Heterogeneous changes in action potential and intracellular Ca\textsuperscript{2+} in left ventricular myocyte sub-types from rabbits with heart failure

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Abstract

Objective: Myocardial cellular electrophysiology and intracellular Ca\textsuperscript{2+} regulation are altered in heart failure. The extent of these changes may vary within the layers of the ventricular wall. To examine this, cell size, action potential and intracellular Ca\textsuperscript{2+} transient characteristics (Fura-2) were measured in single cardiac myocytes from sub-epicardial, mid-myocardial, and sub-endocardial regions of the left ventricle of rabbits with heart failure.

Methods: Myocytes were isolated from animals with heart failure induced by chronic coronary artery ligation and from sham operated controls. Trans-membrane potential was measured using high resistance microelectrodes (30 M\textOmega; 2 M KCl). Fura-2 was loaded into cells by incubation with the AM form. Subsequent fluorescence measurements were used to measure intracellular Ca\textsuperscript{2+} concentration at a range of stimulus frequencies.

Results: Resting cell length was significantly greater in the heart failure group; \textbf{115\% of control values in sub-epicardial and mid-myocardial cells, and 108\% in sub-endocardial cells.} Using criteria described by previous studies on other mammalian hearts, functional M cells were identified by a higher maximum rate of depolarisation and longer action potential duration at 90\% repolarisation (APD\textsubscript{90}) compared to the two other myocyte sub-types. In the heart failure group, APD\textsubscript{90} and Ca\textsuperscript{2+} transient duration (CaD\textsubscript{90}) were prolonged in sub-epicardial and M cells but shortened in sub-endocardial myocytes. These changes were significant at lower stimulus frequencies, but the relative effect diminished at higher frequencies (3 Hz). Peak systolic [Ca\textsuperscript{2+}] was reduced in sub-epicardial and M cells but \textit{increased} in sub-endocardial cells in the heart failure group compared to controls. At higher stimulus frequencies, end diastolic Ca\textsuperscript{2+} levels were lower in sub-epicardial cells but higher in sub-endocardial myocytes of the heart failure group compared with controls. In general, changes were greater in heart failure animals with more severe in vivo ventricular dysfunction (ejection fraction \textless 44\%).

Conclusions: Heart failure was associated with an increased cell size throughout the left ventricle, but the form of the changes in electrophysiology and Ca\textsuperscript{2+} transient were dependent on the myocyte sub-type. In particular sub-endocardial cells displayed markedly different changes compared to the other myocyte sub-types. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Calcium (cellular); Heart failure; Hypertrophy; Membrane potential; Myocytes

1. Introduction

In both human heart failure and animal models chronic mechanical stress causes enlargement of myocytes (hypertrophy) and changes in their electrophysiological and mechanical properties [1,2]. These changes are thought to contribute both to the mechanical dysfunction and the increased risk of arrhythmias in failing myocardium [3].

Changes in myocyte shape depend on the nature of the mechanical stress within the heart [4], and there may also be differential changes depending on the transmural position of the myocyte [5]. Myocytes from the sub-endocardial and sub-epicardial regions of the mammalian left ventricular wall differ in cell size, electrical and mechanical properties [6,7]. A third sub-type with distinct electrophysiological characteristics, the M cell [8], has been found in the mid-myocardial region of the ventricle in human, dog, cat, rat and guinea-pig. No studies have
examined whether the electrophysiological changes during hypertrophy are common to all three ventricular myocyte sub-types. Prolongation of action potential duration appears to be a consistent feature of cells isolated from the sub-epicardial region of hypertrophied hearts [9–12]. However, conflicting results have been reported for cells isolated from sub-endocardial regions, ranging from a prolongation of action potential duration [11,13], no change [9] to a marked shortening [10].

Abnormalities of intracellular $[\text{Ca}^{2+}]$ accompany the electrophysiological changes associated with myocyte hypertrophy and may contribute to both the electrical and mechanical dysfunction in heart failure. $\text{Ca}^{2+}$ transients of reduced amplitude and prolonged time course are commonly observed in human heart failure [14,15] and in animal models [16,17]. However, several studies suggest either unchanged [18,19], or increased intracellular $[\text{Ca}^{2+}]$ in failing myocardium [16,20]. These inconsistencies may be attributed to interspecies variation or to different forms of response according to the underlying stimulus to hypertrophy or failure. An alternative hypothesis, that there are transmural differences in the hypertrophic response, is addressed in the present study.

2. Methods

2.1. Animal model

A well-characterised model of heart failure induced by chronic left ventricular infarction in the rabbit was used in this study [21–25]. Procedures were undertaken in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and conforms 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 1996). New Zealand White male rabbits aged approximately 12 weeks and weighing 2.5–3 kg were anaesthetised with fentanyl citrate (Hypnorm) and maintained with halothane and nitrous oxide/oxygen. A left thoracotomy was performed and the large circumflex branch of the left coronary artery was identified and ligated midway between the left atrial appendage and the cardiac apex. This gives rise to a large homogeneous infarct due to the limited collateral circulation in the rabbit. Sham-operated animals underwent thoracotomy with the heart manipulated in a similar fashion to the heart failure group but the artery was not tied.

Left ventricular function was assessed by echocardiography [21] [22] 8 weeks after surgery. The coronary ligated animals showed significant haemodynamic dysfunction in terms of increased left ventricular end-diastolic dimension (LVEDD) and left atrial dimension (LAD) and decreased ejection fraction (EF) (Table 1). Evidence of congestion was manifest in significant increases in lung and liver wet weight present at post-mortem examination. Previous work has shown that this animal model shows significant cardiac hypertrophy, evident as a 20–30% increase in heart weight and left ventricular dry weight [21,22]. In vivo haemodynamic measurements reveal a reduced cardiac output, raised end diastolic pressure and reduced response to an increased pre-load in this model [21]. Increased inducibility of arrhythmias and lowered fibrillation threshold observed in vitro [25] suggest accompanying electrophysiological dysfunction.

In the present study, the heart failure group displayed a range of haemodynamic dysfunction. EF ranged from 56 to 34% suggesting a non-uniform infarct size at the end of the 8 weeks post-ligation period. In a separate study, hearts from a different cohort of animals were sectioned and the infarct perimeter studied. This did show a large variation, which was correlated with the severity of the in vivo LV dysfunction (Burton and MacPhaden, unpublished observation). Consequently, the heart failure group was dichotomised on the basis of the median value of EF for the whole group (44%). The sub-group with an EF≤44% had greater mean LAD, LVEDD, liver and lung weights than the sub-group with an EF>44%, although these differences were not statistically significant.

2.2. Cell isolation

At 8 weeks post-operation, the rabbits were given an

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Mean echocardiographic parameters and post-mortem organ weights in sham and heart failure groups*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham (n=14)</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>3.5±0.02</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>71.6±1.5</td>
</tr>
<tr>
<td>LAD (mm)</td>
<td>11.4±0.3</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>17.0±0.5</td>
</tr>
<tr>
<td>Liver wet weight (g)</td>
<td>82.9±5.1</td>
</tr>
<tr>
<td>Lung wet weight (g)</td>
<td>12.0±0.4</td>
</tr>
</tbody>
</table>

* The heart failure (HF) group is subdivided on the basis of ejection fraction (EF) to those less than or equal to 44% (EF≤44%), and those greater than 44% (EF>44%). Left atrial dimension (LAD) and left ventricular ends diastolic dimension (LVEDD). Values are expressed as mean±S.E.M.

** Significant difference between Sham and HF group at the level of P<0.05. ** Significant difference between Sham and HF groups at the level of P<0.001.
intravenous injection of 500 U heparin together with an overdose of sodium pentobarbitone (100 mg/kg). Isolated hearts were perfused retrogradely (25 ml/min, 37°C) with a nominally Ca²⁺-free Krebs–Henseleit solution for 10 min. This was followed by perfusion with re-circulated Krebs–Henseleit solution supplemented with 0.6 mg/ml collagenase (type 1, Worthington Chemical Co.), 0.1 mg/ml protease (type XIV, Sigma Chemical Co) and 80 μM CaCl₂ for 10–17 min. The left ventricular free wall was isolated, and the infarct and neighbouring myocardium (2.5–3 mm border) was carefully dissected away. Based on measurements of the size of the border zone from a histological study [26], this procedure ensured that the remaining myocardium did not contain myocytes from the peri-infarct zone [27]. The sub-endocardial, mid-myocardial and sub-epicardial layers of the free wall were dissected from the remaining tissue by dissecting a 1–1.5-mm layer from the epicardial and endocardial surfaces. These two layers of tissue and the remaining intervening mid-myocardial layer were incubated separately for 5 min in enzyme solution containing 80 μM CaCl₂ and 4% bovine serum albumin (BSA, fraction V, Sigma). The cell suspensions obtained at the end of the incubation period were filtered into Krebs–Henseleit solution containing 0.1 mM CaCl₂ and 1.5% BSA, and the [Ca²⁺] was increased to 1.5 mM progressively over 30 min. The cells were transferred to Petri dishes containing Medium 199 (Gibco) plus supplements of taurine (5 mM), creatine (5 mM) and BSA (0.2%) and kept at room temperature until use. No difference in the percentage yield of cells was observed whether either myocardium from different regions or between experimental groups.

2.3. Experimental procedures

Myocytes were superfused with the physiological salt solution at 36–37°C in a chamber mounted on the stage of an inverted microscope. Transmembrane action potentials were recorded using 2 M KCl filled glass microelectrodes with resistances of 15–30 MΩ. Micro-electrode with similar characteristics have been used by other groups to study action potential characteristics of mammalian cardiac cells [9]; this configuration minimises intracellular dialysis by the electrode solution. Action potentials were elicited in bridge mode by injecting 2–5 ms threshold current pulses (Axoclamp 2A amplifier, Axon Instruments, Foster City, CA, USA). Membrane potential was recorded to magnetic tape (18.5 KHz bandwidth) for later offline analysis. Action potential duration was measured from the rapid upstroke of the AP following the stimulus to 90% (APD₉₀) repolarisation. Resting membrane potential, maximum upstroke velocity (Vₘ₉₀) and action potential amplitude were measured from the digitised signal using National Instruments A/D board (PC-Lab) controlled by CMAP software developed by Dr J. Dempster, (Strathclyde University). Resting membrane potential and action potential shape were stable over the duration of the measurements suggesting that the dialysis of the intracellular contents by KCl from the recording microelectrode was not significant.

In a separate group of experiments, simultaneous action potential and intracellular [Ca²⁺] transients were recorded in myocytes from sham and failing hearts. Intracellular [Ca²⁺] was measured from Fura-2 fluorescence signals using a dual wavelength spectrophotometric method described previously [28]. Fura-2 was incorporated into the cells as the acetoxymethyl (AM) ester, by incubating them with 3 μM Fura-2 AM at 37°C for 20 min. Minimal Fura-2 loading protocols were used and the recorded Ca²⁺ transients were averaged to give sufficient signal-to-noise ratio. This precaution was used to minimise the possibility of intracellular Ca²⁺ buffering by the indicator. The loaded cells were placed in the recording chamber and superfused with physiological saline. Fluorescence measurements (at >500 nm) from sequential illumination with light at 340 and 380 nm at 60 Hz were made using a spinning wheel spectrophotometer (Cairn Research Ltd). The ratio measurement of fluorescence (340:380 nm) provides a direct measure of intracellular [Ca²⁺] [29]. The minimum and maximum fluorescence ratios (Rₘᵢₙ and Rₘₐₓ) were determined using a previously published protocol [30]. The values of Rₘᵢₙ and Rₘₐₓ were not significantly different in cells from different regions or between sham and heart failure groups. These values were Rₘᵢₙ (sham) = 0.25 ± 0.02; Rₘᵢₙ (HF) = 0.24 ± 0.03; Rₘₐₓ (sham) = 4.3 ± 0.2; Rₘₐₓ (HF) = 4.4 ± 0.4. As with previous studies [17, 31] the range of Rₘᵢₙ and Rₘₐₓ values represents a much lower dynamic range than that measured in vitro, possibly due to additional fluorescence components from non-cytosolic forms of the indicator. The intracellular [Ca²⁺] was calculated assuming a dissociation constant of 200 nM [17, 32] in all cell types and in both experimental groups. The mean peak systolic Ca²⁺ and minimum diastolic Ca²⁺ were measured from each cell and expressed as mean values. Ca²⁺ transient duration was measured at 50% of the transient amplitude (CaD₅₀).

Cells were stimulated by progressive step increases in stimulus frequencies. Each test frequency was maintained until action potential and Ca²⁺ transients reached a steady state (1–5 mins), before increasing the test frequency. Only cells that returned to a stable baseline after this protocol were used. Measurements were made from averages of 56 sequential steady state records with 0.1, 0.3 1.0, 2.0 and 3.0 Hz stimuli. As described in detail in the results, cells isolated from the sub-endocardial and mid-myocardial regions exhibited electrophysiological characteristics of either M cells or sub-endocardial cells (see below). M cells were distinguished from other cell types by prolonged action potential duration at low stimulus rates and high rates of depolarisation. Some cells isolated from the mid-myocardium showed non-M cell (possibly sub-endocardial or sub-epicardial) characteristics. These cells were not included in the electrophysiological analysis.
In parallel with electrophysiological studies, cell length and width measurements using an eye-piece graticule were made on approximately 20 cells from the sub-endocardial, mid-myocardial and sub-epicardial regions in each animal. Cell dimension measurements did not allow sub-endocardial cells to be distinguished from M cells (see above). Therefore this group is termed ‘mid-myocardial myocytes’ to distinguish this group from cells which were identified as ‘M-cells from their electrophysiological characteristics. The dimensions of cells used for electrophysiological measurements were recorded separately.

2.4. Solutions

The Krebs–Henseleit solution used in the cell isolation contained, in mM: NaCl, 130; KCl, 5.4; NaH₂PO₄, 0.4; MgCl₂, 3.5; Heps, 5; taurine, 20; creatine, 10; and glucose, 11.1 (pH 7.25, equilibrated with 100% O₂). The superfusate solution used for the experiments contained, in mM: NaCl, 144; KCl, 5.4; NaH₂PO₄, 0.3; MgCl₂, 1; Heps, 5; glucose, 11.1 and CaCl₂, 1.8. All chemicals were obtained from Sigma with the exception of Fura-2 AM and DMSO (6) which were obtained from Molecular Probes and Fluka, respectively.

2.5. Statistical analysis

Measurements were made from cells from sub-endocardial, mid-myocardial and sub-epicardial regions of each heart as described above. The mean values of the electrophysiological and Ca²⁺ transient parameters from two or more cells from each sub-group (typically three) were calculated for each animal. These individual median values were used to calculate the average ± standard error of the mean (S.E.M.) for each experimental group based on the number of animals. This method was used instead of pooling all the measurements together, as this latter technique would give unequal weight to the hearts from which the most measurements were available [33]. Comparisons of values from different layers and from different experimental groups were performed using one-way ANOVA followed by Tukey-Kramer multiple comparisons post test. Correlations were examined by linear regression analysis. \( P<0.05 \) was considered significant.

### Table 2

Average left ventricular cell dimensions from sham and heart failure (HF) groups

<table>
<thead>
<tr>
<th></th>
<th>Cell dimensions (μm)</th>
<th>Sham</th>
<th>HF</th>
<th>HF(EF&gt;44%)</th>
<th>HF(EF≤44%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sub-epicardial cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( n_1 )</td>
<td></td>
<td>14</td>
<td>25</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>( n_2 )</td>
<td></td>
<td>344</td>
<td>276</td>
<td>146</td>
<td>130</td>
</tr>
<tr>
<td>Length (μm)</td>
<td></td>
<td>134.8±2.7</td>
<td>153.8±3.9**</td>
<td>150.4±5.8*</td>
<td>157.0±5.3**</td>
</tr>
<tr>
<td>Width (μm)</td>
<td></td>
<td>27.3±1.0</td>
<td>31.0±1.1*</td>
<td>30.6±1.4*</td>
<td>31.5±1.7*</td>
</tr>
<tr>
<td><strong>Mid-myocardial cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( n_1 )</td>
<td></td>
<td>14</td>
<td>25</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>( n_2 )</td>
<td></td>
<td>368</td>
<td>325</td>
<td>169</td>
<td>156</td>
</tr>
<tr>
<td>Length (μm)</td>
<td></td>
<td>135.1±3.8</td>
<td>155.8±4.3*</td>
<td>150.3±4.7*</td>
<td>157.7±7.6*</td>
</tr>
<tr>
<td>Width (μm)</td>
<td></td>
<td>26.4±1.3</td>
<td>28.8±1.1</td>
<td>28.0±1.5</td>
<td>29.5±1.6</td>
</tr>
<tr>
<td><strong>Sub-endocardial cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( n_1 )</td>
<td></td>
<td>14</td>
<td>25</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>( n_2 )</td>
<td></td>
<td>368</td>
<td>325</td>
<td>169</td>
<td>156</td>
</tr>
<tr>
<td>Length (μm)</td>
<td></td>
<td>140.4±2.6</td>
<td>151.9±4.2*</td>
<td>150.2±5.6*</td>
<td>154.0±4.2*</td>
</tr>
<tr>
<td>Width (μm)</td>
<td></td>
<td>29.5±1.1</td>
<td>31.8±1.2</td>
<td>31.1±1.4</td>
<td>32.5±2.0</td>
</tr>
</tbody>
</table>

\( n_1 \) is the number of animals, \( n_2 \) is the total number of cells in each experimental group. The mean and standard errors are calculated using animal number. The HF group is subdivided on the basis of ejection fraction (EF).

\( ^* \) Indicates significant difference between myocyte sub-type and sub-endocardial cells \( P<0.05 \). * Significant difference between Sham and HF groups at the level of \( P<0.05 \). ** Significant difference between Sham and HF groups at the level of \( P<0.001 \).
cardial myocytes (~108%). The effect was greater in cells from animals with severe left ventricular dysfunction (EF≤44%) for all regions. A significant increase in cell width (to ~110% of control) was observed only in sub-epicardial cells. The dimensions of cells used for measurements of membrane potential and intracellular [Ca$^{2+}$] ($\sim$30 cells per group, results not shown) were not significantly different to those made on the larger samples ($\sim$350 cells per group) from each of the three ventricular regions. In a separate study on the same animal model (M. McIntosh, unpublished observations), similar increases of cell length were accompanied by a significant increased cell electrical capacitance of both sub-endocardial (sham: 147±9 pF, n=11; HF: 188±9 pF, n=5) and sub-epicardial cells (sham: 129±7 pF, n=10; HF: 165±18 pF, n=5). This supports the conclusion that the cell shape changes are a result of cellular hypertrophy.

3.2. Action potential characteristics

Electrophysiological measurements were made on ~30 cells from each region in the sham and heart failure groups. About half of the cells in each group were loaded with Fura-2 AM for simultaneous measurement of intracellular [Ca$^{2+}$]. Comparison of action potential characteristics indicated no effects of loading cells with Fura-2. Resting membrane potential was not significantly different between the sham and heart failure groups in any myocyte sub-type at any frequency of stimulation, the results for 0.3 Hz stimulus rate are shown in Table 3. There were no significant differences in action potential amplitude (APA) between the sham and heart failure groups in any region, with the exception of the values in sub-epicardial cells at 0.3 Hz (Table 3). This pattern also applied when comparing sham values with the heart failure sub-group with severe ventricular dysfunction (EF≤44%, results not shown).

3.2.1. Upstroke velocity

As has been reported in a number of other mammalian species, the maximum rate of depolarisation ($V_{\text{max}}$) was significantly greater in M cells than in the other two myocyte sub-types over the range 0.3 to 3 Hz. The values measured at 0.3 Hz are shown in Table 3. Similarly, $V_{\text{max}}$ was significantly greater in M cells than the other two cell sub-types in the heart failure group (Table 3). There were no significant differences in $V_{\text{max}}$ values between sham and heart failure groups in corresponding ventricular regions, or in the sub-group with more severe ventricular dysfunction (EF≤44%, results not shown).

3.2.2. Action potential duration

Fig. 1 shows traces of action potentials and Ca$^{2+}$ transients recorded at 0.3 and 3 Hz from cells isolated from the three ventricular layers in the two experimental groups. The records have been normalised for amplitude to highlight the differences in action potential duration (APD$_{90}$) and Ca$^{2+}$ transient duration (CaD$_{50}$). The mean values are shown in Fig. 2, with the results from the HF group dichotomised on the basis of ejection fraction. Comparing APD$_{90}$ values from different regions within the sham group reveals that the APD$_{90}$ in sub-endocardial cells was longer than in sub-epicardial cells at 0.1–1 Hz stimulation frequency. The APD$_{90}$ of M cells were longer than those of sub-epicardial myocytes at stimulation frequencies 0.1–2 Hz and longer than those of sub-endocardial myocytes at 0.1 and 0.3 Hz. The prolonged APD$_{90}$ at low frequencies (and higher $V_{\text{max}}$) is characteristic of M cells in other mammalian species.

There is an overall increase in APD$_{90}$ in sub-epicardial cells and decrease in sub-endocardial cells in heart failure. These changes reduce the endo-epicardial difference in APD$_{90}$ values. In the severe ventricular dysfunction sub-group there was no significant difference in APD$_{90}$ between cells from sub-epicardial and sub-endocardial regions at all stimulation frequencies apart from 0.1 Hz. At this frequency the normal pattern is reversed and sub-epicardial APD$_{90}$ is now significantly longer than in sub-endocardial myocytes. M cell APD$_{90}$ was significantly longer than sub-epicardial and sub-endocardial cells at stimulus frequencies ≤1 Hz.

Comparing each region, the mean APD$_{90}$ of sub-epicardial cells in the HF group (EF ≤44%) was longer than those from sham hearts at all stimulation frequencies apart from 3 Hz. A similar trend was seen in the M cells from the HF group (EF ≤44%) but the difference was only significant at 1 and 2 Hz. In contrast, the APD$_{90}$ was shorter in sub-endocardial cells from the HF group compared with shams at 0.3 and 0.1 Hz.

### Table 3

<table>
<thead>
<tr>
<th>Sub-epicardial cells</th>
<th>Sham</th>
<th>HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>14</td>
<td>22</td>
</tr>
<tr>
<td>$E_a$ (mV)</td>
<td>-82.4±0.6</td>
<td>-80.3±0.9</td>
</tr>
<tr>
<td>APA (mV)</td>
<td>113.6±1.1</td>
<td>108.4±1.6</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (mV/s)</td>
<td>112.7±8.1</td>
<td>125.1±9.1*</td>
</tr>
<tr>
<td>M cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td>$E_a$ (mV)</td>
<td>-79.4±1.6</td>
<td>-83.4±0.8</td>
</tr>
<tr>
<td>APA (mV)</td>
<td>120.9±1.5</td>
<td>117.4±1.3</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (mV/s)</td>
<td>158±18</td>
<td>169.9±18</td>
</tr>
<tr>
<td>Sub-endocardial cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>$E_a$ (mV)</td>
<td>-80.9±0.5</td>
<td>-80.9±0.5</td>
</tr>
<tr>
<td>APA (mV)</td>
<td>117.8±0.8</td>
<td>107.5±2.1</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (mV/s)</td>
<td>126.2±6.6</td>
<td>122.0±4.9*</td>
</tr>
</tbody>
</table>

* $n$ is the number of animals in each experimental group. The mean and standard errors are calculated using animal number.

* Indicates significant difference between sub-group and M cell group at the level of $P<0.05$.

* Indicates significant difference between Sham and HF groups at the level of $P<0.05$. 
Fig. 1. Averaged and normalised records of action potential and associated Ca$^{2+}$ transients from single cardiac myocytes isolated from the left ventricle of sham and heart failure rabbit hearts (●). In all, 56 separate records (1-s duration) of simultaneous recordings of membrane potential and Fura-2 fluorescence were averaged from a single cell during stimulation at: (i) 0.3 Hz, and (ii) 3 Hz. Myocyte sub-types are shown above the record: panel A, sub-epicardial; panel B, M cell; and panel C, sub-endocardial cell.

3.2.3. Ca$^{2+}$ transient duration

Averaged Ca$^{2+}$ transient records were normalised by matching peak systolic and end diastolic fluorescence ratio values. Superimposed records from sub-epicardial, M and sub-endocardial cells isolated from sham and failing hearts are illustrated in Fig. 1. Mean durations are shown in Fig. 3, with the results from the heart failure group dichotomised on the basis of ejection fraction. Between different regions within the sham group, CaD$_{50}$ in sub-endocardial cells was significantly longer than in sub-epicardial cells at 0.1–1 Hz stimulation rates. The CaD$_{50}$ of M cells were longer than sub-epicardial myocytes and sub-endocardial cells at frequencies ≤1 Hz.

As with APD$_{90}$ values, there is an overall increase in CaD$_{50}$ in sub-epicardial cells and a decrease in sub-endocardial cells in heart failure. These changes reduce the endo-epicardial difference in CaD$_{50}$ values observed normally. In the sub-group with more severe ventricular dysfunction there was no significant difference in the CaD$_{50}$ between cells from sub-epicardial and sub-endocardial regions. However, CaD$_{50}$ of M cells were significantly longer than that of sub-epicardial and sub-endocardial cells at stimulus frequencies ≤1 Hz.

Between corresponding regions, the mean CaD$_{50}$ of sub-epicardial cells in the heart failure group (EF≤44%) was longer than those from sham hearts only at 1 Hz. There was no significant difference in CaD$_{50}$ of M cells from heart failure group. In contrast, the CaD$_{50}$ was shorter in sub-endocardial cells from the HF group compared with shams at 0.3 and 0.1 Hz.

3.2.4. Peak systolic and end diastolic [Ca$^{2+}$]

Fig. 4 shows the overall mean values (±S.E.M.) of peak systolic and end diastolic [Ca$^{2+}$]. All cells from sham hearts showed an increased peak systolic [Ca$^{2+}$] and end diastolic [Ca$^{2+}$] as stimulation frequency increased. Comparing these parameters from regions within the sham group revealed no significant difference between the three myocyte sub-types apart from a significantly higher peak systolic [Ca$^{2+}$] at 0.1 and 1 Hz in M cells. In the severe ventricular dysfunction sub-group the peak systolic [Ca$^{2+}$] was significantly higher in sub-endocardial cells than sub-epicardial and M cells. This shift in characteristics is due to the decreased peak systolic [Ca$^{2+}$] in sub-epicardial and M cells in the severe heart failure group while peak systolic [Ca$^{2+}$] is increased in sub-endocardial cells. In the sub-group of cells with low ejection fraction, end diastolic [Ca$^{2+}$] was significantly reduced in sub-epicardial cells stimulated at 2 and 3 Hz and significantly higher in sub-endocardial cells stimulated at 3 Hz.

3.3. Correlation between electrophysiological parameters and intracellular [Ca$^{2+}$]

As described above, the APD$_{90}$ and CaD$_{50}$ show a complex dependency on stimulation frequency in both sham and heart failure groups. In particular, non-uniform changes in these variables occur in sub-epicardial and sub-endocardial cells. At any one stimulation frequency, the heart failure group had an increased APD$_{90}$ and CaD$_{50}$ in sub-epicardial and M cells and a decreased APD$_{90}$ and
Fig. 2. The relationship between stimulus frequency and action potential duration at 90% repolarisation (APD\textsubscript{90}) in sub-epicardial cells (panel A), M cells (panel B) and sub-endocardial cells (panel C). Data are presented as mean (±S.E.M.) APD\textsubscript{90} for sham group and heart failure group dichotomised on the basis of ejection fraction (EF). * Indicates significant difference between myocyte sub-type and sub-endocardial cells. † Indicates significant difference between sub-type and M cells. ‡ Indicates significant difference between sham and heart failure groups. Number animals (n) are indicated in each panel. Note different scale of y-axis in panel B.

CaD\textsubscript{50} in sub-endocardial cells compared to the sham group. This suggests a linkage between APD\textsubscript{90} and CaD\textsubscript{50} in cells throughout the myocardium, which was confirmed by plotting APD\textsubscript{90} against CaD\textsubscript{50} for individual cells in the heart failure group at one stimulation frequency (0.3 Hz) as shown in Fig. 5A. There is a strong linear relationship common to cells from all three myocyte sub-types (r=...
Fig. 4. The relationship between stimulus frequency and peak systolic and end diastolic \([Ca^{2+}]\) in sub-epicardial cells (panel A), M cells (panel B) and sub-endocardial cells (panel C). Data is presented as mean (±S.E.M.) for sham group and heart failure group dichotomised on the basis of ejection fraction (EF). * Indicates significant difference between myocyte sub-type and sub-endocardial cells. Indicates significant difference between sub-type and M cells. Indicates significant difference between sham and heart failure groups. Number of animals (n) are indicated in each panel.

0.93, P<0.001). A similar relationship exists for cells from sham hearts and for both groups at other stimulation frequencies, however the correlation was weaker at higher stimulation frequencies due to a limited range of values.

Another association to be made is the relationship between the CaD\(_{50}\) and the amplitude of the Ca\(^{2+}\) transient. As shown in Fig. 5B, a higher peak systolic \([Ca^{2+}]\) is correlated with a shorter Ca\(^{2+}\) duration in cells from the three ventricular regions. While sub-endocardial cells and sub-epicardial cells appear to have a similar relationship, M cells have significantly prolonged CaD\(_{50}\) values for a comparable peak systolic \([Ca^{2+}]\).

4. Discussion

The aim of this study was to characterise and interrelate the effects of chronic myocardial infarction on cell size, electrophysiology and intracellular \([Ca^{2+}]\) in ventricular myocyte sub-types.
4.1. Cell size

The epicardial cell length difference evident in sham hearts was not present in the heart failure group where cells from all regions were 150–155 μm long. In this model, the hypertrophic stimulus to sub-epicardial and mid-myocardial cells results in a greater increase in length (~115%) than in sub-endocardial cells (~108%). The same baseline pattern and extent of changes in cell dimension have been observed in the cardiac hypertrophy model associated with hyperthyroid-induced volume overload [5]. In contrast, pressure overload hypertrophy in response to hypertension results in a marked increase in cell cross sectional area but no increase in cell length [34–36], and the increase in cross sectional area was greatest in the endocardial region [35]. Therefore, the site and form of cellular hypertrophy appears to be dependent upon the nature of the pathological stress. In human heart failure, increases in both cell length and width are observed [37] with an approximately 60% lengthening of myocytes observed in dilated cardiomyopathy [38]–a greater degree of hypertrophy than that observed in this study.

4.2. Electrophysiology of normal (sham) myocytes

The shorter APD90 in myocytes from the sub-epicardium compared to sub-endocardial cells reported here confirms earlier work on rabbit [39,40] and other mammalian species [8]. Work on larger mammals has indicated that sub-epicardial cells possess a characteristic spike and dome action potential morphology [5,41]. Although action potentials with this morphology were recorded in rabbit myocytes, they were not exclusive to the sub-epicardial region. APD90 in sub-epicardial cells remained significantly shorter than in sub-endocardial cells over most of the frequency range studied (0.1–2 Hz). Sub-epicardial myocyte APD90 increased with frequency of stimulation in this and a previous study in rabbit [42], in contrast to the flat rate dependence observed in sub-endocardial cells [39,43].

A distinct myocyte type was observed in samples taken from the sub-endocardial and the mid-myocardial regions. These cells were characterised by an extremely long APD20 of mainly sub-endocardial cells. Cells from all regions were 150–155 μm long. So in isolated myocytes from the rabbit, although recordings from M cells in the rabbit ventricular slice preparation have been reported [44]. The finding of mixed populations of M cells and sub-endocardial cells in the isolates from the sub-endocardial and mid-myocardial regions has important implications, since it cannot be assumed that cells isolated from these regions form a homogeneous population. Characterisation of each cell by its action potential characteristics is necessary to avoid confusion.

4.3. Electrophysiology of heart failure myocytes

The most striking finding of the present study is the transmural difference in the hypertrophic response to heart failure, resulting in opposite changes in the characteristics of sub-epicardial and M cells compared with sub-endocardial cells.

As summarised in Table 4, APD90 was prolonged in sub-epicardial and M cells in heart failure. The effect was more pronounced in cells from hearts with severe left ventricular dysfunction (EF ≤44%). The results are consistent with epicardial monophasic action potential measurements made in Langendorff perfused preparations of this heart failure model [45]. The large variability of M cell APD90 reduces the ability to distinguish differences between cells from the sham and heart failure groups. However, a statistically significant prolongation in APD90 was observed at 1 and 2 Hz in M cells belonging to the sub-group of rabbits with severe left ventricular dysfunction.

Prolongation of APD is the most common observation in previous studies on myocytes isolated from animal models and from failing human hearts [2]. The origin of the myocytes is not specified in the majority of these studies. However, the few animal studies using myocytes from specific ventricular regions have reported prolonged APDs in sub-epicardial myocytes [9–12]. No previous study has specifically examined M cells in heart failure. Cells from the mid-myocardium of mildly hypertrophied guinea-pig left ventricle exhibited a prolonged APD, but as noted by

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Table 4

Summary of changes in action potential and Ca²⁺ transient characteristics in the rabbit model of heart failure

<table>
<thead>
<tr>
<th>Changes observed in heart failure group*</th>
<th>Sub-epicardial cells</th>
<th>M-cells</th>
<th>Sub-endocardial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Action potential duration</td>
<td>↑*</td>
<td>↑*</td>
<td>↓*</td>
</tr>
<tr>
<td>Ca²⁺ transient duration</td>
<td>↑*</td>
<td>↑*</td>
<td>↓*</td>
</tr>
<tr>
<td>Peak systolic [Ca²⁺]</td>
<td>↓*</td>
<td>↓*</td>
<td>↑*</td>
</tr>
<tr>
<td>End diastolic [Ca²⁺]</td>
<td>↔</td>
<td>↔</td>
<td>↑*</td>
</tr>
</tbody>
</table>

* Increases and decreases in the parameters are indicated by ↑ and ↓, respectively. ↔ indicates no significant change.

* Indicates that significant differences between sham and heart failure group was only evident at low stimulus frequencies (0.1 and 0.3 Hz). " Indicates that significant differences between sham and heart failure groups were evident at high stimulus frequencies (2–3 Hz).
the authors [9], the action potential did not exhibit the increased \( V_{\text{max}} \) and long APD characteristic of M cells.

In contrast to the changes in sub-epicardial and M cells, sub-endocardial myocytes from the heart failure group in the present study showed consistently shorter APD values than in the sham group. Sub-group analysis indicated that more severe failure was associated with a more pronounced shortening of APD sub-epicardial cells (statistically significant at 0.1 and 0.3 Hz), while the APD values in mild failure were very similar to the sham values. Thus the shortening of APD sub-epicardial in sub-endocardial cells is dependent on the severity of ventricular dysfunction. A previous study has reported shorter APD values associated with hypertrophy in sub-endocardial cells of the rat [10].

### 4.4. Intracellular Ca\(^{2+}\) Transients in Normal (sham) Myocytes

The time-course of the Ca\(^{2+}\) transients recorded in this study are similar to those observed in previous reports on isolated rabbit [46] and guinea-pig myocytes [17] using the Ca\(^{2+}\) indicator Fura-2. Apart from an increase at 0.3 Hz, CaD sub-epicardial values shortened as stimulation frequency was increased in all cell types. This shortening of CaD sub-epicardial paralleled the APD sub-epicardial shortening observed in M cells, but was in contrast to the lengthening of APD values in sub-epicardial cells and the flat APD sub-endocardial-frequency response in sub-endocardial cells. The close correlation between APD sub-epicardial and CaD sub-epicardial across the frequency range for M cells is as expected from voltage clamp studies in isolated rat myocytes, which show that shortening the clamp duration reduced the Ca\(^{2+}\) transient duration [47]. This effect is thought to be mediated by the sarcolemmal Na\(^+\)/Ca\(^{2+}\) exchanger. However, other membrane currents appear to be important in determining the relationship between APD sub-epicardial and CaD sub-epicardial at different stimulus frequencies in sub-epicardial and sub-endocardial rabbit myocytes.

In all myocyte sub-types, peak systolic [Ca\(^{2+}\)] and end diastolic [Ca\(^{2+}\)] increased as stimulus frequency increased. The values of end diastolic [Ca\(^{2+}\)] and peak systolic [Ca\(^{2+}\)] (at 0.3 Hz stimulation) were similar to those recorded at 0.5 Hz using Indo-1 in rabbit myocytes [48] and guinea pig myocytes at 1 Hz stimulation [17]. No significant differences in peak systolic and end diastolic [Ca\(^{2+}\)] were obvious in any of the sham myocyte sub-types, in agreement with previous work [7]. However, there was a tendency for a lower systolic [Ca\(^{2+}\)] in sub-endocardial cells, which is consistent with a lower intracellular sodium concentration in sub-endocardial cells [40]. The results of the current study are in contrast to in situ intracellular [Ca\(^{2+}\)] measurements in rat heart [49] indicating that peak systolic [Ca\(^{2+}\)] and end diastolic [Ca\(^{2+}\)] was higher in the sub-endocardial region than the sub-epicardial region. This difference may have a basis in the marked APD differences between rat and rabbit myocytes, or alternatively, the differential mechanical stresses experienced in the sub-endocardial and sub-epicardial regions in situ compared to dissociated single cells.

### 4.5. Intracellular Ca\(^{2+}\) Transients in Heart Failure

As summarised in Table 4, sub-epicardial and M cells from this rabbit model of heart failure appear to conform to the generally observed hypertrophic behaviour of prolonged action potential and Ca\(^{2+}\) transient duration, coupled with a reduced peak systolic [Ca\(^{2+}\)] and a poor inotropic response to increased stimulus frequency [1]. These results are in contrast to those from sub-endocardial cells in heart failure, which displayed a shortened CaD sub-endocardial and increased peak systolic [Ca\(^{2+}\)] in comparison to sham hearts. Also, as indicated in Fig. 5B, the cells with shortened CaD sub-endocardial are associated with an increased peak systolic Ca\(^{2+}\) and appear to belong to the same relationship observed in sub-epicardial cells. This is thought to be due in part to the relationship between the rate of Ca\(^{2+}\) uptake by the SR and systolic [Ca\(^{2+}\)] [30,50]. A combination of decreased action potential duration and increased Ca\(^{2+}\) transient amplitude noted in sub-endocardial cells from heart failure group is observed experimentally in raised extracellular Ca\(^{2+}\) [51] or isoprenaline [52]. This suggests that an increased Ca\(^{2+}\) release from the SR may be the primary change that alters the action potential duration via Ca\(^{2+}\) activated currents. Further work is required to determine the cellular basis of the action potential and Ca\(^{2+}\) transient changes observed in the sub-endocardial cells of this heart failure model.

In summary, myocytes from the heart failure group displayed marked differences in APD sub-epicardial and CaD sub-epicardial when compared to the sham group (Table 4). At higher stimulus rates (3 Hz), however, the differences were not significant, although small differences may exist which cannot be detected because of the inherent variability of single cell measurements. In support of this, epicardial monophasic action potential and Ca\(^{2+}\) transient duration were approximately 20 ms longer in the heart failure group in Langendorf perfused hearts from the same rabbit model (3 Hz stimulation rate) [22,45]. This is comparable to the differences in APD sub-epicardial and CaD sub-epicardial values observed in sub-epicardial cells in this study and suggests that small APD and CaD duration differences persist in epicardial cells at physiological stimulus rates. The present study indicates that heart failure related differences in APD persist in sub-epicardial cells isolated from the electrotonic interaction of adjacent areas of myocardium. Furthermore, the improved optical signals from single cell experiments allowed a direct quantification of diastolic and systolic [Ca\(^{2+}\)] indicating that the APD differences were accompanied by changes in diastolic and systolic intracellular [Ca\(^{2+}\)].

Unlike APD sub-epicardial and CaD sub-epicardial measurements, the differences in peak systolic Ca\(^{2+}\) in the heart failure group were most
prominent at the highest stimulus rates. This study is the first to report contrasting changes in peak systolic Ca$^{2+}$ in myocytes from different sites of failing myocardium. Sub-endocardial cells from the heart failure group displayed an increased peak systolic [Ca$^{2+}$] in contrast to the decreased values observed in sub-epicardial cells and M cells. These results may explain the previous range of disparate results in the literature including both a decreased [14,53,54] and enhanced [16,20] peak systolic [Ca$^{2+}$]. Studies on trabeculae or papillary muscles would be using myocardium that is predominately sub-endocardial, while in a dissociated left ventricle, only approximately 15% of the cells would be of sub-endocardial origin [55,56].

4.6. Electrical and mechanical consequences

It is difficult to predict the net effect of differential changes in ventricular action potentials and Ca$^{2+}$ transients on the electrical stability and mechanical function of the whole heart. At physiological stimulus rates (~3 Hz), the APD$_{90}$ and CaD$_{50}$ values were similar to normal. As discussed above, the inherent variability of single cell measurements may prevent small consistent changes from being observed. The results of this study indicate that the normal endocardial–epicardial differences in APD$_{90}$ would be reduced in heart failure particularly at sub-physiological heart rates. This feature has been observed in other heart failure models [9], the resulting altered pattern of transmural repolarisation may have pro-arrhythmic consequences. Among the postulated mechanisms for arrhythmogenesis in heart failure are single cell arrhythmic mechanisms, particularly triggered activity due to early or delayed after depolarisations [57]. These events may be more frequent in cells with increased intracellular [Ca$^{2+}$]. Based on the results presented in this study, the increased intracellular [Ca$^{2+}$] observed in sub-endocardial cells in heart failure may predispose these cells to arrhythmic events. In support of this, Pogwizd [58] showed that the sub-endocardial region was the site of premature ventricular complexes in a rabbit model of heart failure; separately Vermeulen et al. [43] demonstrated delayed after-depolarisations in surface cells of papillary muscles from failing hearts. The larger than normal Ca$^{2+}$ transients observed in failing sub-endocardial cells in the present study suggest an increased SR Ca$^{2+}$ content. This can arise from altered sarcolemma Ca$^{2+}$ flux pathways or an up-regulation of SR Ca$^{2+}$ overload in areas (e.g. sub-endocardium) where the Ca$^{2+}$ transient amplitude is increased in sub-endocardial cells in the heart failure group. However, Ca$^{2+}$ transient amplitude was reduced in sub-epicardial and M cells, so the overall change in the Ca$^{2+}$ transient within the viable myocardium of the left ventricle will depend on the proportion of these three myocyte types. Based on estimates from other species [55,56], M cells and sub-epicardial cells may constitute 60–75% of the left ventricular free wall [8]. However, no estimates exist for rabbit heart. Also, the contractility of the myocyte depends critically on the properties of the myofilaments. However, studies on the rabbit infarct model have failed to reveal altered myofilament Ca$^{2+}$-sensitivity or force production [59].

In summary, the two novel findings of the present study are: (1) In a chronic myocardial infarct model, sub-endocardial cells of the left ventricle showed an increased amplitude of the Ca$^{2+}$ transient and a shortened action potential duration. (2) M cells, identified by electrophysiological criteria, showed a decreased Ca$^{2+}$ transient amplitude and prolonged action potential duration. Similar changes in Ca$^{2+}$ transient and action potential characteristics were observed in sub-epicardial cells. The extent of these changes was related to the severity of left ventricular dysfunction in vivo. Comparable information concerning endocardial-epicardial differences in human heart failure is not available. The marked regional differences in rabbit myocardium described in this study may represent a distinct stage in heart failure relatively early (8 weeks) after the formation of an infarct and in the absence of underlying hypertension.

The primary cause of reduced intracellular Ca$^{2+}$ observed in human heart failure and in most animal models is thought to be depressed sarcoplasmic reticulum (SR) function [60], although a variety of Ca$^{2+}$ handling proteins are known to be affected: in particular the Na/Ca$^{2+}$ exchanger [61]. There is a widespread interest in the SR as a target for therapeutic intervention in heart failure [62]. The results of this study suggest that up-regulation of SERCA2 function may restore cardiac contractility by increasing the amplitude of the Ca$^{2+}$ transient in M cells and sub-epicardial cells. However, this might also result in SR Ca$^{2+}$ overload in areas (e.g. sub-endocardium) where function was normal or even up-regulated, predisposing these areas to the development of after-depolarisations and triggered arrhythmias.

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