Non-uniform prolongation of intracellular $\text{Ca}^{2+}$ transients recorded from the epicardial surface of isolated hearts from rabbits with heart failure

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Abstract

Objectives: To study the time course of $\text{Ca}^{2+}$ transients recorded from the left ventricular epicardial surface of isolated hearts from rabbits with heart failure and to correlate the results with mechanical function. Methods: Heart failure was induced in the rabbit 8 weeks after coronary ligation ($n = 17$) with 13 controls. Echocardiography was used to assess in vivo left ventricular dysfunction. The fluorescent indicator Indo-1 was loaded into isolated Langendorff-perfused hearts and $\text{Ca}^{2+}$ transients were recorded from 15 sites over the left ventricular epicardial surface using a single core light guide. The time course of the $\text{Ca}^{2+}$ transients was analysed and the duration measured and correlated with in vitro mechanical function. Results: Significant mechanical dysfunction was produced in this model of heart failure. The mean duration of the $\text{Ca}^{2+}$ transients obtained from failing hearts was prolonged 156.2 ± 3.2 ms when compared to controls 124.9 ± 2.6 ms, $P < 0.001$. Delayed relaxation as measured by the maximum rate of intraventricular pressure decay was significantly correlated with the prolonged $\text{Ca}^{2+}$ transients ($r = -0.63$, $P < 0.001$). In addition, there was increased variation of the $\text{Ca}^{2+}$ transient duration in the failing hearts. Conclusions: Coronary artery ligation-induced heart failure is associated with changes in the surviving myocardium which result in a non-uniform prolongation of $\text{Ca}^{2+}$ transient duration. This suggests that there is a regional heterogeneity to the abnormal intracellular $\text{Ca}^{2+}$ handling in heart failure. © 1998 Elsevier Science B.V.

Keywords: Heart failure; Intracellular $[\text{Ca}^{2+}]$; $\text{Ca}^{2+}$ transient; Indo-1; Langendorff-perfused heart; Rabbit

1. Introduction

Heart failure is a clinical syndrome characterised by a reduced pump efficiency of the heart resulting in haemodynamic disturbances and a cascade of local and systemic changes [1]. The syndrome can have a variety of pathological origins with the common feature of chronic mechanical stress in the heart. This is normally followed by myocardial hypertrophy as a compensatory or an adaptive mechanism [2]. However, in heart failure, these adaptive processes are inadequate leading to mechanical dysfunction with reduced contractile performance [3] and an abnormal relaxation profile [4]. The mechanisms underlying the systolic and diastolic dysfunction are unknown, but changes have been noted in the extracellular matrix and in the structure and function of the myocyte [5,6]. Recent studies have focused on abnormalities of excitation–contraction coupling in heart failure. In particular, pronounced changes have been noted in the amplitude and time course of the change in intracellular $[\text{Ca}^{2+}]$ (the $\text{Ca}^{2+}$ transient) in isolated cells or muscle preparations from both animal models of heart failure and failing human myocardium [7–10]. In general, the systolic $[\text{Ca}^{2+}]$ is lower than normal, and the duration of the $\text{Ca}^{2+}$ transient is prolonged. These changes have been considered to be the basis of the mechanical abnormalities observed in heart failure [11]. However, in the majority of studies, the measurements were made at abnormally low stimulus rates and temperatures. It is difficult to extrapolate from these results to predict the behaviour of the intracellular $[\text{Ca}^{2+}]$ and its
impact on mechanical function under more physiological conditions. Furthermore, the degree of heterogeneity in the time course of Ca\textsuperscript{2+} transients within the remodelled, hypertrophied myocardium has not received consideration. In this study, the fluorescent indicator Indo-1 was used to investigate the time course and regional characteristics of the intracellular Ca\textsuperscript{2+} transient in conjunction with the mechanical performance of the left ventricle of isolated hearts from rabbits with experimentally induced heart failure.

2. Methods

2.1. Coronary artery ligation

A rabbit coronary artery ligation model of heart failure was used for this study. The procedures, which have been described before [12,13], were undertaken in accordance with the Animals (Scientific Procedures) Act 1986 and conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

Adult male New Zealand White rabbits (2.5–3.0 kg) were given premedication with 0.4 ml · kg\textsuperscript{-1} intramuscular Hypnorm (fentanyl citrate (0.315 mg · ml\textsuperscript{-1}); fluanisone (10 mg · ml\textsuperscript{-1}); Janssen Pharmaceuticals). Anaesthesia was induced with 0.25–0.5 mg · kg\textsuperscript{-1} midazolam (Hypnovel, Roche) given via an indwelling cannula in the marginal ear vein. The rabbit was intubated and ventilated using a Harvard small-animal ventilator with a 1:1 mixture of nitrous oxide and oxygen containing 1% halothane at a tidal volume of 50 ml and a frequency of 40 min\textsuperscript{-1}. Pre-operative antibiotic prophylaxis was given with 1 ml of Amfipen (ampicillin 100 mg · ml\textsuperscript{-1}; Mycofarm UK Ltd.) intramuscularly. A left thoracotomy was performed through the fourth intercostal space. Quinidine hydrochloride 10 mg · kg\textsuperscript{-1} (Sigma Pharmaceuticals) was administered intravenously prior to coronary artery ligation to reduce the incidence of ventricular fibrillation. The marginal branch of the left circumflex coronary artery, which supplies most of the left ventricular free wall, was ligated halfway between the atroventricular groove and the cardiac apex to produce an ischaemic area of 30–40% of the left ventricle. As there is relatively little collateral circulation in the rabbit, a homogenous apical infarct was produced. Ventricular fibrillation occurred in approximately 30% of cases, usually 8–12 min following occlusion and defibrillation was undertaken with a 5–10 J epicardial DC shock. Once the animal was stable, the thoracotomy was closed. The animals were then given 20 ml of isotonic saline intravenously to replace perioperative fluid losses and allowed to convalesce in a warm clean environment with adequate monitoring for any early signs of distress. Analgesia was given with 0.04 mg · kg\textsuperscript{-1} intramuscular Vetgesic (buprenorphine hydrochloride 0.3 mg · ml\textsuperscript{-1}, Reckitt and Colman Products Ltd.) immediately after surgery and the next morning. Heart failure was allowed to develop in these rabbits for 8 weeks prior to killing the animals for the experimental studies. Sham-operated rabbits which underwent thoracotomy without coronary artery ligation were used as controls.

2.2. Echocardiography

Echocardiography was performed 1 week prior to killing the animals for the experimental studies to assess in vivo cardiac function, using a 5 MHz paediatric probe with a Toshiba sonograph (Sonolayer 100). The rabbit was sedated with 0.3 mg · kg\textsuperscript{-1} Hypnorm and a small area of the anterior chest wall was shaved to allow a satisfactory echo window. The animal was placed in the left lateral position and a parasternal long-axis view was obtained to assess the left atrial dimension (LAD) and left ventricular end-diastolic dimension (LVEDD) in the M-mode. Ejection fraction (EF) was assessed in the short-axis view at a level just below the tips of the mitral valve leaflets. Using the end-diastolic and end-systolic frames captured, the endocardial border was marked and the enclosed area automatically computed. EF was calculated as the percentage area reduction with systole.

2.3. Langendorff perfusion

Rabbits were killed with an intravenous injection of 0.5 ml · kg\textsuperscript{-1} Euthatal (sodium pentobarbitone 200 mg · ml\textsuperscript{-1}; Rhône Mérieux) mixed with 500 IU of heparin. The hearts were rapidly excised and perfused in the Langendorff mode with Tyrode’s solution of the following composition (mM): Na 138, K 4.0, Ca 1.8, Mg 1.0, HCO\textsubscript{3} 24.0, H\textsubscript{2}PO\textsubscript{4} 0.4, Cl 121, glucose 11. The pH of the solution was maintained at 7.4 by continuously bubbling with 95% O\textsubscript{2}/5% CO\textsubscript{2} mixture and the temperature was maintained at 37°C. A constant perfusion rate of 50 ml · min\textsuperscript{-1} was maintained using a Gilson Minipuls 3 peristaltic pump. A 6-cm-long 6F polypropylene catheter (Portex, Kent, UK) was inserted at the apex of the left ventricle for draining thebesian venous effluent. Intraventricular pressure was monitored with a fluid-filled latex balloon connected with a 6 F cannula to a pressure transducer (Triton Technology Inc., San Diego, CA) and inserted into the left ventricle via the left atrium. The volume of the balloon was adjusted to give zero end-diastolic pressure. Perfusion pressure was monitored with a second pressure transducer in series with the aortic cannula. Pairs of platinum electrodes (Grass Instruments, USA) were inserted into the right atrial appendage and the epicardial surface of the right ventricular apex for atrial and ventricular pacing respectively. Under control conditions the hearts were paced via the right atrium at a cycle length of 350 ms.
2.4. Fluorescence measurement system

Fluorescence from areas of the left ventricular epicardial surface was measured using a custom-built apparatus shown in Fig. 1A. Illumination was provided by a Nikon 75 W Xenon lamp filtered at 360 ± 10 nm (F1) and focused via a 385 nm long-pass dichroic mirror (D1) onto a 3-mm-diameter liquid light guide (Ultratine Technology, UK) held in contact with the left ventricular epicardial surface of the isolated heart. Emitted light from the preparation was collected by the same light guide and light with wavelengths greater than 385 nm passed through the dichroic mirror (D2) and was subsequently split by a second dichroic mirror (D3). Light with wavelengths greater than 460 nm passed through the second dichroic mirror onto a photomultiplier (Thorn EMI, UK) fitted with an optical band-pass filter centred on 495 ± 20 nm. The remaining light was reflected onto a second photomultiplier with a band-pass filter centred on 405 ± 35 nm. The output of the photomultipliers was passed to an electronic ratio circuit so that an effectively continuous signal of fluorescence intensity ratio \( F_{495}/F_{405} \) could be obtained. This analogue ratio signal, the fluorescence intensity of the individual wavelengths and the electrical signals representing intraventricular pressure and perfusion pressure were displayed continuously on a Gould TA-11 chart recorder and stored on video tape using a CRC VR-100B digital recorder (Instrutech Corp, USA).

Indo-1 fluorescence measurements were made at 15 sites over the left ventricular epicardial surface. The locations of the sites were based on anatomical features (see Fig. 1B) with the left anterior descending artery forming the left border and the posterior descending artery forming the right.

2.5. Indo-1 loading

2.5.1. Protocol

Indo-1 AM (Molecular Probes Inc., OH) stock solution was initially prepared by dissolving 1 mg of Indo-1 AM in 1 ml of dimethyl sulphoxide/25% (w/v) pluronic acid F-127 solution. This was then added to 100 ml of Tyrode’s solution with 5% foetal calf serum and 0.3 mM probenecid to make up a final concentration of 10 μM Indo-1 AM. After an initial equilibration period of 30 min with normal Tyrode’s solution, the hearts were perfused with the Indo-1 AM containing solution at a rate of 25 ml · min⁻¹ at room temperature (25°C) for a period of 60 min. The loading of the myocardium with the fluorescent indicator was observed by illuminating the preparation for 10 s at regular intervals, with the light guide being held at the same position (site 1, Fig. 1B) throughout the loading procedure. At the end of this period, the hearts were perfused with normal Tyrode’s solution for 15 min with the perfusion rate increased back to 50 ml · min⁻¹ and temperature back to 37°C. This ensured that the intracellular Indo-1 AM was fully cleaved to the Ca²⁺-sensitive

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Fig. 1. A: a schematic diagram of the optical arrangement used to measure Indo-1 fluorescence from the epicardial surface of isolated Langendorff-perfused rabbit heart. D₁ is a 385 nm long-pass dichroic mirror and D₂ is a 460 nm long-pass dichroic mirror. F₁, F₂ and F₃ are optical filters with peak transmission wavelengths and band-widths as shown on the diagram. The representation of an isolated rabbit heart is shown with the right ventricular outflow tract (RVOT), left atrium (LA), right atrium (RA) and left ventricle (LV) detailed. B: a representation of the left ventricular epicardial surface illustrating the 15 sites used for fluorescence measurements. The anatomical features used as points of reference are shown: atrio-ventricular (AV) groove, anterior descending artery, posterior descending artery, marginal branch of the left circumflex artery, and apex.
Comparison of Indo-1 loading characteristics between failing HF and control Sham hearts

During Indo-1 loading, the rise in fluorescence routinely reached a plateau after about 45 min with the ratio signal reaching a steady state at the same time. Typically, the $F_{405}$ increased by $1.37 \pm 0.05$ times background level while an increase of $3.08 \pm 0.24$ times background level was seen at $F_{495}$. Phasic changes in the $F_{405}/F_{495}$ ratio were discernible after 10 min loading and the signal-to-noise ratio improved as loading progressed.

The fluorescence due to Indo-1 loading alone could be obtained at site 1 by subtracting the background fluorescence for each wavelength. The time courses of the rise in $F_{405}$ and $F_{495}$ were fitted to an exponential curve and the time to half-maximal fluorescence noted for individual wavelengths. An exponential time course describes the increase in fluorescence satisfactorily, with approximately the same half-time for each wavelength. This analysis allowed the loading characteristics of failing and sham hearts to be compared. These results are summarised in Table 1 and show that background fluorescence at site 1 and the extent of loading with Indo-1 were similar in the two groups. The values for the half-times indicate that Indo-1 loading occurred with a slightly faster time course in the control group when compared to failing hearts, but this difference did not reach statistical significance.

### 2.6. Measurement of intracellular Ca$^{2+}$ transient

Fig. 2 illustrates the individual fluorescence signals ($F_{405}$ and $F_{495}$) measured from site 1 on the surface of the left ventricle and the simultaneously recorded isovolumetric pressure record in a typical experiment. The fluorescence records prior to loading with Indo-1 showed no phasic changes and the fluorescence ratio was constant with a value of approximately 4. These recordings illustrate that this particular optical arrangement did not generate significant movement artefact in the fluorescence signal from the contracting myocardium below the light guide. After the loading procedure, the fluorescence of the epicardial surface had increased significantly at both wavelengths and as indicated in Fig. 2A, phasic signals were evident at both 405 and 495 nm. The value of the fluorescence ratio was now approximately 2, due to the relatively greater increase in fluorescence at 495 nm. In addition to this, the signal contained a significant phasic component as shown on an expanded scale in Fig. 2B. The fluorescence ratio increased rapidly to a peak and fell gradually to steady level and represents the time course of the changes of intracellular [Ca$^{2+}$] within the myocardium at site 1. As mentioned above, there were no phasic changes prior to Indo-1 loading that could be attributed to movement artefact. It is, therefore, very unlikely that the limited increases in fluorescence of the myocardium after Indo-1 loading would have introduced a movement artefact sufficient to affect the time course of the signal due to Ca$^{2+}$.

Conventionally, the fluorescence ratio is calculated after the subtraction of background fluorescence, and the values of maximum and minimum fluorescence ratio represent the systolic and diastolic intracellular [Ca$^{2+}$] respectively. However, in this study the subtraction of background fluorescence for all 15 sites was not feasible for two reasons. (i) Precise background fluorescence measurements would have to be made at the 15 sites, either prior to Indo-1 loading or after quenching the Ca$^{2+}$-sensitive signal at the end of the experiment. Due to the optical system used in these experiments, the magnitude of background fluorescence was approximately the same as that of the Indo-1 fluorescence. Imprecise positioning of the light guide would generate errors in the estimation of background fluorescence leading to large errors in the estimation of the Indo-1 fluorescence ratio values. This was confirmed by preliminary measurements of Indo-1 fluorescence ratio which showed large variations in value when comparing a number of sites over the epicardial surface of one heart or the same site over a number of individual hearts within one experimental group (results not shown). (ii) As shown in Fig. 2A, fluorescence signals at 405 and 495 nm consisted of small variations of photomultiplier current on top of a substantial baseline values. Background subtraction at 15 sites would have to be performed off-line on digitised data. The resolution of the A/D converter (12 bit) limits the precision of the measurements of small signals on top of a large base line value and therefore substantially degrades the computed ratio signal. For these

<table>
<thead>
<tr>
<th></th>
<th>Background fluorescence (A)</th>
<th>Increase in fluorescence (A)</th>
<th>Half-time of loading (min)</th>
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<tbody>
<tr>
<td></td>
<td>405 nm</td>
<td>495 nm</td>
<td>405 nm</td>
</tr>
<tr>
<td>HF ($n = 17$)</td>
<td>1.32 ± 0.05</td>
<td>0.39 ± 0.02</td>
<td>0.48 ± 0.09</td>
</tr>
<tr>
<td>Sham ($n = 15$)</td>
<td>1.31 ± 0.03</td>
<td>0.35 ± 0.01</td>
<td>0.46 ± 0.08</td>
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<tr>
<td>$P$ value</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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Free-acid form and that the Indo-1 AM-containing solution was washed out of the circulation and extracellular space prior to intracellular Ca$^{2+}$ measurements. There was no change in the amplitude or slowing of the time course of the intraventricular pressure during the loading period indicating that there was no significant buffering of intracellular Ca$^{2+}$ by Indo-1.
Fig. 2. A: records of fluorescence at 405 nm ($F_{405}$), 495 nm ($F_{495}$), fluorescence ratio ($F_{405}/F_{495}$) and left ventricular isovolumic pressure (Pressure) from an isolated Langendorff-perfused rabbit heart at 37°C, paced via the right atrium at a cycle length of 350 ms. Records are shown before and after Indo-1 loading procedure. Each trace is an average of 30 recordings. B: sections of the above traces on an expanded vertical and horizontal scale. The individual points (○) superimposed on the $F_{405}/F_{495}$ ratio record are the calculated Indo-1 fluorescence ratio values (right-hand side axis, see text).

two reasons, the fluorescence ratio measurements quoted in this study represent the ratio of the total fluorescence signals provided by an analogue divider circuit. These measurements prevent comparisons of diastolic and systolic [Ca$^{2+}$] values across the epicardial surface which, as described above, are not feasible with this technique. However, the time course of the transient changes in signal can be used to examine the duration of the intracellular [Ca$^{2+}$] signal. To illustrate the similarities in the time course of the two fluorescence ratio signals, the calculated Indo-1 fluorescence ratio is plotted (as individual points) superimposed on the continuous analogue signal of total fluorescence ratio in Fig. 2B. It is clear that both fluorescence ratio values, although different in magnitude, have similar time courses.

2.6.1. Factors potentially affecting time course of Ca$^{2+}$ transients

If the activation of the myocytes within the region sampled by the single light guide (≈ 7 mm$^2$) were not completely synchronous, the time course of the average Ca$^{2+}$ transient may be affected by altered patterns of excitation of the myocardium. One way to test for this is to compare the time course of the Ca$^{2+}$ transient obtained during atrial pacing with that obtained at the same site during direct ventricular pacing. The direct stimulation of the ventricle will generate a different excitation pattern with delay in ventricular activation when compared to atrial pacing which uses the His–Purkinje system [14]. However, when the Ca$^{2+}$ transients recorded during atrial and ventricular pacing were compared, no difference in the time course was evident ($n = 6$, results not shown) suggesting that changes in the excitation pattern of the ventricle do not significantly affect the time course of the Ca$^{2+}$ transients obtained with the 3-mm-diameter single light guide.

Another consideration is the variation in the depth of myocardium from which the signals were recorded. The potential contribution of the endocardium to the fluorescence obtained from the epicardial surface was examined by freezing the inside of the heart ($n = 3$) using a technique described by Allessie et al. [15]. No change in the amplitude or time course of the Ca$^{2+}$ transient signal was evident when only a 0.5–0.7-mm-thick epicardial layer remained viable (results not shown). This indicates that the epicardial Ca$^{2+}$ transients are recorded from a layer ap-
proximately 0.7 mm deep. This is consistent with results described by other studies [16,17]. Another method to reduce the depth of myocardium excited by the incident light is to reduce the intensity of the excitation light. Reducing the light intensity by a factor of 10 (ND 1) or 100 (ND 2) did not alter the time course of the recorded fluorescence ratio (n = 3, results not shown), but the signal-to-noise ratio decreases as the intensity of the excitation light was decreased.

In a separate set of experiments (n = 4), the possibility that fluorescence signals from the vascular endothelium may influence the time course of the fluorescence ratio signals was tested by recording transients before and during perfusion of the heart with 10 \( \mu \text{M} \) bradykinin. As observed in previous studies [18,19], bradykinin caused a sustained, reversible increase in 405 nm and decrease in 495 nm fluorescence signals without significantly affecting the time course of the changes in fluorescence ratio (results not shown).

These measurements indicate that the fluorescence ratio signal accurately reflects the time course of the intracellular \( \text{Ca}^{2+} \) transient and the time course is unaffected by either the pattern of excitation, the depth of myocardium within the region sampled by the single light guide or a contribution of the signal from the vascular endothelium.

2.7. Signal measurements and analysis

Fluorescence measurements were made at each of the 15 left ventricular epicardial sites sequentially. Signal reproducibility was verified by repeated sampling at the initial sites at 30-min intervals. There was a variation of less than 3% in the duration of the \( \text{Ca}^{2+} \) transients measured as CaD-50 (see later) in different samples made at the same sites. Intraventricular pressure recordings indicated the stability of the preparation during the course of the experiments.

Pressure and fluorescence signals were digitised at 1 kHz with 12-bit precision using the program WCP (Dr. J. Dempster, Strathclyde Electrophysiology Software). Using this software, 30 successive sweeps (representing a continuous 30-s recording period) were averaged before measurements of time course and amplitude were made.

2.8. Organ / tissue weights

Liver and lungs were excised from the animals at the time they were killed, blotted to remove excess blood and their wet weights were measured. After each experiment, the heart was removed from the perfusion apparatus, taking care to preserve the aorta and other structures. The heart was blotted and the wet weight was measured. It was then cut along the atrioventricular groove, separating the atria and great vessels from the ventricles. The right ventricular free wall was carefully dissected away from the left ventricle and both were dried in an oven heated to 50°C for 2 weeks after which their dry weights were obtained.

2.9. Statistics

All data are expressed as mean ± SE. Comparison between groups of data was made with Student’s t-test (paired when appropriate). Two-tailed \( P \) value of less than 0.05 was considered significant.

3. Results

3.1. Characterisation of the animal model

The degree of left ventricular dysfunction produced by coronary artery ligation was quantified by analysing echocardiographic data in terms of ejection fraction (EF), left atrial dimension (LAD) and left ventricular end-diastolic dimension (LVEDD). The results from the heart-
failure group \((n = 17)\) were compared with those from sham-operated animals \((n = 13)\) and are summarised in Table 2.

This shows that the ejection fraction was lower and the cardiac dimensions (LAD and LVEDD) were significantly increased in the heart-failure group when compared to controls. Rabbits with heart failure also had a significantly higher left ventricular dry weight when compared to controls indicating that significant remodelling or compensatory hypertrophy had occurred by 8 weeks after coronary ligation. Right ventricular dry weight was also increased in the heart-failure group suggesting that there had been significant right ventricular overload as a result of left ventricular dysfunction. In addition, lung and liver wet weights were significantly greater in the heart-failure group indicating organ congestion as seen in the clinical syndrome.

### 3.2. Comparison of in vivo and in vitro mechanical function

The reduced ejection fraction measured in vivo indicated that failing hearts had significantly impaired contrac-

<table>
<thead>
<tr>
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<th>HF ((n = 17))</th>
<th>Sham ((n = 13))</th>
<th>(P) value</th>
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</thead>
<tbody>
<tr>
<td>Peak systolic pressure (mmHg)</td>
<td>26.2 ± 3.7</td>
<td>49.0 ± 2.6</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>(-dP/dt_{max}) (mmHg/ms)</td>
<td>0.32 ± 0.04</td>
<td>0.66 ± 0.05</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CaD-50 (ms)</td>
<td>156.2 ± 3.2</td>
<td>124.9 ± 2.6</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
tile function when compared to controls. This was confirmed in vitro by measurements of left ventricular peak systolic pressure. Under conditions of a standard stimulation rate, end-diastolic pressure and perfusion pressure, the peak systolic pressure in the heart-failure group was approximately 50% of that in the sham-operated group (26.2 ± 3.7 vs. 49.0 ± 2.6 mm Hg). The individual values of in vivo ejection fraction were significantly correlated with in vitro peak systolic pressure ($r = 0.73$, $P < 0.001$) as shown in Fig. 3.

### 3.3. Measurement of $Ca^{2+}$ transient duration over the epicardial surface

Fig. 4 illustrates the range of signals measured at 15 epicardial sites from an isolated heart from the sham-operated group (panel A) and from the heart-failure group (panel B). It is clear that the duration of the $Ca^{2+}$ transient varies to a limited extent in sham-operated hearts, the shortest duration was observed at site 7, while the longest duration was observed at site 1 for the heart illustrated in Fig. 4A. The recordings from a heart with a significant infarct had several unusual features. Firstly, records made from within the infarct zone showed no transient changes confirming that this region did not contain significant amounts of viable myocardium. Secondly, the remaining sites showed transient signals with a wide range of time courses, the shortest transient being recorded from site 3, while transients with a particularly long duration were obvious at sites 2 and 8 for the heart illustrated in Fig. 4B.

The $Ca^{2+}$ transient durations were analysed in all 15 sites obtained from control hearts. In failing hearts, since recordings from sites within the infarct zone gave no transient signal, analysis only included recordings from the remaining surviving myocardium with transient signals, the number of viable sites ranging from 7 to 12 (mean of 9).

The prolonged $Ca^{2+}$ transients had a slowed rate of decay whilst the upstroke remained brief. The time between the beginning of the upstroke and 50% of the peak was not different in the heart failure and sham-operated...
groups (10.0 ± 0.5 vs. 9.5 ± 0.3 ms, \( P = \text{NS} \)). The duration of the Ca\(^{2+}\) transient recorded at each site was measured from the beginning of the upstroke to 50\% decay and a mean value (CaD-50) was obtained for each heart. The results are summarised in Table 3. The mean Ca\(^{2+}\) transient duration was 124.9 ± 2.6 ms in control hearts in contrast to a mean duration of 156.2 ± 3.2 ms recorded from the heart-failure group (\( P < 0.001 \)). Multiple comparisons of the Ca\(^{2+}\) transient durations obtained at different sites within each group revealed no consistent relationship between the site of recording and the duration of the Ca\(^{2+}\) transients. Furthermore, the prolongation of Ca\(^{2+}\) transient duration in the heart-failure group was non-uniform and not site-specific. There were regions in hearts from the heart-failure group which had Ca\(^{2+}\) transient durations within the range observed in sham-operated hearts and again there was no consistent pattern to the locality of these areas.

The Ca\(^{2+}\) transient measurements were accompanied by the maximum rate of left ventricular pressure decay (\(-dP/dt_{\text{max}}\)) recorded from the same hearts. It is apparent from Table 3 that hearts from the heart-failure group relaxed slower than those from the sham-operated group. The link between the duration of the Ca\(^{2+}\) transient and the rate of relaxation is made clearer in Fig. 5. The corresponding values of \(-dP/dt_{\text{max}}\) and mean Ca\(^{2+}\) transient duration (CaD-50) showed a clear correlation (\( r = -0.63, \ P < 0.001 \)) indicating a relationship between the two parameters.

Analysis of the Ca\(^{2+}\) transients recorded with this method not only provides a measure of the mean duration but also the variation within each heart. An estimate of the range of Ca\(^{2+}\) transient duration measured in the individual groups are shown using two forms of analysis in Fig. 6A,B. One method of examining the variability of the Ca\(^{2+}\) transient duration is to compare the standard deviation or range of the values from each heart. Fig. 6A uses a box and whiskers plot to compare the average standard deviation and range of the Ca\(^{2+}\) transient duration in each experimental group. This figure shows that the range of

![Graph](image-url)

Fig. 7. A: records of fluorescence ratio \(F_{605}/F_{505}\) from one site on the epicardial surface of an isolated Langendorff-perfused rabbit heart from the control group stimulated at a cycle length of 350 ms via electrodes placed on the right atrium. Traces represent the average of 30 records sampled in the presence of 0.8 mM, 1.8 mM normal and 3.6 mM extracellular Ca\(^{2+}\). B: (i) shows the traces superimposed on a common baseline, (ii) shows normalised traces superimposed and aligned at the upstroke.
Table 4
Effect of altered extracellular [Ca\textsuperscript{2+}] on peak systolic pressure, maximum rate of pressure decay (-dP/dt\textsubscript{max}) and Ca\textsuperscript{2+} transient duration (CaD-50) (n = 4)

<table>
<thead>
<tr>
<th>Condition</th>
<th>0.8 mM [Ca\textsuperscript{2+}]</th>
<th>1.8 mM [Ca\textsuperscript{2+}]</th>
<th>3.6 mM [Ca\textsuperscript{2+}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak systolic pressure (mm Hg)</td>
<td>28.3 ± 2.1</td>
<td>42.6 ± 2.2</td>
<td>54.5 ± 1.3</td>
</tr>
<tr>
<td>-dP/dt\textsubscript{max} (mm Hg·ms\textsuperscript{-1})</td>
<td>0.32 ± 0.04</td>
<td>0.55 ± 0.05</td>
<td>0.64 ± 0.04</td>
</tr>
<tr>
<td>CaD-50 (ms)</td>
<td>139.5 ± 8.8</td>
<td>126.0 ± 9.5</td>
<td>109 ± 9.6</td>
</tr>
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</table>

Ca\textsuperscript{2+} transient durations was increased in the heart-failure group mainly due to the higher maximum value, with only a small increase in the minimum value when compared with the control group. An alternative method of analysis is to measure the dispersion of the Ca\textsuperscript{2+} transient duration in each heart by the difference between the longest and the shortest duration. Fig. 6B shows that dispersion of the Ca\textsuperscript{2+} transient duration was significantly greater in the heart-failure group.

3.4. Effect of altered extracellular [Ca\textsuperscript{2+}] on intracellular Ca\textsuperscript{2+} transient duration in control hearts

In a separate set of experiments, Ca\textsuperscript{2+} transients were monitored from the same site on the epicardial surface of control hearts while the extracellular [Ca\textsuperscript{2+}] was decreased from 1.8 to 0.8 mM, or increased to 3.6 mM. The steady-state Ca\textsuperscript{2+} transients are shown in Fig. 7. Lowering the extracellular [Ca\textsuperscript{2+}] appeared to reduce both the peak systolic and end-diastolic fluorescence ratio. However, the duration of the Ca\textsuperscript{2+} transient was prolonged when compared to that measured in normal extracellular [Ca\textsuperscript{2+}]. Conversely, raising extracellular [Ca\textsuperscript{2+}] increased the peak systolic and end-diastolic fluorescence ratio, while the duration of the Ca\textsuperscript{2+} transient was shorter than normal. Similar results were observed in three other experiments, and the results are shown in Table 4 accompanied by the mean peak systolic pressure and maximum rate of pressure decay.

4. Discussion

This is the first study to show non-uniform prolongation of Ca\textsuperscript{2+} transient duration over the left ventricular epicardial surface of isolated hearts in heart failure. Furthermore, the degree of prolongation was significantly correlated with mechanical dysfunction of the left ventricle.

4.1. Mechanical dysfunction and hypertrophy in failing hearts

The echocardiographic findings presented in this study revealed significant changes in cardiac function and dimensions as a result of chronic myocardial infarction secondary to coronary artery ligation. Ejection fraction was markedly reduced in the heart-failure group. There was significant left ventricular dilatation analogous to that seen in human heart failure as a result of volume overload caused by impaired left ventricular function. Consistent with this was the increased left atrial dimension, indicating raised filling pressure and possible mitral regurgitation. Increased left ventricular dry weight was indicative of significant compensatory hypertrophy. Right ventricular hypertrophy and systemic effects were also observed with organ congestion suggesting a state of decompensation in the experimental group of animals.

These measurements suggest significant contractile dysfunction in the heart-failure group but direct comparison with the control group is difficult with different heart rates and loading conditions which cannot be standardised easily in vivo. In vitro measurements under standard conditions showed significant reduction in contractile function in the heart-failure group with markedly reduced systolic pressure. The link between the in vivo and in vitro contractile function was further strengthened by the correlation between the ejection fraction and peak systolic pressure. These studies suggest that the in vitro contractile behaviour of the Langendorff-perfused heart accurately reflects in vivo contractile dysfunction in heart failure. This is consistent with the results of a more detailed haemodynamic study of this model by Pye et al. [12].

4.2. Characteristics of intracellular Ca\textsuperscript{2+} transient measurement with single light guide

4.2.1. Background fluorescence and indo-1 loading

As indicated in Section 3, background fluorescence at both wavelengths prior to Indo-1 loading were similar in both the control and experimental groups. Fralix et al. [20] showed that the majority of this tissue autofluorescence is due to intracellular metabolite, NADPH. The Indo-1 loading characteristics were similar in the failing hearts and controls with both groups achieving the same level of fluorescence increase above background. However, the half-time in fluorescence increase in the failing hearts was slightly longer (although not statistically significant) suggesting a lower esterase activity in these hearts when compared to controls. Intraventricular pressure monitoring during the loading procedure suggested against significant intracellular Ca\textsuperscript{2+}-buffering by Indo-1 in either group of hearts.

The method of Indo-1 loading described in this study resulted in an increase in fluorescence intensity at both
wavelengths by a factor of 1.4–3.1. This is in contrast to other studies using bifurcated light guides for measuring intracellular \([\text{Ca}^{2+}]\) in intact hearts using Indo-1 where a much higher (up to 20-fold) increase in fluorescence intensity above original tissue autofluorescence was observed \([18,21,22]\). One explanation may be that the background fluorescence measured with the optical arrangement in this study using a single light guide was higher than in other studies leading to a lower relative increase in fluorescence intensity with Indo-1 loading. This was verified by preliminary experiments \((n = 3)\) performed by the authors using a bifurcated liquid light guide in a modified set-up using the same Indo-1 loading protocol which showed a much lower background fluorescence and a 10- to 15-fold increase in fluorescence intensity with Indo-1 loading. Although the reason for this difference in background fluorescence obtained with the two methods is unknown, initial studies would suggest that the contribution of epicardial fluorescence to the background fluorescence was less using a bifurcated light guide. Despite a higher background fluorescence using a single light guide, measurements could be made in a smaller, more discrete area of myocardium and signals were less prone to movement artefacts than signals recorded using a bifurcated light guide. These features are of particular importance in this study for the measurement of fluorescence signals from multiple sites over the epicardial surface of the left ventricle.

4.2.2. Measurement of intracellular \([\text{Ca}^{2+}]\) transient

After Indo-1 loading, the analogue ratio of the total fluorescence at 405 and 495 nm revealed phasic changes with the time course and features characteristic of the intracellular \([\text{Ca}^{2+}]\) signal. While the peak systolic and diastolic values of this signal do not simply reflect systolic and diastolic \([\text{Ca}^{2+}]\), the time course of the fluorescence change reflects the time course of intracellular \([\text{Ca}^{2+}]\) change. This assertion was verified by calculating the Indo-1 fluorescence ratio change (after background subtraction) which followed the time course of the analogue ratio accurately. This shows that the time course of the total fluorescence ratio change is not sensitive to the level of the background fluorescence.

However, the amplitude of the calculated Indo-1 fluorescence ratio was much less than expected on the basis of the Indo-1 spectrum published by Grynkiewicz et al. \([23]\). There are a number of possible reasons for this. (i) The dichroic mirror (385 nm long-pass) will reduce the fluorescence collected by the 405 nm photomultiplier. (ii) The Indo-1 spectrum may be shifted significantly within the myocyte \([24,25]\). (iii) Several studies have shown that there is a non-cytosolic component to the Indo-1 fluorescence due to loading of dye into organelles such as mitochondria and sarcoplasmic reticulum \([19,26,27]\). Miyata et al. \([28]\) showed that fluorescence from Indo-1 located in mitochondria did not affect the beat-to-beat variation of the total fluorescence signal. (iv) There may be a component of unhydrolysed Indo-1 AM in the signal but there is debate about the importance of its contribution to the total fluorescence \([26,27]\). (v) Finally, Lorell et al. \([18]\) suggested that a substantial proportion of the fluorescence signal was due to Indo-1 loading of vascular endothelial cells but this was shown not to affect the time course of the ratio signal which is consistent with the control experiments performed in this study with bradykinin.

All the above-mentioned factors may affect the intensity of the fluorescence signals at the individual wavelengths and make the quantitation of cytosolic \([\text{Ca}^{2+}]\) in intact hearts difficult. Different techniques \([22,25,29]\) have been used which yielded different normal ranges for systolic and diastolic \([\text{Ca}^{2+}]\). We did not make any calibration measurements of the intracellular \([\text{Ca}^{2+}]\) in this study in view of the potential limitations and also because of the aims of the study. Since we were sampling from 15 sites over the left ventricular epicardial surface, measurement of background fluorescence prior to Indo-1 loading at the exact 15 positions would be required for the quantitation of \([\text{Ca}^{2+}]\) at all sites. The main objective of this study was to examine the time course of the \([\text{Ca}^{2+}]\) transients from different sites over the left ventricular epicardial surface rather than the absolute value of \([\text{Ca}^{2+}]\) at each site. The factors described above which affect the intensity of the fluorescence signals and hinder the quantitation of \([\text{Ca}^{2+}]\) have been shown not to affect the time course of the ratio signal.

Another factor which may potentially influence the time course of the recorded fluorescence signal is the conduction delay over the epicardial surface. The absence of difference between the time course of \([\text{Ca}^{2+}]\) transients on atrial and ventricular pacing suggests that conduction delay over the epicardial surface studied with the light guide would not account for any change in \([\text{Ca}^{2+}]\) transient duration. This is further supported by the fact that the time from the beginning of the \([\text{Ca}^{2+}]\) transient to 50% of the peak was about 10 ms in both groups which is in line with published data made on isolated myocytes \([30,31]\) and multicellular preparations \([32,33]\).

4.3. Measurement of \([\text{Ca}^{2+}]\) transient duration over the epicardial surface

It was shown in this study that the mean duration (CaD-50) of \([\text{Ca}^{2+}]\) transients recorded from the left ventricular epicardial surface of hearts from the control group was 124.9 ± 2.6 ms. Individual hearts exhibited \([\text{Ca}^{2+}]\) transients with a limited range of durations and there was no consistent relationship between the site position and the \([\text{Ca}^{2+}]\) transient duration. The cause of this variation is unknown, regional differences in excitation pattern are not thought to be a factor since \([\text{Ca}^{2+}]\) transients of identical time course were obtained on atrial or ventricular stimulation at the same site recorded with the single light guide. A physiological dispersion of repolarisation is recognised.
[34] and this may be related to the regional variation in Ca$^{2+}$ transient duration observed in the control hearts.

Analysis of Ca$^{2+}$ transient duration recorded from the heart-failure group revealed a significantly longer mean duration (156.2 ± 3.2 ms). A causal link between the duration of the Ca$^{2+}$ transient and the mechanical function of these hearts was suggested by the significant correlation between CaD-50 value and the maximum rate of left ventricular pressure decay (Fig. 5). The smaller systolic pressures observed in failing hearts were naturally associated with slower maximum rates of relaxation (see Table 3). The limitations of the optical techniques described above prevented the systolic and diastolic intracellular [Ca$^{2+}$] being measured but it would not be unreasonable to assume that the slower rates of decay of the Ca$^{2+}$ transients in failure hearts were accompanied by a reduced peak systolic [Ca$^{2+}$]. A number of investigators have demonstrated a reduced peak systolic [Ca$^{2+}$] in isolated muscle preparations or myocytes from failing hearts [8–10]. These studies also demonstrated a marked prolongation of the Ca$^{2+}$ transient. However, this was noted at low stimulation rates and there appears to be little information on Ca$^{2+}$ transient duration in heart failure at close to physiological stimulation rates. This study is the first to show a direct relationship between prolonged Ca$^{2+}$ transients and an abnormal mechanical profile in heart failure in the intact heart.

Further scrutiny of Ca$^{2+}$ signals from the heart-failure group reveals that the longer mean duration was a result of abnormally prolonged Ca$^{2+}$ transients in a number of sites in each heart. Despite recordings being available from fewer sites due to the presence of an apical infarct when compared to controls, the variation of Ca$^{2+}$ transient duration was greater in each heart from the heart-failure group. This is obvious from measurements of the range of values (Fig. 6A) or from the measure of the dispersion of Ca$^{2+}$ transient duration (Fig. 6B). Therefore, changes in the myocardium in this model of heart failure in the rabbit appear heterogeneous. Some regions showed pronounced prolongation of the Ca$^{2+}$ transient while the duration of the transients in others were close to normal. As with the normal myocardium, there appeared to be no consistent relationship between site position and the duration of the Ca$^{2+}$ transient. In particular, prolonged Ca$^{2+}$ transients were not specifically located at the border between normal and infarcted myocardium. This study appears to be the first to report a heterogeneous change in the time course of the Ca$^{2+}$ transient in heart failure.

4.4. Effects of altered extracellular [Ca$^{2+}$] on intracellular Ca$^{2+}$ transient duration

As discussed above, the heart-failure group was characterised by a significantly reduced peak systolic pressure. When the reduced peak systolic force was mimicked in control hearts by reducing the extracellular [Ca$^{2+}$] to 0.8 mM, this was accompanied by a prolongation of the Ca$^{2+}$ transient measured at one site on the epicardial surface. In contrast to this, increasing extracellular [Ca$^{2+}$] to 3.6 mM caused an increased peak systolic pressure and a shortening of the duration of the Ca$^{2+}$ transient. Changes in Ca$^{2+}$ transient duration as a result of inotropic changes have been extensively studied in multicellular [32,33] and single cell preparations [30,31]. The basis for the effect on time course is thought to be the increased rate of Ca$^{2+}$ uptake by the sarcoplasmic reticulum. While this may be the explanation for the changes in Ca$^{2+}$ transient duration observed in this study, a detailed analysis of the rate of decay of the Ca$^{2+}$ transient in isolated rabbit myocytes has yet to be performed at physiological stimulation rates (= 3 Hz).

The contractile performance of normal hearts perfused with 0.8 mM extracellular Ca$^{2+}$ appears to be very similar to the average contractile performance of the heart-failure group in terms of peak systolic pressure and maximum rate of decay. However, the Ca$^{2+}$ transient duration under conditions of low extracellular [Ca$^{2+}$] in control hearts was not prolonged to the same extent as that seen in heart failure (see Table 3 and 4). The fact that the measurement of Ca$^{2+}$ transient was performed at only one site, weakens this comparison but it would be expected that the effects of lowering extracellular [Ca$^{2+}$] would have uniform effects on the intracellular [Ca$^{2+}$] across the ventricle. These results suggest that the abnormally long Ca$^{2+}$ transients observed in heart failure may be partly explained by a reduced peak systolic intracellular [Ca$^{2+}$].

4.5. Abnormal Ca$^{2+}$ handling in heart failure

Numerous investigators have demonstrated abnormalities in the processes involved in excitation-contraction coupling in heart failure. Depressed sarcoplasmic reticulum function is observed in human heart failure and most animal models [35–37]. Levels of the ryanodine-sensitive Ca$^{2+}$-release channel are thought to be reduced in heart failure [38,39] as are the levels of sarcoplasmic reticulum Ca$^{2+}$-pump [39–43] and the regulator protein phospholamban [39,44] although these findings are not universal [45–47]. Depressed sarcoplasmic reticulum function is consistent with the results described in this study since the abnormally long Ca$^{2+}$ transients recorded are probably the result of reduced sarcoplasmic reticulum Ca$^{2+}$ release or re-uptake in heart failure. The increased variation in Ca$^{2+}$ transient duration over the epicardial surface in failing hearts suggests that the alteration in excitation-contraction coupling may be a heterogeneous process in the whole heart. The correlation between the prolonged Ca$^{2+}$ transient duration and abnormal intraventricular pressure measurements suggests that spatial and temporal variation in the mechanical profile of regions of the whole heart may contribute towards the impairment of the overall performance of the failing heart.
In conclusion, coronary artery ligation-induced heart failure is associated with changes in the surviving myocardium which result in a non-uniform prolongation of Ca\(^{2+}\) transient duration. This suggests that there is a regional heterogeneity to the abnormal intracellular Ca\(^{2+}\) handling in heart failure.

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**References**


