. These abnormalities are slowing of diastolic Ca^{2+} re-uptake, slowed systolic Ca^{2+} release and reduced amplitude of systolic Ca^{2+} release. The consequences of these abnormalities will be in both diastolic and systolic contractile dysfunction. Endocardial myocytes are more affected than epicardial cells, probably as a result of greater wall stress during ventricular remodelling. The mechanisms of the observed changes in Ca^{2+} handling are most likely to include reduced activity of SERCA 2A, although it is difficult to explain all of the changes seen without invoking other mechanisms.

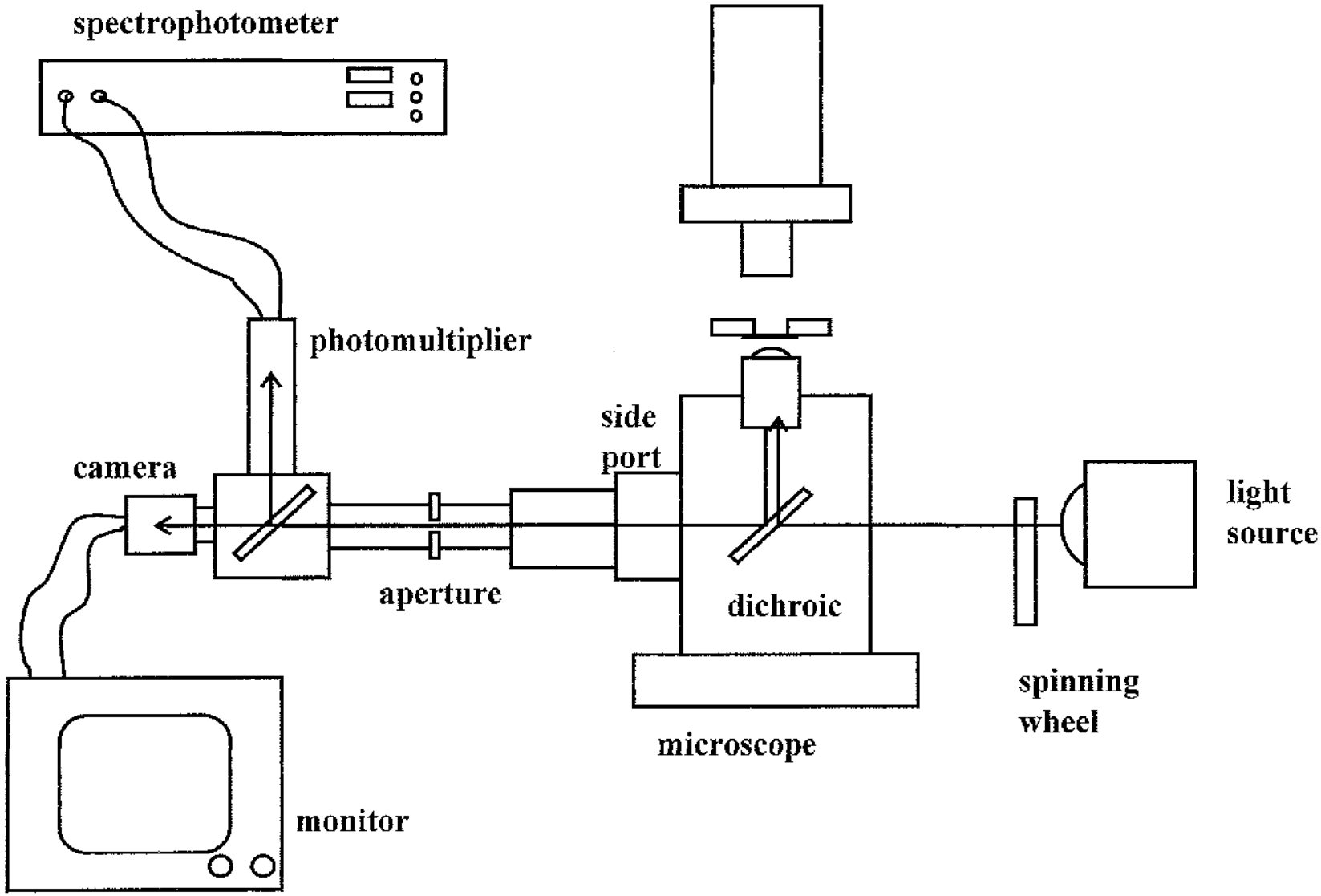

Fig. 2.1 Experimental set-up for recording Ca^{2+} signals from isolated myocytes.

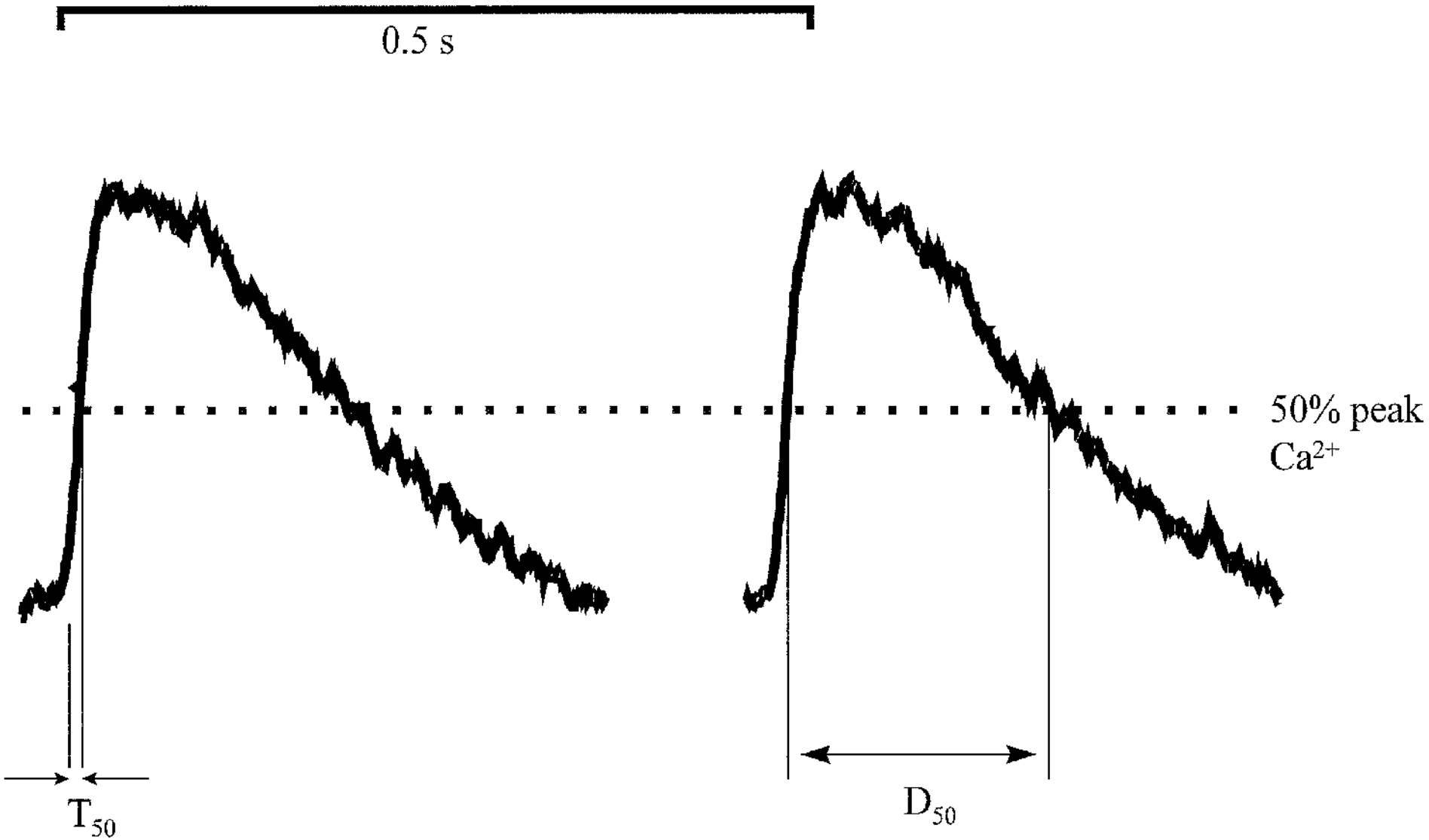

Fig. 2.2 Sample Ca^{2+} transients showing measurment of T_{50} and D_{50}

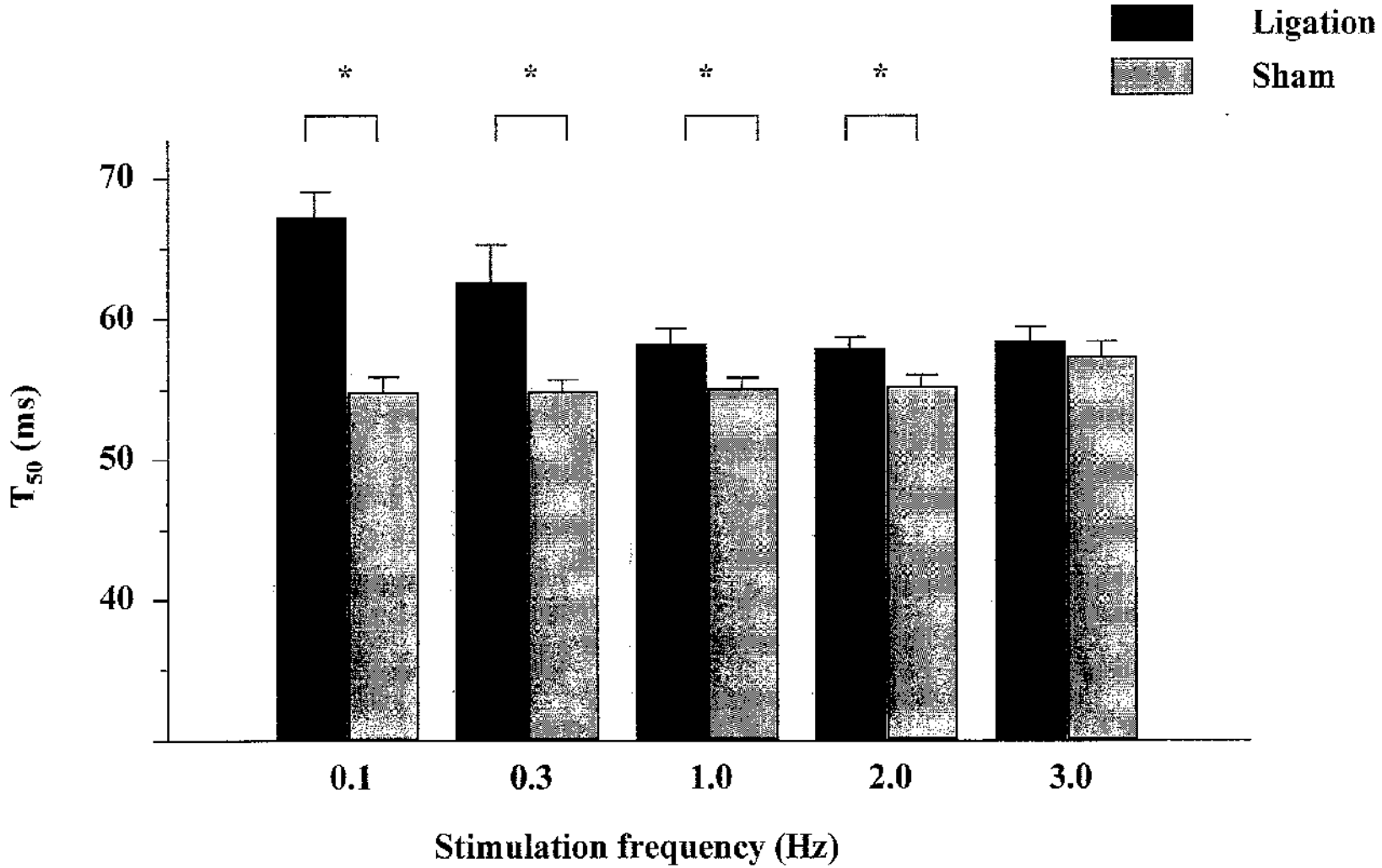

Fig. 2.3: Rate of Ca^{2+} rise in endocardial myocytes.
(* : $p < 0.05$)

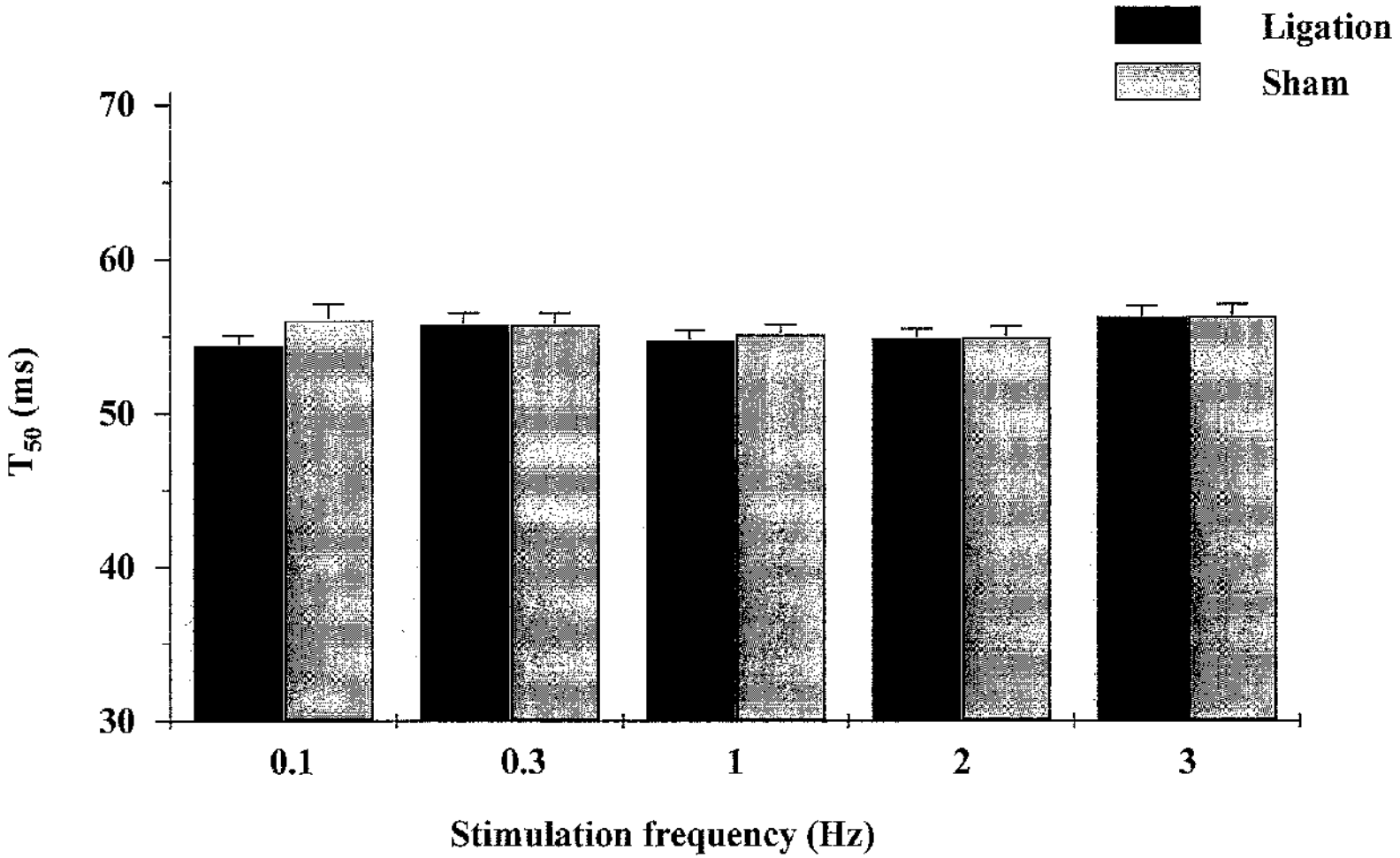

Fig. 2.4: Rate of Ca^{2+} rise in epicardial myocytes.

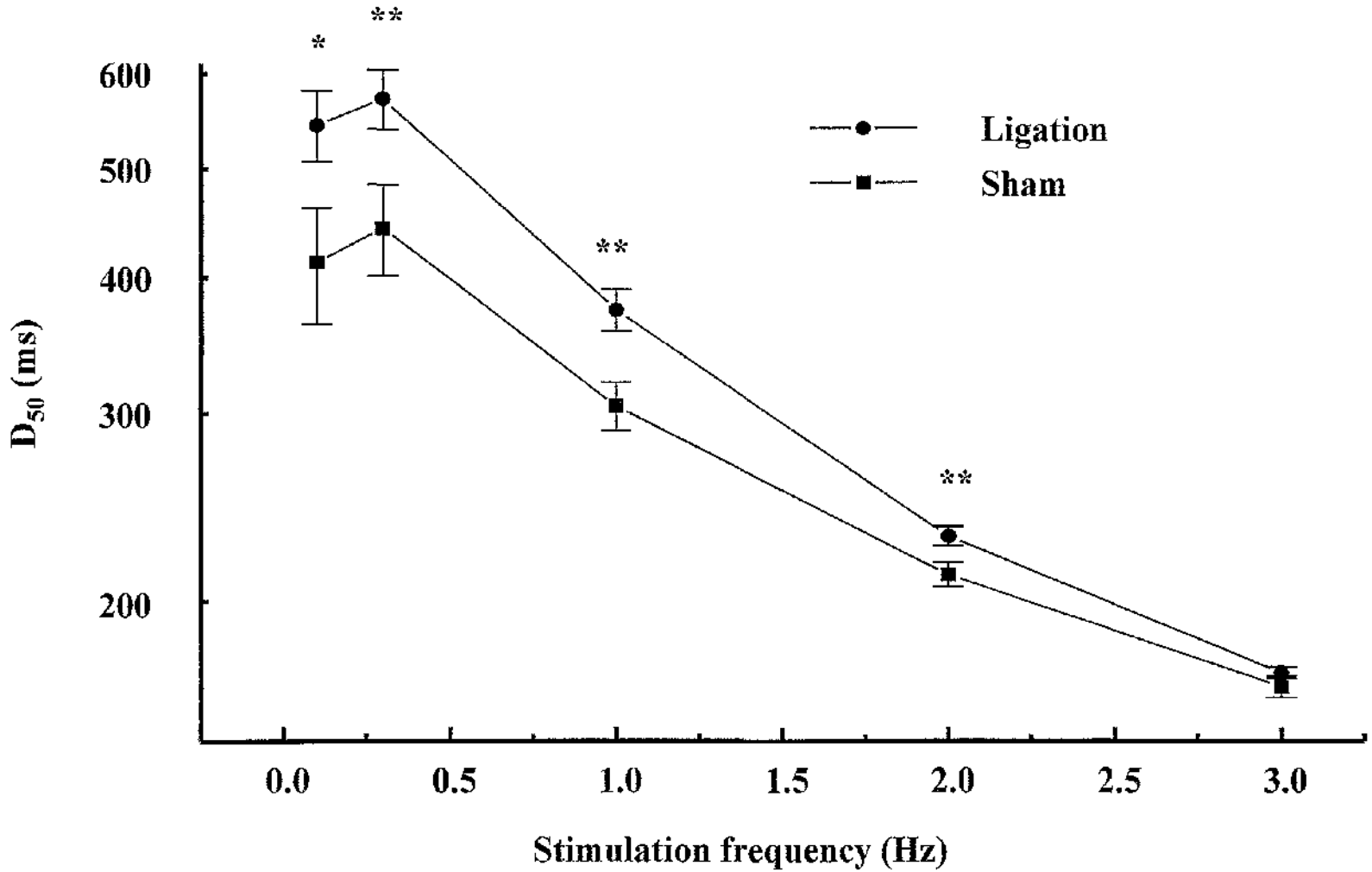

Fig. 2.5: Duration of Ca^{2+} transients in endocardial myoytes.
(* : $p < 0.05$, ** : $p<0.01$)

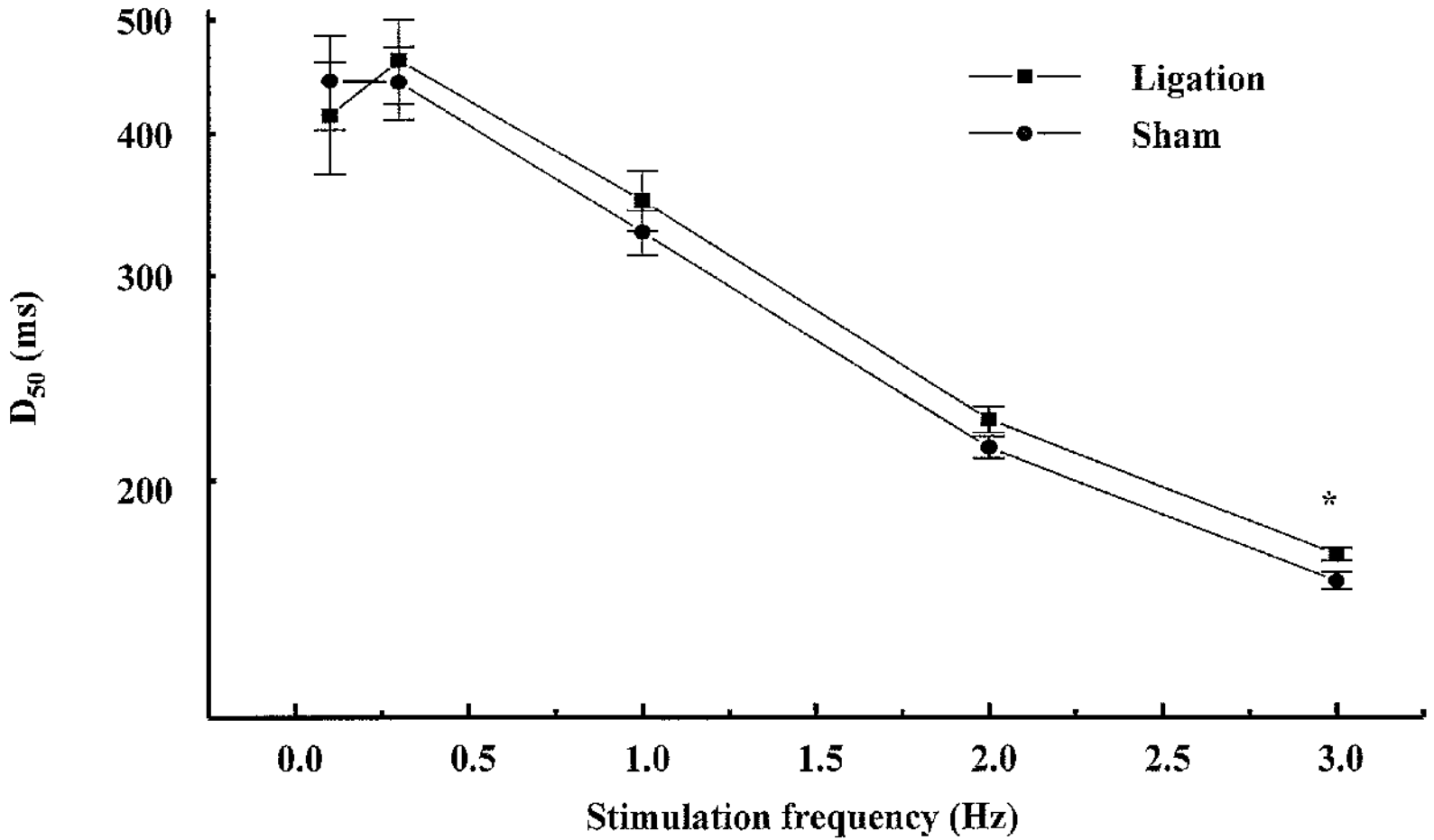

Fig. 2.6: Duration of Ca^{2+} transients in epicardial myocytes.
(* : $p < 0.05$)

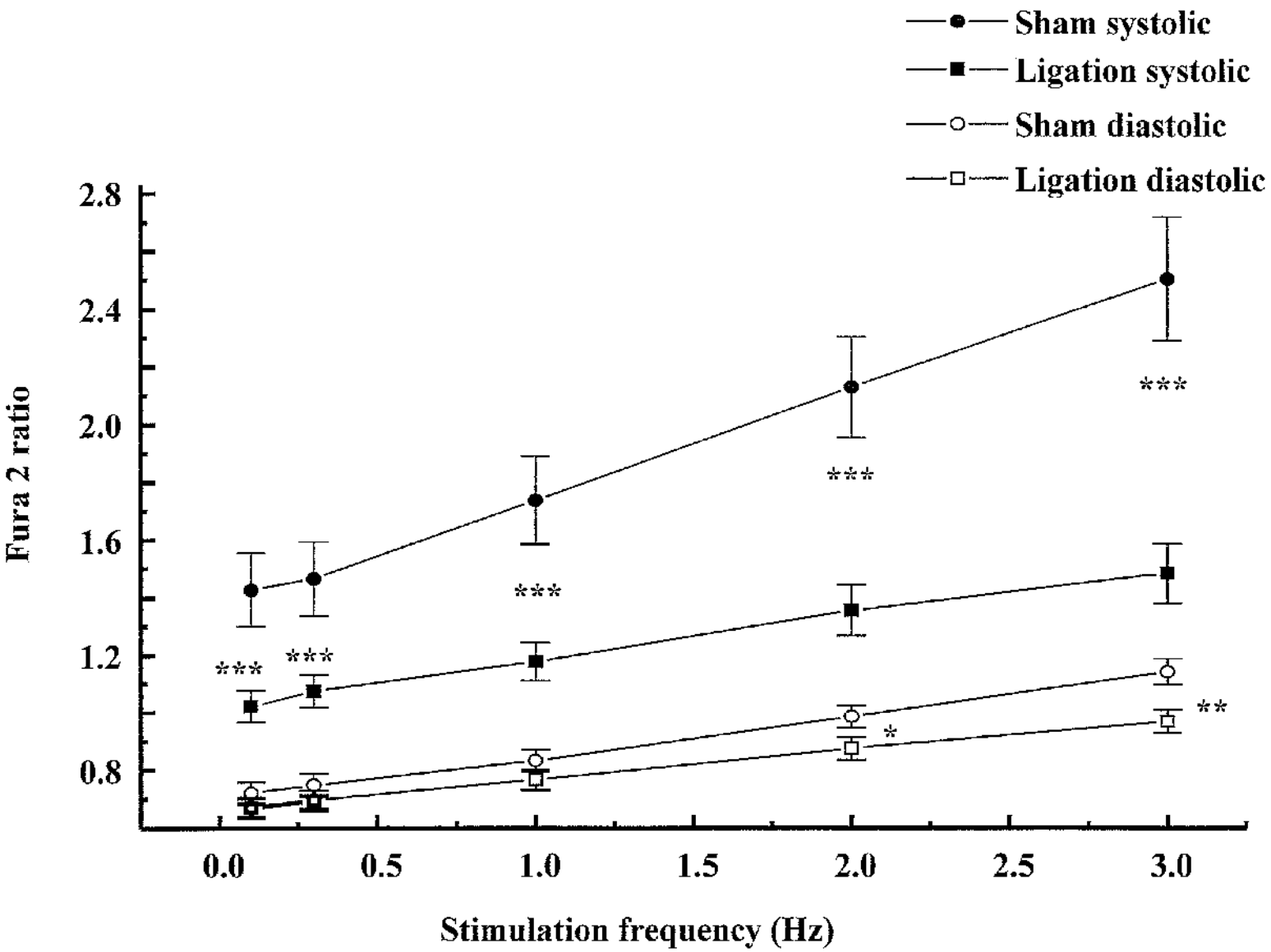

Fig. 2.7: Systolic and diastolic Fura 2 ratios in endocardial myocytes.
(* : $p < 0.05$, ** : $p < 0.01$, *** : $p < 0.001$)

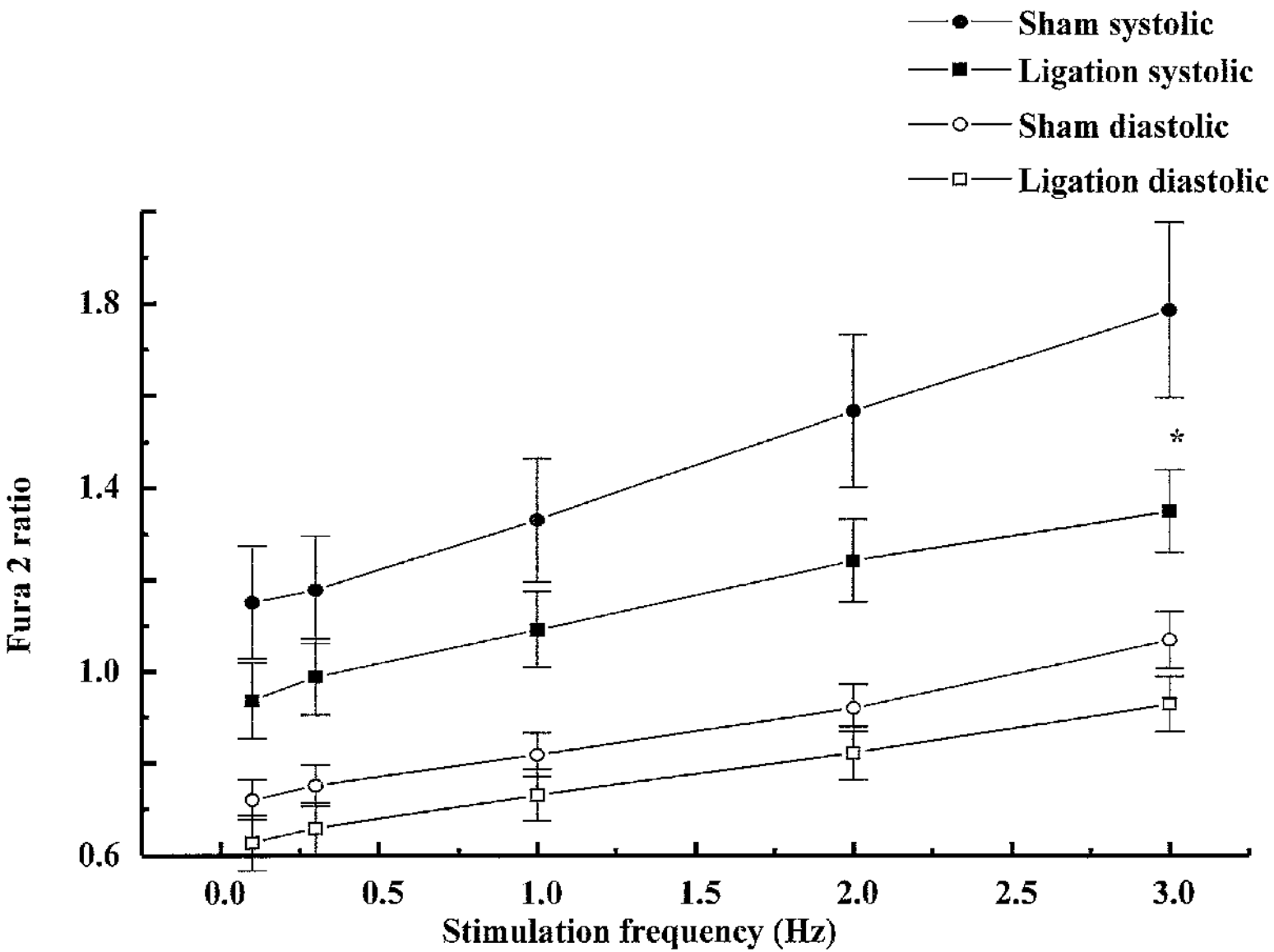

Fig. 2.8: Systolic and diastolic Fura 2 ratios in epicardial myocytes.
(* : $p < 0.05$)

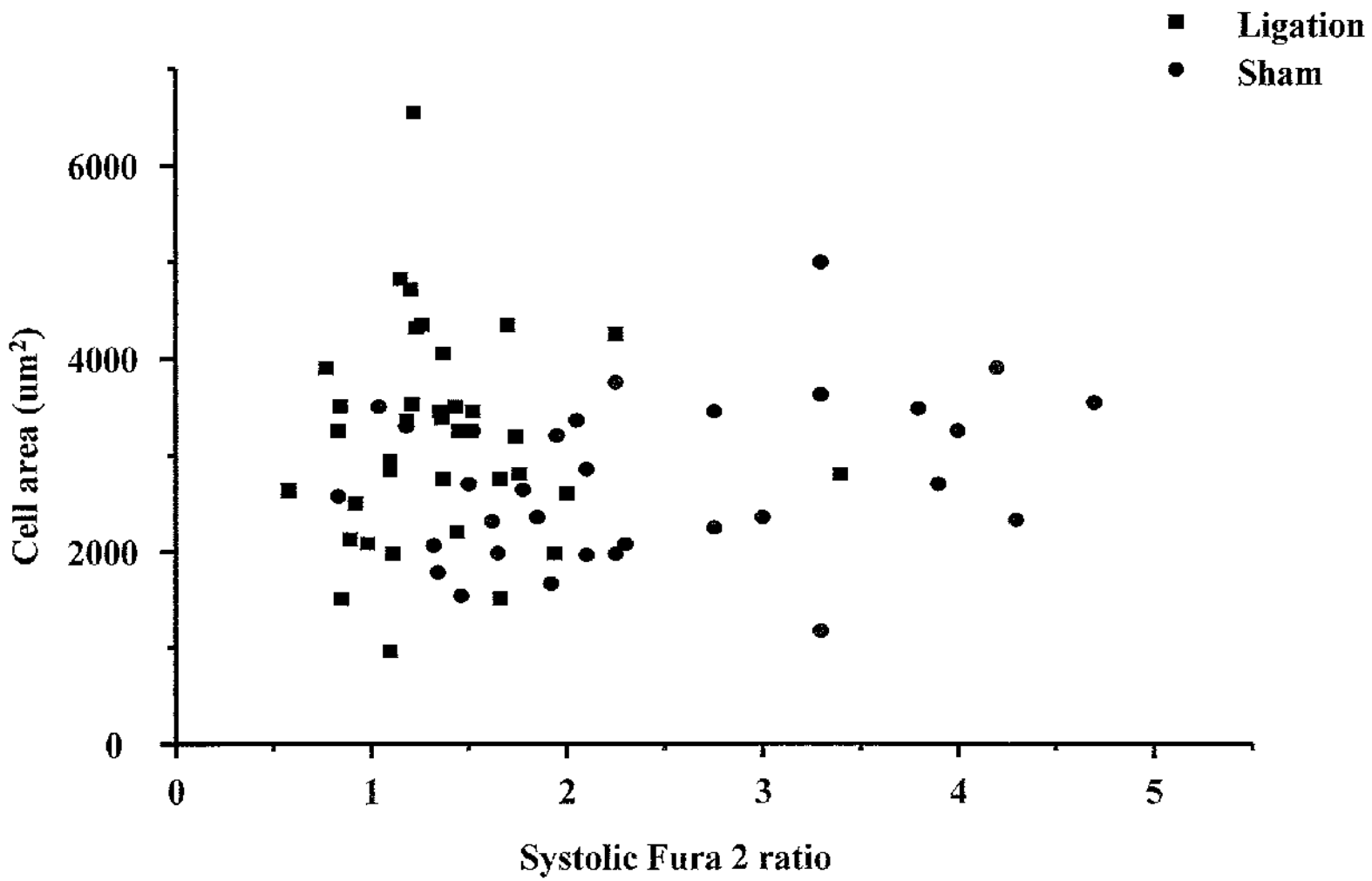

Fig. 2.9 Lack of relationship between cell size and peak systolic [Ca^{2+}]
(Data shown is for endocardial cells stimulated at 3Hz.)

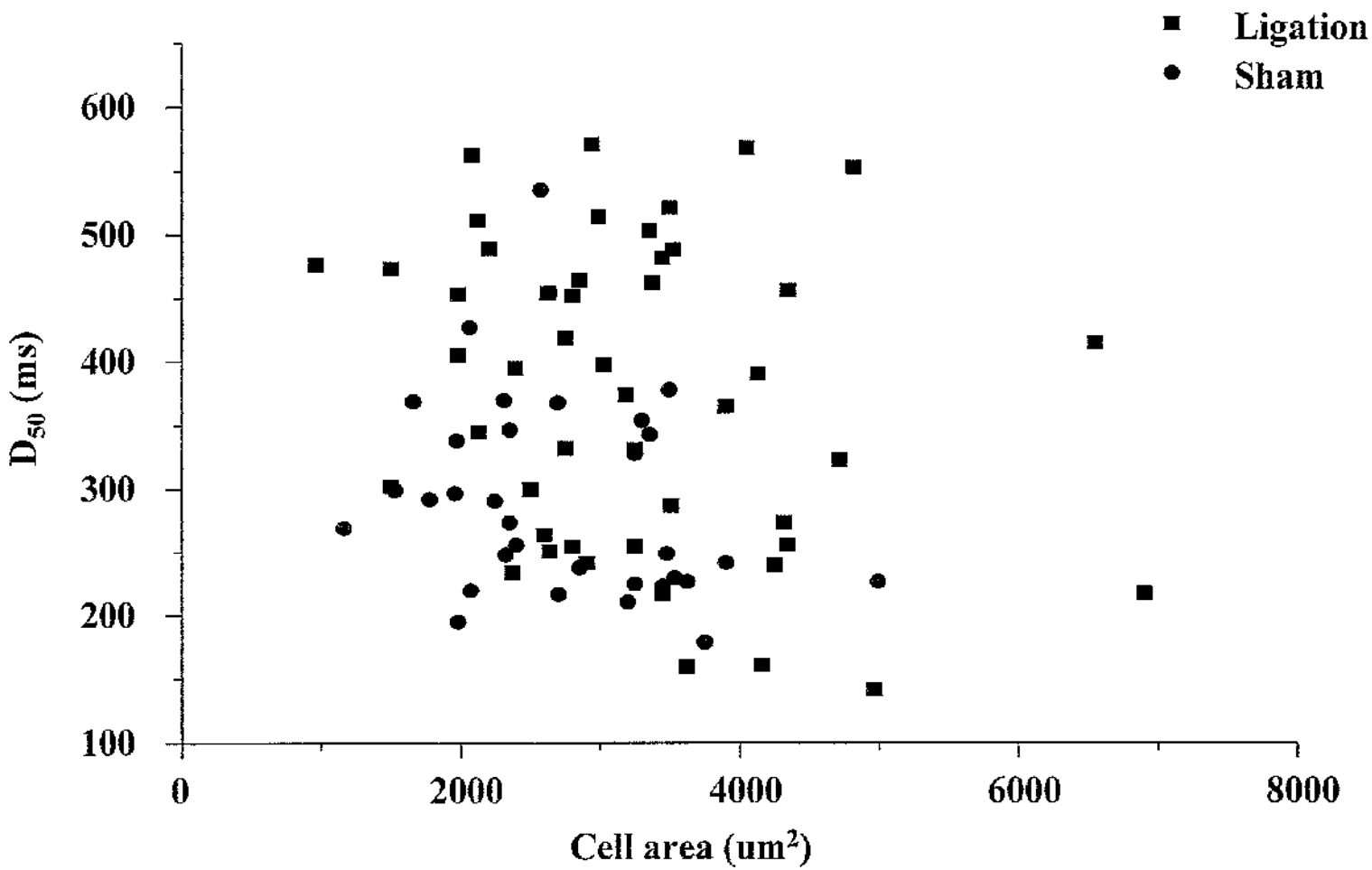

Fig. 2.10 : Lack of relationship between cell size and Ca^{2+} transient duration.
(Data shown is for endocardial cells stimulated at 1Hz.)

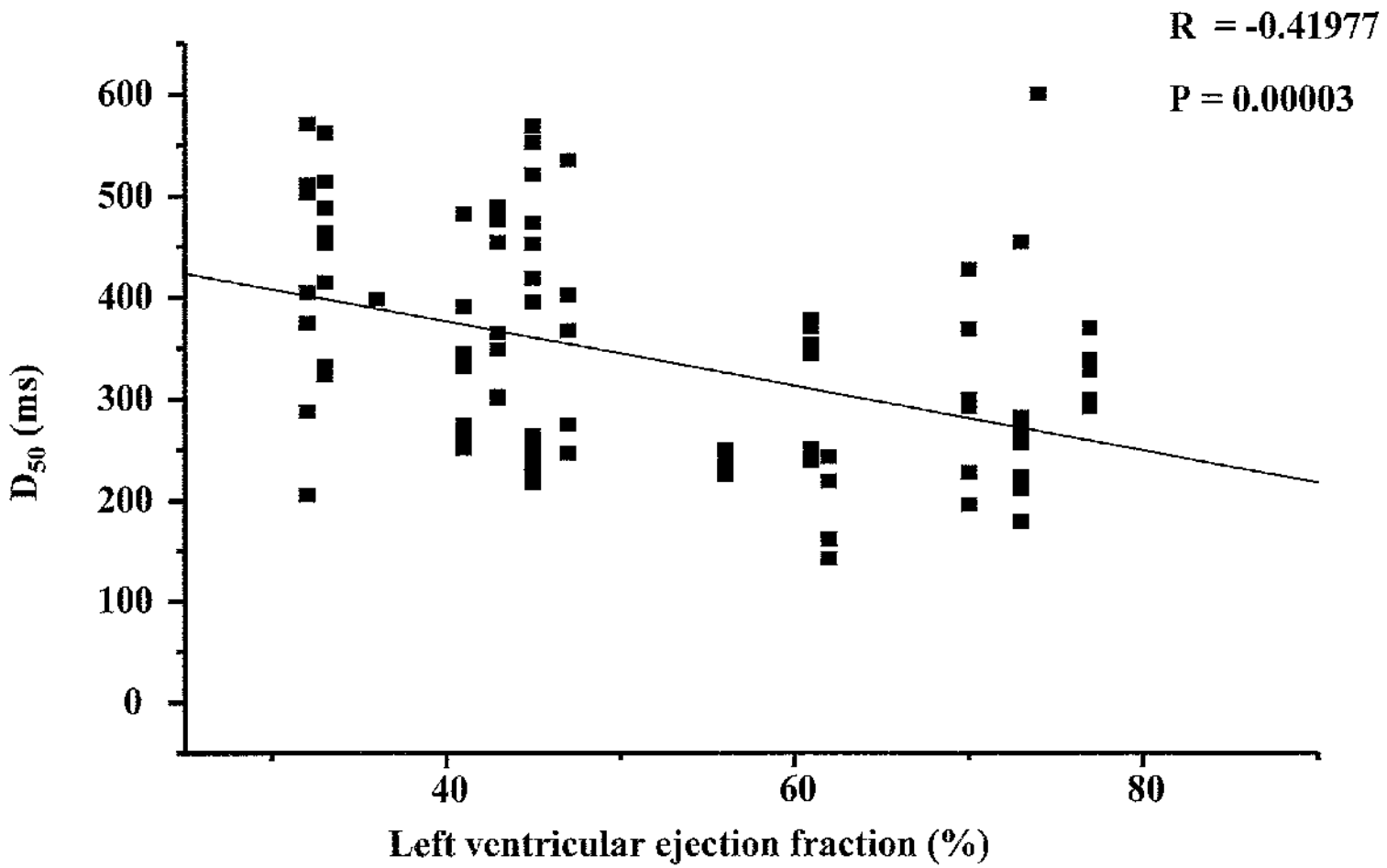

Fig. 2.11 : Relationship of Ca^{2+} transient duration to ejection fraction.
(Data shown is from failure and sham endocardial cells stimulated at 1Hz.)

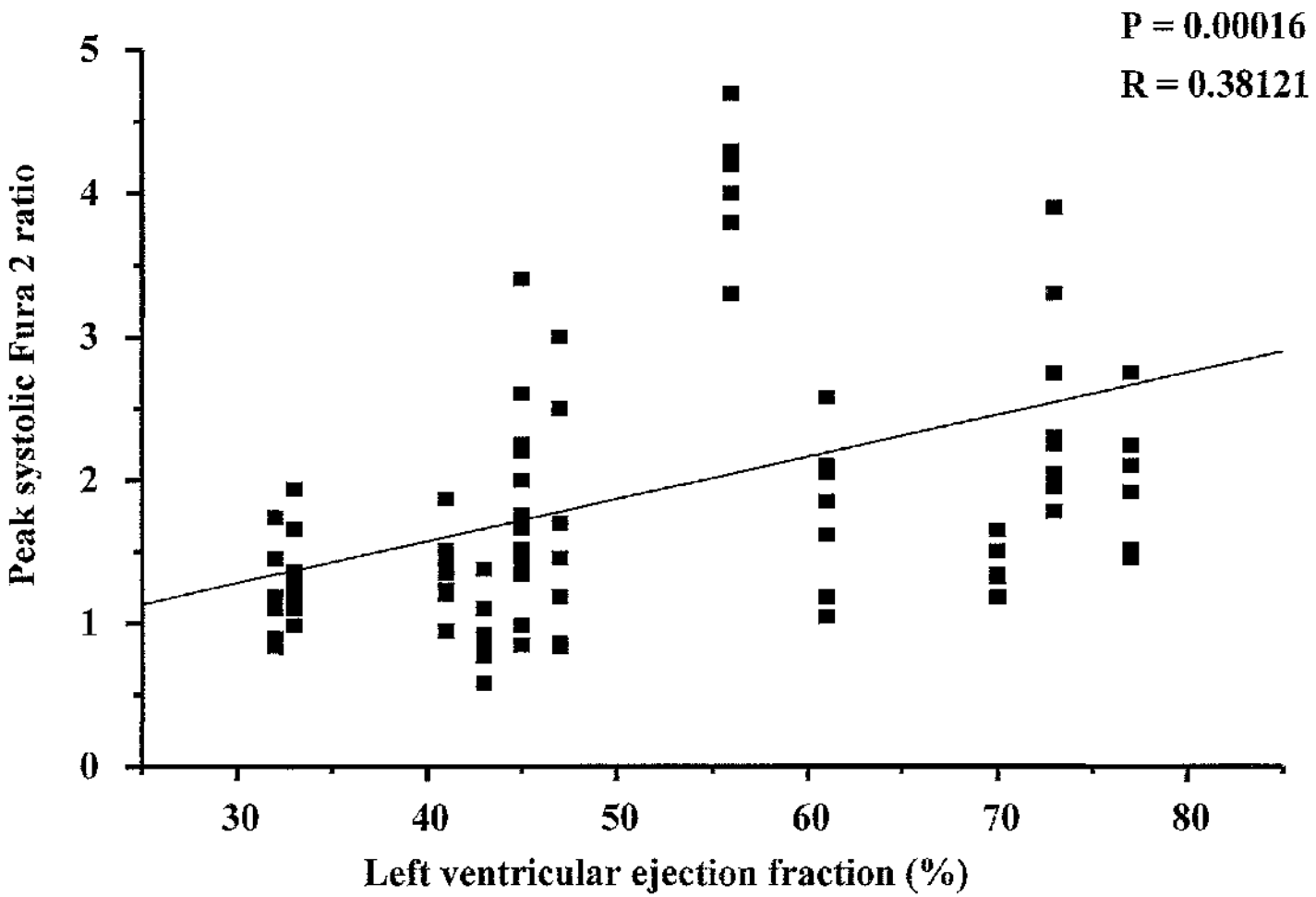

Fig. 2.12: Relationship of peak Ca^{2+} release to ejection fraction.
(Data shown is from failure and sham endocardial cells stimulated at 3Hz.)

Chapter 3 - Studies of SR load in permeabilised myocytes.

3.1 Background.

Studies in intact myocytes have therefore demonstrated abnormal Ca^{2+} handling in the rabbit coronary artery ligation model of left ventricular dysfunction at the level of the whole cell. These studies do not allow firm conclusions as to the mechanism of abnormal Ca^{2+} handling to be drawn. In particular it is not possible to differentiate between abnormalities of sarcolemmal Ca^{2+} transport and SR function using this experimental preparation. As discussed previously, evidence from studies in several species, using various experimental preparations suggests that the most likely cause of abnormal Ca^{2+} handling in heart failure is a reduction in activity of SERCA 2A, the SR Ca^{2+} pump. Following the characterisation of the abnormalities of Ca^{2+} handling at the whole cell level, the next stage of this work was to investigate the mechanisms underlying these changes. Since SR dysfunction was the most likely mechanism, it was decided to examine SR function in isolation from sarcolemmal function in this model of heart failure.

Several experimental preparations have been used to study SR function in heart failure. Ca^{2+} uptake in isolated SR vesicles has been measured in several studies with conflicting results (Ohkusa *et al.* 1997; Movsesian *et al.* 1989; Schwinger *et al.* 1995). Many studies have also inferred SR dysfunction from alterations in measured mRNA and protein expression in heart failure (Stein *et al.* 1996; Matsui *et al.* 1995; Kiss *et al.* 1995; Schwinger *et al.* 1995; Arai *et al.* 1993; Mercadier *et al.* 1990). While useful, the disadvantage of these types of experiment is that SR function is studied in isolation from the excitation contraction process, and the normal anatomy of the SR is disrupted. An alternative preparation which has been used extensively to

study SR function in both smooth and skeletal muscle is the permeabilised or skinned tissue preparation (Isac *et al.* 1988; Suko *et al.* 1976; Endo *et al.* 1982). This preparation has several advantages. Mechanical or chemical skinning either removes or permeabilises the sarcolemma, without disrupting the intracellular architecture. This allows the metabolic and ionic conditions of the cytosol next to the SR to be controlled, and therefore loading of the SR can be measured at precisely controlled pH, Ca^{2+}concentration and other metabolic conditions. Since the intracellular architecture is intact, the cell retains the ability to contract and relax. Skinned preparations are therefore useful for studying the mechanisms of excitation-contraction coupling distal to the sarcolemma.

Skinned cardiac muscle has been studied using trabeculae and other multicellular preparations (eg(Steele & Smith, 1993; Smith & Steele, 1992). Denvir has studied SR loading in permeabilised ventricular trabeculae from rabbits with left ventricular dysfunction (Denvir *et al.* 1996) and from humans with heart failure (Denvir *et al.* 1995). The use of multicellular preparations, however, is hampered by limitations of diffusion which become significant at >50μm diameter. In effect, multicellular skinned preparations behave as a chemical syncytium, without the normal, rapid electrical coupling. In addition to this, individual cells within multicellular preparations can display heterogeneity of SR function and of metabolic conditions, for example in the core of trabeculae.

Permeabilised single myocytes do not suffer from these limitations. There have been previous reports of the use of skinned single cardiac myocytes. Fabiato (Fabiato, 1982) developed techniques for the mechanical skinning of single cardiac myocytes, but these techniques were difficult to apply. Bers (Hove-Madsen & Bers,

1993b; Hove-Madsen & Bers, 1993b) has studied SR Ca^{2+} uptake in suspensions of chemically skinned myocytes. This thesis is believed, however, to be the first substantive work on chemically skinned single cardiac myocytes.

The permeabilised single myocyte preparation is suited to the study of SR function in isolation from changes in sarcolemmal Ca^{2+} transport. As an integrated subcellular preparation it is more physiological than isolated SR vesicle preparations, but is simpler and has fewer confounding factors than trabeculae or other multicellular cardiac preparations. Studies of SR function were therefore undertaken in permeabilised single myocytes from the rabbit model of left ventricular dysfunction described in chapter 2.

3.2 Methods.

3.2.1 Dissociation of ventricular myocytes.

Single ventricular myocytes were dissociated from experimental and sham-operated rabbit hearts as described in section 2.1.2. Cells were isolated from all regions of the left ventricular myocardium and not separated into endocardial, epicardial and mid-myocardial fractions. Following dissociation, cells were suspended in 0.1 relaxing solution (0.1R), a mock intracellular solution with Ca^{2+} concentration buffered to around 50nM with EGTA at pH 7.0 (see appendix 1 for exact composition), and stored at room temperature until use.

3.2.2 Permeabilisation of myocytes.

Cells were then placed in a 250µl perfusion chamber mounted on an inverted Nikon Diaphot microscope as before, and perfused with 1ml min^{-1} of 0.1R at room temperature. In early experiments it was found that permeabilised cells were less

adherent to the cover slip than intact cells, and tended to float out of the field of vision during perfusion and particularly when caffeine was injected. To prevent this, cells were held in place by anchoring with a blunt, sealed micro-electrode positioned onto the cell with a micromanipulator (Narashige Instruments, Japan). Adhesion of the cell to the electrode was improved by coating the electrode in Celltak (Collaborative Biomedical Products). This also formed a barrier on the electrode which prevented leeching of Ca^{2+} from the glass of the electrode. The experimental apparatus is shown in fig. 3.1. A micropipette was placed within the field of vision of the microscope close to the cell. This micropipette was attached to four syringe drivers controlled from a personal computer using the program DT (Francis Burton 1990). The first syringe contained 0.1R with 100μg ml^{-1} β-escin (final concentration 90nM). β-escin is a saponin ester which permeabilises cardiac sarcolemma to low molecular weight molecules by removing cholesterol moieties (Itoh *et al.* 1991). β-escin has little or no effect on the membranes of intra-cellular organelles since these membranes contain little cholesterol and therefore permeabilised cells retain an active SR component (Konishi & Watanabe, 1995). Permeabilisation of the sarcolemma of the myocyte was achieved by rapid injection of 5-10μL of the β-escin solution close to the cell surface. In early experiments the effect of β-escin was monitored by pre-incubating myocytes with membrane permeant Fura-2 AM. Permeabilisation of the sarcolemma was confirmed in these cells by a loss of fluorescence. The lack of effect of β-escin on the SR membrane was also confirmed in an early experiment by exposing permeabilised cells to 1% Triton-X (Sigma chemicals) showing an increase in fluorescence as Ca^{2+} stores were released from the SR on exposure to Triton but not β-escin.

3.2.3 Stimulation of SR Ca^{2+} release.

Skinned preparations allow study of SR function in isolation from sarcolemmal function. Since the sarcolemma is permeabilised by the action of β-escin, the membrane potential is abolished, and myocytes cannot be stimulated electrically. SR Ca^{2+} release and hence cellular contraction can be stimulated in skinned preparations by exposure to millimolar concentrations of caffeine, which causes Ca^{2+} release by increasing the open probability of the ryanodine receptor (Sitsapesan & Williams, 1990). In these experiments SR Ca^{2+} release was stimulated by microinjecting 2-3μL of 10mM caffeine close to the cell via the micropipette. To avoid causing an injection artefact, this injectate had to have the exact composition of the perfusing solution in terms of Ca^{2+} and Fura-2 concentrations. Since the experimental protocol measured caffeine -induced SR Ca^{2+} release at three different cytosolic Ca^{2+} concentrations, it was necessary to use three syringe drivers connected to the same micropipette with a very small terminal deadspace, so that different solutions could be injected through the same pipette with minimal mixing.

3.2.4 Experimental protocol.

Throughout all experiments cells were superfused at 1ml min^{-1} with 0.1R containing 10μM Fura-2. Ca^{2+} concentration was varied during the experiment by the addition of 10 mM $CaCl_2$ (0-4 μl ml^{-1} - giving solutions of 50-250 nM Ca^{2+} concentration, referred to as solutions 0,1,2,3,and 4). Fluorescence was measured using a spectrophotometer at 30 Hz as described in section 2.1.3 and recorded on VHS videotape and chart recorder. The signal was digitised and recorded to disc using the program New Tape (Francis Burton 1990). Video image of cell contraction was simultaneously monitored on screen and recorded to videotape.

Myocytes with clear cross striations and minimal membrane blebbing were selected for study. The field of view of the microscope was constrained to the area of the cell being studied by a diaphragm on the output port of the microscope. The contribution to the signal of the bulk solution above the cell did not appear to depend on the depth of the bath, probably because the cell was in the focal plane of the microscope. Following anchoring, each cell was superfused for 2 minutes with solution 1 (100nM [Ca^{2+}]) before being permeabilised by brief exposure to β-escin. A test injection of 10mM caffeine confirmed successful permeabilisation if cellular contraction was accompanied by a Ca^{2+} transient, since Fura 2 does not cross the sarcolemma of intact cells. SR Ca^{2+} release and cellular contraction were then stimulated at 2 min intervals by injection of 2-3μl 10 mM caffeine via the micropipette described above. This protocol continued until reproducible caffeine-induced Ca^{2+} transients were elicited. Caffeine-induced Ca^{2+} transients were then measured in solution 0 (50nM [Ca^{2+}]) and then solution 2 (150nM [Ca^{2+}]) again at 2 minute intervals until reproducible transients were obtained. Ca^{2+} concentration in the injected caffeine solution was always equal to that in the perfusate to avoid injection artefact. Thus caffeine-stimulated SR Ca^{2+} release was measured at cytosolic Ca^{2+} concentrations from 50-150 nM. At higher cytosolic Ca^{2+} concentration, spontaneous SR Ca^{2+} release occurred, and caffeine stimulation was unnecessary. Spontaneous Ca^{2+} transients were then recorded for 2 mins during perfusion with solutions 3 and 4 (200nM and 250nM [Ca^{2+}]). Spontaneous Ca^{2+} transients were therefore measured at 200-250 nM cytosolic Ca^{2+} concentration, but in most cells spontaneous Ca^{2+} release first occurred at cytosolic Ca^{2+} concentrations of 100-150nM. Thus for many cells results were available to compare caffeine-induced and spontaneous Ca^{2+} release at

the same cytosolic Ca^{2+} concentrations in solutions 1 and 2. A representative experimental trace showing caffeine-induced and spontaneous Ca^{2+} transients is shown in fig. 3.2.

3.2.5 Calibration of cytosolic Ca^{2+} concentration

Use of Fura 2 acid in permeabilised cells allows precise measurement of Ca^{2+} concentration, since calibration curves can be plotted of Fura 2 ratio against Ca^{2+} concentration. The relationship of Ca^{2+} concentration to Fura 2 ratio is sigmoid, and is described by the equation:

$$Ca^{2+} = K_D \times \left(\frac{R - R_{min}}{R_{max} - R} \right) \qquad [1]$$

where **R** is the measured Fura 2 ratio, R_{min} is the ratio in Ca^{2+}-free solution, R_{max} is the ratio at saturating Ca^{2+} concentration, and K is constant (Grynkiewicz *et al.* 1985).

A calibration curve of Fura 2 ratios against Ca^{2+} concentration was plotted. Ca^{2+} concentration was varied by combining different ratios of 10 mM EGTA and 10 mM CaEGTA. Ca^{2+} concentration in these solutions was calculated using the program React (Godfrey Smith 1990). A curve was then fitted to the data points using the equation:

$$Y = \frac{\left(\frac{X}{K_D} \right) \times (R_{max} + R_{min})}{1 + X \times K_D} \qquad [2]$$

(from (Grynkiewicz *et al.* 1985)). Here Y (Fura 2 ratio) is a function of X (Ca^{2+} concentration) dependant on the constants R_{max}, R_{min} and K_D. The curve was

fitted by iterative variation of these constants until the best fit was found. This was done using the program Origin (Microcal Software). Using the constants calculated from this curve fitting, Ca^{2-}concentration could then be calculated from any given Fura 2 ratio using equation [1]. The calibration curve is shown in fig. 3.3. The constants given by this curve were:

$\mathbf{R_{min}}$ 0.531

$\mathbf{R_{max}}$ 9.068

$\mathbf{K_D}$ 1.517×10^{-6}

3.3 Results.

3.3.1 Measurement of background Ca^{2+}concentration.

EGTA was used in all solutions at a concentration of 0.1mM to buffer Ca^{2+}concentration to very low levels. Free Ca^{2-}concentration in these experiments was designed to range from sub-diastolic levels (around 50nM) to levels approaching those seen during systole (200-300nM). Using the program REACT the theoretical free Ca^{2+}concentration of 0.1R with no added Ca^{2+} was calculated to be of the order of 50nM. Adding 1µl ml^{-1} 10mM $CaCl_2$ was calculated to raise free Ca^{2+}concentration by around 50nM, giving a range of free Ca^{2+}concentration for the experiment from 50-250nM by adding 0-4 µl ml^{-1} 10mM $CaCl_2$. (For the purposes of these calculations, the contaminant Ca^{2+}concentration of de-ionised water in this laboratory has previously been measured using a pH metric technique (Moisescu & Pusch, 1975) to be 5.6 ± 0.35µM and using similar techniques the purity of EGTA has been measured to be 97%. Free $[Mg^{2+}]$ for these experiments was 1mM).

Using the Fura-2 Ca^{2+}concentration calibration curve described above, Ca^{2+}concentration in all experimental solutions was measured. The mean results for solutions used for ligation and sham experiments are shown in table 3.1.

	Ligation		Sham		
Calcium	Mean	SEM	Mean	SEM	p
0	16.0nM	5.7nM	16.6nM	6.7nM	0.47
1	50.1nM	6.8nM	50.6nM	9.9nM	0.48
2	86.0nM	9.3nM	74.2nM	15.2nM	0.25
3	154.6nM	15.9nM	129.1nM	28.1nM	0.2
4	217.0nM	19.8nM	195.4nM	38.4nM	0.29

Tab 3.1: Mean Ca^{2+}concentration measured by Fura-2 fluorescence for solutions used in skinned cell experiments. Calcium is added in aliquots of 0-4 $\mu l\ ml^{-1}$ of 10mM $CaCl_2$.

As can be seen from table 3.1, Fura-2 measurements of Ca^{2+}concentration consistently underestimated theoretical calculated Ca^{2+}concentration, however there were no significant differences between the mean Ca^{2+}concentration of solutions used for ligation and sham experiments. The differences between measured Ca^{2+} and theoretical calculated Ca^{2+} may reflect buffering of Ca^{2+} by cells themselves (which were not present in calibration solutions), or small changes in solution composition due to cellular metabolites.

3.3.2. Caffeine-induced Ca^{2+} transients.

Peak Ca^{2+}concentration following caffeine injection was measured for all cells in solutions containing 0,1 and 2 $\mu l\ ml^{-1}$ 10mM $CaCl_2$. These will be referred to as

solution 0,1 and 2 respectively. The amplitude of the Ca^{2+} transient produced by caffeine injection was calculated as the rise in measured Ca^{2+}concentration. This measurement rather than an integral of Ca^{2+} release throughout the transient was used since the duration of the Ca^{2+} transient produced by caffeine injection depended upon the length of time the cell was exposed to caffeine. Exposure time of the cell to caffeine depended upon the rate at which caffeine diffused away from the cell following injection. This varied between experiments and depended upon the depth of the bath and the exact placement of the injection cannula with respect to the cell. These factors could not be standardised between experiments, but should not have affected the amplitude of the Ca^{2+} transient, in particular, small movements of the injection cannula did not qualitatively affect peak Ca^{2+} release. For these reasons, and because some SR Ca^{2+} re-uptake can occur even in the presence of caffeine (Smith & Steele, 1998), measurement of the peak Ca^{2+} release rather than the integral of Ca^{2+} release was used as a surrogate measure of SR load. Experiments were first undertaken in stock animals to investigate the validity of this approach. Figure 3.4 shows that within free Ca^{2+} concentrations studied in these experiments, there is an approximately linear relationship between cytosolic Ca^{2+}concentration and the amplitude of caffeine-induced Ca^{2+} transients, suggesting that amplitude of Ca^{2+} release is a reasonable measure of SR load.

3.3.3 Caffeine response in heart failure and sham cells.

Experiments were undertaken in 37 cells from 11 experimental hearts, and in 38 cells from 13 sham hearts. In 8 cells (21.6%) from experimental hearts caffeine-induced Ca^{2+} transients could not be elicited at any of the three cytosolic Ca^{2+}concentrations studied. This was the case in only one cell (2.6%) from sham

operated hearts. Successful permeabilisation was confirmed in all of these cells by observing spontaneous cell contraction with associated Ca^{2+} transients in higher Ca^{2+}concentration solutions. Failure of these cells to respond to caffeine may therefore have reflected poor placement of the caffeine-injection cannula, although in all but one experiment, other cells produced adequate transients with the same placement of the cannula. There was therefore a significantly higher percentage of cells from experimental hearts which failed to respond to caffeine ($p<0.01$). This difference did not remain significant if the experiment where no cells from one heart responded to caffeine was removed from the analysis ($p=0.06$).

In addition to the cells which did not respond to caffeine at any cytosolic Ca^{2+}concentration, there were a number of cells from both failure and sham hearts that did not respond to caffeine at all cytosolic Ca^{2+} concentrations. Cells were most likely to respond to caffeine in higher Ca^{2+}concentration solutions. Excluding cells which did not respond to caffeine at any Ca^{2+}concentration, 62.1% of cells from ligation hearts responded to caffeine in solution 0. A significantly greater fraction of cells from sham operated hearts responded to caffeine in solution 0 (83.8% $p<0.05$). For solutions 1 and 2, similar numbers of cells from experimental and sham hearts responded to caffeine (96.6% vs 91.9% for solution 1 and 96.6% vs 97.3% for solution 2).

There are two possible explanations for cells which did not respond to caffeine at low cytosolic Ca^{2+}concentration. The first explanation is that this represents a true failure to respond to caffeine, reflecting low SR Ca^{2+} load. It is difficult, however, to discount the possibility that the amplitude of Ca^{2+} transients elicited by caffeine-injection in these cells was too low to be detected above the background noise.

Visual inspection of these cells for contraction was difficult as during caffeine injection there was a small movement artefact of the cell, and so small contractions could also be missed.

3.3.4 Amplitude of caffeine-induced Ca^{2+} transients.

The amplitude of caffeine-induced Ca^{2+} transients for experimental and sham cells for solutions 0, 1 and 2 are shown in table 3.2. Data shown are mean ± SEM for 37 cells from 11 experimental hearts and from 38 cells from 13 sham operated hearts. Mean amplitudes of caffeine-induced Ca^{2+} transients were significantly greater in cells from sham hearts at all three cytosolic calcium concentrations. As with preliminary results from stock animals, in sham cells there was a positive relationship between cytosolic Ca^{2+}concentration and the amplitude of caffeine-induced Ca^{2+} transients. This relationship was blunted in cells from experimental hearts. This is also shown in fig. 3.5.

	Ligation		Sham		
Calcium	Mean	SEM	Mean	SEM	p
0	22.3nM	3.4nM	34.1nM	3.8nM	<0.05
1	35.5nM	6.0nM	56.5nM	6.5nM	<0.05
2	39.3nM	6.3nM	61.4nM	9.5nM	<0.05

Table 3.2: mean amplitude of caffeine-induced Ca^{2+} transients in cells from ligation and sham hearts.

3.3.5 Spontaneous activity.

Again data were available from 37 cells from 11 experimental hearts and from 38 cells from 13 sham operated hearts. Any cells which did not respond to caffeine

and did not show any spontaneous activity were discarded and not included in any analysis. Spontaneous contraction with associated Ca^{2+} transients was not seen in any cells in solution 0. No spontaneous activity was seen in 9 cells from sham hearts (23.7%) at any cytosolic Ca^{2+}concentration. Similarly there was no spontaneous activity at any Ca^{2+}concentration in 10 cells from experimental hearts (27.0%, p = n.s.). The integrity of the contractile response in these cells and their successful permeabilisation was confirmed by the presence of contraction with associated Ca^{2+} transients during caffeine injection.

Spontaneous activity was observed in increasing number of cells from both failing hearts and sham operated hearts as cytosolic Ca^{2+}concentration increased. Excluding cells with no spontaneous activity at any Ca^{2+}concentration, 48.3% of sham cells were spontaneously active in solution 1 compared to 14.8% of experimental cells ($p<0.01$). Figures for solution 2 were 75.9% and 44.4% ($p<0.01$). There was no difference between sham and experimental cells in solution 3 (93.1% vs 88.9%, p = n.s.) or solution 4 (93.1% vs 96.3%, p = n.s.).

3.3.6 Frequency of spontaneous activity.

The mean frequency of spontaneous Ca^{2+} release and contraction for cells from experimental and sham operated hearts is compared in table 3.3 and shown in fig. 3.6. Frequency increases with increasing cytosolic Ca^{2+}concentration in cells from both experimental and sham hearts. At low cytosolic Ca^{2+}concentration frequency of spontaneous Ca^{2+} release is significantly greater in cells from sham hearts. This difference is not apparent at higher cytosolic Ca^{2+}concentration (solutions 3 and 4).

and did not show any spontaneous activity were discarded and not included in any analysis. Spontaneous contraction with associated Ca^{2+} transients was not seen in any cells in solution 0. No spontaneous activity was seen in 9 cells from sham hearts (23.7%) at any cytosolic Ca^{2+}concentration. Similarly there was no spontaneous activity at any Ca^{2+}concentration in 10 cells from experimental hearts (27.0%, p = n.s.). The integrity of the contractile response in these cells and their successful permeabilisation was confirmed by the presence of contraction with associated Ca^{2+} transients during caffeine injection.

Spontaneous activity was observed in increasing number of cells from both failing hearts and sham operated hearts as cytosolic Ca^{2+}concentration increased. Excluding cells with no spontaneous activity at any Ca^{2+}concentration, 48.3% of sham cells were spontaneously active in solution 1 compared to 14.8% of experimental cells ($p<0.01$). Figures for solution 2 were 75.9% and 44.4% ($p<0.01$). There was no difference between sham and experimental cells in solution 3 (93.1% vs 88.9%, p = n.s.) or solution 4 (93.1% vs 96.3%, p = n.s.).

3.3.6 Frequency of spontaneous activity.

The mean frequency of spontaneous Ca^{2+} release and contraction for cells from experimental and sham operated hearts is compared in table 3.3 and shown in fig. 3.6. Frequency increases with increasing cytosolic Ca^{2+}concentration in cells from both experimental and sham hearts. At low cytosolic Ca^{2+}concentration frequency of spontaneous Ca^{2+} release is significantly greater in cells from sham hearts. This difference is not apparent at higher cytosolic Ca^{2+}concentration (solutions 3 and 4).

	Ligation		Sham		
Calcium	Mean	SEM	Mean	SEM	p
1	0.011hz	0.006	0.034hz	0.007	<0.05
2	0.031hx	0.009	0.056hz	0.009	<0.05
3	0.08hz	0.014	0.094hz	0.013	>0.05
4	0.135hz	0.023	0.139hz	0.019	>0.05

Table 3.3: frequency of spontaneous Ca^{2+} release in cells from experimental and sham hearts.

3.3.7 Amplitude of spontaneous Ca^{2+} transients.

The amplitudes of spontaneous Ca^{2+} transients for experimental and sham cells for solutions 1, 2, 3 and 4 are shown in table 3.4. Mean amplitude ± SEM was calculated only from cells which were spontaneously active at the given cytosolic Ca^{2+}concentration. The number of cells active and therefore used for calculation purposes is shown in the table. Mean amplitudes of transients were greater at all cytosolic Ca^{2+}concentration in cells from sham hearts than from experimental hearts. This reached statistical significance for solutions 2, 3 and 4. As with caffeine-induced Ca^{2+} transients, in sham cells there was a positive relationship between cytosolic Ca^{2-}concentration and the amplitude of spontaneous Ca^{2+} transients. This relationship was blunted in cells from experimental hearts This is shown in fig. 3.7, and amplitude of spontaneous Ca^{2+} release is compared to that for caffeine-induced release in fig. 3.8.

	Ligation.			Sham.			
Calcium	Mean	SEM	N	Mean	SEM	N	p
1	27.2nM	7.2nM	4	49.4nM	7.4nM	14	>0.05
2	37.5nM	4.3nM	12	68.4nM	9.2nM	22	<0.05
3	48.7nM	4.1nM	25	73.4nM	10.4nM	27	<0.05
4	65.6nM	6.3nM	28	106.7nM	14.1nM	28	<0.01

Table 3.4 mean amplitude of spontaneous Ca^{2+} transients in cells from experimental and sham hearts.

3.4 Discussion.

3.4.1 Experimental preparation.

Experiments in intact myocytes confirmed the presence of abnormal Ca^{2+} handling in this coronary artery ligation model of heart failure. The experiments described in this chapter were designed to provide more specific information on the site or mechanism of abnormal Ca^{2+} handling. The permeabilised myocyte preparation allowed the investigation of SR function in isolation from any possible changes in sarcolemmal Ca^{2+} transport.

While permeabilisation of the sarcolemma allowed specific study of SR function, this also led to the main drawback of this preparation, the inability to electrically stimulate the preparation. This meant that experiments in permeabilised cells could not be directly analogous to those in intact cells. In intact cells the effect of increasing the frequency of stimulation was studied, and showed that in normal cells increasing stimulation frequency caused an increase in the amplitude of Ca^{2+}

transients, analogous to the positive force-frequency relationship which has previously been correlated to increasing systolic Ca^{2+} release (Pieske *et al.* 1995).

In permeabilised cells the effect of increasing stimulation frequency can not be directly assessed. In the current experiments what is investigated is the ability of the SR to load at different cytosolic Ca^{2+} concentrations. These experiments show that as cytosolic Ca^{2+} concentration increases, the amplitude of Ca^{2+} release rises, and there is an increased frequency of spontaneous Ca^{2+} release. This is relevant to the positive force frequency relationship as one of the mechanisms of increased contractile force at higher stimulation frequencies is a rise in diastolic Ca^{2+} which promotes a rise in SR Ca^{2+} content (reviewed in (Bers, 1991)). Diastolic Ca^{2+} rises since increased stimulation frequency increases Ca^{2+} influx through L-type Ca^{2+} channels. There is a proportional reduction in the time spent in diastole and therefore reduced Ca^{2+} efflux. From this it follows that the force frequency relationship depends upon the integrity of function of the SR Ca^{2+} pump, SERCA 2A. If abnormal function of this pump underlies the abnormal Ca^{2+} handling seen in heart failure as seems most likely from the studies in intact cells described above and from evidence discussed in the introduction to this thesis, then investigation of the ability of the SR to load under different conditions of cytosolic Ca^{2+}concentration is reasonable.

The nature of the permeabilised myocyte preparation also means that the duration of Ca^{2+} transients which was seen to be abnormal in intact cells could not be investigated in these experiments. In intact cells the decay of the Ca^{2+} transient is effected by both SERCA 2A and Na^{+}-Ca^{2+} exchange. In permeabilised cells, however, the decay depends upon both SR Ca^{2+} uptake mediated by SERCA 2A and diffusion of Ca^{2+} away from the cell studied. Since the fraction of the decay mediated

by diffusion cannot be quantified and this fraction will vary between experiments due to alterations in bath kinetics, it was not possible to make comparisons of the duration of Ca^{2+} transients between cells using this preparation.

An additional benefit of this preparation however, was the opportunity to study spontaneous SR Ca^{2+} release. Spontaneous SR Ca^{2+} release is thought to reflect SR Ca^{2+} overload (Lakatta, 1992), and may cause ventricular arrhythmias by the production of delayed afterdepolarisations (Marban *et al.* 1986). Arrhythmias produced by this mechanism may be particularly important in heart failure (Vermeulen *et al.* 1994). Since cytosolic Ca^{2+} concentration can be controlled directly in the permeabilised myocyte preparation, SR Ca^{2+} overload and spontaneous SR Ca^{2+} release can be readily produced. This preparation therefore allowed the relationship between SR Ca^{2+} load and spontaneous SR activity to be compared in normal and failing tissue.

3.4.2 Loading characteristics of the SR in heart failure.

Caffeine-induced contracture of myocytes and trabeculae have been used previously to assay the Ca^{2+} content of the SR (Bers, 1987). Implicit in these measurements is the assumption that caffeine causes the release of a high fraction of stored Ca^{2+} and that this fraction is reproducibly released. Given the good agreement between caffeine-induced Ca^{2+} release and rapid cooling induced contracture results (Bers, 1987), these assumptions appear reasonable. In the current experiments the *amplitude* of the caffeine-induced Ca^{2+} transient has been used as a measure of SR Ca^{2+} load or content. The alternative measure would have been to integrate the area beneath the Ca^{2+} transient, but this method cannot be used since this area is critically dependent on bath flow, the characteristics of which vary between experiments. The

linear relationship between cytosolic Ca^{2+}concentration and Ca^{2+} transient amplitude as a measure of SR load shown in fig. 3.4 suggests that this is a valid approach. Thus although SR Ca^{2+} content can not be directly measured using these techniques, since amplitude of caffeine-induced transients depends upon SR Ca^{2+} content, it can be used to compare SR Ca^{2+} content in different cells under the same loading conditions. Fig. 3.8 shows that at the same loading conditions caffeine-induced Ca^{2+} transients are generally larger than spontaneous transients, indicating that a greater proportion of SR Ca^{2+} is released during caffeine-stimulation than during spontaneous release. These differences are not significant, however, and mean amplitude of spontaneous release is also a valid comparative measure of SR load.

Figs 3.5 and 3.7 show that under the same loading conditions, spontaneous and caffeine-induced Ca^{2+} transients have significantly greater amplitude in sham cells compared to cells from failing hearts. The ratio of sham transient amplitudes to failure amplitudes remains fairly constant across all loading conditions as shown in table 3.5.

Ca2+ soln:	ratio (sham : failing)
0 (caff)	1.529
1 (caff)	1.593
1 (spont)	1.817
2 (caff)	1.561
2 (spont)	1.825
3 (spont)	1.508
4 (spont)	1.626
mean	1.577 ± 0.050

Table 3.5 Ratios of mean amplitude of Ca^{2+} release in cells from sham and failing hearts in different Ca^{2+} solutions.

Amplitude of Ca^{2+} release is therefore in the order of 50-60% greater at all loading conditions in sham cells compared to failing cells. As discussed above, the implication of this is that SR Ca^{2+} content is greater by the same magnitude in sham cells than failing cells. The only alternative explanation of this would be that the fractional release of SR Ca^{2+} stores during caffeine stimulation or during spontaneous oscillation was different in heart failure.

Is it possible to differentiate between these two possibilities? A reduction in the fractional release of Ca^{2+} during contraction could be mediated by a reduction in the numbers of SR Ca^{2+} release channels. As discussed in chapter 1, there is contradictory evidence for such a reduction with some studies finding a reduction in mRNA (Go *et al.* 1995; Arai *et al.* 1993) and cDNA (Brillantes *et al.* 1992) for the ryanodine receptor with others finding unchanged protein levels (Meyer *et al.* 1995;

Movsesian *et al.* 1994). Since the amplitude of Ca^{2+} release is approximately 60% higher in sham cells, it is possible to estimate the change in fractional Ca^{2+} release in heart failure needed to explain this. If SR Ca^{2+} content is equivalent in heart failure and sham cells, and it is assumed that caffeine causes an 80% release of stored Ca^{2+} in normal tissue, this figure must be reduced to 50% in heart failure to account for the reduced Ca^{2+} release seen. Similarly if it is assumed that 70% of stored Ca^{2+} is released during a spontaneous contraction in normal cells, 43.8% would be released in failing cells. Brillantes (Brillantes *et al.* 1992) reported a 28% reduction in cDNA for the ryanodine receptor in heart failure, which is of the order necessary to account for the reduction in fractional Ca^{2+} release that would be necessary to explain the smaller Ca^{2+} transients observed here. This seems unlikely, however, since studies measuring actual ryanodine receptor protein levels have reported no change (Meyer *et al.* 1995; Movsesian *et al.* 1994). However, studies in ferret myocytes have shown that fractional release of stored Ca^{2+} from the SR depends upon SR Ca^{2+} content, and that the fraction of Ca^{2+} released rises as stored Ca^{2+} increases (Bassani *et al.* 1995). This suggests that if SR Ca^{2+} content was reduced in heart failure, there might be an additive effect on Ca^{2+} release since a smaller fraction of the SR Ca^{2+} store would be released.

A reduction in the fraction of SR Ca^{2+} released as a single explanation for the observed reduction in amplitude of Ca^{2+} transients seems unlikely when considered with the findings in this study with regard to spontaneous SR activity, however. Denvir found that spontaneous SR Ca^{2+} release was seen in skinned trabeculae following long loading periods at high loading Ca^{2+} concentration, suggesting that spontaneous activity depended upon SR Ca^{2+} overload (Denvir *et al.* 1995). In the

current experiments, heart failure cells were significantly less likely to be spontaneously active than control cells, and in cells which were spontaneously active, frequency of oscillation was significantly lower in heart failure cells at low Ca^{2+}concentration, and equivalent at higher Ca^{2+}concentration (as shown in fig. 3.6). If heart failure cells had equivalent SR Ca^{2+} content but released less Ca^{2+} per oscillation, the time required for the SR to accumulate sufficient Ca^{2+} to cause overload would be less than in control cells. Thus if the main determinant of spontaneous Ca^{2+} release was SR Ca^{2-} overload, heart failure cells would be expected to have higher frequencies of spontaneous oscillation. The fact that this is not the case argues against the hypothesis that Ca^{2+} transient amplitude is lower in heart failure due to a reduced fraction of SR Ca^{2+} load being released.

It therefore seems probable that the reduction in systolic Ca^{2+} release in heart failure cells is caused by a lower SR Ca^{2+} content in these cells. Since previous studies have found no difference in levels of the SR Ca^{2+} storage protein calsequestrin (Arai *et al.* 1993; Meyer *et al.* 1995; Movsesian *et al.* 1994), it seems unlikely that this reduction in SR Ca^{2+} content depends upon reduced ability of the SR to store Ca^{2+}. Furthermore, if the assumption that caffeine stimulation causes the release of most of the stored Ca^{2+} from the SR is correct, it follows that there must be abnormal SR Ca^{2+} uptake, whether there is a defect in SR Ca^{2+} storage ability or not.

The working hypothesis suggested both by the weight of previous investigation and the results of experiments on intact cells was that the abnormal Ca^{2+} handling seen in heart failure resulted from a reduction in the activity of the SR Ca^{2+} pump, SERCA 2A. Evidence for a reduction in protein expression of SERCA 2A is strong, particularly in animal models of heart failure (Stein *et al.* 1996; Matsui *et al.* 1995;

Kiss *et al.* 1995), and as discussed in chapter 2, the most likely explanation for the prolongation of Ca^{2+} transients and the reduction in systolic release of Ca^{2+} in intact myocytes was a reduction in the activity of SERCA 2A. The present data from permeabilised myocytes support this hypothesis. A reduction in the activity of SERCA 2A is consistent with the findings of reduced SR Ca^{2+} release during caffeine-induced or spontaneous contractions. This reduction in activity of SERCA 2A would cause a reduction in SR Ca^{2+} content, and reduced Ca^{2+} release on stimulation. Since SR Ca^{2+} uptake would be slower, if SR volume or storage capacity was unchanged, SR Ca^{2+} overload would take longer and the frequency of spontaneous SR Ca^{2+} release would be lower.

A reduction in the activity of SERCA 2A could be caused by a reduction in the expression of SERCA 2A as suggested by previous measurements of this protein in heart failure (Stein *et al.* 1996; Matsui *et al.* 1995; Kiss *et al.* 1995). The same result would be achieved by an increase in activity of phospholamban, the inhibitory regulator of SERCA 2A, but this seems unlikely, as previous measurements of this protein have found that levels are reduced in heart failure (Meyer *et al.* 1995; Kiss *et al.* 1995) or unchanged (Bohm *et al.* 1994; Movsesian *et al.* 1994; Schwinger *et al.* 1995), and phosphorylation levels of phospholamban are increased in heart failure (Currie & Smith, 1996).

Finally, an apparent reduction in SR Ca^{2+} uptake could be caused by an increased Ca^{2+} leak from the SR in the presence of normal activity of SERCA 2A. Bassani and Bers have measured low rates of SR Ca^{2+} leak from intact myocytes during diastole or rest which they attributed to occasional openings of the ryanodine receptor (Bassani & Bers, 1995). The Ca^{2+} flux due to these elemental openings or Ca^{2+} sparks

is low compared to other transmembrane Ca^{2+} currents or SR Ca^{2+} fluxes, but may be more significant when integrated over a longer timecourse as in the current experiments when cells may be quiescent for long periods of time (i.e. up to two minutes during this protocol). Thus if the rate of Ca^{2+} leak via this pathway was larger in heart failure cells than control cells it is conceivable that this would have the effect of reducing SR Ca^{2+} content in heart failure cells. The theoretical basis for a possible increase in Ca^{2+} leak through this pathway is that β-adrenergic stimulation has been shown to cause phosphorylation of the ryanodine receptor (Yoshida *et al.* 1992). Since phosphorylation may increase the open probability of the ryanodine receptor (Hain *et al.* 1995) (although one study has found the opposite (Lokuta *et al.* 1995b)), and sympathetic overdrive occurs as a consequence of heart failure (Homcy *et al.* 1991), this seems a reasonable hypothesis although there is no hard evidence to support it. However, since background leak in normal SR has been measured to be 0.15-0.33% of peak SR Ca^{2+} uptake (Bassani & Bers, 1995), any increase in heart failure would have to be around 1 to 2 orders of magnitude to have an effect of reducing SR Ca^{2+} content to the degree seen in these studies. An effect of increased leak may, however, when integrated over longer timecourses, have some effect on SR Ca^{2+} content, particularly at high SR Ca^{2+} load, when SERCA 2 activity will be reduced.

In summary, the results of SR loading experiments in permeabilised myocytes have shown that there is significantly less Ca^{2+} release in response to caffeine and during spontaneous SR activity in cells from ligation hearts than control hearts. Taking into account the results of studies described in chapter 2 in intact myocytes, and earlier studies reviewed in the introduction to this thesis, the most satisfactory

explanation for this is that the SR Ca^{2+} content is reduced in heart failure, rather than that a reduced fraction of SR Ca^{2+} content is released during each contraction. The most likely explanation of this reduced SR Ca^{2+} content is a reduction in the activity of the SR Ca^{2+} pump, SERCA 2A, rather than an increased leak of Ca^{2+} from the SR. Whilst these results are best explained by a reduction in the activity of SERCA 2A, it is not possible from these experiments to exclude smaller contributions from the other mechanisms discussed to the SR dysfunction seen in this model of heart failure. In support of this is the finding in ferret myocytes that the degree of fractional release of stored Ca^{2+} from the SR depends upon SR Ca^{2+} content, and that the fraction of Ca^{2+} released rises as stored Ca^{2+} increases (Bassani *et al.* 1995). In this heart failure model, therefore, with reduced SR Ca^{2+} content secondary to reduced activity of SERCA 2A, the amplitude of systolic Ca^{2+} release would be reduced secondary to reduced SR Ca^{2+} content, and further reduced compared to control since a reduced fraction of these SR Ca^{2+} stores would be released per contraction.

3.4.3 Implications of reduced SR Ca^{2+} load in heart failure.

Eisner (Eisner *et al.* 1998) has recently reviewed mechanisms for the control of Ca^{2+} release from the SR, concluding that changes in myocardial force of contraction are largely mediated via changes in systolic Ca^{2+} release, and that the predominant mechanism for increasing systolic Ca^{2+} release is increasing SR Ca^{2+} content. SR Ca^{2+} content may be increased by increasing frequency of stimulation which results in cellular gain of Ca^{2+} due to greater influx of Ca^{2+} through L-type Ca^{2+} channels in systole than Ca^{2+} efflux through Na^{+}-Ca^{2+} exchange during diastole. SR Ca^{2+} content may also be increased by adrenergic stimulation which results in phosphorylation of

phospholamban which reduces inhibition of SERCA 2A, as well as increasing Ca^{2+} influx via L-type Ca^{2+} channel phosphorylation.

Thus two important mechanisms for increasing force of myocardial contraction, (the force-frequency relationship and adrenergic stimulation) rely upon the ability of the SR to increase Ca^{2+} load. Several investigators have noted the absence of a positive force-frequency relationship in heart failure (Davies *et al.* 1995; Schwinger *et al.* 1993; Mulieri *et al.* 1992) and blunted responses to adrenergic stimulation (Schwinger *et al.* 1993; Harding *et al.* 1990). Whilst there may be several pathophysiological mechanisms for contractile dysfunction in heart failure, the reduced ability of the SR to load Ca^{2+} seen in this model of heart failure is consistent with these findings, and suggests that SR dysfunction is an important mediator in the development of contractile dysfunction.

3.4.4 Implications of spontaneous SR activity to arrhythmogenesis in heart failure.

Spontaneous SR Ca^{2+} release has been described as defining feature of SR Ca^{2+} overload (Stern *et al.* 1988). As SR Ca^{2+} load increases, the gain function of Ca^{2+}-induced Ca^{2+} release increases (Janczewski *et al.* 1995), and therefore unitary releases of Ca^{2+} from single ryanodine receptors ("calcium sparks") are more likely to propagate and produce generalised SR Ca^{2+} release and cellular contraction. Furthermore it is likely that sparks themselves occur more frequently as SR Ca^{2+} load increases. Spontaneous SR Ca^{2+} release therefore occurs as a function of increasing cytosolic Ca^{2+} concentration and increasing time intervals between stimulation. There are species differences in the occurrence of spontaneous SR Ca^{2+} release, and these differences depend upon factors such as the gain of Ca^{2+}-induced

Ca^{2+} release (Stern *et al.* 1988). Spontaneous Ca^{2+} release is more common in rats and relatively less common in rabbits and humans, but seems to occur in all species studied to some extent (Stern *et al.* 1988).

It is interesting in the present studies that increasing cytosolic Ca^{2+} concentration caused an increase not only in the frequency of spontaneous release, but also in the amplitude. It might be expected from the above discussion that once a threshold SR Ca^{2+} content was reached spontaneous release would occur, and this would preclude further SR Ca^{2+} loading. This hypothesis is supported by experiments in intact rat myocytes where it was found that increases in cytosolic Ca^{2+}concentration affected frequency but not amplitude of spontaneous Ca^{2+} release (Diaz *et al.* 1997). It is possible that these opposite results reflect species differences between rat and rabbit cells, since the gain function of the ryanodine receptor is higher in rat SR than rabbit (Stern *et al.* 1988). It is also possible that small increases in SR load may produce larger increases in Ca^{2+} release as fractional Ca^{2+} release increases with increasing SR load (Bassani *et al.* 1995).

Spontaneous SR Ca^{2+} release has been suggested to be arrhythmogenic due to the production of delayed after depolarisations (Vermeulen *et al.* 1994; Marban *et al.* 1986). Other suggested consequences have been an increase in diastolic tone due to myofilament activation, and reduced systolic Ca^{2+} release due to SR Ca^{2+} depletion (Stern *et al.* 1988). Denvir found increased frequencies of spontaneous Ca^{2+} release in trabeculae from the coronary artery ligation model of heart failure in the rabbit (Denvir *et al.* 1996) and suggested that spontaneous Ca^{2+} release may underlie some of the increased incidence of arrhythmias in heart failure. In human tissue the same group found that spontaneous SR Ca^{2+} release was more common in hearts from

patients with ischaemic rather than dilated cardiomyopathy, and in moderate rather than severe heart failure (Denvir *et al.* 1995).

The results from this current study are in contradiction to those found in Denvir's earlier study in the same rabbit model of heart failure (Denvir *et al.* 1996). Spontaneous SR Ca^{2+} release was less likely to occur in cells from heart failure animals than shams, and when it did occur, frequency of release was lower. At high cytosolic Ca^{2+}concentration these differences were less marked, but at no Ca^{2+}concentration was spontaneous activity more likely to occur in heart failure cells. Given the mechanism for the production of spontaneous SR Ca^{2+} release, this finding is not surprising. At all cytosolic Ca^{2+}concentrations studied in these experiments, cells from heart failure animals showed reduced SR Ca^{2+} content. If spontaneous SR Ca^{2+} release stems from SR Ca^{2+} overload, it would be expected that spontaneous activity would be less common in heart failure cells with lower SR Ca^{2+} content.

Although Denvir's study was in the same model of heart failure used in this study, the experimental preparation studied was skinned ventricular trabeculae, and Ca^{2+} release was not measured directly, but inferred from changes in tension production. Spontaneous SR Ca^{2+} release has been reported previously in multicellular preparations (Stern *et al.* 1988), but has been described as a "chaotic squirming motion" of "asynchronous subcellular waves of contraction". Denvir describes "spontaneous tension oscillations". In skinned preparations spontaneous SR Ca^{2+} release must propagate from the site of initiation by chemical diffusion, since membrane potential is abolished. In single cells rate of propagation has been estimated at 100μm s^{-1} (Stern *et al.* 1988) and in multicellular preparations such as trabeculae with diameters of 100-200 μm and lengths of 2-3mm, diffusion rates will

clearly affect the behaviour of the preparation as a whole during spontaneous activity. It seems unlikely therefore that spontaneous activity in these preparations would be synchronous. However Denvir's findings that spontaneous activity was more common in heart failure was consistent with the proposed mechanism of generation of spontaneous SR Ca^{2+} release, since in his experiments, heart failure rabbits showed enhanced SR loading, and in these circumstances increased spontaneous activity would be expected.

There is no clear explanation for the fact that Denvir found enhanced SR loading in trabeculae from the same model of heart failure when the experiments reported here conclude that SR loading is significantly impaired in heart failure. Evidence from many different sources discussed in the introduction to this thesis suggests that there is impaired SR function in heart failure. Denvir argued that the enhanced SR function seen in his experiments may have reflected early adaptive responses to impaired ventricular function, for example phospholamban phosphorylation in response to chronic adrenergic stimulation. Whilst the coronary artery ligation model of heart failure in the rabbit is not a severe model, and represents compensated left ventricular dysfunction rather than terminal heart failure, there is no corroborating evidence from other studies to support the hypothesis of enhanced SR function in early heart failure, and Ca^{2+} uptake studies in SR vesicles from this same model also suggest significantly reduced SR function (Currie & Smith, 1997). One possible explanation of the differences reported in this thesis and those reported by Denvir, is that trabeculae are derived almost exclusively from endocardial myocytes, and the experiments described here were performed on myocytes from the full thickness of

the left ventricle and would contain endocardial, epicardial and mid-myocardial myocytes.

The reduction in frequency and incidence of spontaneous SR Ca^{2+} release in cells from ligation hearts seen in these experiments seems to argue against a major role for this mechanism of arrhythmia production in heart failure. It is interesting, however, that at higher cytosolic Ca^{2+} concentrations the frequency of spontaneous release in heart failure cells approaches that seen in control cells. Since spontaneous Ca^{2+} transients have lower amplitude in heart failure cells with the implication that SR Ca^{2+} content is lower in these cells, this may suggest that there is reduced SR volume in these cells, or that spontaneous Ca^{2+} release is more likely at a given SR Ca^{2+} content in heart failure. Circumstances likely to produce high cytosolic Ca^{2+} concentration *in vivo*, for example digitalis treatment and ischaemia, are more common in heart failure than in health. It therefore remains possible that despite the fact that at a given cytosolic Ca^{2+} concentration spontaneous SR Ca^{2+} release occurs less frequently in heart failure cells than in control cells, since high cytosolic Ca^{2+}concentration is reached more commonly in heart failure, paradoxically, spontaneous SR Ca^{2+} release may still be more common in heart failure.

3.5 Summary.

Following the findings of reduced systolic Ca^{2+} release and prolongation of Ca^{2+} transients in intact myocytes in heart failure, studies in permeabilised cells were undertaken to clarify whether SR dysfunction was responsible for these abnormalities. In these experiments, there was a significant reduction in the amplitude of caffeine-induced and spontaneous SR Ca^{2+} release. The most likely

explanation for these findings is a reduction in SR Ca^{2+} content in heart failure, reflecting reduced activity of the SR Ca^{2+} pump. These findings are in keeping with previous observations of a negative force frequency relationship and blunted responses to adrenergic stimulation in heart failure.

The incidence and frequency of spontaneous SR Ca^{2+} release was found to be reduced in cells from heart failure animals. This is in keeping with findings of decreased SR Ca^{2+} content, and may argue against, but not necessarily exclude a major role for this mechanism of arrhythmia generation in heart failure.

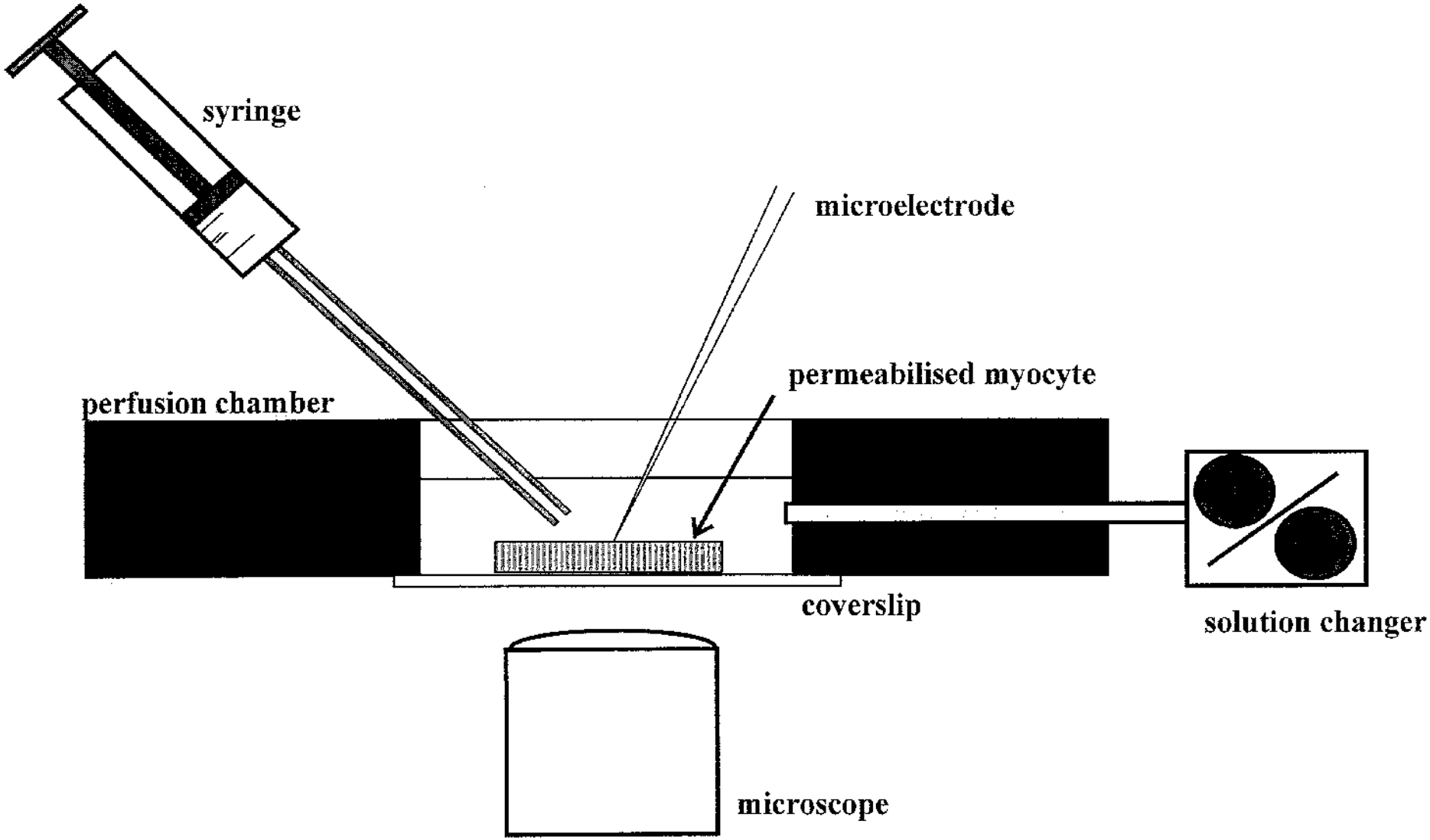

Fig. 3.1: Diagrammatic representation of apparatus for anchoring and injecting onto permeabilised single myocyte.

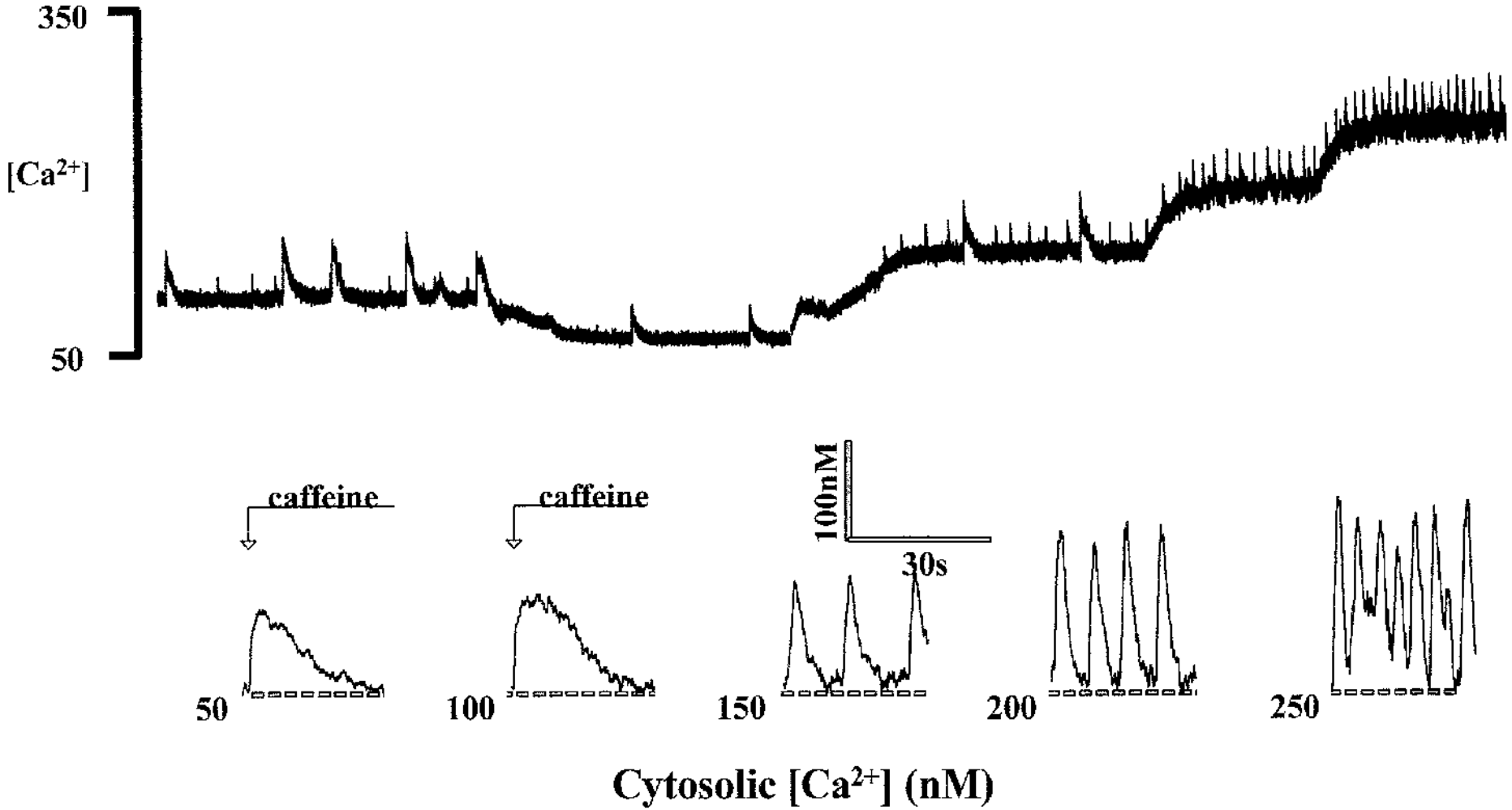

Fig. 3.2 Sample trace from a permeabilised single myocyte preparation.

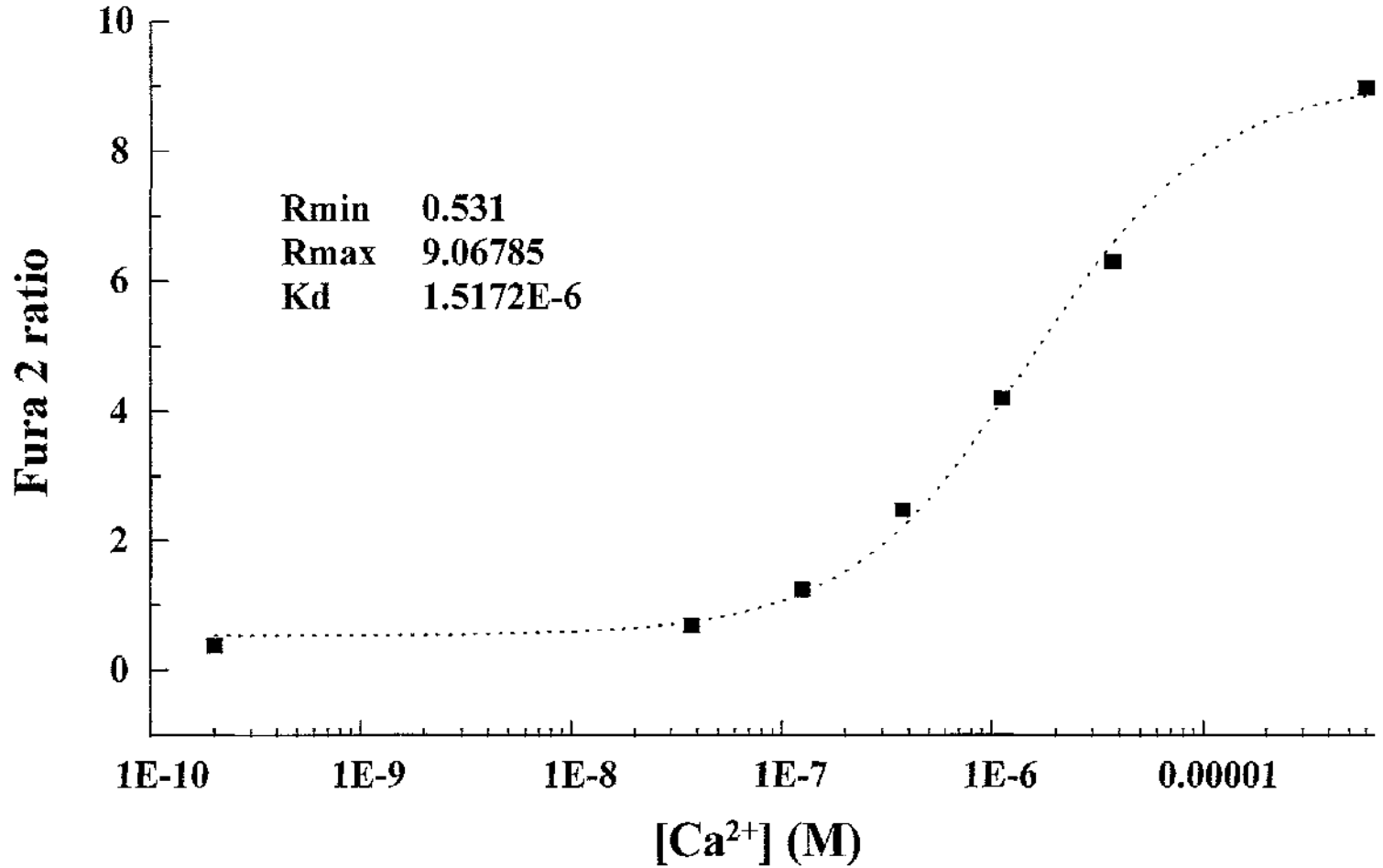

Fig. 3.3 Fura-2 calibration curve.

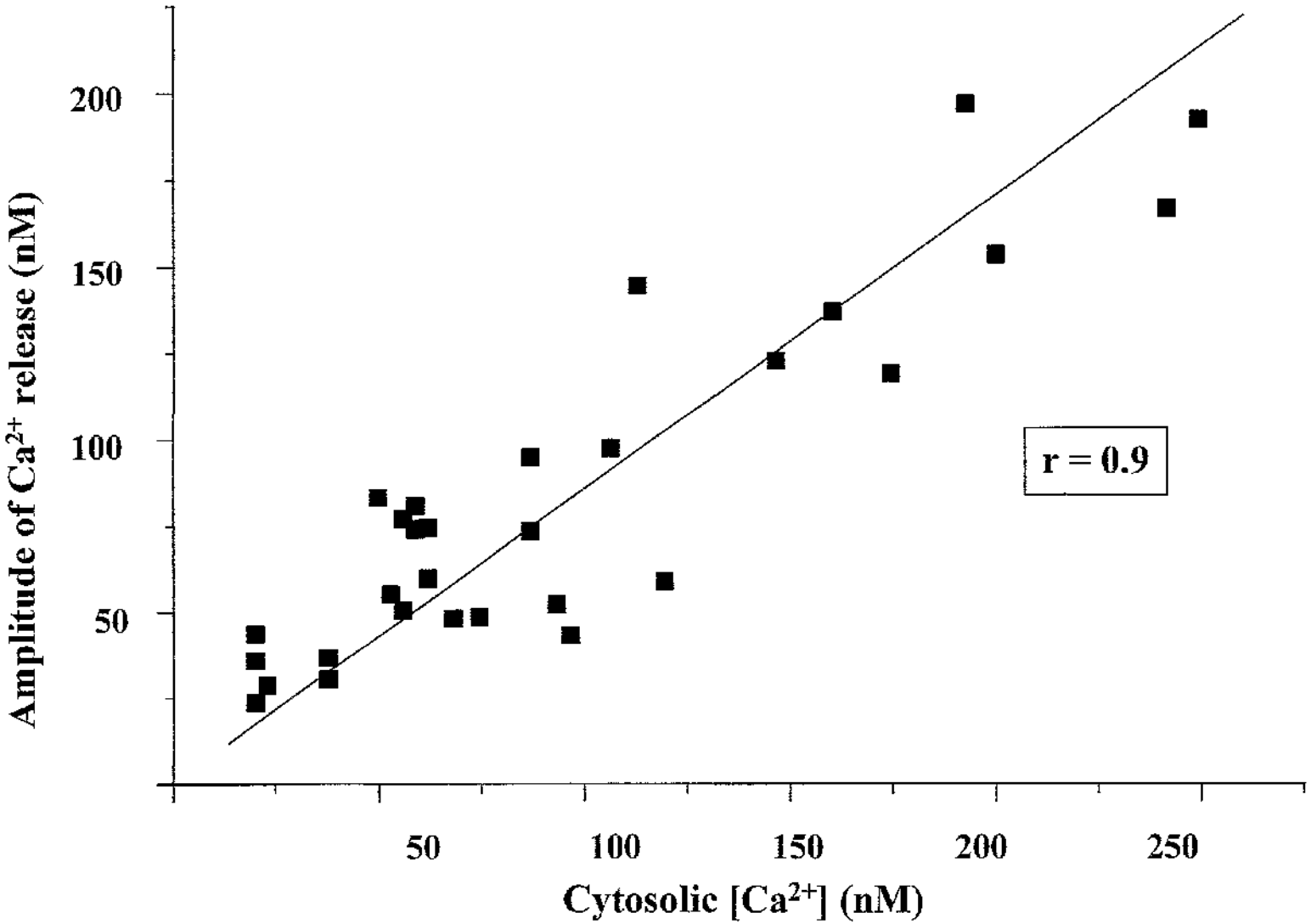

Fig. 3.4 Rrelationship of caffeine-induced SR Ca^{2+} release to cytosolic [Ca^{2+}] in skinned rabbit myocytes

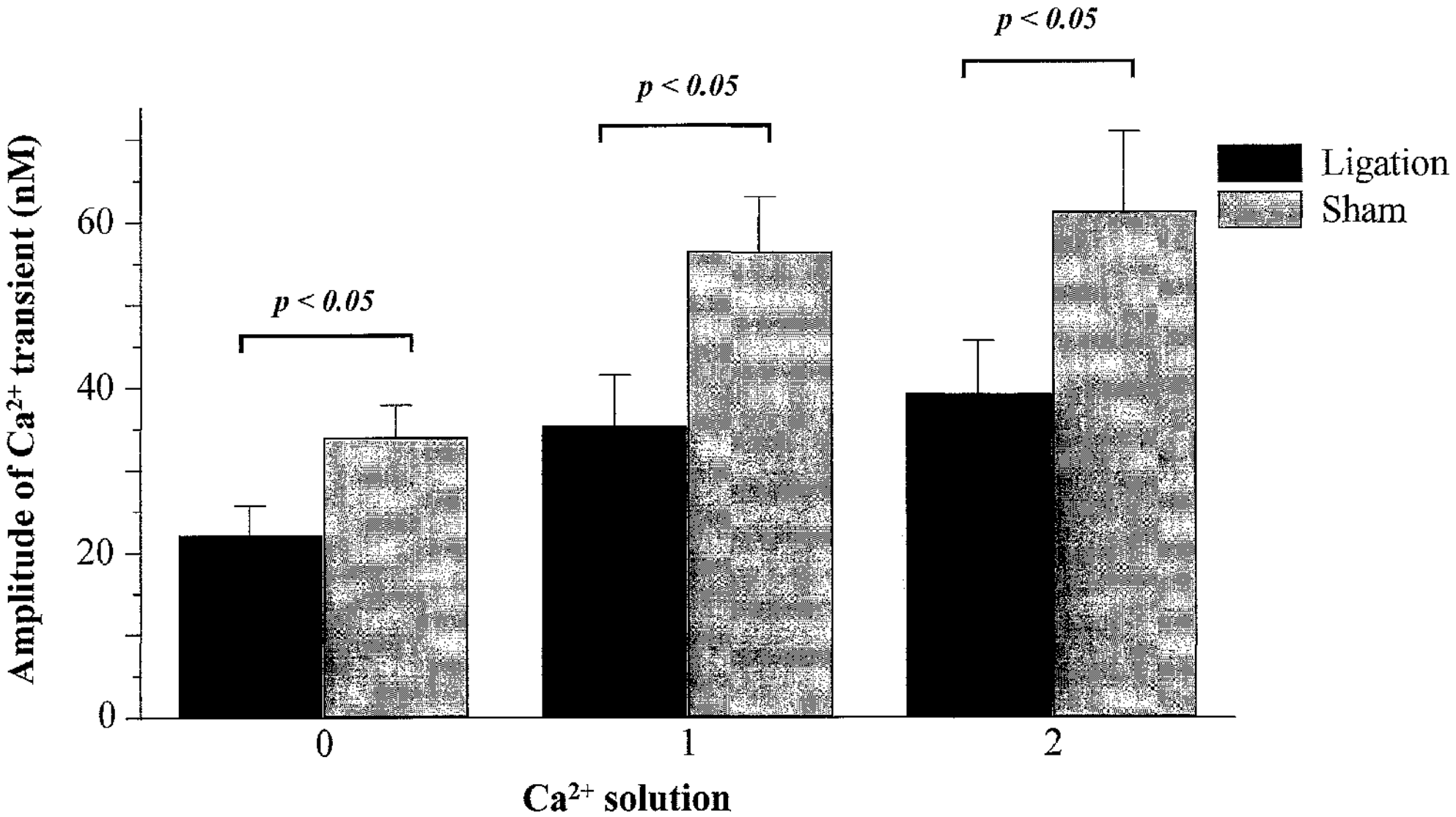

Fig. 3.5: Amplitude of caffeine-induced Ca^{2+} transients in cells from experimental and sham hearts.See text for details of solutions.

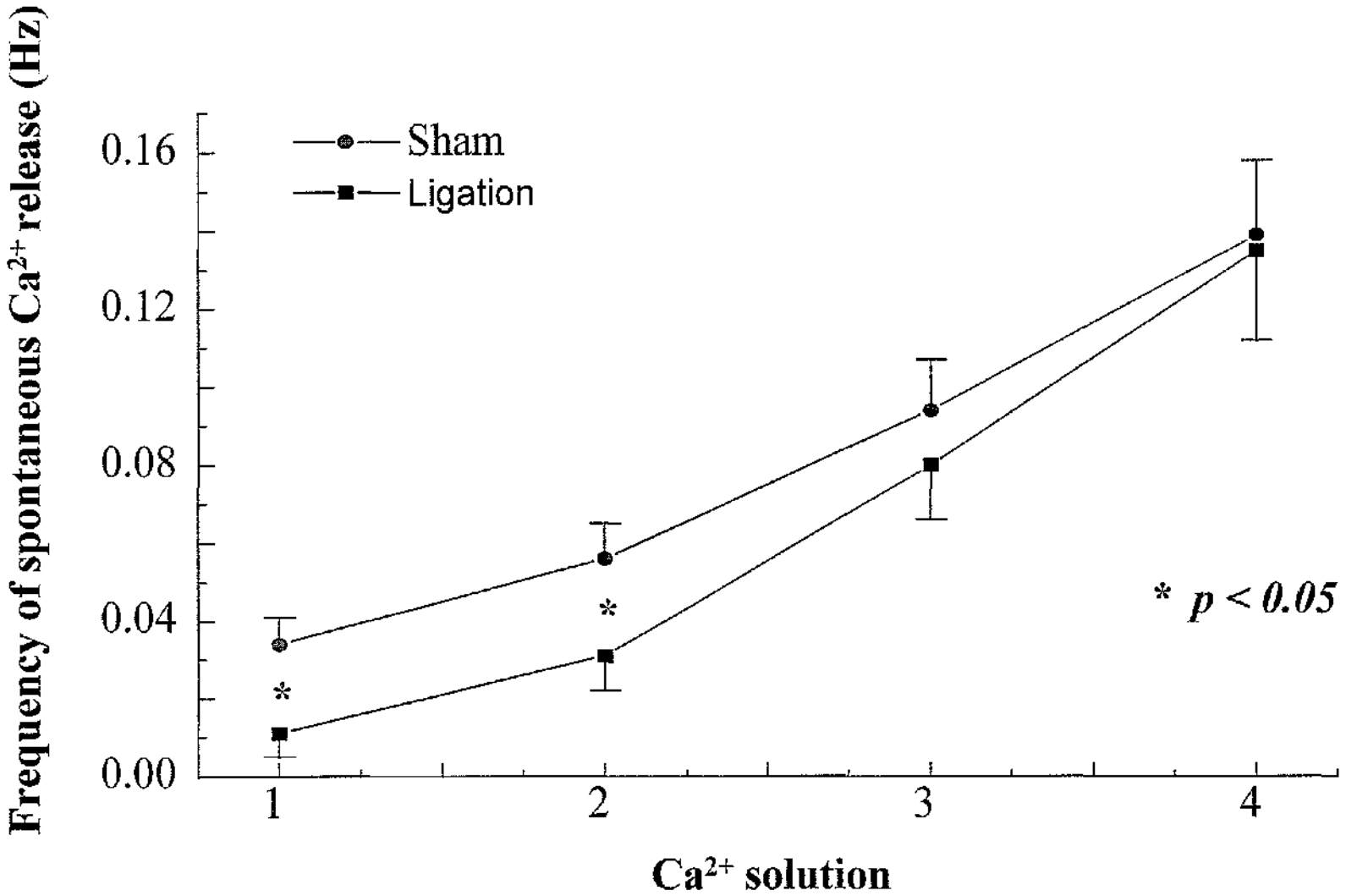

Fig. 3.6 Frequency of spontaneous Ca^{2+} release in cells from experimental and sham hearts. See text for details of Ca^{2+} solutions.

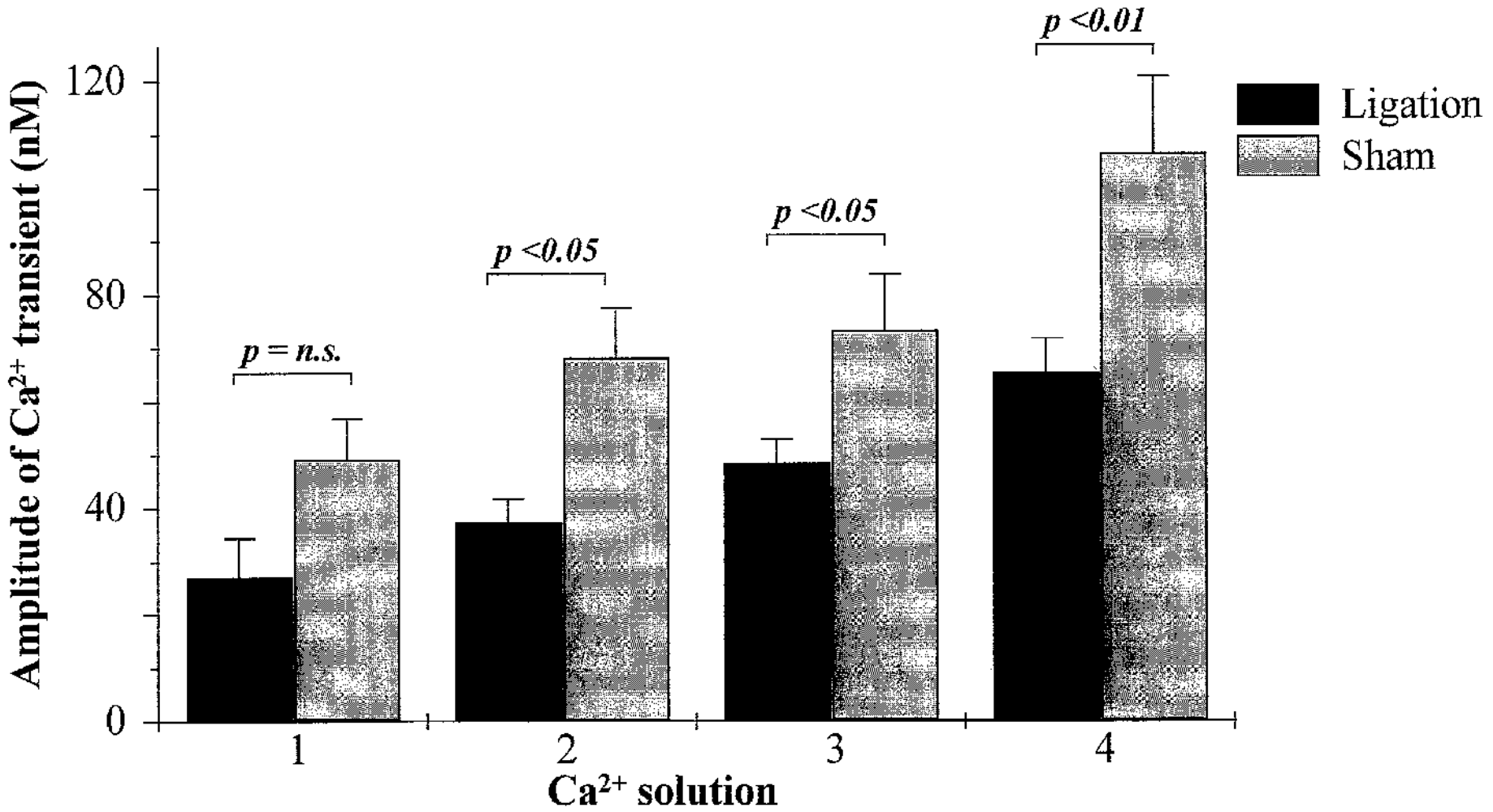

Fig. 3.7 Amplitude of spontaneous Ca^{2+} transients in cells from experimental and sham hearts.See text for details of solutions.

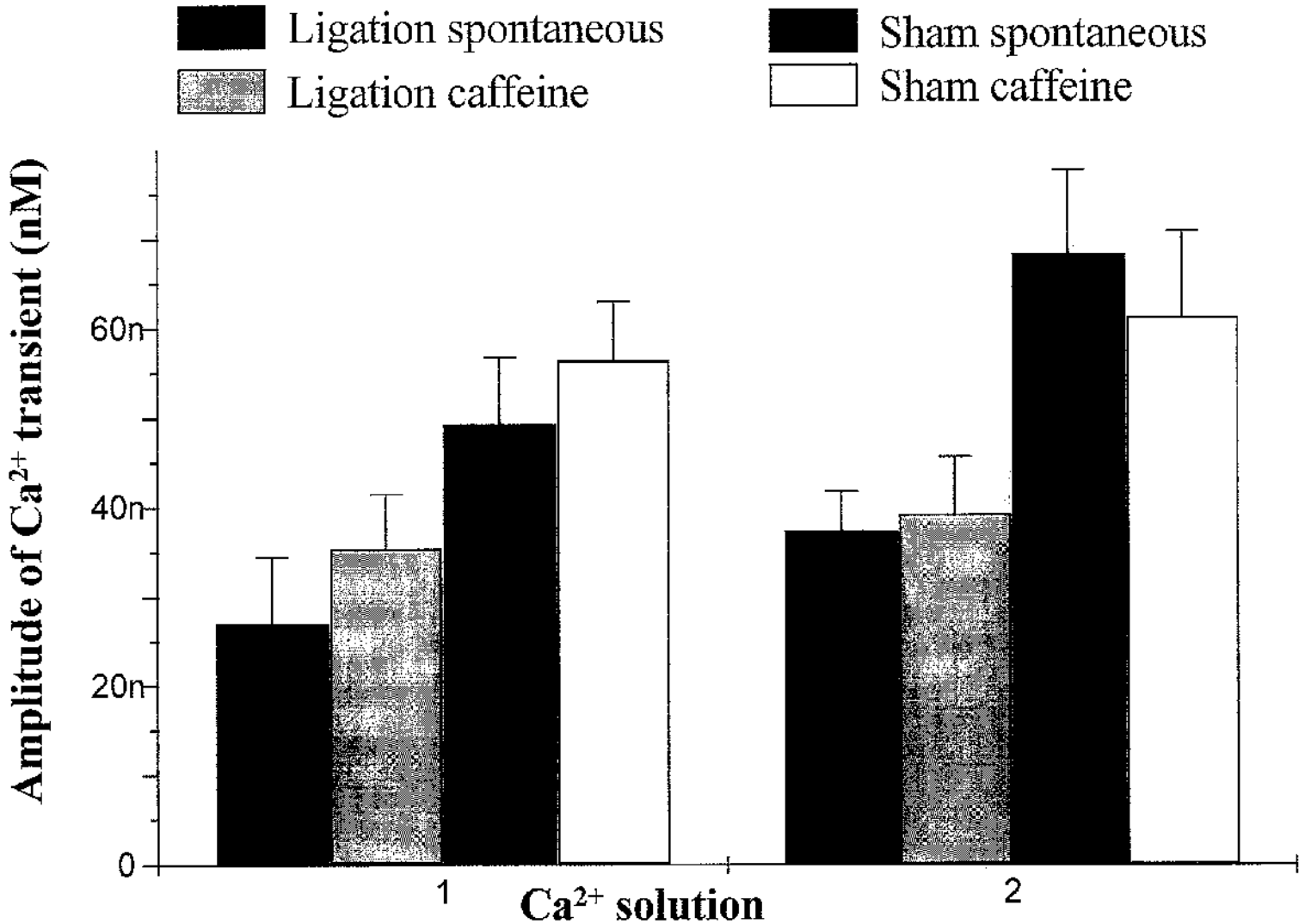

Fig. 3.8 Comparison of the amplitudes of caffeine-induced and spontaneous Ca^{2+} transients. There are no significant differencesbetween caffeine and spontaneous transients in sham or ligation cells.

Chapter 4 - Studies of SR Ca^{2+} uptake in cuvette-based experiments.

4.1 Background.

Following the studies described in chapter 2 confirming abnormal Ca^{2+} handling in the coronary artery ligation model of heart failure in the rabbit, and the experiments in single permeabilised myocytes described in chapter 3 suggesting that this abnormal Ca^{2+} handling was caused at least in part by SR dysfunction, studies were designed to look specifically at SR Ca^{2+} uptake. In chapter 3, studies on single permeabilised myocytes were described, and the chief finding was that SR Ca^{2+} release was reduced in heart failure cells at all loading Ca^{2+} concentrations studied. The implication of this result was that SR Ca^{2+} loading was impaired in these heart failure cells, and the likeliest cause of this was reduced SR Ca^{2+} uptake, although as discussed it was not possible to exclude an increase in SR Ca^{2+} leak.

The experimental preparation chosen in investigate SR Ca^{2+} uptake was again the permeabilised myocyte, but in the studies to be described in this chapter, suspensions of isolated myocytes rather than single cells were used. This followed similar descriptions of the measurement of SR Ca^{2+} uptake in this preparation in normal tissue by Hove-Madsen and Bers (Hove-Madsen & Bers, 1993b; Hove-Madsen & Bers, 1993b). Briefly, a standardised concentration of myocytes is suspended in a cuvette and permeabilised using β-escin. Ca^{2+} concentration within the SR is maintained at a low level by loading with oxalate and cytosolic Ca^{2+} concentration is raised by the addition of $CaCl_2$ to the cuvette. Cytosolic Ca^{2+} concentration is monitored using Fura-2, and SR Ca^{2+} uptake is stimulated by adding ATP. Following addition of $CaCl_2$ the rate of SR Ca^{2+} uptake is measured from the decay in cytosolic Ca^{2+} concentration.

Measurement of SR Ca^{2+} uptake in this preparation is analogous to experiments in SR vesicle preparations described in the introduction to this thesis eg(Ohkusa *et al.* 1997; Movsesian *et al.* 1989), but this preparation has the advantage that the intracellular architecture is preserved, and there is also no confusion over "sidedness" of the SR component. The advantage of this preparation over the single permeabilised myocyte experiments described in chapter 3 is that SR Ca^{2+} uptake can be directly measured rather than inferred from SR Ca^{2+} release, and since SR uptake is measured in a large number of myocytes simultaneously, individual variations in cell function have less effect on results. Finally, in this preparation, SR Ca^{2+} leak can be blocked without affecting measurement of SR Ca^{2+} uptake, and therefore differences in both SERCA 2A function and SR Ca^{2+} leak can be differentiated in heart failure and control cells.

4.2 Methods.

4.2.1 Overview

In these experiments the rate of SR Ca^{2+} uptake in suspensions of myocytes was measured. Myocyte concentration was standardised to allow comparisons of the rate of SR Ca^{2+} uptake in heart failure and control cells. The range of Ca^{2+} concentrations over which uptake was measured was also standardised.

The rate of SR Ca^{2+} uptake in this preparation would have been expected to be limited by Ca^{2+} concentration within the SR. For this reason all experiments were done in the presence of oxalate, which acts as a Ca^{2+}-precipitating anion within the SR, and prevents Ca^{2+} concentrations within the SR rising to levels which would inhibit SR Ca^{2+} uptake. In the presence of oxalate, therefore the rate of SR Ca^{2+}

uptake is determined by free Ca^{2+} concentration outside the SR (Hove-Madsen & Bers, 1993b). In the absence of oxalate, Ca^{2+} uptake was very slow.

Measures of the rate of SR Ca^{2+} uptake in this preparation depended upon the activity of the SR Ca^{2+} pump (SERCA 2A), but also depended upon the rate of Ca^{2+} leak from the SR through the ryanodine receptor. The magnitude of the Ca^{2+} leak through the ryanodine receptor would be a non-linear function of extra-SR Ca^{2+} concentration due to Ca^{2+}-induced Ca^{2+} release and hence would be expected to affect both the steady state Ca^{2+} concentration outside the SR and the net rate of SR Ca^{2+} uptake. For this reason SR Ca^{2+} uptake was measured in all experiments in the absence and presence of ruthenium red, a high affinity blocker of this leak pathway (Ma, 1993).

4.2.2 Myocyte isolation.

Myocytes were isolated from coronary artery ligated and sham rabbit hearts as described in section 2.1.2. Since maximum numbers of functioning myocytes were required for the experimental protocol, myocytes were taken from the full thickness of the left ventricle, and not divided into endocardial, epicardial and mid-myocardial fractions. Following isolation myocytes were suspended in 0.05 relaxing solution (0.05R), a mock intracellular solution with Ca^{2+} concentration buffered to low levels with 50μM EGTA at pH 7.0 (see appendix 1 for exact composition), and stored at room temperature until use.

4.2.3 Myocyte counting.

In order to make comparative measurements of SR Ca^{2+} uptake rates in myocytes from heart failure and control hearts, it was necessary to standardise the preparation since myocyte yield varied between dissociations. Measurement of Ca^{2+} uptake in SR

vesicle preparations has previously been standardised by weight of the SR component (Movsesian *et al.* 1989). In the current experiments it was decided to standardise the concentration of rod shaped myocytes within the cuvette. This was done by counting the number of dissociated rod shaped cells in a given volume following dissociation in a cytometer, the Improved Neubauer cytometer (Weber, England). This method of standardisation relies on the assumption that SR Ca^{2+} uptake is mediated solely or principally by rod shaped myocytes. However, dissociation of single myocytes produces both rod shaped myocytes and cells which are morphologically balled-up. As will be discussed later, the mechanism by which these cells become contracted is uncertain, but may reflect Ca^{2+} overload and hypercontracture due to membrane damage during dissociation. However, since it was not clear whether these cells contained any functioning SR material the number of these myocytes was also counted. Since the number of rod shaped cells and contracted cells varied independently, it was not possible to standardise concentrations of both types of cell. Following counting of myocytes, the suspension was then either concentrated by centrifugation or diluted to give a solution containing 2.5×10^5 rod shaped myocytes ml^{-1} and then counting was repeated to confirm myocyte concentration.

4.2.4 Experimental protocol.

Following counting, myocytes were transferred to 2ml cuvettes containing 10mM oxalate, and stirred continuously with a magnetic stirrer to prevent settling. The rate of stirring was not changed between experiments. The experimental apparatus is shown in fig. 4.1.

Ca^{2+} concentration in the cuvette (and outside the SR) was measured by addition of 10μM Fura-2. Fluorescence evoked at 340nm and 380nm was measured using a Cairn spectrophotometer as described in section 2.1.3, and a ratiometric measurement of free Ca^{2-} concentration was made and recorded to disc using the program New Tape (Francis Burton 1990) and to chart recorder.

Following a 2 min equilibration period 10-20μL of 10mM $CaCl_2$ was added to the cuvette, raising the free Ca^{2-} concentration to 1-2 μM. There was some variation between experiments in the amount of $CaCl_2$ required to raise Ca^{2+} concentration to this level. This varied with the buffering power of the solution which in turn depended on the total concentration of myocytes (live and dead) within the solution. The volume of $CaCl_2$ required to raise free Ca^{2+} therefore varied between experiments, and had to be assessed by trial and error at the start of each experiment. Once the volume required was ascertained, it remained fixed for the duration of the experiment. Fura-2 ratio was then measured as free Ca^{2+} concentration decayed to resting levels. Myocytes were then permeabilised by the addition of 90nM β-escin, and a repeat free Ca^{2+} decay curve was recorded after the addition of the previous volume of $CaCl_2$.

This protocol allowed measurement of SR Ca^{2+} uptake rates over a range of free Ca^{2+} concentrations from around 1-2 μM to around 50-100nM. Since Ca^{2+}-induced Ca^{2+} release would be expected to occur at Ca^{2+} concentrations much less than the maximum free concentrations in this range, SR Ca^{2-} uptake was then measured in the presence of ruthenium red (30mM) which blocks SR Ca^{2+} release from the ryanodine receptor, with a K_d of order of < 1μM (Ma, 1993). Ruthenium red blocked both Ca^{2+}-

induced Ca^{2+} release and SR Ca^{2+} leak which is thought to be caused by occasional spontaneous openings of the ryanodine receptor (Bassani & Bers, 1995).

Experiments were performed in suspensions of myocytes from both ligation and control hearts. Thus SR Ca^{2+} uptake could be compared in both intact and permeabilised myocytes from failing and normal hearts, and SR Ca^{2+} uptake could be compared in the presence and absence of SR Ca^{2+} leak. A typical experimental trace showing SR Ca^{2+} uptake in myocytes before and after permeabilisation and then following the addition of ruthenium red is shown in fig. 4.2.

4.2.5 Data analysis and curve fitting.

The relationship of the ratio of Fura-2 fluorescence at 340nm and 380nm to Ca^{2+} concentration was described in section 3.2.5. Essentially the relationship is sigmoid and depends on the constants R_{min} R_{max} and K_d. These constants vary depending upon several properties of the system in use, in particular, the optical properties of the system and the presence of other chemicals within the solution. For these reasons it was necessary to plot a calibration curve for these experiments. This was done as previously described by combining different ratios of 10mM EGTA and 10mM CaEGTA to create a range of Ca^{2+} concentrations, and measuring the ratio of fluorescence at 340 and 380nM stimulation. Free Ca^{2+} concentration was calculated for these solutions using the program React (Godfrey Smith 1990). As shown in fig. 4.3, the addition of ruthenium red to the solution significantly affected the fluorescence properties of Fura-2. Ruthenium red had a quenching effect shown by a reduction in fluorescence at both wavelengths which was not completely symmetrical. For this reason calibration curves were plotted in the presence and absence of ruthenium red.

The values calculated from these calibration curves were:

	ruthenium red:	No ruthenium red:
R_{min}	0.83	0.94
R_{max}	13.55	13.19
K_d	1.51×10^{-6}	8.37×10^{-7}

Raw data were plotted in real time on chart paper, and the output voltage from the spectrophotometer for individual wavelengths and ratio was stored on hard disc. All analysis of Ca^{2+} decay curves was performed on personal computer. Areas of interest within data sets were selected using the program QA (Francis Burton 1990) and ratiometric measurements were then converted into Ca^{2+} concentrations. Following conversion, each Ca^{2+} decay curve was then plotted using the program Origin (Microcal software) and a bi-exponential curve was fitted to the portion of the decay curve below 600nM Ca^{2+} concentration. From this plot, 2 time constants for each decay curve were calculated, reflecting a fast and slow phase of Ca^{2+} uptake. The fast time constant reflected Ca^{2+} uptake at higher cytosolic Ca^{2+} concentrations, and was more a more consistent measure of the rate of Ca^{2+} uptake, and comparisons of Ca^{2+} uptake between heart failure and control preparations were therefore made using this measurement. Other methods of analysis were possible, for example plotting the differential of changes in free Ca^{2+}, but this method was chosen for simplicity. An example of this curve fitting is shown in figure 4.4.

4.3 Results.

Ca^{2+} uptake measurements were made in cell suspensions from 7 ligation hearts and 8 control hearts. Time constants from bi-exponentially fitted decay curves were therefore available for Ca^{2+} uptake in intact cells (prior to the addition of β-escin),

skinned cells, and in skinned cells following the blocking of Ca^{2+} leak with ruthenium red. Representative decay curves for a heart failure preparation and a sham preparation are shown together in fig. 4.5. It can be seen that the rate of Ca^{2+} uptake was slower in heart failure preparations.

Table 4.1 shows the mean time constants for heart failure and sham preparations. It can be seen that the rate of Ca^{2+} uptake is reduced in heart failure cells, although the difference between mean time constants was only statistically significant for skinned cells before the addition of ruthenium red. In sham cells, the time constant for Ca^{2+} uptake was significantly less in skinned than intact cells ($p=0.03$), and in ruthenium red treated cells than skinned cells ($p=0.01$). In failure cells there was no significant difference in the time constant in intact and skinned cells ($p=0.3$) but time constants were significantly shorter when SR Ca^{2+} leak was blocked with ruthenium red ($p=0.01$). The ratio of mean time constants of skinned to intact cells was 0.78 in sham cells and 0.81 in failure cells ($p=0.09$) The ratio of mean time constants of ruthenium red treated cells to skinned cells was 0.77 in sham cells and 0.56 in failure cells ($p=0.04$). These results are shown in fig. 4.6.

	Sham			Expt		
	I	S	RR	I	S	RR
mean (s)	54.72	42.43	32.55	90.64	74.04	41.81
SD (s)	27.54	13.81	13.07	46.41	29.02	11.57
SEM (s)	8.71	4.37	4.13	13.99	8.75	3.49
p	0.052	0.009	0.086			

Table 4.1 Mean time constants for Ca^{2+} uptake in suspensions of heart failure and sham cells. (I = intact, S = skinned, RR = ruthenium red).

The analysis of these results was based on the premise that SR Ca^{2+} uptake was mediated principally by cells that remained rod shaped following the isolation procedure, and numbers of these cells were standardised between experiments. Numbers of cells which had undergone hypercontracture and appeared balled up could not be standardised at the same time as rod shaped cells since the yield of the two types of cells varied in each experiment. This is shown in table 4.2. As can be seen, although there was no difference in the total yield between experimental and sham hearts, there was a significantly higher yield of rod shaped myocytes from sham hearts. Since the experimental protocol standardised the number of rod shaped myocytes between experiments, this means that the total number of all myocytes (rod shaped and contracted) was lower in sham experiments than heart failure experiments.

	Sham				Failure			
Expt.	Date	rods	balls	total	Date	rods	balls	total
1	3.6.97	6.3	17.4	23.7	19.6.97	5	23.1	28.1
2	12.6.97	7.7	13.3	21	26.6.97	4.6	21.7	26.3
3	24.6.97	6.3	21.1	27.4	31.7.97	4	12.5	16.5
4	29.7.97	5.4	22	27.4	21.8.97	3.6	10.9	14.5
5	7.8.97	3.6	5.6	9.2	3.7.97	3.6	28.1	31.7
6	28.8.97	5.7	15.3	21	17.7.97	6.9	15.2	22.1
7	9.9.97	8.3	23	31.3	5.8.97	3.4	13	16.4
8	19.8.97	6	12.6	18.6				
	mean	6.16	16.29	22.44	mean	4.44	17.79	22.23
	SEM	0.43	1.76	2.05	SEM	0.37	1.97	2.01
	p	0.014	0.323	0.476				

Table 4.2. Numbers of rod shaped and hypercontracted myocytes following isolated. Statistical tests compare sham and heart failure experiments. All numbers are x10^5.

Given the results described in the previous chapter it was surprising that the differences in the rate of Ca^{2+} uptake in the cuvette preparation were so small. This raised the question of whether the original premise that Ca^{2+} uptake was mediated totally or principally by rod shaped myocytes. This question was being investigated independently at this time by Lorraine Bruce (PhD student under supervision of Professor Smith). Data from experiments in control tissue showed that rates of Ca^{2+}

uptake were predicted better by the total number of cells in the preparation, and that there was a poor correlation between uptake rate and concentration of rod shaped myocytes The best correlation with uptake rate was with total protein concentration within the cuvette, although total cell concentration was more accurate in predicting uptake rate than rod shaped myocyte concentration. This suggested that SR material in contracted cells contributed to Ca^{2+} uptake, and that cell counting was less discriminating in estimating the amount of functioning SR in the preparation than protein measurement.

Protein measurement had not been undertaken in these experiments, but it was possible to re-analyse the data standardised to the time constant for decay per $1x10^5$ total cells. These data are shown in table 4.3 and fig 4.7.

	Sham			Expt.		
	Ic	Sc	RRc	Ic	Sc	RRc
mean	496.59	382.56	287.32	1161.42	1031.41	552.51
SEM	96.57	50.15	35.36	218.16	214.07	92.33
p	0.023	0.013	0.02			

Table 4.3. Time constants for Ca^{2+} uptake corrected to $1x10^5$ total cells in intact (Ic), permeabilised (Pc) and ruthenium red (RRc) cells. Statistical comparisons are between heart failure and sham experiments.

Comparisons of time constants for Ca^{2+} uptake in intact, skinned and ruthenium red treated cells in this analysis shows that in sham cells, the time constant for Ca^{2+}

uptake was significantly shorter in skinned than intact cells (p=0.03), and in ruthenium red treated cells than skinned cells (p=0.02). In failure cells there was no significant difference in the time constant in intact and skinned cells (p=0.4) but time constants were significantly shorter when SR Ca^{2+} leak was blocked with ruthenium red than in skinned cells (p=0.02). The ratio of mean time constants of skinned to intact cells was 0.77 in sham cells and 0.88 in failure cells (p=0.09) The ratio of mean time constants of ruthenium red treated cells to skinned cells was 0.75 in sham cells and 0.54 in failure cells (p=0.04).

In summary then, the results presented in this chapter show:

(i) When data are analysed normalised to rod shaped myocyte numbers, there is a general small, non-significant trend towards lower time constants for Ca^{2+} uptake in sham compared to failure cells.

(ii) This difference is significant in intact, skinned and ruthenium red treated cells when the data are normalised to total cell numbers rather than rod shaped myocyte numbers.

(iii) In sham cells there is a significant increase in the rate of Ca^{2+} uptake when myocytes are skinned, and again when SR Ca^{2+} leak is blocked with ruthenium red. In failure cells the difference between intact and skinned cells does not reach significance.

(iv) The relative increase in Ca^{2+} uptake with the addition of ruthenium red is greater in failure cells than in sham cells.

4.4 Discussion.

The purpose of the experiments described in this chapter was to directly measure SR Ca^{2-} uptake and identify whether this was slowed in heart failure cells compared to control cells. Studies in single permeabilised myocytes described in chapter 3 suggested that SR Ca^{2+} load was reduced in heart failure cells, and the most obvious cause of this was a reduction in SERCA 2A mediated Ca^{2+} uptake. It was not possible to exclude changes in SR Ca^{2+} release or storage as a cause of the apparent decrease in SR load, however. These problems were overcome in these studies by directly measuring SR Ca^{2+} uptake in large numbers of myocytes.

4.4.1 Normalisation of results.

As discussed earlier, in planning these experiments, it was assumed that the SR component of hypercontracted cells was not active, and therefore, SR Ca^{2+} uptake would be mediated by rod shaped myocytes. For this reason, myocyte concentration in cuvettes was standardised to numbers of rod shaped myocytes. This assumption was critical, since there was a significant difference in the relative yields of rod and contracted myocytes from failing and control hearts. Subsequent experiments from this laboratory contradict this assumption, and for this reason data were re-analysed, and normalised to the total number of cells within each preparation. This analysis did not change the trend of the results in intact, skinned or ruthenium red treated cells, but affected the magnitude and significance of the differences in failing and control cells. Further refinement of these results would have been possible if rates of Ca^{2+} uptake could have been normalised to total protein content. Information on the predictive value of protein estimation came from control tissue, however, and it is possible that protein levels in heart failure cells would differ from those in control

cells, and may not therefore have been the most appropriate measure of cell concentration in these studies. Normalising Ca^{2+} uptake to total cell numbers seems therefore to be the best practical solution.

It is possible that this is an oversimplification, however, since this analysis assumes that SR activity in contracted cells is equivalent to that in rod shaped myocytes. It is possible that there is at least a degree of SR damage in these cells, and there would be a range in activity of SR function. The mechanism of cellular damage is important in this context, since hypercontracture may be generated by both hypoxia causing a depletion of high energy metabolites, or by direct damage to the sarcolemma in the dissociation process. In the former case, SR activity may be restored by the availability of ATP and creatine phosphate in the bathing solution. These questions could only be fully assessed by measuring SR Ca^{2+} uptake in pure suspensions of rod shaped and contracted myocytes, but at present there is no method for separating these two fractions. It remains possible, therefore, that normalisation of SR Ca^{2+} uptake to total cell number is an overcorrection, which artificially increases the difference in SR uptake in failure and control cells. If this were the case, the true difference would lie somewhere between the corrected and uncorrected data.

4.4.2 Uptake in intact myocytes.

Uptake was first measured in all experiments in intact myocytes. In these circumstances free Ca^{2+} was measured in the extracellular space, since Fura 2 is not membrane permeant. SR Ca^{2+} uptake in these experiments depended on both sarcolemmal Ca^{2+} uptake (principally via the Na-Ca^{2+} exchanger following a concentration gradient) and thereafter SR uptake mediated by SERCA 2A. There are

a number of theoretical problems in the analysis of these results. Firstly enzymatic dissociation of myocytes is thought to damage the sarcolemma in some cells, causing increased membrane permeance. These cells undergo hypercontracture due to Ca^{2+} overload from extracellular sources, and appear balled up on light microscopy. It has already been suggested, however, that these cells retain an active SR component, and thus SR Ca^{2+} uptake measurements in intact cells probably constitute an element of uptake in both intact and leaky (or permeable) cells. Since the relative yield of rod and ball shaped myocytes varied between experiments, and there was a significantly greater proportion of rod shaped cells from sham hearts, there is an inherent bias in the analysis. However, it would be expected that the presence of a higher fraction of permeable cells in failure experiments would tend to increase the rate of Ca^{2+} uptake, since in these cells Ca^{2+} uptake would be a single step process, and trans-sarcolemmal Ca^{2+} transport would be bypassed. These arguments tend to strengthen the finding that in intact myocytes Ca^{2+} uptake is slower in heart failure cells.

In control cells there was a significant decrease in the time constant for Ca^{2+} uptake between intact and skinned cells. This difference was not significant in failure cells, and the ratios of 0.77 (control cells normalised to total cell numbers) and 0.88 (failure cells) were significantly different. This may reflect the different numbers of permeable cells in the preparations, since if higher numbers of cells were already permeable in the failure group it would be expected that there would be smaller increase in the rate of Ca^{2+} uptake following chemical permeabilisation. An alternative explanation is that sarcolemmal Ca^{2+} transport was less of a barrier to SR Ca^{2+} uptake in heart failure cells due to an upregulation of Na-Ca^{2+} exchange, which has been suggested earlier. It is not possible to separate these two hypotheses.

A further problem in intact myocyte studies, is the site of oxalate mediated Ca^{2+} buffering. Control experiments in the absence of cellular material showed that following addition of Ca^{2+} boluses, Ca^{2-} concentration did not decay, suggesting that Ca^{2+} concentration was not sufficiently high in the extracellular compartment to precipitate with oxalate. It is also unlikely that cytosolic Ca^{2+} concentration would be high enough to cause precipitation, although this cannot be excluded. The fact that Ca^{2+} uptake was consistently higher in skinned cells, however, argues against this happening to any major extent.

4.4.3 Uptake in skinned myocytes.

The difficulties in interpreting results in the studies described above are less problematic in permeabilised cells. Following permeabilisation, the cytosol becomes a single compartment with the extracellular space, and control studies have not shown any significant Ca^{2+} oxalate precipitation here. Similarly, the theoretical problem of contracted cells having a permeable sarcolemma is also irrelevant following chemical permeabilisation. As would be expected, in control cells, SR Ca^{2+} uptake is significantly faster in permeabilised cells than in intact cells. The magnitude of this increase in rate of uptake is around 25%, suggesting that sarcolemmal Ca^{2} transport is a less significant barrier to SR Ca^{2+} uptake than SERCA 2A mediated uptake in itself. This seems lower than might have been expected, since there will be a tenfold maximum trans-sarcolemmal Ca^{2+} gradient in these experiments (≈1μM [extracellular]: ≈100nM [cytosolic]). If this is compared with the concentration gradient seen if vivo, with ionised Ca^{2+} in the millimolar range in extracellular fluid it can be seen that Na-Ca^{2-} exchange has a substantially lower driving gradient in these experiments.

The main conclusion from measurements of SR Ca^{2+} uptake in skinned cells is that uptake is significantly faster in control compared to failure cells. These experiments are analogous to results in intact and skinned single cells, in that they allow the conclusion to be made that the abnormal Ca^{2+} handling in this model stems from SR dysfunction rather than changes in Na-Ca^{2+} exchange, although additional changes in Na-Ca^{2+} exchange are not excluded. The difference is that in these experiments SR Ca^{2+} uptake is measured directly rather than inferred from measurements of Ca^{2+} release.

Ca^{2+} uptake was 43% faster in skinned cells from control compared to failure hearts. This figures rises to 63% when the data are normalised to total cell numbers. This compares well with the findings in single cells described in chapter 3 that SR load was 50-60% greater in control than failure cells.

4.4.4 Blocking Ca^{2+} release at the ryanodine receptor.

While this experimental protocol measures SR Ca^{2+} uptake in permeabilised cells, net Ca^{2+} uptake represents a balance between Ca^{2+} uptake and release at the ryanodine receptor. Since cytosolic Ca^{2+} concentration at the beginning of uptake experiments is high (1-2μM), Ca^{2+}-induced Ca^{2+} release occurs, and may significantly reduce the rate of net Ca^{2+} uptake. There are several reasons why the rate of SR Ca^{2+} leak may not be equivalent in failure and control preparations. Evidence for differences in protein expression of the ryanodine receptor in heart failure was discussed in chapter 1, but briefly, some investigators have found reduced mRNA expression (Arai *et al.* 1993) and ryanodine binding (Go *et al.* 1995). Sensitivity of the ryanodine receptor is also modulated by phosphorylation (Lokuta *et al.* 1995a). Little is known of the state of ryanodine receptor phosphorylation in heart failure although alterations are

possible since phosphorylation may be dependant on β-adrenergic stimulation (Yoshida *et al.* 1992), and other β-adrenergic dependent modulations are altered in heart failure (Harding *et al.* 1990). There is also evidence for a ryanodine-independent SR Ca^{2+} leak (Smith *et al.* 1998), although nothing is known as whether this leak is altered in heart failure.

The presence of oxalate within the SR will, however, reduce the amplitude of both ryanodine-dependent and independent SR Ca^{2+} leak, since free Ca^{2+} concentration in the SR will be in the order of 1μM, approximately one hundred times lower than normal. In control tissue, however, Ca^{2+} uptake is significantly faster following the addition of ruthenium red, signifying that there is an appreciable SR Ca^{2+} leak despite the presence of oxalate.

The ratio for the time constant for Ca^{2+} uptake in control tissue following addition of ruthenium red to uptake in skinned myocytes was 0.77, and when normalised to total cell number this ratio was virtually unchanged at 0.75. This increase in rate of uptake following blocking of the ryanodine-dependent leak was therefore equivalent to the change seen following skinning of myocytes. This confirms that SR Ca^{2+} leak has a significant effect on the rate on net SR Ca^{2-} accumulation. This underestimates the true effect of SR Ca^{2+} leak, since the magnitude of the ryanodine-independent leak has not been quantified.

In failure cells, the effect of ruthenium red is still more pronounced. Here the ratio of time constants for Ca^{2+} uptake following ruthenium red was 0.56. This was significantly lower than in control cells ($p = 0.04$), indicating that ryanodine dependent Ca^{2-} leak has a greater effect on net Ca^{2+} accumulation in heart failure

cells. Results are similar in the normalised analysis, with the ratio in heart failure being 0.54, again significantly lower than in control cells ($p = 0.04$).

Since the fractional increase in rate of Ca^{2+} uptake following ruthenium red is greater in heart failure than control cells, it follows that the magnitude of the ryanodine-dependent leak is greater in heart failure. This is an important finding, as this is the first result in this series of experiments to indicate that abnormalities of Ca^{2+} handling in heart failure cannot be explained purely by a reduction in the activity of SERCA 2A.

The mechanism of this increased SR Ca^{2+} leak must depend on a modulation of the ryanodine receptor in heart failure, since all evidence of protein expression, mRNA expression and ryanodine binding suggests that levels of the ryanodine receptor are either unchanged or reduced in heart failure. The increased leak at the ryanodine receptor cannot be a function dependent on intraluminal Ca^{2+} concentration, since studies in skinned single myocytes described in chapter 3 show that SR Ca^{2+} load is decreased in heart failure, and the sensitivity of the ryanodine receptor is increased by higher rather than lower SR Ca^{2+} load (Janczewski *et al.* 1995). It is also not possible to explain the increased leak in terms of raised cytosolic Ca^{2+} concentration in heart failure, since in the skinned cell preparation, cytosolic Ca^{2+} concentration is controlled by the bathing medium, rather than any function of cell Ca^{2+} handling. It therefore follows that the mechanism of the increased Ca^{2+} leak must depend either on a functional alteration of the ryanodine receptor itself, or modulation of the receptor by external control mechanisms (eg phosphorylation). These changes must cause either an increase in the inherent leak characteristics of the ryanodine receptor, or increase its sensitivity to Ca^{2+} induced Ca^{2+} release. An

increase in the sensitivity of Ca^{2+}-induced Ca^{2+} release could be a secondary, compensatory change following either a reduction in the numbers of ryanodine receptors, or reduced SERCA 2A mediated SR Ca^{2+} loading.

The finding of an increased SR Ca^{2+} leak, possibly secondary to a compensatory increase in the sensitivity of Ca^{2+}-induced Ca^{2+} release may seem to be at variance with the finding of reduced spontaneous SR Ca^{2+} release in skinned single cells described in the previous chapter. Spontaneous SR Ca^{2+} release was measured, however, at the whole cell level. It is likely that an increased SR Ca^{2+} leak would manifest as an increased frequency of Ca^{2+} sparks, but for this to be seen at the whole cell level, spark propagation would need to occur throughout the cell. A reduction in SR Ca^{2+} load would decrease the likelihood of spark propagation, even in circumstances where spark frequency was increased. In support of this, a number of cells were recorded from heart failure experiments where local contractions were seen at high cytosolic Ca^{2+} concentrations, but co-ordinated cell contractions were absent, and changes in fluorescence were not large enough to constitute Ca^{2+} transients. This could only be fully investigated if confocal microscopy were available to record the frequency and propagation of Ca^{2+} sparks in heart failure and control cells.

The second important conclusion from uptake experiments in the presence of ruthenium red is that the absolute rate of uptake is reduced in heart failure cells. The mean time constant for uptake was 22% lower in sham than failure cells ($p = 0.09$). This difference was significant following normalisation of results (52%, $p = 0.02$). Since the ryanodine-dependent Ca^{2+} leak is abolished in these experiments, measurement of net Ca^{2+} uptake largely reflects SERCA 2A activity. The small,

ryanodine-independent leak is a possible confounding factor, but there is no evidence that this leak is altered in heart failure. The mechanism of this leak is uncertain, although it is possible that it reflects reverse activity of SERCA 2A in a proportion of Ca^{2+} pumps (Smith *et al.* 1998). Since the suggested cause of decreased SERCA 2A activity in heart failure is a reduction in the expression of SERCA 2A eg. (Kiss *et al.* 1995), it might be expected that there would be a concomitant decrease in both forward and reverse activity of SERCA 2A in these circumstances, and a consequent reduction of the ryanodine-independent leak. This hypothesis would serve to strengthen the results presented in this chapter. Finally, if the cause of the ryanodine-independent leak was reverse activity of SERCA 2A, and this leak were increased in heart failure, it could be argued that this in itself constitutes a reduction in the net activity of SERCA 2A.

It is therefore reasonable to conclude that in this heart failure model, there is a reduction in the activity of SERCA 2A of the order of 50%. This again is consistent with the findings in skinned single cells of a reduction in the SR Ca^{2+} load of 50-60% in heart failure.

4.5 Summary.

The main findings from these experiments measuring Ca^{2+} uptake in suspensions of permeabilised myocytes are that there is a reduction in the activity of SERCA 2A and an increased ryanodine-dependent SR Ca^{2+} leak in heart failure. This second finding may be a compensatory change. These findings are entirely consistent with earlier results in intact and skinned myocytes, but allow firm conclusions as to the mechanism of abnormal Ca^{2+} handling and SR dysfunction in heart failure. For the first time,

however, it has been shown that the changes in Ca^{2+} handling seen in this model are not purely explicable in terms of a reduction in the activity (or expression) of SERCA 2A.

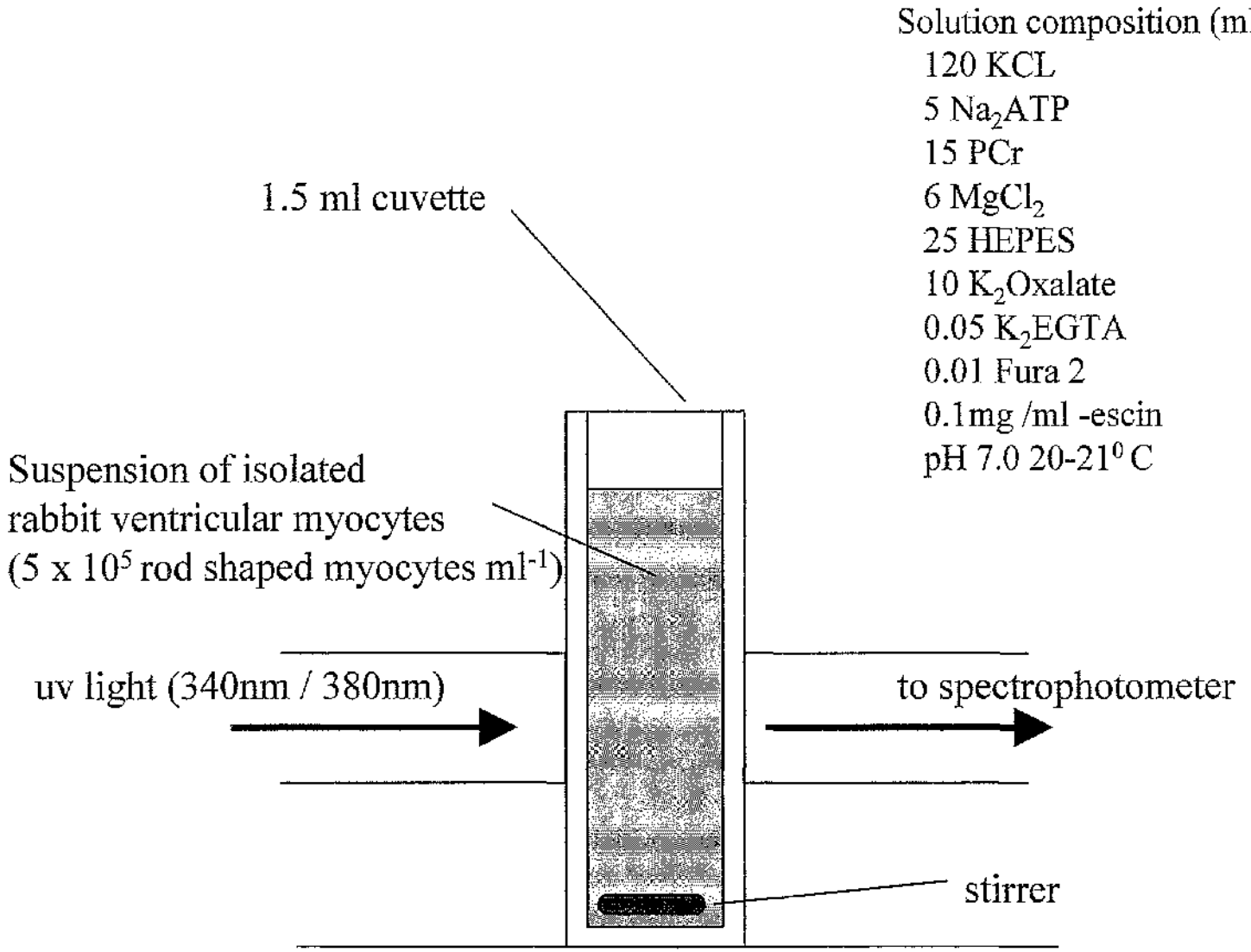

Fig. 4.1 Experimental apparatus to measure Ca^{2+} uptake in suspensions of permeabilised myocytes.

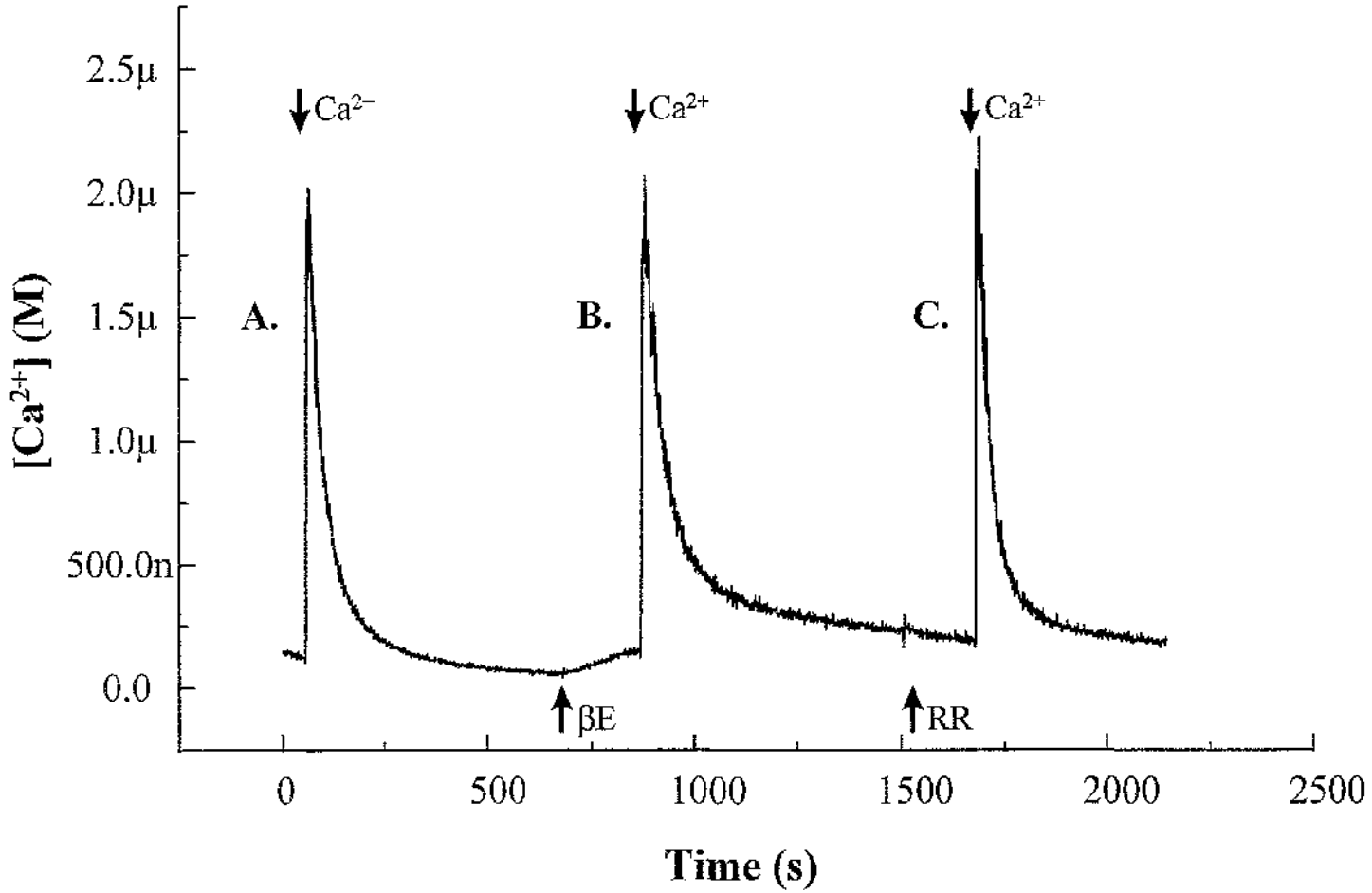

Fig. 4.2 Typical experimental trace showing timecourse of SR Ca^{2+} uptake in A: intact myocytes, B: permeabilised myocytes and C: myocytes with SR Ca^{2+} leak blocked. (βE : β-escin, RR : ruthenium red.)

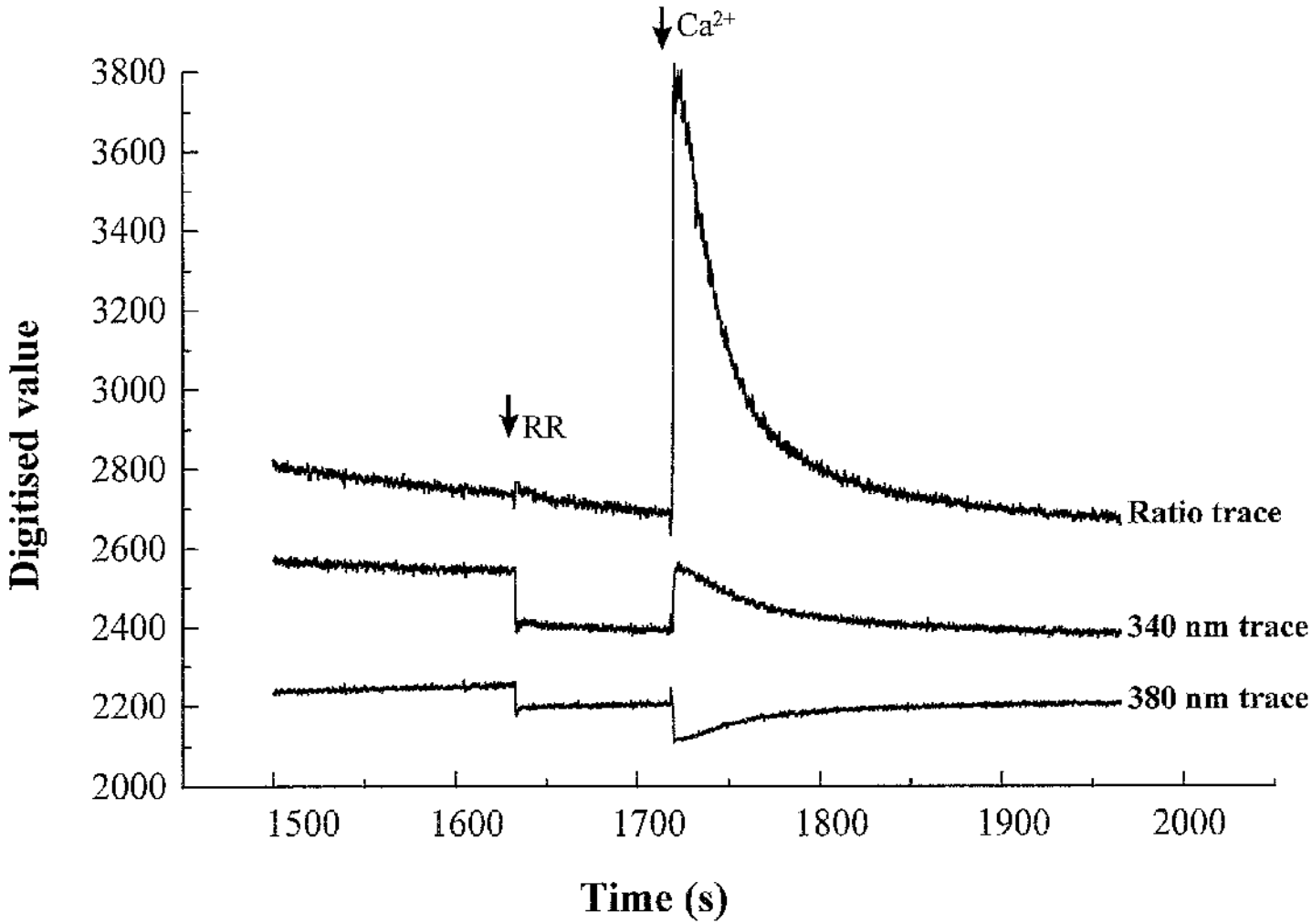

Fig. 4.3 Raw digitised data showing effect of ruthenium red on Fura-2 fluorescence.

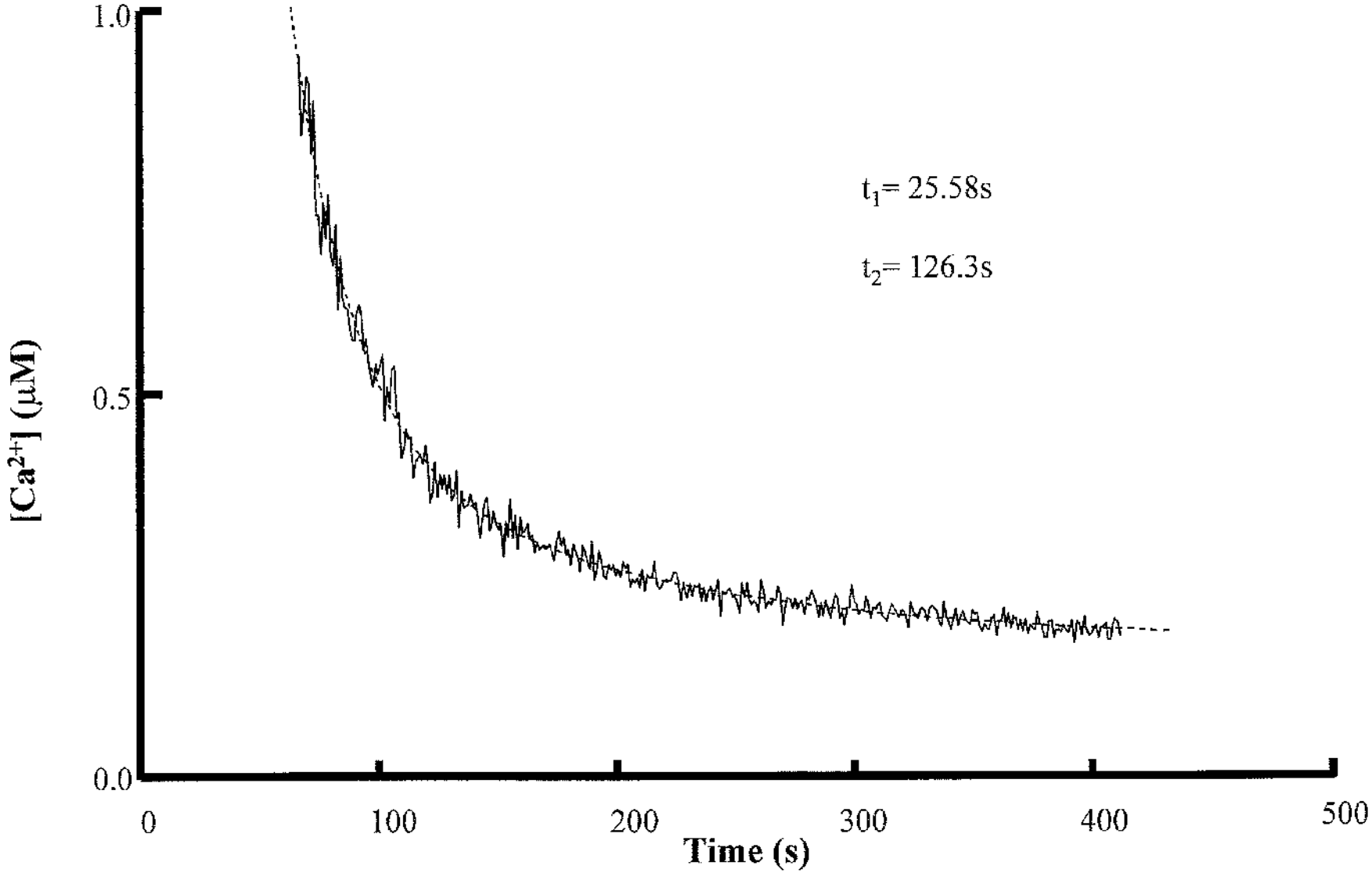

Fig. 4.4 Bi-exponential curve fitted to typical Ca^{2+} uptake curve over the range 800-200 nM. The time constants for this curve were 25.58s and 126.3s.

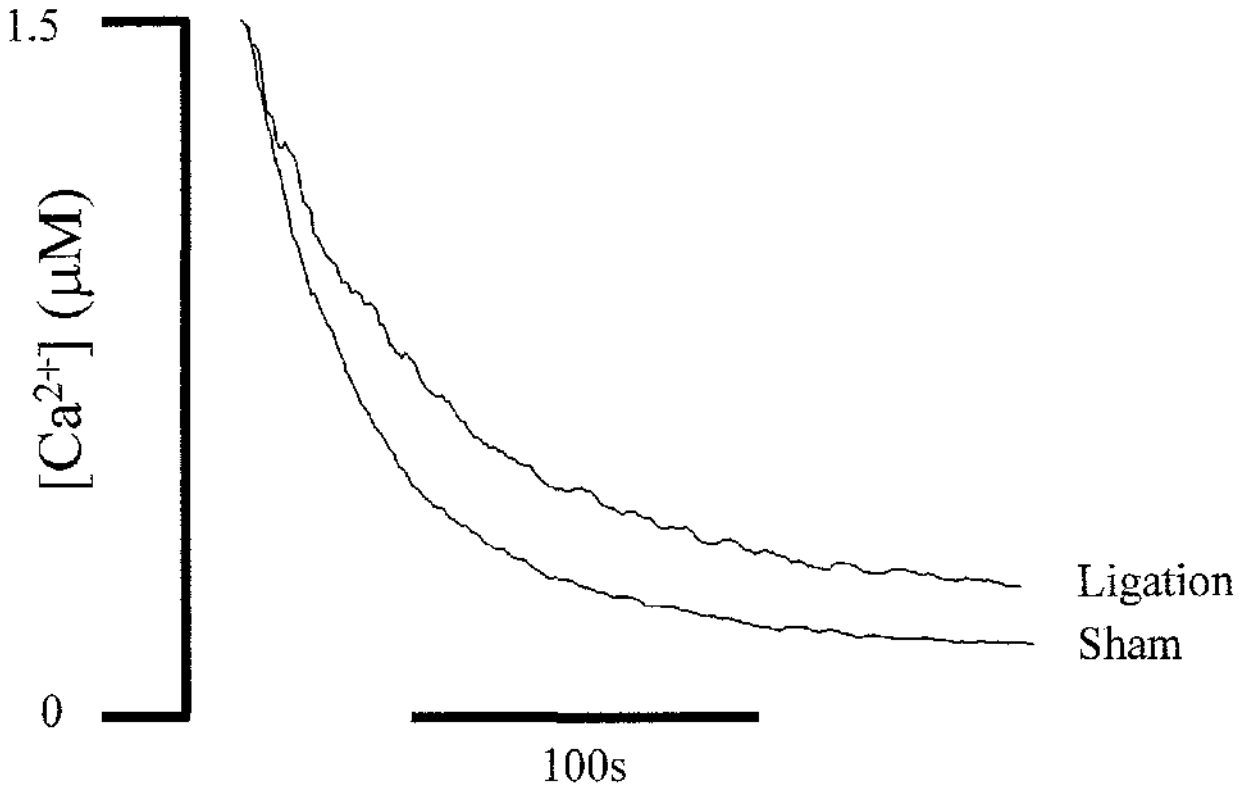

Fig 4.5 Reduced rate of SR Ca^{2+} uptake in suspensions of myocytes from failing hearts.

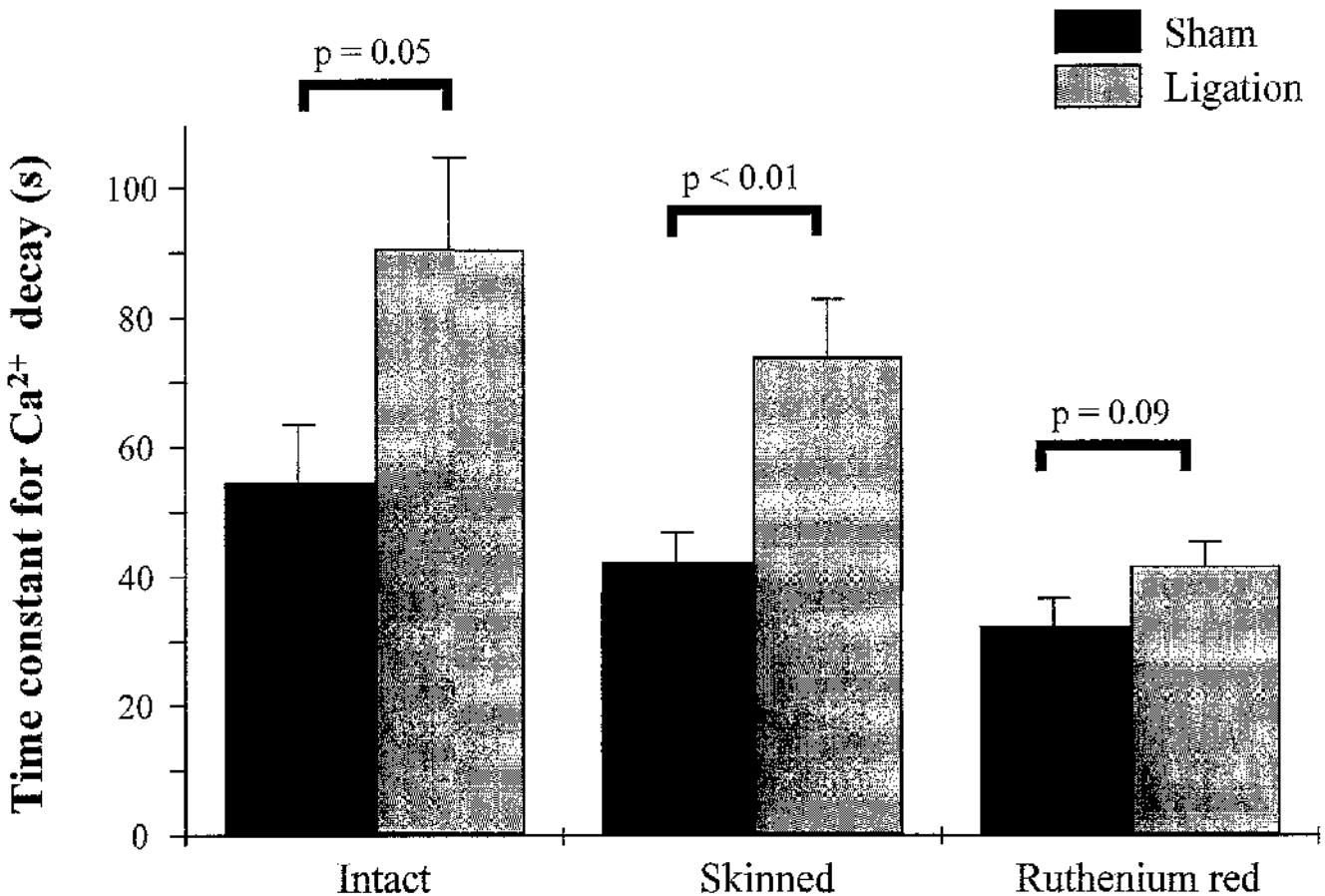

Fig. 4.6 Time constants for Ca^{2+} uptake in suspensions of $5x10^5$ rod shaped myocytes.

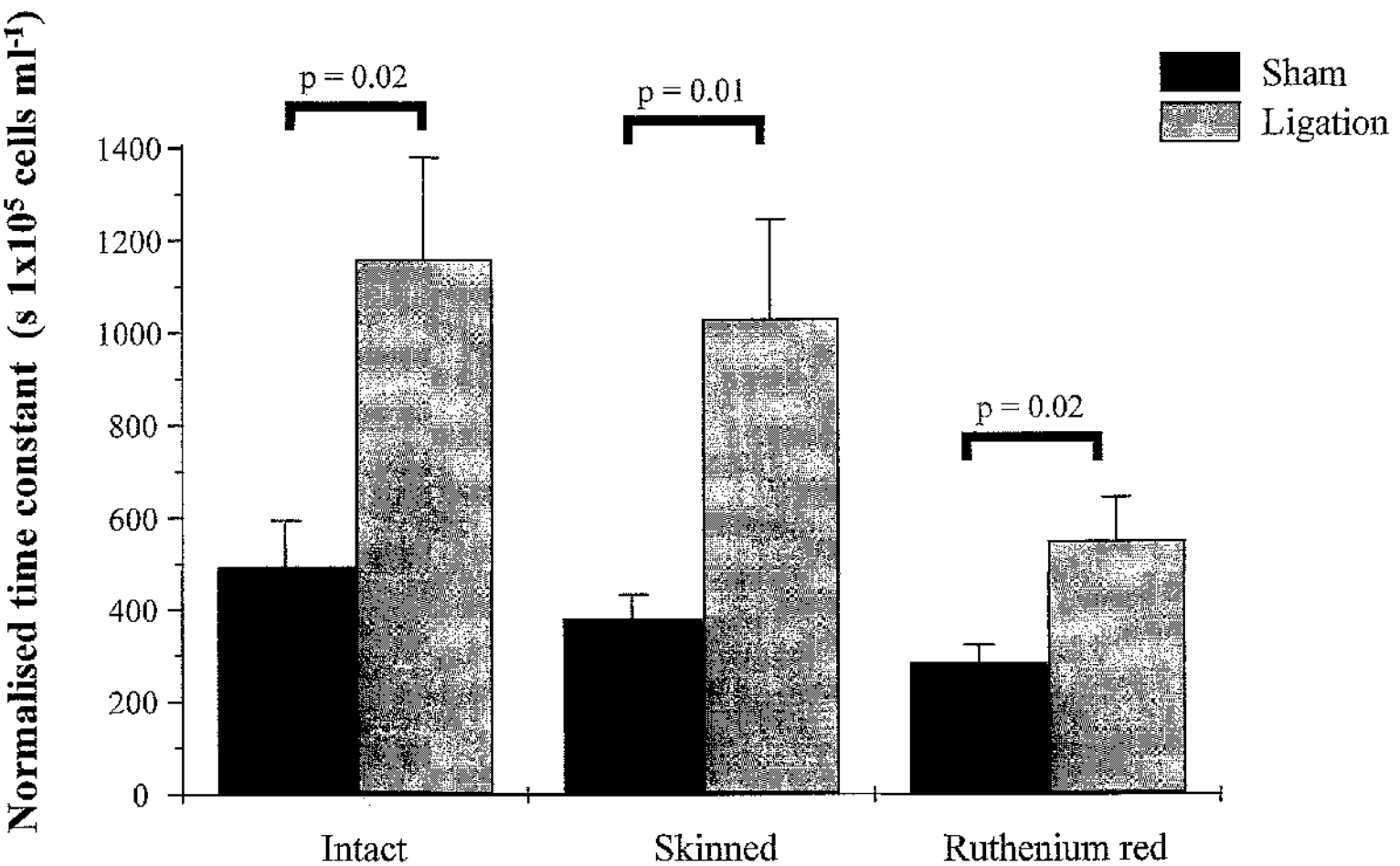

Fig. 4.7 Time constants for Ca^{2+} uptake normalised to $1x10^5$ cells ml^{-1}.

Chapter 5: Pharmacological modelling of SR dysfunction seen in heart failure.

5.1 Introduction.

Pharmacological modelling of the changes in SR function described in the previous chapters is attractive for a number of reasons. Since heart failure is a complex syndrome, with many pathophysiological features, the finding of a single physiological abnormality does not necessarily imply that this mechanism plays a causative role in the pathogenesis of heart failure. In addition, any abnormalities found may be compensatory mechanisms acting to offset other known or unknown changes. If a drug whose pharmacological action is known can be shown to produce the phenotypic abnormality seen in heart failure, this can be considered as a positive control experiment. If the action of such a drug does not produce the required phenotype, this is strong evidence against the proposed pathophysiological mechanism. If the drug produces a phenotype which resembles that seen in heart failure, but changes are different in quality or degree, this suggests that the proposed mechanism is over simplistic.

Several compounds are known to have specific actions on SR Ca^{2+} uptake and release processes. Unlike the clinical picture of heart failure, where there may be multiple perturbations of myocyte function, the action of these compounds can be relatively specific in their effect on SR Ca^{2+} handling. Thus modelling of SR dysfunction in normal myocytes may be able to dissect out the effects of abnormalities of these functions, without directly affecting other control mechanisms. By studying the effects of compounds which alter SR functions, the potential benefits of pharmacological therapy to offset changes in SR function in heart failure can also be addressed.

Possible sites for disruption of SR Ca^{2+} handling processes are shown in fig. 5.1. The compounds which were studied in this group of experiments were:

(i) Tetracaine.

(ii) Caffeine.

(iii) Thapsigargin.

(iv) Ionomycin.

5.1.1 Tetracaine.

Tetracaine, like other local anaesthetics blocks fast Na^+ channels in excitable tissue. It is membrane permeant, however, and has been found to inhibit Ca^{2+} release through the ryanodine receptor in skeletal muscle (Xu *et al.* 1993) and cardiac muscle (Gyorke *et al.* 1997a). Evidence that there was reduced expression of the ryanodine receptor in heart failure (eg (Brillantes *et al.* 1992; Arai *et al.* 1993)) suggested that abnormal Ca^{2+} release through the ryanodine receptor may contribute to the altered Ca^{2+} handling seen in heart failure. Although this hypothesis does not seem to explain the abnormalities of diastolic Ca^{2+} re-uptake seen in many studies (eg (Gwathmey *et al.* 1987)), it is possible that reduced Ca^{2+} release contributed to the small Ca^{2+} transients seen in both intact and permeabilised myocytes described in earlier chapters of this thesis. Tetracaine was thus chosen to test the hypothesis that inhibition of Ca^{2+} release at the ryanodine receptor would model the abnormalities of frequency and amplitude of spontaneous Ca^{2+} release seen in permeabilised myocytes from failing hearts.

The mechanism of action of tetracaine on SR Ca^{2+} release has been studied in purified ryanodine preparations in lipid bilayers from rabbit skeletal muscle (Volpe *et*

al. 1983; Xu *et al.* 1993) and in spontaneously active rat myocytes (Overend *et al.* 1997; Studer *et al.* 1994). The effects of other local anaesthetics including procaine has been studied in these preparations and in sheep cardiac SR (Tinker & Williams, 1993). Possible mechanisms of action of tetracaine include reducing single channel conductance of the ryanodine receptor, reducing the open probability of the receptor, decreasing the sensitivity of the receptor to cytosolic Ca^{2+} (decreased sensitivity of Ca^{2+}- induced Ca^{2+} release) or reducing the sensitivity of the ryanodine receptor to lumenal Ca^{2+} (i.e. reduced sensitivity to increasing SR Ca^{2+} load). (The presence of a mechanism sensing SR Ca^{2+} load and promoting Ca^{2+} release through the ryanodine receptor is suggested by the finding that the gain function of Ca^{2+} -induced Ca^{2+} release is linearly related to SR Ca^{2+} load (Janczewski *et al.* 1995)). These possible mechanisms may not be mutually exclusive.

In bilayer experiments from rabbit skeletal muscle, 150 μM tetracaine reduced the open probability of ryanodine receptors by around 50% (Xu *et al.* 1993). A Hill coefficient of 2 suggested that tetracaine bound at 2 or more co-operative sites. Single channel recordings showed that tetracaine (up to 500μM) did not affect channel conductance unless lumenal [Ca^{2+}] was above 10mM. Tetracaine had the same action when applied to both the cytoplasmic or SR lumenal side of the bilayer. The authors suggested that the action of tetracaine depended on binding to two or more regulatory sites near or within the SR membrane resulting in allosteric modification of the ryanodine receptor, with the possibility of a further low affinity binding site within the channel causing direct blockade. Despite tetracaine acting when placed on either the cytosolic or lumenal phase of the SR membrane, evidence for a time dependence on the action of local anaesthetics depending on membrane

permeability suggests that the site of the putative regulatory binding sites is either within the membrane or on the lumenal side (Volpe *et al.* 1983). In contrast, experiments in sheep cardiac muscle showed that local anaesthetics procaine, QX314 and QX222 acted to reduce Ca^{2+} release by reducing open probability, but only when present at the cytosolic face of the SR membrane (Tinker & Williams, 1993). This contradiction in the effect of local anaesthetics on the lumenal face of the SR membrane is important, since it is less likely that the mechanism of action of tetracaine is via an intra-SR Ca^{2+} sensing pathway if the tetracaine binding site is outside the SR.

The action of tetracaine has been investigated at the level of the whole cell in spontaneously contracting rat cardiac myocytes (Overend *et al.* 1997). Application of 25-200μM tetracaine decreased the frequency of spontaneous SR Ca^{2+} release, but increased the amplitude of release events. This was associated with an increase in SR Ca^{2+} content, which is readily understandable since in the presence of tetracaine Ca^{2+} will continue to load into the SR, but the threshold SR Ca^{2+} content necessary to initiate Ca^{2+} release will be higher. It is interesting that the amplitude of each release also increased, despite inhibition by tetracaine. This finding is consistent with the previous result that tetracaine reduces the open probability of the ryanodine receptor but does not reduce channel conductance (Overend *et al.* 1997). Using confocal microscopy, tetracaine has been shown to initially reduce the frequency and magnitude of Ca^{2+} sparks in rat cardiac myocytes, but in these experiments this was followed by a delayed potentiatory phase where spark frequency, amplitude and duration were all increased (Gyorke *et al.* 1997b). The action of tetracaine was again associated with an increase in SR Ca^{2+} content. In lipid bilayer experiments the

application of high concentrations of Ca^{2+} overcame the inhibition of channel activity induced by tetracaine. In these experiments the dose of tetracaine was significantly higher, 0.25-1.25 mM.

In summary, sub-millimolar doses of tetracaine have been shown to inhibit Ca^{2+} release through the ryanodine receptor, although the mechanism underlying this and the site of action of tetracaine remain unproved.

5.1.2 Caffeine.

Caffeine is thought to bind to a specific site on the ryanodine receptor and increases the open probability of the channel without affecting the duration of opening events (Sitsapesan & Williams, 1990). Caffeine thus acts to potentiate Ca^{2+} -induced Ca^{2+} release (Trafford *et al.* 1998). Since studies described in the preceding chapters of this thesis have shown a reduction in the systolic release of Ca^{2+} in heart failure, caffeine was used to test the hypothesis that by sensitising Ca^{2+} -induced Ca^{2+} release, opposite changes in SR function to those seen in heart failure could be produced. Such a result would raise the possibility that drugs such as caffeine could be used to reverse the abnormalities of SR function seen in heart failure.

At millimolar doses caffeine is thought to cause complete release of SR Ca^{2+} stores, and has therefore been used to assay SR Ca^{2+} content (Varro *et al.* 1993; Bers, 1987). The effect of caffeine is dose dependent in this range, however. Sustained application of caffeine causes a concentration dependent release of Ca^{2+} from the ryanodine receptor, and the ability of the SR to re-accumulate Ca^{2+} is inversely dependent on the concentration of caffeine applied in the range 5 - 40mM (Smith & Steele, 1998). At high concentrations, caffeine therefore has the effect of causing SR

Ca^{2+} depletion, since the rate of SR Ca^{2+} release is greater than the maximum rate of SERCA 2A mediated SR Ca^{2+} re-uptake.

Since the effects of caffeine are dose dependent, however, it remains possible that low doses of sustained caffeine application may stimulate Ca^{2+} release without jeopardising the ability of the SR to re-accumulate Ca^{2+} during the diastolic or rest interval, particularly as reduced SR lumenal Ca^{2+} concentration is known to stimulate SERCA 2A mediated SR Ca^{2+} uptake (Inesi & de Meis, 1989). It is therefore possible that caffeine could cause a sustained increase in SR Ca^{2+} cycling and hence force development during myocyte contraction and relaxation. In intact rat myocytes 100-200μM caffeine caused only a transient increase the amplitude of the Ca^{2+} transient (Trafford *et al.* 1998). This was accompanied by an increase in Na^{+}-Ca^{2+} exchange current, and a decrease in SR Ca^{2+} content. In steady state the amplitude of the Ca^{2+} transient returned to control levels despite the reduced SR Ca^{2+} content. This data confirms that low dose caffeine has a sustained effect on SR function, since the predicted effect of reduced SR Ca^{2+} content would be a reduction in amplitude of the Ca^{2+} transient. Since the reduction in SR Ca^{2+} content was secondary to increased cellular efflux of Ca^{2+} via the Na^{+}-Ca^{2+} exchanger, the effect of caffeine in permeabilised cells without the compounding influence of the Na^{+}-Ca^{2+} would be to increase both SR Ca^{2+} release and uptake.

5.1.3 Thapsigargin.

Thapsigargin is a naturally occurring sesquiterpine lactone which interferes with intracellular Ca^{2+} control in many cell types. In cardiac and skeletal muscle thapsigargin reduces systolic SR Ca^{2+} release and contraction without directly affecting sarcolemmal Ca^{2+} currents (Kirby *et al.* 1992). In permeabilised myocytes

and SR vesicles, thapsigargin blocks oxalate-supported SR Ca^{2+} uptake at concentrations of ~10nM (Sagara & Inesi, 1991; Kirby *et al.* 1992). No effect on Ca^{2+}-induced Ca^{2+} release in ryanodine receptor enriched SR vesicles is seen with thapsigargin (Kirby *et al.* 1992).

Thapsigargin acts in cardiac muscle by inhibiting the SR Ca^{2+} ATPase SERCA 2A. Ion transport with SERCA 2A is accomplished by Ca^{2+} binding when the enzyme is in a high affinity state (E_1 state) and release with the enzyme in a low affinity state (E_2 state). The shift in state from E_1 to E_2 is caused by hydrolysis of ATP via the formation of a phosphorylated intermediate (EP). Thapsigargin acts to inhibit the E_2 form of the enzyme (Kijima *et al.* 1991). The sensitivity of the Ca^{2+} ATPase to thapsigargin is lower in cardiac muscle than skeletal muscle, but sensitivity in cardiac muscle is increased by phosphorylation. Since phospholamban also binds to the E_2 form of SERCA 2A, and phospholamban is modulated by phosphorylation, it has been suggested that the binding sites of thapsigargin and phospholamban may be close to each other (Kijima *et al.* 1991).

The central hypothesis of this thesis has been that abnormal Ca^{2+} handling in heart failure results from SR dysfunction, which in turns stems from a reduction in the activity of SERCA 2A. As discussed in the introduction to this thesis, there is substantial evidence that there is a reduction in expression of SERCA 2A in heart failure. In addition, the findings in intact and permeabilised myocytes presented in earlier chapters that there is a reduction in SR Ca^{2+} release and SR Ca^{2+} load are most readily explained by a reduction in SERCA 2A activity. For these reasons, the effect of thapsigargin in modelling the changes in SR function seen in heart failure was crucial to the support of this hypothesis.

5.1.4 Ionomycin.

Ionomycin is a carboxylic acid ionophore which is relatively unselective for the transport of several divalent and trivalent cations (Wang *et al.* 1998; Liu & Hermann, 1978). One calcium ion is transported across membranes complexed to one ionomycin molecule, and each calcium ion is exchanged for 2 protons so that the process is electroneutral. The driving force for transport is therefore a transmembrane Ca^{2+} or proton gradient (Erdahl *et al.* 1995).

Little experimental work has been published on the effect of ionomycin on cardiac myocytes. In vascular smooth muscle low doses of ionomycin cause release of Ca^{2+} from the SR, an effect which is abolished if the SR is depleted prior to addition of ionomycin (Smith *et al.* 1989). Higher doses of ionomycin increase cytosolic Ca^{2+} by increasing sarcolemmal Ca^{2+} influx. The basis for the selectivity of action of ionomycin at low doses is probably the relatively low affinity of ionomycin for Ca^{2+}; higher Ca^{2+} concentration within the SR compared to extracellular concentrations means that the predominant effect of ionomycin is to transport Ca^{2+} across SR membranes rather than the sarcolemma.

In other tissues, the effect seems more complex. In myometrium, ionomycin induces phasic contractions which can be attenuated by inhibiting the production of inositol 1,4,5-triphosphate (IP_3), by inhibiting Ca^{2+}-induced Ca^{2+} release or by blocking sarcolemmal Ca^{2+} channels (Phillippe *et al.* 1995). The induction of phasic contractions was associated with an increased production of both inositol 1-monophosphate (IP_1) and IP_3.It is therefore difficult to explain the action of ionomycin in this system purely in terms of a release effect on SR Ca^{2+} stores, and it

is possible that in addition to its action as a Ca^{2+} ionophore, ionomycin acted directly or indirectly on the phosphotidylinositol signalling pathway.

For the purposes of the studies described in this thesis, the action of ionomycin was initially assumed to be simply that of a Ca^{2+} ionophore. It seemed likely that the action of ionomycin in cardiac myocytes would resemble that in vascular smooth muscle, rather than the more complex action described in myometrium. If this assumption was correct ionomycin would increase SR Ca^{2+} leak through a ryanodine-insensitive pathway. This would reduce SR Ca^{2+} content through a mechanism other than the inhibition of SERCA 2A. This pharmacological manipulation was necessary since results in permeabilised myocytes described in chapter 3 suggested that SR Ca^{2+} content was lower in heart failure cells. Since some authors had found unchanged levels of SERCA 2A in heart failure (eg (Schwinger *et al.* 1995; Cory *et al.* 1994)), it was important to explore other potential mechanisms for producing the heart failure phenotype. While there is no evidence currently available that SR Ca^{2-} leak is increased in heart failure, and studies of SR Ca^{2+} uptake and leak in oxalate-loaded myocytes described in chapter 4 suggested that in fact the ryanodine insensitive SR Ca^{2+} leak was *reduced* in heart failure, these experiments seemed worthwhile for completeness.

5.2 Methods.

All experiments in this section were performed on cells from healthy animals which had not undergone coronary artery ligation or sham operation. Left ventricular function was not measured, but was assumed to be normal. Ventricular myocytes were dissociated from stock New Zealand White rabbits as described in chapter 2. They were then stored as previously at room temperature until experimentation. Two

types of experiment were performed. In single cell experiments rate and amplitude of spontaneous oscillation and SR Ca^{2+} load assayed by Ca^{2+} release stimulated by high dose caffeine were compared before and after perfusion with the compound under study. In a second set of experiments, SR Ca^{2+} uptake was measured in oxalate-loaded suspensions of myocytes to confirm the mechanism of action of some of the compounds.

5.2.1 Single cell experiments.

Single ventricular myocytes were perfused with O.1R (see appendix for composition) at 1 ml min^{-1} in a perfusion chamber on an inverted Nikon microscope. Cells were anchored using a blunt, sealed microelectrode coated in Celltak to improve adhesion. The initial perfusate contained 1µl ml^{-1} 10mmol $CaCl_2$, with 10µM Fura-2. Permeabilisation was achieved by briefly exposing the cell to 100µg ml^{-1} β-escin (final concentration 90nM). Ca^{2+} concentration in the perfusate was then raised to a solution containing 3.5 1µl ml^{-1} 10mmol $CaCl_2$. This concentration was chosen since at this concentration, most cells in the previous experiments had been spontaneously active, while above this concentration, the signal to noise ratio deteriorated. Permeabilisation was confirmed when Ca^{2+} transients were recorded from spontaneously active cells, showing that cytosolic Ca^{2+} concentration had risen, and that the sarcolemma was permeable to Fura-2. In those cells where spontaneous activity was absent after 2mins in the high Ca^{2+} solution, or cells that were active but Ca^{2+} transients were not seen, a further exposure to β-escin was tried. Cells which continued not to respond were discarded. Fluorescence was measured using a Cairn spinning wheel spectrophotometer operating at 30Hz. Previous calibration values for R_{max}, R_{min}, and K_D were used.

The rate and amplitude of spontaneous activity were measured for a period of two minutes once a steady state for amplitude and frequency of oscillation had been reached. In tetracaine and caffeine experiments, SR Ca^{2+} content was measured after this two minute period by microinjecting a solution of 10mmol caffeine which contained the same concentration of Ca^{2+} and Fura-2 to avoid injection artefact. SR Ca^{2+} load was measured on 3-4 occasions at 2min intervals until reproducible Ca^{2+} transients were obtained. The perfusate was then changed to one containing the compound for study. Rate and amplitude of spontaneous Ca^{2+} release was measured as the mean of all releases for 30s bins following the change of solution. The experiment proceeded until a new steady state was reached, either of cessation of spontaneous activity, or for new steady values for amplitude and frequency were achieved. Experiments continued for at least 10 mins if no changes in frequency or amplitude were apparent. In tetracaine and caffeine experiments, measurement of SR load using high dose caffeine injection were performed at 2 min intervals once steady state had been achieved. Dose ranging studies were performed for all compounds by starting at relatively high concentrations and reducing until no effect was seen. The dose ranges used were:

(i) Tetracaine: 50 - 100μM

(ii) Caffeine: 100μM

(iii) Thapsigargin: 100nM - 100pM

(iv) Ionomycin: 15μM - 15nM

5.2.2 Oxalate-supported Ca^{2+} uptake measurements.

Oxalate-supported Ca^{2+} uptake was measured in the presence of tetracaine. As before myocytes resuspended in 0.05R (see appendix for details) containing 10mM oxalate to buffer intra-SR Ca^{2+} concentration. They were then counted, and the concentration adjusted to give a final concentration of 2.5 10^5 ml^{-1} rod shaped myocytes. Myocytes were transferred to 2ml cuvettes and 10μM Fura-2 and 90nM β-escin added. Fluorescence was measured using a Cairn spinning wheel spectrophotometer at 30 Hz. Following a 2min incubation, 10-20 μl of 10mM $CaCl_2$ was added to raise cytosolic Ca^{2+} concentration to approximately 1-2μM. Ca^{2+} decay curves were then recorded as Ca^{2+} concentration fell due to SR uptake mediated by SERCA 2A.

In the case of tetracaine experiments, SR Ca^{2+} uptake was then measured following addition of 75μM tetracaine, and then again in the presence of tetracaine plus 30μM ruthenium red. The protocol was then repeated in a new batch of cells and Ca^{2+} uptake was measured again in the absence of both tetracaine and ruthenium red. Ca^{2+} uptake was then measured in the presence of ruthenium red, and finally in the presence of both tetracaine and ruthenium red. This allowed any ruthenium red-independent action of tetracaine to be measured, since at this concentration, ruthenium red can be considered a total blocker of Ca^{2+} release at the ryanodine receptor. In ionomycin experiments, uptake was measured following the sequential addition of ruthenium red and ionomycin. The concentrations of ionomycin and tetracaine were chosen from experiments in single cells to be in the range where the compound studied had a noticeable steady state effect, but did not cause complete inhibition of spontaneous SR Ca^{2+} release.

Results were analysed as previously. 2nd order exponential decay curves were fitted to the data, and time constants calculated from these curves. In the case of ionomycin experiments, further analysis of SR Ca^{2+} leak was performed.

5.3 Results.

5.3.1 Effect on tetracaine on spontaneous Ca^{2+} release in single cells.

The effect of tetracaine on permeabilised single cells was measured at doses of 50μM, 75μM and 100μM. In total, experiments were done on 34 cells from 10 rabbits. A typical experimental record in a spontaneously active cell is shown in fig. 5.2. Here 75μM tetracaine was added to the perfusate of a spontaneously active single cell in steady state. Prior to the addition of tetracaine, SR Ca^{2+} content was assayed by rapid injection of 10mM caffeine. The trace shows that after addition of tetracaine, there is a decrease in frequency of spontaneous Ca^{2+} release. This is associated with a rise in the amplitude of spontaneous Ca^{2+} release. The reason for the rise in amplitude is shown by the increase in the amplitude of the caffeine induced Ca^{2+} transient, indicating increased SR Ca^{2+} content. Following the removal of tetracaine, there is a transient burst of high frequency spontaneous Ca^{2+} release, before the cell returns to control activity.

The effect of 100μM tetracaine on frequency of spontaneous release in 14 cells from 7 rabbit hearts is shown in table 5.1. The was a reduction in the mean frequency of spontaneous SR Ca^{2+} release from 0.149 ± 0.012 Hz to 0.028 ± 0.010 Hz, a reduction of 77.5 ± 8.1% ($p = 9.5 \times 10^{-6}$) on addition of tetracaine. 100μM tetracaine abolished spontaneous activity in 50% of all cells. The mean frequency of spontaneous release in those cells where tetracaine abolished spontaneous release

was non-significantly higher than in the other cells (0.176 ± 0.013 Hz, $p > 0.05$). If the analysis is restricted to the cells in which there was ongoing spontaneous SR Ca^{2+} release, the effect of tetracaine was to reduce frequency by 55 ± 7.6%.

Expt. No.	Cell no.	Control Frequency (Hz)	Tetracaine Frequency (Hz)	Normalised Frequency (%)
1	1	0.2	0	0
	2	0.13	0.025	19.2
2	3	0.1	0	0
3	4	0.2	0	0
	5	0.15	0	0
4	6	0.217	0	0
	7	0.233	0	0
5	8	0.1	0.05	50
6	9	0.15	0.058	38.9
	10	0.125	0.033	26.7
	11	0.108	0.017	15.4
7	12	0.12	0.106	88
	13	0.13	0.1	76.9
	14	0.129	0	0
	mean	0.149	0.028	22.5
	SD	0.045	0.037	30.2
	SEM	0.012	0.01	8.1
	p	9.52 x 10-6		

Table 5.1: Effect of 100μM tetracaine on the frequency of spontaneous Ca^{2+} release. P value shown is calculated from a paired t-test of control and tetracaine frequency.

The effect of 75μM tetracaine on frequency of spontaneous release in 7 cells from 3 rabbit hearts is shown in table 5.2. The was a reduction in the mean frequency of spontaneous SR Ca^{2+} release from 0.250 ± 0.022 Hz to 0.114 ± 0.033 Hz, a reduction of 55.2 ± 12.2% ($p = 0.002$) on addition of tetracaine. 75μM tetracaine abolished spontaneous activity in only 1 of 7 cells. In the cells which remained spontaneously active the effect of tetracaine was to reduce frequency by 47.7 ± 10.6%.

Expt. No.	Cell no.	Control frequency (Hz)	Tetracaine frequency (Hz)	Normalised frequency (%)
1	1	0.2	0.005	2.5
	2	0.18	0.1	55.6
2	3	0.23	0.2	87
3	4	0.23	0.13	56.5
	5	0.35	0.23	65.7
	6	0.28	0	0
	7	0.28	0.13	46.4
	mean	0.25	0.114	44.8
	SD	0.058	0.088	32.3
	SEM	0.022	0.033	12.2
	p	0.002		

Table 5.2: Effect of 75μM tetracaine on the frequency of spontaneous Ca^{2+} release. P value shown is calculated from a paired t-test of control and tetracaine frequency.

The effect of 50μM tetracaine on frequency of spontaneous release in 13 cells from 5 rabbit hearts is shown in table 5.3. There was a reduction in the mean frequency of spontaneous SR Ca^{2+} release from 0.169 ± 0.018 Hz to 0.137 ± 0.018 Hz, a reduction of 20.1 ± 2.9% ($p = 2.23 \times 10^{-6}$) on addition of tetracaine. This dose of tetracaine did not abolish spontaneous activity in any of the cells studied.

Tetracaine thus reduced the frequency of spontaneous SR Ca^{2+} release in a dose dependent manner. At high doses, spontaneous activity was abolished in 50% of cells and markedly reduced in all others. At lower doses the effect was reduced. Spontaneous activity was not abolished, and frequency was reduced by around 20%. The dose dependence of the effect of tetracaine is shown in figure 5.3.

Expt. No.	Cell No.	Control frequency (Hz)	Tetracaine frequency (Hz)	Normalised frequency (Hz)
1	1	0.23	0.2	87
	2	0.23	0.2	87
2	3	0.258	0.233	90.3
	4	0.3	0.267	88.9
3	5	0.142	0.083	58.8
	6	0.083	0.075	90
	7	0.17	0.117	68.6
4	8	0.108	0.085	78.5
	9	0.15	0.114	76.2
	10	0.107	0.077	72.1
5	11	0.167	0.133	80
	12	0.117	0.108	92.9
	13	0.133	0.092	68.8
	mean	0.169	0.137	79.9
	SD	0.066	0.065	10.5
	SEM	0.018	0.018	2.9
	p	2.22 x 10-6		

Table 5.3: Effect of 50μM tetracaine on the frequency of spontaneous Ca^{2+} release. P value shown is calculated from a paired t-test of control and tetracaine frequency.

Table 5.4 shows the effect of 100μM tetracaine on the amplitude of spontaneous SR Ca^{2+} release in 6 cells in which spontaneous SR Ca^{2+} release was not abolished. There was a significant increase in the amplitude of release in these cells from 35.4 ± 3.1 nM to 39.2 ± 3.2 (p=0.0013). This represented an increase of 11.2 ± 2.2%. The amplitude of spontaneous Ca^{2+} release in those cells where this dose of tetracaine abolished spontaneous activity was 38.0 ± 5.1 nM.

Expt. No.	Cell No.	Control frequency (Hz)	Tetracaine frequency (Hz)	Normalised frequency (Hz)
1	1	33.6	40	118.81
2	2	32.4	34.2	105.62
	3	31.6	35.8	113.31
	4	49.6	54	108.78
3	5	28	32.3	115.39
	6	36.9	38.9	105.44
	mean	35.4	39.2	111.22
	SD	7.6	7.8	5.48
	SEM	3.1	3.2	2.24
	p	0.0013		

Table 5.4: Effect of 100μM tetracaine on the amplitude of spontaneous Ca^{2+} release. P value shown is calculated from a paired t-test of control and tetracaine amplitudes.

Table 5.5 shows the effect of 75μM tetracaine on the amplitude of spontaneous SR Ca^{2+} release in the 5 cells in which spontaneous SR Ca^{2+} release was not abolished. There was a non-significant increase in the amplitude of release in these cells from 62.4 ± 8.4 nM to 65.2 ± 8.2 (p=0.220). This represented an increase of 6.2 ± 6.3%.

Expt. No.	Cell No.	Control frequency (Hz)	Tetracaine frequency (Hz)	Normalised frequency (Hz)
1	1	42	48.7	115.82
	2	45.5	54.6	120.01
2	3	84.9	86.9	102.33
3	4	62.6	52.6	84.1
	5	77	83.8	108.86
	mean	62.4	65.3	106.2
	SD	18.8	18.4	14.1
	SEM	8.4	8.2	6.3
	p	0.22		

Table 5.5: Effect of 75μM tetracaine on the amplitude of spontaneous Ca^{2+} release. P value shown is calculated from a paired t-test of control and tetracaine amplitudes.

Table 5.6 shows the effect of 50μM tetracaine on the amplitude of spontaneous SR Ca^{2+} release in the 13 cells from 5 experiments. There was a highly significant increase in the amplitude of release in these cells from 36.6 ± 2.6 nM to 40.2 ± 2.9 ($p = 6.58 \times 10^{-5}$). This represented an increase of 10.1 ± 2.6%.

Expt. No.	Cell No.	Control frequency (Hz)	Tetracaine frequency (Hz)	Normalised frequency (Hz)
1	1	29.3	31.6	107.85
	2	34.4	36.9	107.45
2	3	52	59	113.48
	4	57.9	60.3	104.01
3	5	38.1	46.4	121.78
	6	35.1	39.5	112.5
	7	33.6	37.6	112.04
4	8	30.2	34.4	113.97
	9	36.8	36.7	99.72
	10	23.6	25.8	109.25
5	11	30.2	32.2	106.69
	12	33.7	35.7	105.9
	13	40.5	47.2	116.51
	mean	36.6	40.2	110.09
	SD	9.3	10.3	5.78
	SEM	2.6	2.9	1.6
	p	6.58E-05		

Table 5.6: Effect of 50μM tetracaine on the amplitude of spontaneous Ca^{2+} release. P value shown is calculated from a paired t-test of control and tetracaine amplitudes.

In contrast to the effect of tetracaine on frequency of spontaneous SR Ca^{2+} release, the effect on amplitude of spontaneous release was therefore not shown to be dose dependent. This is shown in fig. 5.4.

The cause of increased amplitude of spontaneous SR Ca^{2+} release was investigated by measuring the effect of tetracaine on SR Ca^{2+} content. This was done by measuring caffeine-induced SR Ca^{2+} release in a subgroup of cells for concentrations of tetracaine of 100 μM and 75 μM. In 4 cells from 3 experiments 100 μM tetracaine increased the amplitude of caffeine induced Ca^{2+} transients in 75% of cells. The mean amplitude rose from 53.4 ± 3.7 nM to 61.2 ± 8.5 nM, an increase of 13.3 ± 11.9 % ($p = 0.2$). A clearer effect was seen with 75 μM tetracaine. In experiments on 5 cells from 3 rabbit hearts, tetracaine increased the amplitude of caffeine-induced Ca^{2+} transients in all cells. Mean amplitude rose from 63.9 ± 8.8 nM to 82.0 ± 11.4 nM, an increase of 28.4 ± 2.5 % ($p = 0.002$). While numbers in these experiments were small, and no experiments looked at the effect of 50 μM tetracaine, as with the amplitude of spontaneous SR Ca^{2+} release, the effect of tetracaine did not appear to be dose dependent.

5.3.2 Effect on tetracaine on SR Ca^{2+} uptake and leak in cuvette experiments.

The mid-range dose of 75μM tetracaine was chosen for these experiments. The effect of tetracaine on SR Ca^{2+} uptake in oxalate-loaded myocytes was measured in 4 experiments. Representative traces showing the effect on tetracaine prior to the addition and following the addition of ruthenium red are shown in figure 5.5. These traces show that compared to control conditions, tetracaine caused an increase in the rate of Ca^{2+} uptake in these myocytes. Ruthenium red caused a similar increase in the rate of Ca^{2+} uptake. These traces also show that tetracaine had an small additive action to ruthenium red, and that ruthenium red had a similar additive action to tetracaine.

Table 5.7 shows the action of tetracaine and ruthenium red on mean time constants and rate constants for Ca^{2+} uptake. In the absence of ruthenium red, tetracaine increased the rate constant for Ca^{2+} uptake by 81.6 ± 19.4% (p = 0.02). Addition of ruthenium red increased the rate constant by a further 53.0 ± 15.7% (p = 0.03). Similarly, in the absence of tetracaine, ruthenium red increased the rate constant for Ca^{2+} uptake by 164.7 ± 34.2% (p = 0.01) and addition of tetracaine increased the rate constant by a further 7.9 ± 2.4% (p = 0.02).

Both tetracaine and ruthenium red significantly increased the rate of SR Ca^{2+} uptake in oxalate loaded permeabilised myocytes. The effect of 30nM ruthenium red was greater than that of 75μM tetracaine. Both compounds had an additive effect to each other, and again the addition effect of ruthenium red was greater than the additional effect of tetracaine. This is shown in fig. 5.6. In this figure, panel A(i) shows the effect of tetracaine normalised to control rate constants. Panel A(ii) shows the additional effect of ruthenium red normalised to the rate constants in the presence of tetracaine. Panel B shows the reverse effects of the addition of ruthenium red to control and then of tetracaine to ruthenium red.

(a)	Time constants (s).					
Expt.	Control	Tetracaine	Tet. + RR	Control	RR	RR + Tet.
1	48.7	25.7	19.59	47.2	18.2	16.6
2	70.3	31.3	20.4	63.4	16.8	15.9
3	43.9	22.7	11.5	23.1	11.5	11.2
4	127	106.9	82.2	139.6	63.1	55.5
mean	72.48	46.65	33.42	68.33	27.4	24.8
SD	33.01	34.92	28.38	43.58	20.76	17.85
SEM	16.51	17.46	14.19	21.79	10.38	8.92
(b)	Normalised time constants (%).					
Expt.	Control	Tetracaine	Tet. + RR	Control	RR	RR + Tet.
1	100	52.77	40.23	100	38.56	35.17
2	100	44.52	29.02	100	26.5	25.08
3	100	51.71	26.2	100	49.78	48.48
4	100	84.17	64.72	100	45.2	39.76
mean	100	58.29	40.04	100	40.01	37.12
SD		15.27	15.19		8.76	8.44
SEM		7.64	7.59		4.38	4.22
p	0.009	0.0037		0.0006	0.0299	
(c)	Rate constants (s-1).					
Expt.	Control	Tet.	Tet. + RR	Control	RR	RR + Tet.
1	0.021	0.039	0.051	0.021	0.055	0.06
2	0.014	0.032	0.049	0.016	0.06	0.063
3	0.023	0.044	0.087	0.043	0.087	0.089
4	0.008	0.009	0.012	0.007	0.016	0.018
mean	0.016	0.031	0.05	0.022	0.054	0.058
SD	0.006	0.013	0.026	0.013	0.025	0.026
SEM	0.003	0.007	0.013	0.007	0.013	0.013
(d)	Normalised rate constants (%).					
Expt.	Control	Tet.	Tet. + RR	Control	RR	RR + Tet.
1	100	189.49	248.6	100	259.34	284.34
2	100	224.6	344.61	100	377.38	398.74
3	100	193.39	381.74	100	200.87	206.25
4	100	118.8	154.5	100	221.24	251.53
mean	100	181.57	282.36	100	264.71	285.22
SD		38.71	88.37		68.35	71.17
SEM		19.36	44.19		34.18	35.58
p	0.0177	0.03		0.0125	0.0158	

Table 5.7: effect of tetracaine (Tet.) and ruthenium red (RR) on time and rate constants for Ca^{2+} uptake in permeabilised myocytes.

5.3.3. Effect of caffeine on spontaneous SR Ca^{2+} release.

The effect of 100μM caffeine on spontaneous SR Ca^{2+} release was tested on 13 cells from 8 rabbit hearts. Following addition of caffeine the mean frequency of spontaneous SR Ca^{2+} release fell from 0.21 ± 0.02 Hz to 0.20 ± 0.03 Hz. This represented a reduction of 5.2 ± 7.45%. This effect was non-significant. There was a small accompanying reduction in the amplitude of spontaneous release from 61.0 ± 5.8nM to 52.6 ± 5.5nM, a reduction of 13.6 ± 8.9% ($p = 0.049$).

5.3.4. Effect of thapsigargin on spontaneous SR Ca^{2+} release.

The effect of thapsigargin at concentrations ranging from 100nM to 15nM was measured. Fig 5.7 shows a typical experimental trace of the effect of 30nM thapsigargin on a spontaneously active single myocyte. In this experiment thapsigargin reduced both the frequency and amplitude of spontaneous SR Ca^{2+} release.

(i) Effect on frequency of spontaneous release.

Thapsigargin had a slow onset of action at low concentrations. For this reason recordings from spontaneously active cells were made for significantly longer periods (up to 30mins) than for experiments using other drugs. It was necessary therefore in these experiments to study a control population of cells to ascertain whether there was a natural run down of spontaneous activity over the course of this time period in the absence of any pharmacological manipulation.

Spontaneous activity was recorded in 13 control cells from 6 rabbit hearts. Spontaneous activity ceased within 15mins in only 1 of 13 cells. 11 of 13 cells remained spontaneously active after 20mins. The mean frequency of spontaneous activity after 15 mins was 72.8 ± 7.2% of control value after 15mins and 58.6 ± 6.4%

after 21 mins. In cells in which recording was continued for 30 mins, final frequency of spontaneous release was 52.2 ± 16.4% of control value. There was therefore some diminution of activity, but in the majority of cells activity was relatively stable over the course of 15-20 mins.

High concentrations of thapsigargin had the effect of abolishing spontaneous SR Ca^{2+} release. Thus 100nM thapsigargin abolished spontaneous activity in all cells after 15mins, and 50nM thapsigargin abolished spontaneous activity in 50% of cells over the same timescale. In contrast all cells exposed to 30nM or lower concentrations of thapsigargin remained spontaneously active beyond 15mins. The effect of 100nM - 100pM thapsigargin at 15mins is shown in fig 5.8. Compared to the change in frequency in control cells at 15mins, there was a significant reduction in frequency of spontaneous Ca^{2+} release in 100nM (100%, $p < 0.001$), 50nM (95.0 ± 5.0%, $p < 0.01$), 30nM (67.8 ± 7.8%, $p < 0.001$) and 10nM (43.6 ± 5.6%, $p < 0.05$) thapsigargin. Reductions in frequency caused by 1nM and 100pM were not significant.

The change in frequency of spontaneous SR Ca^{2} release over time compared to control cells for all concentrations of thapsigargin is shown in fig. 5.9. Differences in frequencies of spontaneous activity are apparent for different concentrations of thapsigargin after 3mins of exposure suggesting that the rate of onset of action of thapsigargin is rapid. The curves continue to diverge for different concentrations of thapsigargin, but after 9 minutes the rate of decay in frequency of spontaneous release appears similar for all concentrations, suggesting that the action of thapsigargin is maximum at this point. Further reductions in frequency beyond 9

minutes are of the order of those seen in control cells and therefore seem to represent the natural tendency for the frequency to decline over time.

Thapsigargin concentration	0	100nM	50nM	30nM	10nM	1nM	100pM
No of experiments	6	1	1	7	5	2	2
No of cells	13	3	2	9	9	5	4
Control frequency (Hz)	0.21	0.15	0.09	0.19	0.15	0.18	0.17
(+/-) SEM	0.03	0.04	0.03	0.02	0.02	0.03	0.02
3min frequency (% control)	98.2	73.9	82.3	88.6	95.5	92.2	83.3
(+/-) SEM	5.2	5.8	2.3	2.6	1.9	5.3	3.5
6min frequency (% control)	91.2	24.2	50.6	71.7	88.8	85.3	68.7
(+/-) SEM	6.5	15.1	4.4	5.7	3	4.9	4.9
9min frequency (% control)	83.7	9.1	22.7	58.7	76.5	75	59.5
(+/-) SEM	7.8	8	7.3	5.3	3.4	7	8.1
12min frequency (% control)	70.7	2.8	10	42.1	62.9	66.2	57.3
(+/-) SEM	8.3	2.8	10	8.1	4.9	8.2	7.2
15min frequency (% control)	72.8	0	12.5	32.2	56.4	55	50.5
(+/-) SEM	7.2		12.5	8.8	5.6	8.2	12
18min frequency (% control)	64.6	0	5	39.2	60.6	41.4	46.9
(+/-) SEM	8		5	8.9	5.8	10	11.8
21min frequency (% control)	58.6	0	2.5	30.5	40.4	30	44.7
(+/-) SEM	6.4		2.5	13.4	3.3	8.5	17.4

Table 5.8 Effect of 100nM - 100pM thapsigargin on frequency of spontaneous SR Ca^{2+} release.

(ii) Effect on amplitude of spontaneous release.

Given the slow onset of action of thapsigargin, the effect was again compared to a control group of cells where no pharmacological manipulation was made. This group of cells was studied to ascertain whether there was any natural diminution in the amplitude of spontaneous SR Ca^{2+} release over the timecourse of the experiment. The amplitude of spontaneous Ca^{2+} release in this group of cells was 108.6 ± 3.4% of the

initial value at 15 mins, and 102.6 ± 6.9% at 21 mins. In contrast to the reduction in frequency of spontaneous release over time, there was therefore no reduction in the amplitude of spontaneous SR Ca^{2+} release over 20 mins in this group of cells in the absence of thapsigargin. Any reduction in amplitude seen in the experimental group could therefore be attributed to the action of thapsigargin.

The effect of thapsigargin at concentrations from 100nM to 100pM is shown in table 5.9. At high concentration thapsigargin abolished spontaneous SR Ca^{2+} release. At 100nM, thapsigargin abolished spontaneous activity in all cells within 15 mins. 50nM thapsigargin abolished activity in all but one cell within the same timescale. As can be seen from table 5.9, lower concentrations of thapsigargin caused a dose dependent steady state reduction in the amplitude of spontaneous SR Ca^{2+} release. Thus at 15 mins the percentage of control amplitude for 30nM, 10nM, 1nM and 100pM thapsigargin was 63.1 ± 5.2 % ($p = 0.001$), 82.1 ± 8.2 % ($p = 0.02$), 97.3 ± 7.8% (p = ns) and 100.2 ± 9.9% (p = ns). The dose dependency of the effect of thapsigargin is shown in figure 5.10.

Thapsigargin concentration	0	100nM	50nM	30nM	10nM	1nM	100pM
No of experiments	6	1	1	7	5	2	2
No of cells	13	3	2	9	9	5	4
Control amplitude (nM)	39.8	53.5	33.1	53.7	42.8	31.7	47.1
(+/-) SEM	6.8	6.9	3.2	6.1	4.4	4.7	8.7
3min amplitude (% control)	107	81.3	93.4	93.9	97.7	109.7	95.6
(+/-) SEM	2.3	11.1	0.4	1.5	5	5.2	4.8
6min amplitude (% control)	106.4	70.2	77.5	84.6	96.2	106.7	85.8
(+/-) SEM	2.1	-	1.2	2.7	5.9	7.5	4.4
9min amplitude (% control)	106.2	51.3	61.6	75	93.4	103.4	87.6
(+/-) SEM	2	-	3	5.1	5.9	10.7	5.8
12min amplitude (% control)	104.8	40.3	57.5	68.5	89.6	103.3	91.4
(+/-) SEM	2.6	-	-	6	7.1	8.6	7.7
15min amplitude (% control)	108.6	0	35.9	63.1	82.1	97.3	100.2
(+/-) SEM	3.4		-	5.2	8.2	7.8	9.9
18min amplitude (% control)	109.1	0	35.8	61	73.2	84.4	90.7
(+/-) SEM	5		-	6.9	9.2	5.3	13.9
21min amplitude (% control)	102.6	0	35.8	71.3	46.8	89.4	92.3
(+/-) SEM	6.9		-	2.5	4.6	10.4	9.4

Table 5.9 Effect of 100nM - 100pM thapsigargin on amplitude of spontaneous SR Ca^{2+} release.

5.3.5. Effect of ionomycin on spontaneous SR Ca^{2+} release.

The effect of ionomycin at concentrations ranging from 1.5mM to 15nM was measured. Fig 5.11 shows a typical experimental trace of the effect of 15nM ionomycin on a spontaneously active single myocyte. In this experiment ionomycin reduced the frequency of spontaneous SR Ca^{2+} release, but had no apparent action on the amplitude of Ca^{2+} release.

(i) Effect on frequency of spontaneous release.

Ionomycin had a significant effect on the frequency of spontaneous SR Ca^{2+} release. This is shown in table 5.10. At higher concentrations of 1.5μM and 150nM, ionomycin abolished spontaneous SR Ca^{2+} release within 4 mins in all cells studied. The action of ionomycin was rapid in these cells, and a significant reduction in frequency was observed 2 mins after the addition of ionomycin before total cessation of spontaneous activity within 4 mins. The effect of 15nM ionomycin, the lowest concentration studied was to abolish spontaneous activity in some cells, and reduce the frequency of spontaneous release in the group. Thus spontaneous activity ceased by 10 mins in 1 of 6 cells, and by 12 mins in 3 of 6 cells. In contrast a significant reduction in frequency was apparent 4 mins after addition of ionomycin, although no effect was apparent after 2 mins. These data are also shown in fig. 5.12.

Ionomycin cont.	Expts	Cells		Control Freq. (Hz)	2 min (%)	4 min (%)	6 min (%)	8 min (%)	10 min (%)	12 min (%)
1.5mM	3	5	mean	0.165	35.2	0				
			SEM	0.019	7.5	0				
			p		<0.0001	<0.0001				
150nM	4	5	mean	0.093	47.5	0				
			SEM	0.023	18.8	0				
			p		<0.05	<0.01				
15nM	3	6	mean	0.092	96.9	70.3	65.4	50.1	36	17.3
			SEM	0.017	5.9	3.5	8.2	4.2	9.1	9.6
			p		>0.05	0.001	0.01	0.002	0.001	<0.05

Table 5.10: Effect of ionomycin on frequency on spontaneous SR Ca^{2+} release. (Frequencies shown normalised to control, p values calculated from paired t tests on absolute frequencies compared to control).

Ionomycin thus had a concentration dependent effect on the frequency of spontaneous SR Ca^{2+} release. At high concentrations the effect was rapid and abolished activity within 4mins. At lower concentration, the effect was also rapid: there was a significant reduction in frequency of spontaneous release within 2 mins compared to control. The magnitude of this effect increased over the course of 10 mins.

(ii) Effect on amplitude of spontaneous release.

Ionomycin at concentrations of 1.5μM and 150nM abolished spontaneous SR Ca^{2+} release within 4 mins in all cells, and so no steady state effect on amplitude of spontaneous release could be assessed. At 15nM ionomycin spontaneous activity was abolished in only 1 cell of 6 from 3 hearts, but in the other cells there was no change in amplitude within 10 mins. This is shown in table 5.11. In summary, there did not seem to be any steady state reduction in amplitude of spontaneous SR Ca^{2+} release; spontaneous activity was either abolished or continued at the same amplitude despite a reduction in frequency of release.

Ionomycin conc	Expts	Cells		Control amp. (nM)	2 min (%)	4 min (%)	6 min (%)	8 min (%)	10 min (%)
1.5 mM	3	5	mean	38.6	93.8	0	0	0	0
			SEM	3.5	2.8				
			p		<0.05				
150 nM	4	5	mean	30.8	93.9	0	0	0	0
			SEM	6.5	5.1				
			p		>0.05				
15 nM	3	6	mean	35	101.8	100	99.7	99.2	99.9
			SEM	3	3.5	5	3.4	5.2	4.2
			p		>0.05	>0.05	>0.05	>0.05	>0.05

Table 5.11 Effect of 15 nM - 1.5 μM ionomycin on the amplitude of spontaneous SR Ca^{2+} release over a 10min timecourse.

5.4 Discussion.

The primary purpose of the studies described in this chapter was to attempt to model the changes in SR function seen in heart failure by pharmacological manipulation of SR function. Further information on normal SR function may also be gained from this type of study, however, as well as insight into the action of the four compounds under study.

5.4.1 Modelling heart failure.

The characteristics of spontaneous SR Ca^{2+} release in permeabilised myocytes from failing and normal hearts were described in chapter 3. Essentially, both the frequency and amplitude of spontaneous SR Ca^{2+} release were reduced in cells from failing hearts. The direction of the effect of the four compounds studied on amplitude and frequency of spontaneous SR Ca^{2+} release is compared to the changes seen in heart failure in table 5.12. In terms of direction of change thapsigargin was the only

compound to model the decrease in both amplitude and frequency seen in heart failure.

	Mechanism of action:	Effect on Amplitude	Effect on Frequency
Heart Failure	?	⇓	⇓
Tetracaine	-ve RyR	⇑	⇓
Caffeine	+ve RyR	⇓ ⇒	⇒
Thapsigargin	-ve SERCA	⇓	⇓
Ionomycin	+ve leak	⇒	⇓

Table 5.12 Comparison of the major effects of tetracaine, caffeine, thapsigargin and ionomycin on the amplitude and frequency of spontaneous SR Ca^{2+} release, in relation to the changes in these parameters in heart failure. (RyR: ryanodine receptor, SERCA: SR Ca^{2+} ATPase).

A bipolar plot of the percentage change in amplitude and frequency for heart failure (compared to sham) and the four compounds is shown in figure 5.13. In heart failure cells there was a reduction in frequency of spontaneous SR Ca^{2+} release of 44.6 ± 16.1% for Ca^{2+} solution 2 (see section 3.2.4 for details) and 14.9 ± 14.0% for Ca^{2+} solution 3, giving a mean reduction in frequency of 29.8 ± 15%. There was a reduction in amplitude of 45.3 ± 6.3% and 33.7 ± 5.6% for the same solutions giving a mean reduction of 39.4 ± 5.9%. These figures can be compared with thapsigargin, the only compound to significantly reduce both amplitude and frequency of

spontaneous release. Direct comparison is difficult given the range of effects of thapsigargin at different doses and the fact that the magnitude of effect of thapsigargin increases with time of exposure, but if a moderate dose of thapsigargin is chosen and a median time point is examined (i.e. 30 μM at 12 mins) the reduction in frequency of $57.9 \pm 8.1\%$ and amplitude of $31.5 \pm 6.0\%$ approximates to the changes seen in heart failure, although there may be a slightly greater effect on amplitude compared to frequency in heart failure, with the reverse seen with thapsigargin. As can be seen from fig 5.13, the changes induced with the other compounds modelled the changes in heart failure poorly.

Close modelling of the changes in SR function seen in heart failure by thapsigargin supports the hypothesis that abnormal Ca^{2+} handling in heart failure results from a reduction in the activity of SERCA 2A. However possible explanations as to why thapsigargin did not produce the exact changes seen in heart failure need to be considered. There are three possible explanations for this:

(i) SR dysfunction in heart failure results from multiple alterations in the function of SR components.

(ii) The extent of changes in SR function modelled by thapsigargin was within the bounds of experimental error in comparison to the changes in heart failure.

(iii) Mechanisms other than those considered above result in SR dysfunction in heart failure, and the close modelling of these changes by thapsigargin is coincidental.

These explanations are not mutually exclusive.

Perhaps the weakness of this pharmacological approach to studying SR dysfunction is that it is assumed that changes in SR function result from a single mechanism. In fact, given the cyclical nature of the processes of SR Ca^{2+} uptake and release, it would be surprising if one component of SR function could be altered without a concomitant change in other processes. It is possible from the data above to postulate the effects of multiple pharmacological manipulations. However the necessary effect to add to that of thapsigargin would be one that reduced amplitude of spontaneous release without altering frequency. In this way a smaller thapsigargin effect with this postulated second effect would more accurately model heart failure. The difficulty of this is that no other compound had this effect, and so with the current data, the effect of thapsigargin could not be better matched to heart failure. Since other possible modulations of SR function are possible and have not been tested, however, this approach remains possible.

The possibility remains that these experiments were not accurate enough to reliably distinguish the effects of heart failure and thapsigargin. As has been discussed, the actions of thapsigargin at different concentrations and time points were measured, and changes in the effect of thapsigargin were seen, making comparisons difficult. However, any changes in frequency and amplitude of spontaneous Ca^{2+} release induced by thapsigargin tended to vary in a parallel manner, and no concentration or time point chosen would have given relatively different magnitudes of effect on amplitude compared to frequency. This contrasts, however, with the findings in heart failure cells, described in chapter 3. The amplitude of Ca^{2+} release in heart failure cells was reduced to a similar extent compared to sham cells at all cytosolic Ca^{2+} concentrations, there was a non-linear relationship between cytosolic

Ca^{2+} concentration and the difference between frequency of Ca^{2+} release in heart failure and sham cells.

The possibility that there are other theoretical modulations of SR function that have not been considered here can also not be discounted, but this is a hypothesis that can never be tested by this type of experiment. The corollary of this argument is that for the a reduction in the activity of SERCA 2A not to be the underlying cause of SR dysfunction in heart failure, it is necessary to invoke hypothetical changes in SR function which are not based upon currently understood physiology.

5.4.2 Insights into SR function.

The simplest paradigm of spontaneous SR Ca^{2+} release is a system where Ca^{2+} is taken up into the SR by SERCA 2A until SR Ca^{2+} overload occurs and spontaneous release causes emptying of stored Ca^{2+}. The effects of ionomycin, tetracaine and thapsigargin, as well as the observed changes in heart failure confirm that this is an oversimplification of the system.

The theoretical effect of reducing the activity of SERCA 2A, and hence the rate of SR Ca^{2+} accumulation would be to reduce the frequency of spontaneous SR Ca^{2+} release, since the length of time taken for the intraluminal Ca^{2+} concentration to reach threshold for spontaneous release would be longer. In this simple system there is no mechanism for the alteration in amplitude of Ca^{2+} release that is observed both in heart failure and with thapsigargin. Here there is a concomitant decrease in both frequency and amplitude of Ca^{2+} release. In control cells the reverse of this occurs; as cytosolic Ca^{2+} concentration is raised both amplitude and frequency increase. The unifying feature in these circumstances is that the amplitude of Ca^{2+} release seems to depend upon the rate of SR Ca^{2+} accumulation.

The effect of ionomycin complicates the system further. By increasing the SR Ca^{2+} leak, ionomycin should have the same theoretical effect as thapsigargin, in that the rate of SR Ca^{2+} accumulation will be reduced, although the mechanism of this reduction is different. In the presence of ionomycin, however, there is a reduction in frequency of SR Ca^{2+} release as expected, but amplitude is unaffected. This suggests that modulation of the amplitude of spontaneous Ca^{2+} release is not absolutely dependent of the rate of SR Ca^{2+} accumulation. An attractive hypothesis is that the modulation of amplitude of Ca^{2+} release depends not on the simple rate of SR Ca^{2+} accumulation, but on SERCA 2A activity. This suggests a link between SR Ca^{2+} uptake and release.

The effect of tetracaine is also interesting. Tetracaine acts to inhibit SR Ca^{2+} release at the ryanodine receptor. As discussed earlier, possible mechanisms of action of tetracaine include reducing single channel conductance of the ryanodine receptor, reducing the open probability of the receptor, decreasing the sensitivity of the receptor to cytosolic Ca^{2+} or reducing the sensitivity of the ryanodine receptor to lumenal Ca^{2+}. In this experimental system, tetracaine reduced the frequency of spontaneous Ca^{2+} release, but increased the amplitude. If it is assumed that tetracaine has no action on SERCA 2A, then a reduction in frequency of spontaneous SR Ca^{2+} release means that lumenal Ca^{2+} concentration is higher in the presence of tetracaine prior to release, and this increased lumenal Ca^{2+} overcomes the action of tetracaine. When SR Ca^{2+} release occurs the amplitude of this release reflects the increased SR load. This suggests that tetracaine is not acting to reduce single channel conductance, since the effect is on the likelihood of release rather than the degree of release.

A possible paradigm of spontaneous SR Ca^{2+} release is as follows. In the presence of high cytosolic Ca^{2+} concentration in unstimulated cells, lumenal Ca^{2+} concentration rises due to SERCA 2A mediated SR Ca^{2+} uptake. At low lumenal Ca^{2+} concentration the open probability of the ryanodine receptor is low, and therefore unitary Ca^{2+} release episodes are infrequent. These infrequent unitary releases of Ca^{2+} are insufficient to trigger Ca^{2+}-induced Ca^{2+} release. As lumenal Ca^{2+} concentration rises the open probability of the ryanodine receptor increases. Greater frequency of unitary Ca^{2+} release occurs, and there is a greater likelihood that sufficient unitary release events occur to trigger Ca^{2+}-induced Ca^{2+} release. In this paradigm tetracaine could act in any of the three ways not yet discounted. Tetracaine could reduce open probability, reduce the sensitivity of open probability to lumenal Ca^{2+} concentration, or reduce the sensitivity of Ca^{2+}-induced Ca^{2+} release. In any of these cases an increase in lumenal Ca^{2+} concentration would overcome the effect of tetracaine and the present data therefore cannot separate these possibilities.

As was discussed previously, the effect of thapsigargin on both frequency and amplitude of SR Ca^{2+} release suggested that there may be a functional link between SR Ca^{2+} uptake and release beyond that mediated by lumenal Ca^{2+} concentration. In the experiments with tetracaine, while there is no evidence that inhibition of Ca^{2+} release at the ryanodine receptor has an inhibitory effect on SR Ca^{2-} uptake, such an effect cannot be excluded. In uptake experiments the effect of inhibiting Ca^{2+} release with tetracaine was to increase the rate of net SR Ca^{2+} accumulation. This does not, however give any direct information on possible changes in SERCA 2A activity brought about by inhibiting Ca^{2+} release. The fact that tetracaine caused an additional

increase in the rate of SR Ca^{2+} accumulation independently of the action of ruthenium red may suggest that tetracaine has a direct stimulatory action on SERCA 2A.

5.5 Summary.

In summary, inhibition of SERCA 2A mediated SR Ca^{2+} uptake most closely mimicked the changes on amplitude and frequency of spontaneous SR Ca^{2+} release seen in heart failure. This suggests that a reduction in the activity of SERCA 2A underlies abnormal SR function in heart failure, rather than changes in Ca^{2+} release characteristics of the ryanodine receptor.

Differences in the actions of thapsigargin and ionomycin suggest that there are processes in the SR which regulate Ca^{2+} release independently of lumenal Ca^{2+} concentration. These regulatory processes may link the activity of SERCA 2A to the Ca^{2+} release characteristics of the ryanodine receptor. Whether there is a bi-directional effect can neither be confirmed or excluded. Tetracaine may have a direct action on SERCA 2A.

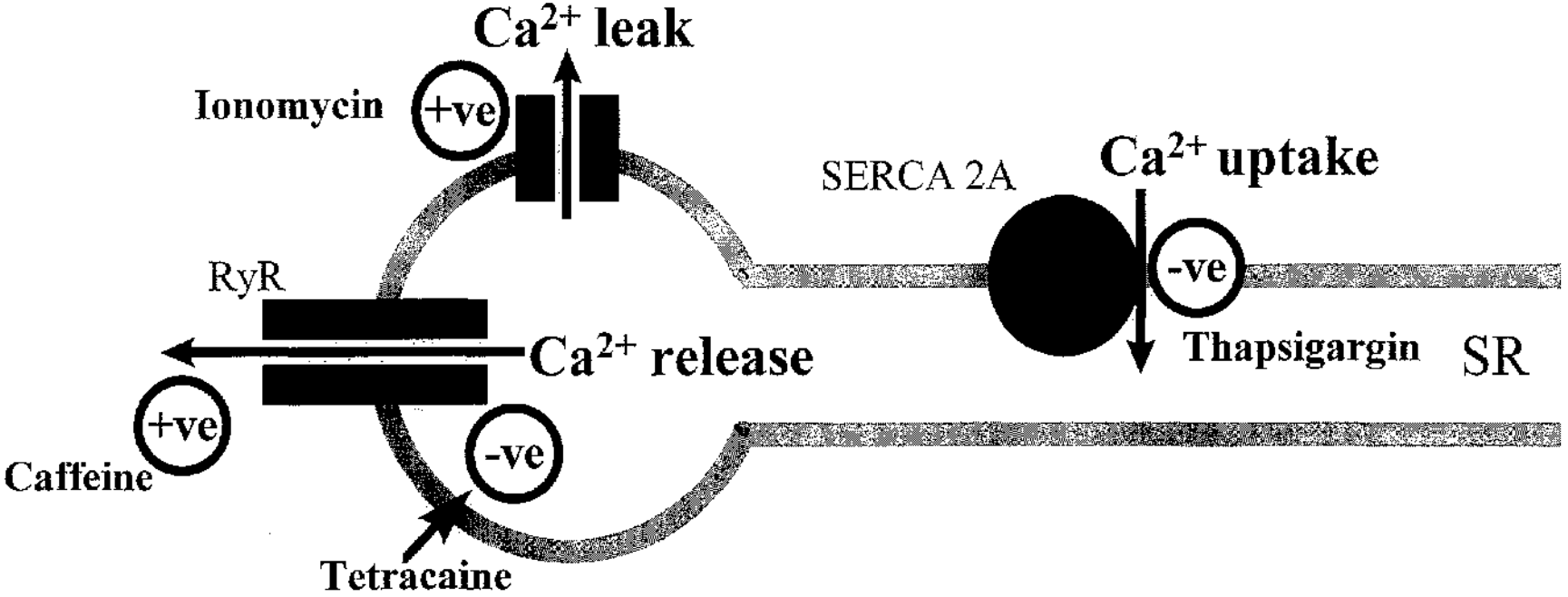

Fig. 5.1: Sites of pharmacological manipulation of SR Ca^{2+} control.
(RyR - ryanodine receptor)

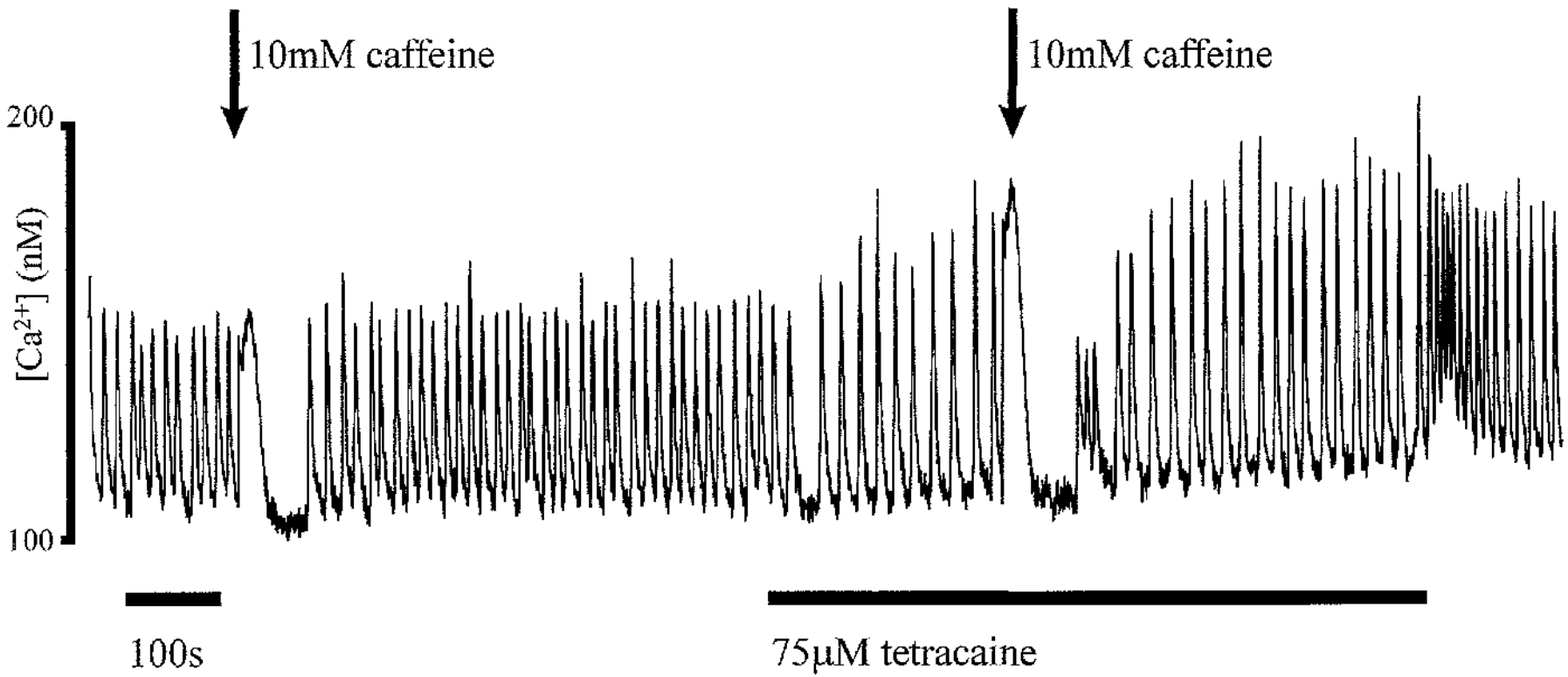

Fig. 5.2: Effect of 75μM tetracaine on spontaneous SR Ca^{2+} release and SR load in a permeabilised single myocyte. Arrows show time of rapid injection of caffeine to assay SR Ca^{2+} content.

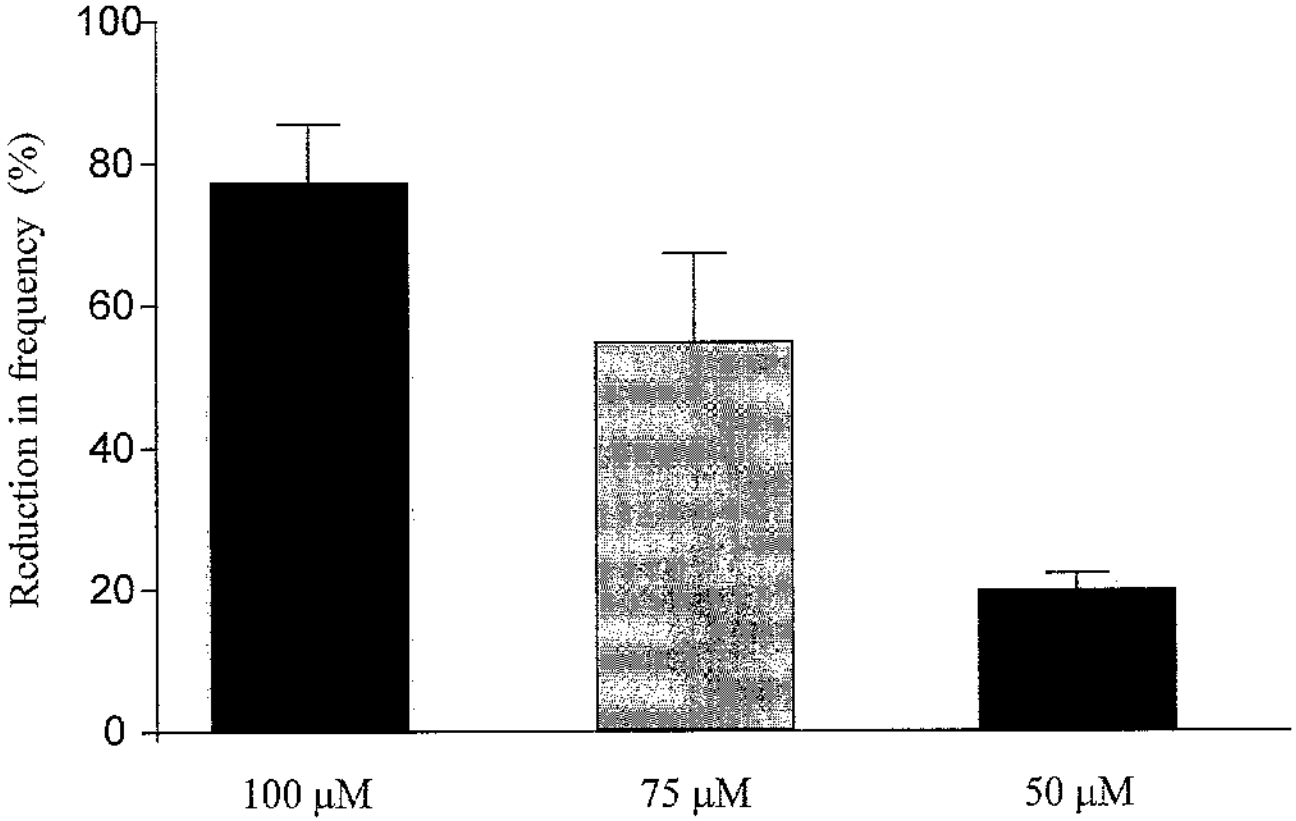

Fig. 5.3: Dose dependence of the effect of tetracaine on the reduction in frequency of spontaneous SR Ca^{2+} release.

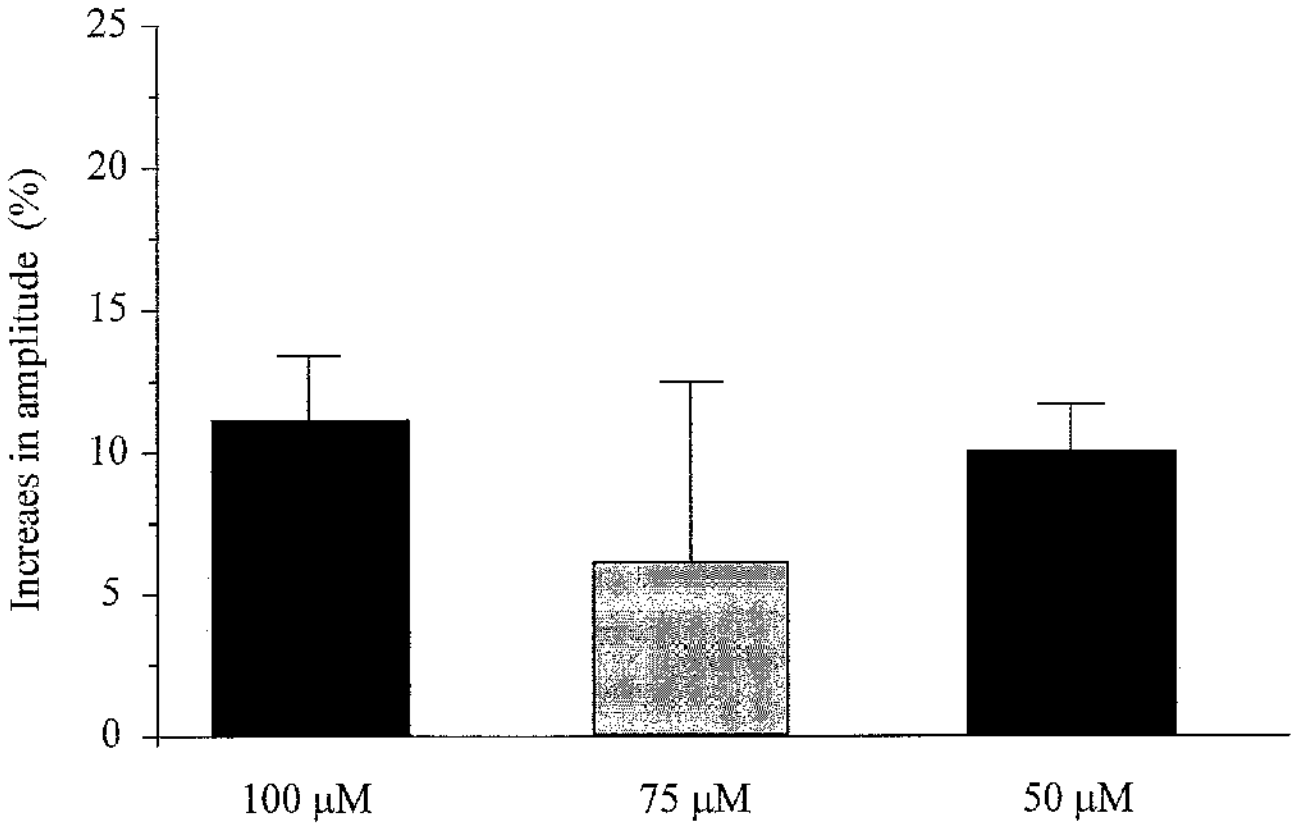

Fig. 5.4: Lack of dose dependence of the effect of tetracaine on the increase in amplitude of spontaneous SR Ca^{2+} release.

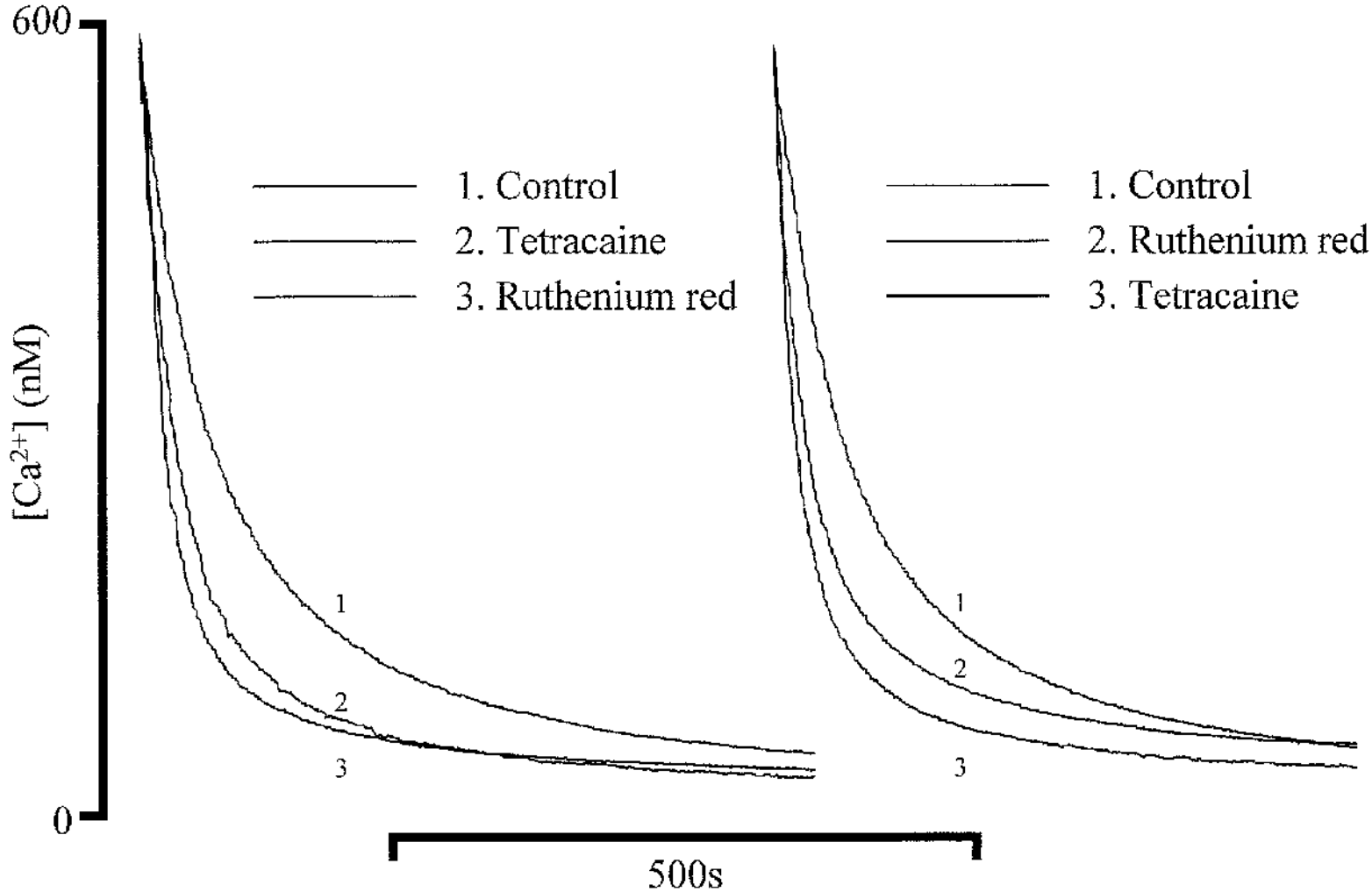

Fig. 5.5: Independent effects of tetracaine and ruthenium red on Ca^{2+} uptake in oxalate loaded permeabilised myocytes.

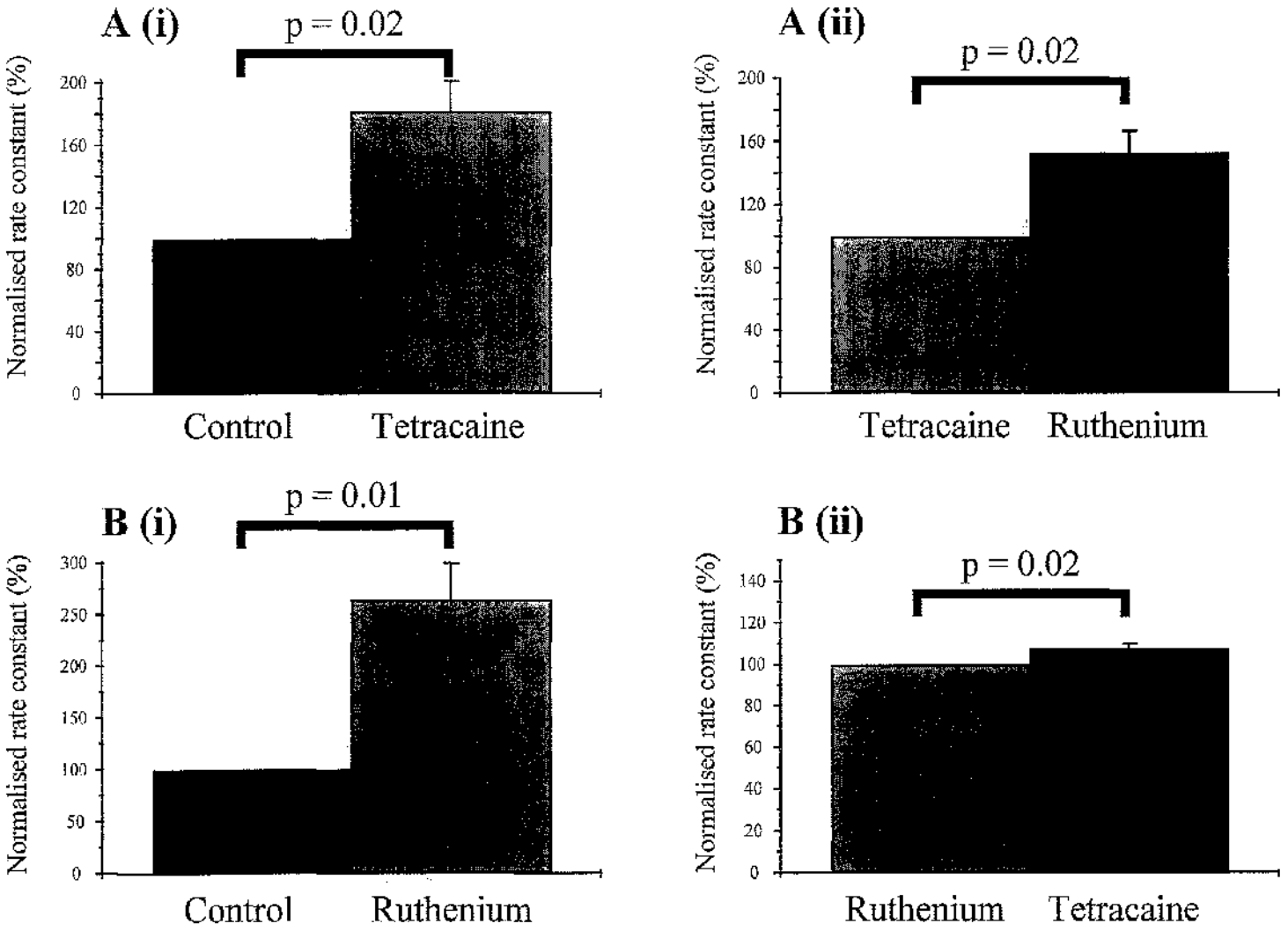

Fig. 5.6: Mean effects of tetracaine and ruthenium red on Ca^{2+} uptake in oxalate loaded permeabilised myocytes. (see text for details).

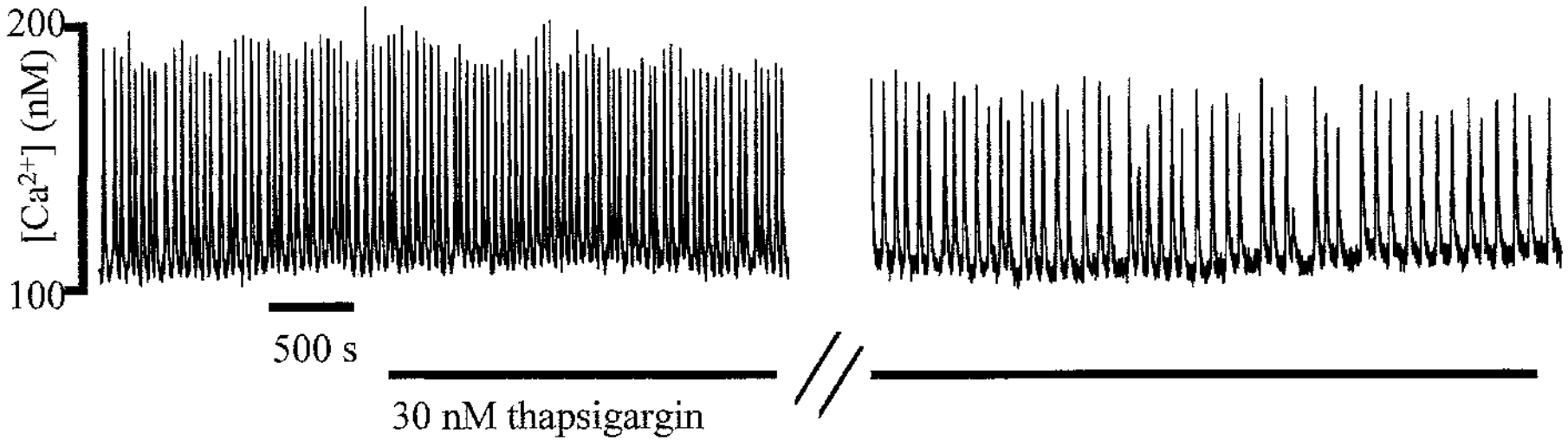

Fig. 5.7: Effect of 30nM thapsigargin on frequency and amplitude of spontaneous SR Ca^{2+} release in a permeabilised single myocyte.

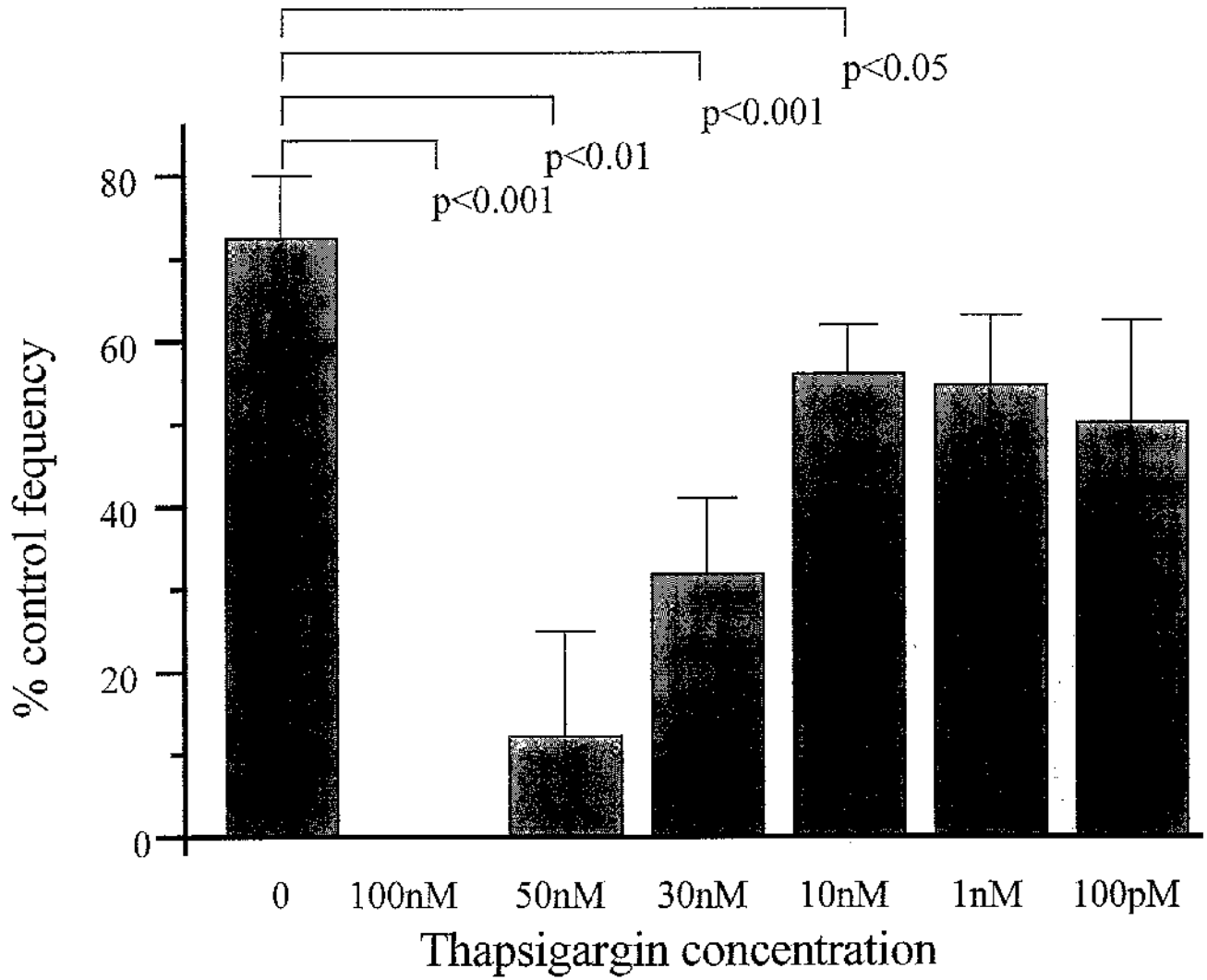

Fig. 5.8: Effect of thapsigargin on frequency of spontaneous SR Ca^{2+} release at 15 mins.
(See text for details of statistical analysis)

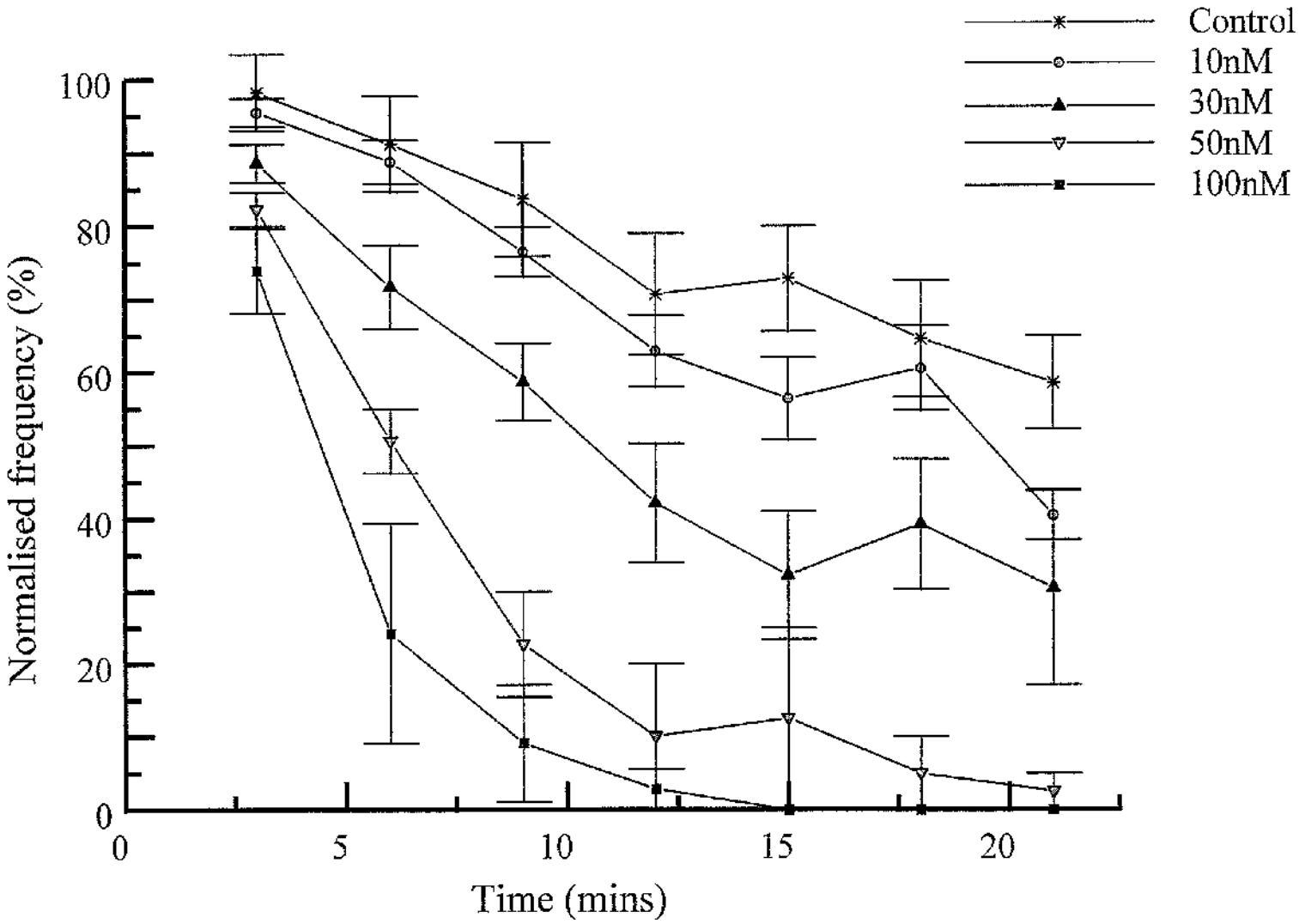

Fig. 5.9: Effect of thapsigargin on frequency of spontaneous SR Ca^{2+} release over time.

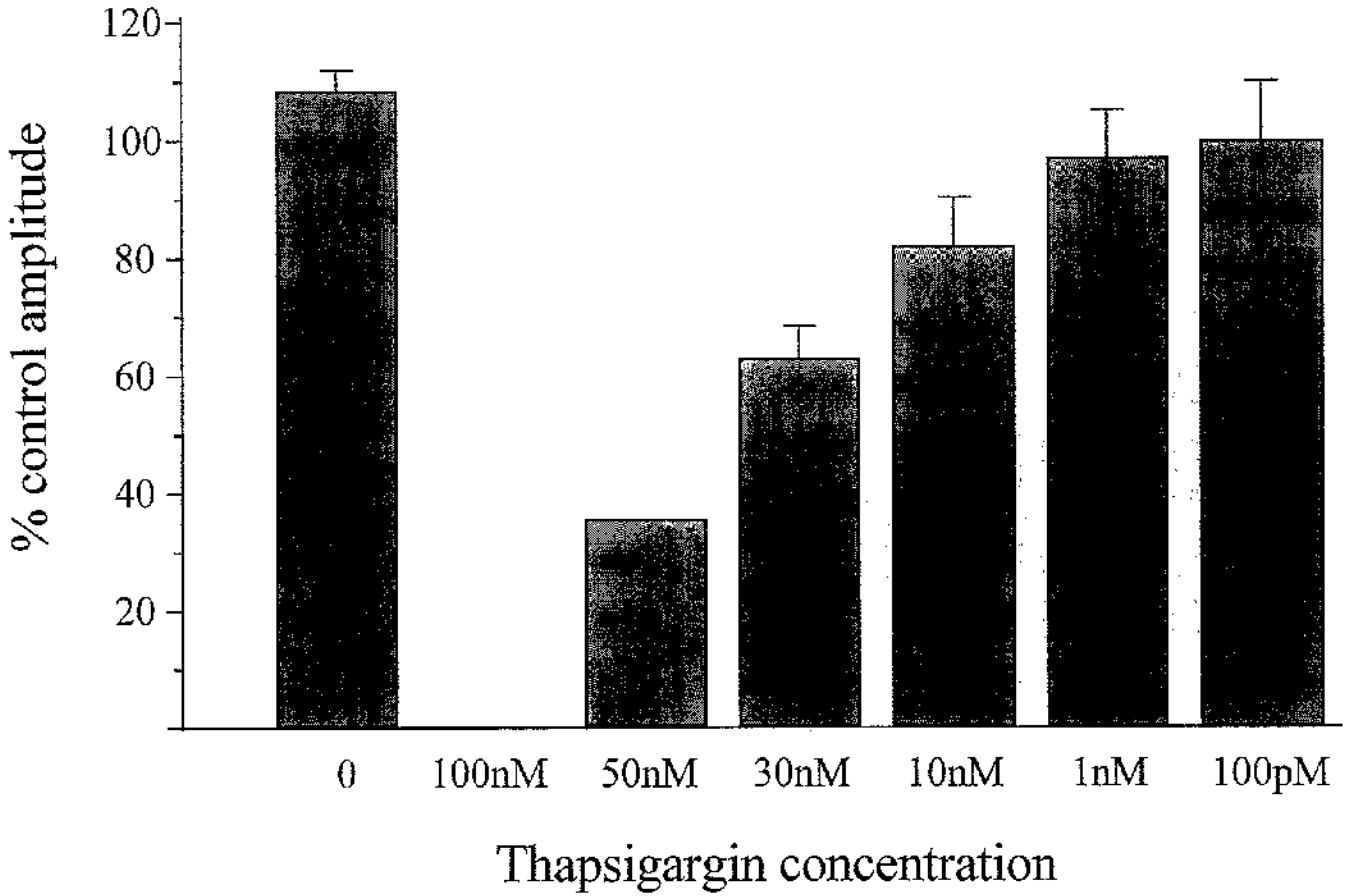

Fig. 5.10: Effect of thapsigargin on amplitude of spontaneous SR Ca^{2+} release at 15 mins. (Statistical analysis is described in the text).

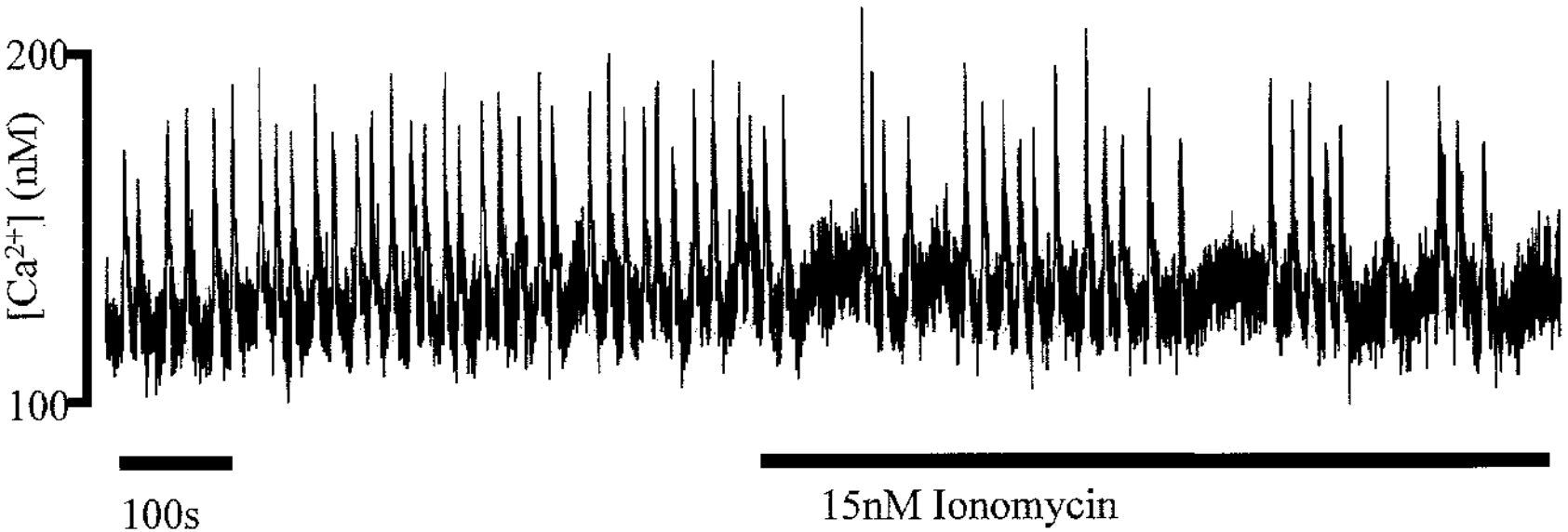

Fig. 5.11: Effect of 15nM ionomycin on frequency and amplitude of spontaneous SR Ca^{2+} release in a permeabilised single myocyte.

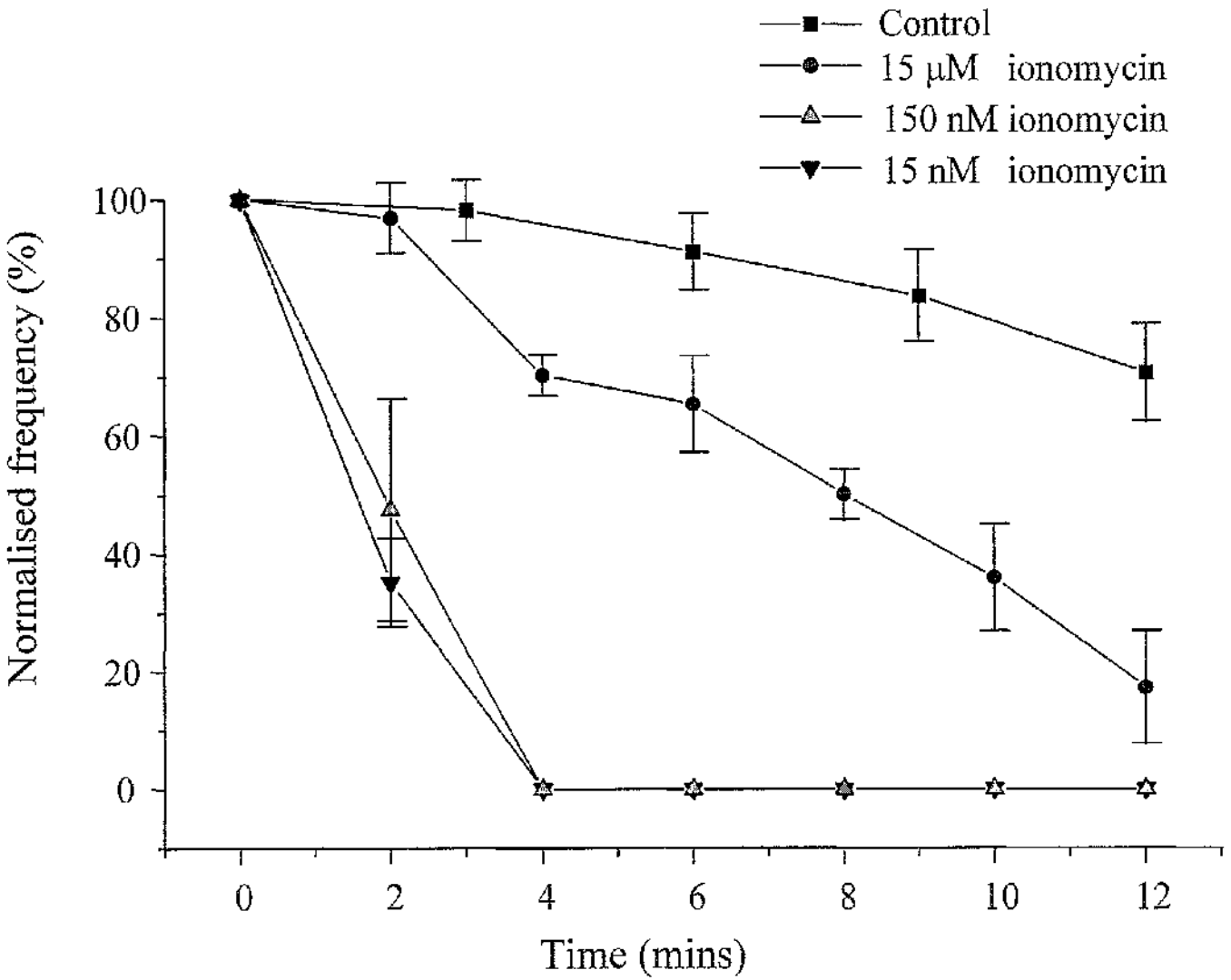

Fig. 5.12: Effect of 15μM - 15nM ionomycin on frequency of spontaneous SR Ca^{2+} release.

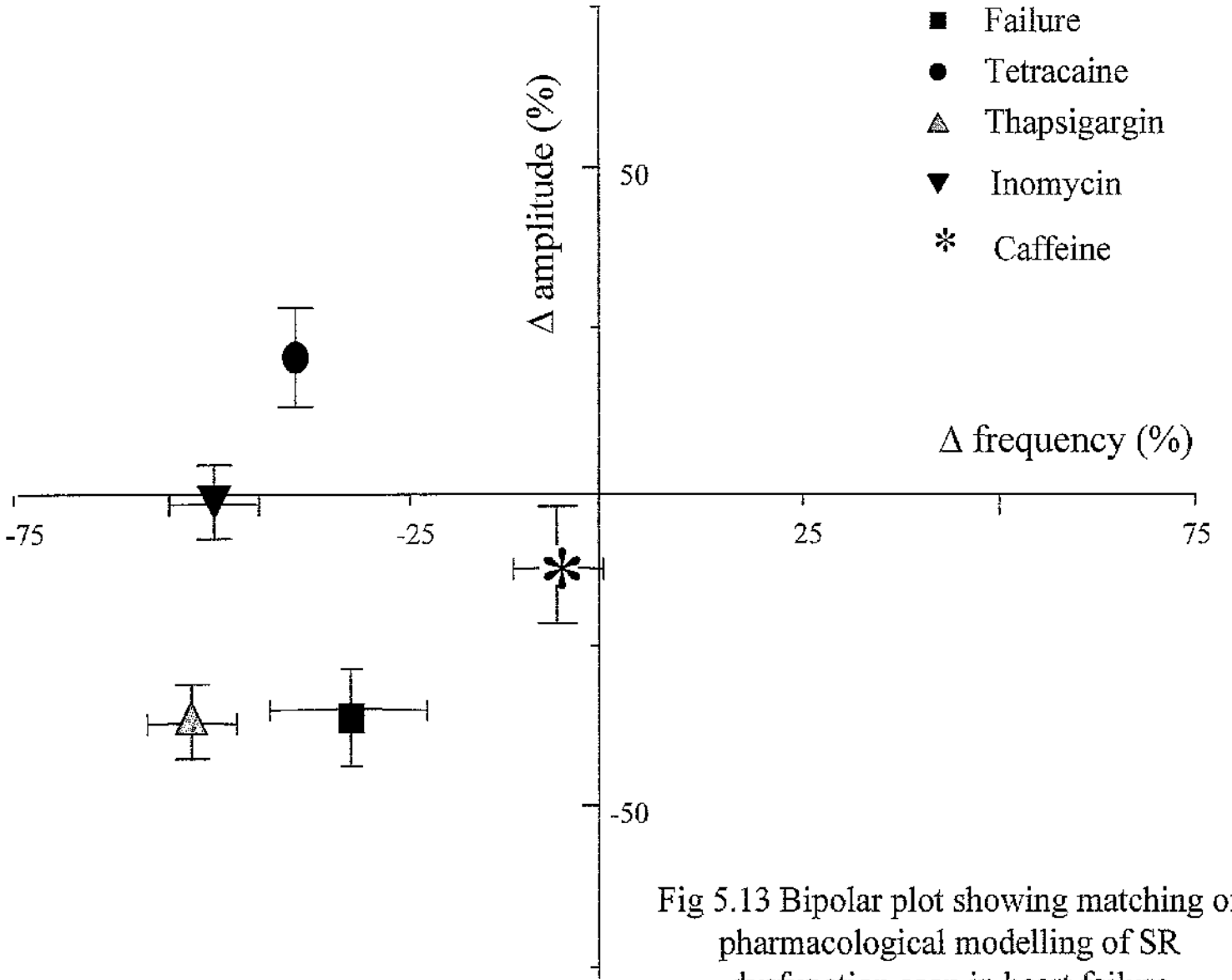

Fig 5.13 Bipolar plot showing matching of pharmacological modelling of SR dysfunction seen in heart failure.

Chapter 6: Studies in human myocytes.

6.1 Background.

The use of animal models of human disease is a powerful tool in the investigation of the pathophysiological processes of disease. Animal models have several clear advantages for scientists, including tissue availability, the absence of co-morbidity or disease modifying treatment, and the ability to produce a relatively homogenous disease population. In contrast, the study of disease processes in human cardiac tissue is made difficult by the frequent co-existence of morbid conditions such as hypertension, diabetes, myocardial ischaemia, pulmonary disease and many others. The untreated patient population is also virtually non-existent, and common cardiological treatments may all have significant effects on the disease process. Thus few patients with significant heart failure will not be treated with combinations of β-blockers, ACE inhibitors, diuretics, Ca^{2+} antagonists and cardiac glycosides. Given the multifactorial aetiology of heart failure, and the pathophysiological interplay between the β--adrenergic system, the renin-angiotensin system, cellular remodelling and E-C coupling, it is clear that pharmacological treatment might confound pathological changes in cellular function.

In addition to these problems, the investigation of human heart failure is further hampered by the difficulty in obtaining human tissue. Since functional study is not possible on post-mortem cardiac specimens, tissue can only be obtained from living donors under special conditions of cardiac surgery or transplantation. Clearly only small tissue samples can be removed from hearts other than at transplantation.

Despite these difficulties, if there is to be any therapeutic gain from this type of research, the study of human tissue is necessary. This research must delineate any

species differences, and establish whether the presence of co-morbidity and medical therapies significantly alter the pathophysiological process. The study of human tissue in heart failure research is therefore an important challenge.

6.1.1 Difficulties in obtaining human myocardial tissue.

Most previous studies have obtained myocardial tissue from explanted hearts taken during cardiac transplantation. In most cases, the pathology in these hearts comprises ischaemic and idiopathic dilated cardiomyopathy, with rarer cases of valvular heart disease and viral myocarditis. Explanted hearts have provided tissue for preparations including isolated myocytes(Harding *et al.* 1992b), trabeculae(Denvir *et al.* 1995), papillary muscles(Schwinger *et al.* 1992) and ventricular muscle strips(Hasenfuss *et al.* 1994). For many of these studies the only control tissue available has come from occasional donor hearts which could not be implanted for technical reasons. In addition to the rarity of this control tissue, donor hearts have frequently been exposed to high doses of inotropic drugs during the period prior to harvesting, which may have significant effects on myocardial physiology. A further problem with the use of explanted hearts is that all experimental tissue comes from patients with end stage heart failure. There is therefore no opportunity to study the pathophysiological process early in the disease process, where therapeutic interventions may be more successful, and in all cases, patients will be on multiple medical therapies.

These problems have led investigators to develop methods for obtaining myocardial tissue from patients undergoing routine cardiac surgery, in most cases coronary artery bypass grafting or valvular surgery. This does not allow tissue to be taken from non-diseased hearts, but provides tissue from patients with normal

ventricular function, and varying degrees of left ventricular dysfunction. This allows the study of heart failure in the early stages of the disease process and during its progression to terminal stages, as well as providing reasonable control tissue. It is not possible to obtain papillary muscle or trabeculae preparations from routine cardiac surgery, but methods for dissociating single myocytes from small atrial and ventricular biopsies have been developed(Peeters *et al.* 1995), and small epicardial ventricular muscle strips can be dissected safely(Hasenfuss *et al.* 1990). Left ventricular volume reduction surgery (the Batista procedure(Batista *et al.* 1996)) provides larger amounts of myocardial tissue for either dissociation of myocytes or dissection of trabeculae, although this procedure is only undertaken in patients with severe heart failure, and myocardium removed is often scarred.

6.1.2 Conflicts between animal and human studies.

In general, studies of force development and shortening in human myocytes and multicellular preparations are in keeping with animal studies. There is a negative force-frequency relationship in papillary muscle preparations (Schwinger *et al.* 1992; Schwinger *et al.* 1993), and this correlates with the degree of left ventricular dysfunction (Schmidt *et al.* 1994). Differences in contractile force are difficult to detect at basal stimulation rates, but are more apparent at higher frequencies and during inotropic stimulation. Similarly, single cell studies have shown a clear blunting in the positive force frequency relationship in cells from failing hearts with significant increases in time to peak contraction, and time to 50% and 90% relaxation (Davies *et al.* 1995), although changes in cell contraction during basal stimulation were difficult to detect (Harding *et al.* 1991).

Changes in the Ca^{2+} transient in human heart failure are also broadly in keeping with animal studies. Gwathmey noted that Ca^{2+} transients in human trabeculae were prolonged (Gwathmey *et al.* 1987), although peak systolic Ca^{2+} levels were unchanged. In general, most studies in human myocytes and trabeculae (Beuckelmann *et al.* 1995; Beuckelmann *et al.* 1992; Pieske *et al.* 1995) have shown a reduction in peak Ca^{2+} availability. While there is general agreement from animal studies that the Ca^{2+} transient is prolonged, the effect of left ventricular dysfunction on systolic Ca^{2+} release is less clear. Studies in a canine pacing-induced induced model (Perreault *et al.* 1992), and a hypertensive rat model (Zhang *et al.* 1995) found no reduction in systolic Ca^{2+} release, whereas peak Ca^{2+} has been found to be reduced in cardiomyopathic hamsters (Kruger *et al.* 1994), post-infarct rats (Capasso *et al.* 1993; Zhang *et al.* 1995). This suggests that alterations in systolic Ca^{2+} handling may depend more on the pathological insult than the particular species, which may have implications for human study, given the broad range of aetiologies of heart failure in some series.

There is greater conflict in human and animal studies when sarcolemmal Ca^{2+} currents have been measured. Several studies have found reduced density of the L-type Ca^{2+} current in animal models of cardiac hypertrophy and failure (Ming *et al.* 1994; Ouadid *et al.* 1995; De Brito Santos *et al.* 1995). In contrast, no reductions in Ca^{2+} current density have been found in myocytes isolated from failing human hearts (Mewes & Ravens, 1994; Beuckelmann *et al.* 1992). There seems to be no consensus on changes in the activity of the Na^{+}/Ca^{2+} exchanger in either animal or human studies.

Changes in expression of SERCA 2A in heart failure are also controversial. Although mRNA for SERCA 2A has been found to be reduced in human heart failure, protein levels have been found to be normal by some groups (eg (Movsesian *et al.* 1994; Schwinger *et al.* 1995)), and reduced in others (eg (Meyer *et al.* 1995; Hasenfuss *et al.* 1994)). In contrast, the majority of investigators have found reduced protein expression of SERCA 2A in several animal models including the rabbit coronary artery ligation preparation(eg (Currie & Smith, 1999; Stein *et al.* 1996; Matsui *et al.* 1995; Kiss *et al.* 1995)).

In summary, it appears that evidence for the pathophysiological mechanisms of altered Ca^{2+} handling in human heart failure is less convincing than in animal models. However, clear evidence in human heart failure might best be described as lacking, rather than being in contradiction to animal studies. This may reflect the inherent difficulties in doing human research, or the possibility of some heterogeneity of mechanism depending upon the aetiology of heart failure, and it would be premature to conclude that there are major differences in the cellular pathophysiology of heart failure in humans and animal models.

6.2 Methods.

The purpose of these studies was to investigate methods for the dissociation of viable, single myocytes, and to assess the feasibility of applying the techniques for measuring Ca^{2+} transients in intact and permeabilised myocytes described in earlier chapters.

6.2.1 Access to myocardial tissue.

Four possible sources of myocardial tissue were identified:

(i) Atrial myocardium taken during the placement of atrial cannulae at the time of cardiopulmonary bypass.

(ii) Epicardial ventricular biopsies during cardiac surgery.

(iii) Ventricular myocardial tissue removed during ventricular volume reduction surgery.

(iv) Ventricular myocardial tissue taken from explanted hearts.

Three cardiac surgeons agreed to collaborate and supply myocardial tissue for these studies. These were:

(i) Mr SK Naik (Glasgow Royal Infirmary).

(ii) Mr GA Berg (Western Infirmary Glasgow).

(iii) Mr K McArthur (Western Infirmary Glasgow).

Relatively large samples of atrial tissue were freely available from all procedures involving cardiopulmonary bypass. The technique of cardiopulmonary bypass involves placing two intravascular cannulae, one in the right atrial appendage and one in the ascending aorta. This forms an extracorporeal bypass circuit where venous blood is removed from the right atrium, oxygenated at a controlled temperature and pumped back into the ascending aorta. The bypass pump therefore performs the work necessary to maintain the circulation with oxygenated blood during cardioplegia and cardiac surgery. The placement of the atrial cannulae opens the atrium and produces an flap of atrial tissue, samples of which can be safely removed. This tissue may be redundant following bypass and can be considered a surgical by product. For this reason patients were not consented for removal of atrial myocardium.

Atrial tissue was used in early experiments to explore methods for dissociation of myocytes, since relatively large samples were available. Since virtually all previous investigation of the pathophysiology of heart failure has studied ventricular myocardium, and changes in the function of atrial myocytes are not well characterised, atrial myocytes were considered unsuitable for detailed experiments.

Most studies were carried out on epicardial biopsies taken from patients undergoing coronary artery bypass grafting. This procedure is undertaken on most days in both local hospitals used to provide tissue, and samples were therefore available as required. Since the removal of a ventricular biopsy was an additional procedure to routine cardiac surgery, all patients who donated samples were approached and gave written, informed consent. The patient information / consent form was designed in conjunction with the medical ethics committees at both hospitals. Two biopsy techniques were evaluated. The first technique used a "trucut" needle. This needle removed a <1mm diameter core of myocardium from the free wall of the left ventricle. The length of the core was up to 1cm depending upon the operator, thus the epicardial and mid-myocardial layers of the ventricle were sampled. The defect did not require suturing and no complications ensued. The second technique involved dissection of the pericardial fat from the free wall of the left ventricle and the removal of a small, superficial chunk of epicardial myocardium. The maximum dimensions of the sample were approximately $3mm^3$. The defect did not require to be sutured, and again no complications ensued.

More rarely, tissue was available from ventricular volume reduction surgery, the Batista procedure (Batista *et al.* 1996). This procedure was carried by Mr Naik on patients with severe heart failure and dilated left ventricles. The basis of this

procedure is that as the removal of a portion of the left ventricular wall causes a reduction in left ventricular dimensions. Applying the law of Laplace, this reduces the developed tension necessary for ventricular contraction and thus reduces myocardial work and energy consumption. The majority of the patients undergoing this type of surgery had ischaemic heart disease, with previous myocardial infarction. Although large sections of the left ventricle were removed during operation, the tissue removed tended to contain large quantities of scar tissue, making myocyte dissociation more difficult. Since the myocardium was removed as part of the operation, patient consent was not sought for the use of part of the excised myocardium.

Finally, tissue was available from explanted hearts as part of the Scottish Cardiac Transplantation program. Approximately 50 transplants per year are carried out at Glasgow Royal Infirmary, and myocardium was removed by dissection as soon as practicable following explantation. Consent was not sought from patients for the removal of this tissue.

6.2.2 Transport of myocardial tissue.

To maximise cardioprotection, epicardial biopsies were always taken following cardioplegic arrest of the heart. This ensured that all myocytes were quiescent, and energy stores were preserved as much as possible. Samples from patients undergoing volume reduction surgery were also removed after cardioplegia. Following removal, myocardial samples were immediately stored in oxygenated medium at 0^0 Celsius and transported to the laboratory as soon as possible. The transport medium composition was (in mM): 120 NaCl, 5.4 KCl, 0.5 $MgSO_4$, 5.0 sodium pyruvate, 20 glucose, 20 taurine, 30 2,3-butanedione monoxime (BDM), 10 N-2-

hydroxyethylpiperazine- N'-2-ethanesulphonic acid (HEPES) and 6 nitrilotriacetic acid (NTA) with pH adjusted to 7.0 with sodium hydroxide. NTA acted as a Ca^{2+} buffer, maintaining Ca^{2+} concentration at sub-micromolar levels. BDM acts on the myofilaments to inhibit Ca^{2+}-activated force production (Steele & Smith, 1993), and thus acts as a cardioprotective agent. Myocardial samples reached the laboratory in 10-30 minutes depending upon which hospital surgery took place in.

6.2.3 Dissociation of myocytes.

Initial attempts to dissociate myocytes followed the methods of Peeters (Peeters *et al.* 1995). Biopsy material was cut into small fragments (~1mm^3) with a scalpel under a 10x dissecting microscope. This procedure was done in the transport medium to prevent the tissue drying out. Peeters had used a mechanical tissue slicer to cut 400μm slices, but epicardial biopsies in these experiments were too small to this to be technically feasible.

Tissue fragments were then transferred to conical flasks containing digestion medium. The composition of the digestion medium was the same HEPES buffered salt solution containing 0.2% bovine serum albumin. NTA was omitted from this solution and Ca^{2+} concentration was raised to 50μM. This solution was maintained at 35-37^0 Celsius.

Myocardial tissue was then digested in 3-4 stages. The first stage used collagenase and protease. The efficacy of two preparations of collagenase were assessed, collagenase type 1a (Sigma pharmaceuticals, St Louis, MO) and collagenase type 1 (Worthington Chemicals). Protease type XIV (Sigma) was used. The concentrations of both protease (0.5-1mg / ml) and collagenase (1-4mg / ml) were varied. Myocardial tissue was gently agitated in this solution by continuous shaking and

periodic tituration. The length of the first digestion was varied from 10-30 mins. No myocytes were successfully dissociated during the first stage of digestion. Tissue fragments were then transferred to a second flask containing identical digestion medium, but with collagenase and hyaluronidase (0.5 mg ml, Sigma). Again, incubation time varied from 10-30 mins. In some experiments, the effect of adding elastase (0.5 mg / ml, type IV, Sigma) was tested. Following this phase of digestion, myocytes had begun to dissociate from tissue fragments. Intact tissue fragments were then gently aspirated from the digestion medium and placed into a flask containing collagenase only, for the third stage of digestion. The supernatant from the second digestion was gently centrifuged at around 100g for 2 mins. All enzyme solution was aspirated, and cells were resuspended in the HEPES buffered salt solution at room temperature. This process was repeated after the third stage of digestion, and in some experiments, an identical fourth digestion took place. Following each of the second, third and fourth digestions, a small sample of the resuspension was examined under a microscope for the presence of rod shaped myocytes. In early experiments, assessment of Ca^{2+} tolerance of myocytes was made by gradually raising Ca^{2+} concentration in the HEPES buffered salt solution, initially to 250μM, and then in stages to 2mM. Unfortunately, most cells were found not to be Ca^{2+} tolerant, and few myocytes remained rod shaped when extracellular Ca^{2+} was raised.

6.2.4 Permeabilised myocyte experiments.

Since most cells were not Ca^{2-} tolerant, analogous experiments to those in intact rabbit myocytes were not possible. It was therefore decided to assess whether human cells could be permeabilised and measurements made of SR load and spontaneous release. Similar protocols were used to those described in chapter 3.

Briefly, myocytes were placed in a perfusion chamber on an inverted Nikon microscope and perfused at 1ml min^{-1} with 0.1R containing 10μM Fura-2. Fluorescence was monitored using a Cairn spinning wheel spectrophotometer. Ca^{2+} concentrations within the perfusate were the same as described in chapter 3, with 0, 1, 2, 3 and 4μl of 10mM $CaCl_2$ added per ml to produce solutions with a range of Ca^{2+} concentrations from approximately 50nM to 250nM. Myocytes were anchored using blunt, sealed microelectrodes, and permeabilised by brief exposure to 90nM β-escin. SR Ca^{2+} release was stimulated by microinjecting 2-3μL of 10mM caffeine close to the cell at low bathing Ca^{2+} concentration (solutions 0, 1 and 2). Spontaneous SR Ca^{2+} release was seen at higher Ca^{2+} concentrations.

6.3 Results.

6.3.1 Dissociation of atrial myocytes.

15 experiments were performed on the dissociation of human atrial myocytes. The mass of atrial tissue removed varied from 80-350μg. Rod shaped myocytes were successfully dissociated from the second experiment onwards. In total dissociation successfully produced myocytes in 80% of experiments. Myocyte numbers varied following dissociation from 4-10 per high-power field.

Atrial myocytes showed reasonable Ca^{2+} tolerance. Ca^{2+} concentration was raised successfully to 2μM. Although numbers of rod shaped myocytes decreased in all experiments when Ca^{2+} concentration was raised, around 50% of myocytes retained their shape and clear cross striations when Ca^{2+} was raised.

6.3.2 Dissociation of ventricular myocytes from myocardial biopsies.

30 experiments were undertaken to dissociate myocytes from epicardial ventricular biopsies. 10 of these were from trucut biopsies, and 20 from epicardial dissection biopsies. Trucut biopsies were of the order of 2mg, dissected biopsies ranged from 1-10mg in size. Biopsies were further dissected into smaller chunks using a dissecting microscope and scalpels. The biopsies were too small to have been mounted for preparation on a tissue slicer. Rod shaped myocytes were successfully dissociated in around 50% of experiments. This figure was similar for dissociations from both trucut and dissected biopsies. Maximum numbers of myocytes dissociated were 3-5 rods per high power field, and mean numbers in successful experiments were 1-3 rods per high power field.

6.3.3 Dissociation of ventricular myocytes from excised myocardium.

3 experiments were undertaken in myocardium taken from ventricular volume reduction surgery. Approximately 50-100g of ventricular myocardium was removed in each procedure. In all three cases, the myocardium removed was extremely fibrotic. Approximately 5g of this tissue was removed for dissociation of cells. Tissue with the lowest apparent scar content was selected. This tissue was then dissected into small chunks. Myocardium from this source would have been suitable for mounting on a tissue slicer if this had been available. Incubation times with digestive enzymes were increased to allow for the high content of fibrous tissue. Small numbers of rod shaped myocytes were dissociated in 2 of three experiments, with fewer than one myocyte per high-power field seen. In these experiments, the

myocytes that were dissociated had a granular appearance. Cells from these dissociations were too few in number to proceed to further study.

6.3.4 Dissociation of ventricular myocytes from explanted hearts.

3 experiments were undertaken in myocardium taken from explanted hearts. In each case, the tissue was from terminally failing hearts, rather than unused donor hearts. Myocardium was taken from both the left and right ventricles. Tissue from the left ventricle tended to consist of more scar and fibrous tissue than the right. Sections of tissue of around 5g were taken. Myocytes were successfully dissociated from 2 of 3 hearts. Maximum numbers of myocytes were 5-10 rods per high power field.

6.3.5 Permeabilisation of ventricular myocytes.

The limiting stage in experiments on permeabilised myocytes was the dissociation process. In addition there was a significant reduction in the numbers of rod shaped myocytes surviving both the dissociation process and storage until permeabilisation experiments could be undertaken. Although the number of experiments on permeabilised myocytes was small, the process of permeabilisation did not cause significant further attrition.

The most successful dissociation experiments were undertaken in myocardium from explanted hearts. Most successful experiments in permeabilised cells where therefore done on cells from this source. Some cells from epicardial biopsies were also successfully permeabilised, but cells from ventricular reduction surgery were of insufficient number and quality for this to be attempted. Atrial cells were also successfully dissociated, but permeabilisation was not attempted in these cells as protocols for permeabilisation had not been developed at this point.

Permeabilised human myocytes behaved qualitatively like permeabilised rabbit myocytes. Cells contracted in response to injection of 10mmol caffeine and Ca^{2+} transients could be recorded simultaneously. Raising cytosolic Ca^{2+} to 150-200nM caused spontaneous oscillatory SR Ca^{2+} release. A typical record from a permeabilised myocyte dissociated from an explanted heart is shown in figure 6.1. Insufficient data were generated to make quantitative comparisons with rabbit myocytes possible.

6.4 Discussion.

These experiments show that investigation of SR function in human myocytes is practicable, though significantly more difficult than work on rabbit myocytes. The chief practical difficulty lies in the dissociation of rod shaped myocytes, rather than in applying methods for permeabilisation and study of SR function developed in rabbit myocytes. Current methods are less successful in producing Ca^{2+} tolerant cells where Ca^{2+} homeostasis can be investigated at the whole cell level. Myocytes are not produced in sufficient numbers to make it feasible to measure oxalate supported SR Ca^{2+} uptake without major adaptation to the protocol used in rabbit myocytes.

There are several possible reasons for the difficulties observed in dissociating human myocytes:

(i) Mass of tissue. The size of ventricular biopsies taken for these experiments is at least an order of magnitude lower than that used by other groups working in this area (Sian Harding, personal communication). The most successful dissociations in these experiments were from explanted hearts where larger amounts of tissue were available. It would be possible to obtain larger biopsies

from operative samples, though this would require further assessment from an ethics and safety perspective.

(ii) Enzymatic tissue access. Rabbit myocytes were dissociated using Langendorff perfusion of the whole heart with proteolytic enzymes. Digestive enzymes were therefore perfused through the capillary network and thus had close access to all myocytes. Digestion of human myocytes was done using the chunk method, and therefore enzymes had to diffuse into the centre of the tissue to access all but the most superficial myocytes. Smaller tissue fragments would alleviate this problem, and thus there would be a clear theoretical advantage to the use of a tissue slicer, if the size of ventricular biopsy allowed this. Tissue slicing would maximise the surface area of tissue available to enzymes and minimise diffusion distances into the centre of tissue. This would allow for the use of shorter incubation times and lower concentrations of enzyme and minimise membrane damage caused by digestive enzymes. The partial success of pre-incubating tissue with enzyme at low temperature (when enzyme will be inactive) suggests that improving tissue access would be beneficial.

(iii) Degree of fibrosis. The fact that dissociation of myocytes from explanted hearts was more successful than from myocardial tissue taken during ventricular volume reduction surgery suggests that the degree of myocardial scarring / fibrosis affects the success of dissociation. This effect can also be seen in the fact that dissociation from explanted hearts was only marginally more successful than dissociation from biopsies, despite the difference in mass of tissue of several orders of magnitude.

This suggests that dissociation would be most successful in non-failing hearts without significant fibrosis, and the mass of tissue required for successful dissociation would be higher in failing hearts.

(iv) Ischaemic time. The mean time delay from taking the biopsy to the beginning of the dissociation process was in the order of 30mins. This compares with a delay of probably under 1 minute from sacrifice to Langendorff perfusion in the rabbit. The fact that successful cardioplegic protection of explanted hearts for transplantation is routine argues against this being a significant problem, but it remains possible that myocytes are more easily damaged by digestive enzymes following an ischaemic period.

6.5 Future work.

These experiments have shown that the methods for studying SR function developed in rabbit single myocytes can be applied to human cells. As discussed in earlier chapters, experiments in rabbit tissue have characterised the abnormalities of SR function in the coronary artery ligation model of heart failure. The future direction of this work should therefore be concentrated on human tissue.

The first priority of any future work would be to improve techniques for dissociation of human myocytes. The most obvious changes in the technique would be to utilise larger biopsies and employ tissue slicing methods to maximise tissue access for the digestive enzymes. Once adequate numbers of myocytes could be dissociated, quantitative studies of SR function could be done to ascertain whether human SR behaves in the same manner to rabbit SR in this permeabilised preparation.

Following these early experiments, SR function could be compared in failing and non-failing hearts. By utilising tissue from biopsies and from explanted hearts, changes in SR function in both mild and severe heart failure could be explored. Material from explanted hearts and from ventricular volume reduction surgery could be used to ascertain whether changes in SR function in heart failure are seen in both endocardial and epicardial regions of the ventricular wall.

Finally, methods for measuring oxalate-supported SR Ca^{2+} uptake could be developed for small masses of human ventricular tissue.

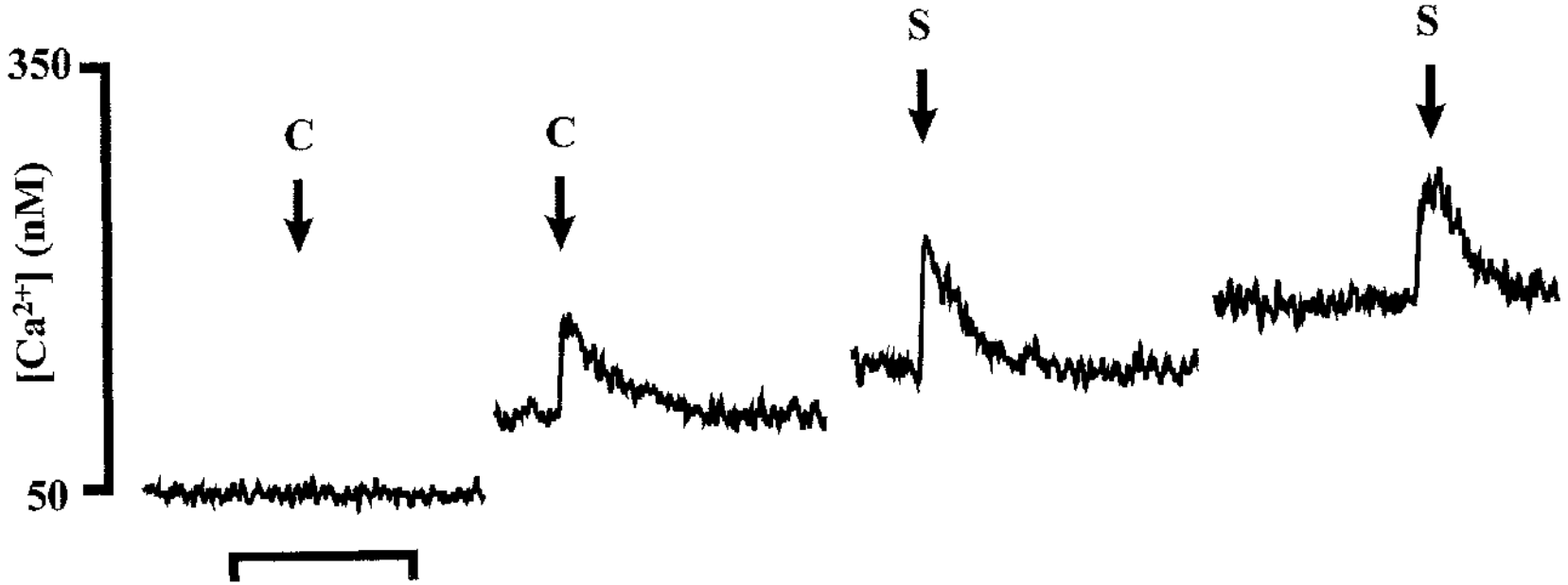

Fig. 6.1: Sample trace from a permeabilised human single myocyte.
(c = caffeine injection, s = spontaneous SR Ca^{2+} release)

Chapter 7: Summary.

Gwathmey showed in 1987 that Ca^{2+} transients were prolonged in heart failure (Gwathmey *et al.* 1987). Evidence that myocyte contractile and lusitropic abnormalities contributes to mechanical dysfunction in heart failure was discussed in the introduction to this thesis. Following Gwathmey's initial findings, other groups have reported abnormalities in sarcolemmal Ca^{2+} transport and in sarcoplasmic reticulum function. More recently abnormalities of expression of sarcoplasmic reticulum proteins have been described, which have been used to explain abnormal Ca^{2+} handling in heart failure. Many of these findings remain controversial, and the both importance of abnormal Ca^{2+} handling in the pathogenesis of heart failure and the exact mechanisms underlying it remain unresolved. Increasingly, manipulation of expression of SR proteins is being used to answer these questions and identify potential therapies.

The initial aim of the studies described in this thesis was to characterise abnormalities of Ca^{2+} handling at the cellular level in the coronary artery ligation model of heart failure in the rabbit. Experiments in intact myocytes confirmed abnormal Ca^{2+} handling in this model. The predominant abnormalities were slowing of diastolic Ca^{2+} re-uptake, slowed systolic Ca^{2+} release and reduced amplitude of systolic Ca^{2+} release. These changes were more apparent in endocardial myocytes than epicardial cells, probably as a result of greater wall stress during ventricular remodelling.

Following this the permeabilised single myocyte preparation was developed to assess the contribution of SR dysfunction to the changes seen in Ca^{2+} handling at the whole cell level. In these experiments, there was a significant reduction in the

amplitude of caffeine-induced and spontaneous SR Ca^{2+} release. The most likely explanation for these findings was a reduction in SR Ca^{2+} content in heart failure cells, reflecting reduced activity of the SR Ca^{2+} pump SERCA 2A.

Experiments were next designed to directly measure SERCA 2A mediated SR Ca^{2+} uptake to test the hypothesis that a reduction in SR Ca^{2+} uptake underlay the reduced SR Ca^{2+} content seen in studies of single permeabilised myocytes. The main finding from these experiments measuring Ca^{2+} uptake in suspensions of permeabilised myocytes confirmed that there was a reduction in the activity of SERCA 2A. In addition these experiments suggested that there was an increase in ryanodine-dependent SR Ca^{2+} leak in myocytes from failing hearts.

The validity of these results was supported by pharmacological modelling of SR function. Inhibition of SERCA 2A -mediated SR Ca^{2+} uptake most closely mimicked the changes on amplitude and frequency of spontaneous SR Ca^{2+} release seen in heart failure. This supported the hypothesis that a reduction in the activity of SERCA 2A underlies abnormal SR function in heart failure, rather than changes in Ca^{2+} release characteristics of the ryanodine receptor.

Differences in the actions of thapsigargin and ionomycin suggest that there are processes in the SR which regulate Ca^{2+} release independently of lumenal Ca^{2+} concentration. These regulatory processes may link the activity of SERCA 2A to the Ca^{2+} release characteristics of the ryanodine receptor. Whether there is a bi-directional effect can neither be confirmed or excluded. Tetracaine may have a direct action on SERCA 2A.

Finally some of the techniques developed for study of SR function in permeabilised myocytes were applied successfully to myocytes isolated from human

left ventricle. Future experimental work will be undertaken to establish whether the pathological mechanisms identified in the rabbit model of heart failure contribute to the pathogenesis of human heart failure.

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Appendix 1: Solutions.

(i) Rat Ringer pH 7.4 37 ^{0}C

NaCl	150mM
KCl	5mM
$CaCl_2$	2mM
$MgCl_2$	1mM
HEPES	5mM
glucose	10mM

(ii) 0.1 Relaxing Solution (0.1R) pH 7.0 20 ^{0}C

EGTA	0.1mM
ATP	5mM
PCR	15mM
HEPES	25mM
KCl	120mM
$MgCl_2$	6mM

(iii) 0.05 Relaxing Solution (0.05R) pH 7.0 20 ^{0}C

EGTA	0.05mM
ATP	5mM
PCR	15mM
HEPES	25mM
KCl	120mM
$MgCl_2$	6mM

(iv) Transport solution for human biopsies pH 7.4 0 ^{0}C

NaCl	120mM
KCl	5.4mM
$MgSO_4$	0.5mM
sodium pyruvate	5mM
glucose	20mM
taurine	20mM
BDM	30mM
HEPES	10mM
NTA	6mM