Imperial College London

The Effects of Adult Progenitor Cell Transplantation on Recipient Cardiomyocyte Excitation-Contraction Coupling

A thesis submitted for the examination for the transfer of status to the degree of Doctor of Philosophy

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14th August 2008
Abstract

Cell transplantation is a promising strategy for treating heart failure but the mechanisms effecting functional improvements remain unknown. The hypothesis that cell transplantation influences the contractile properties and excitation-contraction (EC) coupling of recipient cardiomyocytes by paracrine mechanisms was tested.

Adult rats underwent myocardial infarction and subsequently developed chronic heart failure. They then received intra-myocardial injections of either skeletal myoblasts or bone marrow mononuclear cells which were harvested from transgenic rats constitutively expressing green fluorescent protein. Four weeks after injection, both cell types increased ejection fraction and reduced cardiomyocyte size. Isolated cardiomyocytes emitted low levels of green fluorescence, indicating that they originated from the recipient heart. The cardiomyocytes were then studied using sarcomere length measurements, indo-1 fluorescence and whole-cell patch-clamping techniques. Injection of either bone marrow cells or skeletal myoblasts normalized the impaired contractile performance and the prolonged time-to-peak of the Ca$^{2+}$ transients that were observed in failing cardiomyocytes. The smaller and slower L-type Ca$^{2+}$ current observed in heart failure returned to normal values after skeletal myoblast, but not bone marrow mononuclear cell, transplantation. Analysis of Ca$^{2+}$ sparks in isolated cardiomyocytes using confocal microscopy revealed that SR Ca$^{2+}$ leak had increased in failing cardiomyocytes, but was normalized by skeletal myoblast transplantation.

In order to test the hypothesis that these effects observed \textit{in vivo} are mediated by paracrine substances secreted from the transplanted cells further experiments were performed. Cardiomyocytes were isolated from failing hearts and cultured for 48 hours. Co-culturing with either skeletal myoblasts or bone marrow mononuclear cells during this period improved cardiomyocyte contraction and Ca$^{2+}$ handling.
This effect was maintained even when the different cell populations were mechanically separated by means of a porous membrane, demonstrating that cell-to-cell contact was not required and that soluble substances mediated the effect. Analysis of the supernates obtained from these co-culture experiments identified four candidate substances as possible mediators, but confirmation of their importance requires further experimental investigation.

In addition to the work described above, experiments were performed during the preparation of the whole-cell patch-clamping system. The system was tested by measuring the Na\(^+\)/Ca\(^{2+}\) exchanger current densities in cardiomyocytes isolated from normal rat hearts. Using this system the acute effects of various β-adrenergic agonists was assessed. The results obtained from this separate study are presented in Chapter 6.
Declaration

The study presented in this thesis represents my own work, except where specified. This thesis has not been submitted for a degree to any other academic institution. Some of the experimental findings have been published in scientific journals as abstracts, as below.


Acknowledgements

I am eternally indebted to my supervisor, Dr. Cesare Terracciano, for his kind guidance during my study. Not only did Cesare teach me cell electrophysiology, he continually provided me with invaluable moral support as well as practical suggestions throughout my work. Cesare also performed some of the cell electrophysiology experiments described in Chapter 3.

I would like to express my respect and gratitude to Professor Sir Magdi Yacoub. As well as being an enormous source of inspiration, he has supervised my progress with great interest and has offered valuable suggestions on numerous occasions.

I thank Dr. Mark Stagg, our post-doctoral research fellow, for all his help and advice. Mark performed some of the intracellular pH measurement experiments and calcium sparks studies in Chapter 3, and also wrote the computer script used for the analysis of calcium sparks. I thank my fellow Ph.D. student, Mr. Gopal Soppa. Gopal performed some of the indo-1 fluorescence experiments in Chapter 3. I am particularly grateful to Aalya Malik, who helped me adjust in the laboratory and taught me about cardiac cell electrophysiology. My thanks also go to Dr. Urszula Siedlecka for all her assistance with laboratory work, particularly during the latter days of my study. I am grateful to Dr. Satsuki Fukushima for his initial guidance in operative techniques.

I would like to thank The Magdi Yacoub Institute and The Wellcome Trust for their financial support throughout my study.

I express my gratitude and love to my dear wife, Jaewon. During my years of study Jaewon always offered endless comfort and supported me with great kindness and understanding.
To my parents
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Abbreviations

AC  adenyl cyclase
ACE  angiotensin-converting enzyme
AM  acetoxyethyl (ester)
ANOVA  analysis of variance
AP  action potential
APD  action potential duration
APD$_{50}$  time to 50% repolarization of the action potential
APD$_{90}$  time to 90% repolarization of the action potential
ATP  Adenosine triphosphate
$\beta_{i}$  intracellular buffering capacity
bFGF  basic fibroblast growth factor
$\left[Ca^{2+}\right]_{i}$  intracellular concentration of calcium
$\left[Ca^{2+}\right]_{o}$  extracellular concentration of calcium
CABG  coronary artery bypass graft
cAMP  3',5'-adenosine monophosphate
CaMKII  calmodulin-dependent protein kinase II
CAPSO  3-cyclohexylamino-2-hydroxy-1-propane-sulphonic acid
CICR  calcium induced calcium release
cSEVC  continuous single electrode voltage clamp
DHP  dihydropyridine
DHPR  dihydropyridine receptor. Also known as L-type calcium channel
DMEM  Dulbecco’s modified Eagle’s medium
dSEVC  discontinuous single electrode voltage clamp
$E_{m}$  membrane electrical potential
EC Coupling  excitation-contraction coupling
ECG  electrocardiography
ECL  enhanced chemiluminescence
ECM  extracellular matrix
eGFP  enhanced green fluorescent protein
EGTA  ethylene glycol bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
EF  ejection fraction (of the left ventricle of the heart)
EIPA  5-(N-ethyl-N-isopropyl)amiloride
ELISA  enzyme-linked immunosorbent assay
EPC  endothelial progenitor cell
FBS  foetal bovine serum
FKBP12.6  FK506-binding protein 12.6
FWHM  full width at half-maximal
FDHM  full duration at half-maximal
\( g \)  grammes
\( g \)  gravity
GFP  green fluorescent protein
\( G_i \)  inhibitory guanosine triphosphate binding protein
\( G_s \)  stimulatory guanosine triphosphate binding protein
HBSS  Hank’s balanced salt solution
HEPES  \( N\)-2-hydroxyethylpiperazine-\( N\prime\)-2-ethansulphonic acid
HGF  hepatocyte growth factor
HRP  horse radish peroxidase
\( I_{Ca,L} \)  L-type calcium current
\( I_{K1} \)  inward rectifier potassium current
\( I_{Kr} \)  delayed rectifier potassium current
\( I_{NCX} \)  sodium-calcium exchanger current
\( I_p \)  sodium-potassium ATPase current
\( I_{to} \)  transient outward current
IGF–1  insulin-like growth factor-1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>IL–1α</td>
<td>interleukin-1α</td>
</tr>
<tr>
<td>κ</td>
<td>decay rate constant</td>
</tr>
<tr>
<td>LAD</td>
<td>left anterior descending artery</td>
</tr>
<tr>
<td>LV</td>
<td>left ventricle</td>
</tr>
<tr>
<td>MCP</td>
<td>monocyte chemoattractant protein</td>
</tr>
<tr>
<td>MES</td>
<td>2-((N-morpholino)ethanesulphonic acid</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cell, marrow-derived stromal cell</td>
</tr>
<tr>
<td>NCX</td>
<td>sodium-calcium exchange</td>
</tr>
<tr>
<td>NHE</td>
<td>sodium-hydrogen exchange</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>NYHA</td>
<td>New York Heart Association</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>personal computer</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>pH</td>
<td>potential of hydrogen</td>
</tr>
<tr>
<td>pHᵢ</td>
<td>intracellular potential of hydrogen</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase-A</td>
</tr>
<tr>
<td>PMT</td>
<td>photomultiplier tube</td>
</tr>
<tr>
<td>PTX</td>
<td>pertussis toxin</td>
</tr>
<tr>
<td><strong>real-time PCR</strong></td>
<td>real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>S.E.M</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarcoplasmic &amp; endoplasmic reticulum calcium ATPase</td>
</tr>
<tr>
<td>SNARF</td>
<td>5-(and-6)-Carboxy-SNARF®-1</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>sry</td>
<td>sex-determining region of the Y-chromosome</td>
</tr>
</tbody>
</table>
\( \tau \) decay time constant

\( T_{\text{peak}} \) time to peak (of a transient or a contraction)

\( T_{50} \) time to 50\% of return to a baseline value

\( T_{90} \) time to 90\% of return to a baseline value

TIMP tissue inhibitor of metalloproteinase

TMB 3,3\',5,5\'-Tetramethylbenzidine

VAD ventricular assist device

VEGF vascular endothelial growth factor
Chapter 1

Introduction

1.1 Heart failure

“A state in which the heart fails to maintain an adequate circulation for the needs of the body despite a satisfactory filling pressure” – Paul Wood, 1950.

“Ventricular dysfunction with symptoms” – ANON.

1.1.1 The clinical syndrome

The pattern of symptoms resulting from heart failure depend on which side of the heart is affected (Braunwald 2007). If the left ventricle is performing inadequately blood flow becomes stagnant in the lungs, leading to worsening shortness of breath (dyspnoea) and fatigue. In comparison, symptoms of right-sided heart failure include peripheral œdema, ascites, and those relating to congestive liver failure. It is common for patients to have deteriorating function on both sides of the heart, either because of shared underlying pathology, or because failure of one side of the heart can impose a mechanical burden on the other: congestive cardiac failure.

The manifestations of heart failure are progressive (Pfeffer & Braunwald 1990),
and in its advanced stages can be extremely debilitating. In up to one third of pa-
tients dyspnoea and fatigue can limit exercise tolerance to walking only a few yards. Patients may end up severely dyspnoeic even at rest, and find that they cannot maintain a supine position (orthopnoea). \textit{Paroxysmal nocturnal dyspnoea} is a night time attack of severe breathlessness, usually several hours after going to sleep.

Other symptoms of heart failure include lack of appetite and weight loss, nocturia, impaired cognition, and an increased susceptibility to chest infections. The symptoms lead to a poor overall quality of life, when compared to other chronic diseases (Hobbs et al. 2002). The prognosis for congestive heart failure has been found to correlate closely with decreasing left ventricular ejection fraction, and is very poor in the end stages of the disease (McKee et al. 1971, Ho et al. 1993). Approximately 43% of men and 36% of women die within 1 year of initial diagnosis (Rector & Cohn 1994). The 5 year mortality rates are 75% and 62%, respectively (Levy et al. 2002). To put this in context, congestive heart failure carries a worse prognosis than many common malignancies including those of breast, colorectal, renal, and prostate. Of the deaths in patients with heart failure up to 50% are sudden (\textit{sudden cardiac death}, or SCD). Alternatively, it may be due to worsening pump failure. The proportion due to the latter gradually increases during the later stages of the disease (The DEFIBRILAT Study Group 1991).

\subsection*{1.1.2 Epidemiology}

Heart failure is a common disease in Western countries and represents an enormous burden to society. In the United Kingdom, at least 24,000 people die from the disease annually\textsuperscript{1}. Approximately 2\% of the National Health Service budget is consumed by heart failure, and it accounts for at least 5\% of admissions to general medical and geriatric wards in British hospitals (Davis et al. 2000). These figures are steadily increasing despite advances in medication therapy, probably due to the prevalence of hypertension and obesity in the community and also the large numbers

\textsuperscript{1}Source : British Heart Foundation; The Hillingdon Heart Failure Study 2001
of survivors of acute myocardial infarction who have been spared death in the acute phase (Braunwald 1997).

Heart failure is a disease predominantly affecting the elderly population. A study carried out in Scotland revealed that 78% of patients admitted with heart failure were aged 65 years or more (McMurray et al. 1993). The same study and others showed that men and women are equally at risk (MacIntyre et al. 2000). However, there have also been reports suggesting that the incidence might be higher in men than in women (Cowie et al. 1999). Studies in the United Kingdom (Lip et al. 1997) and those in North America (Dries et al. 1999) showed that black men and women are at a $33 \sim 50\%$ higher risk of developing heart failure.

### 1.1.3 Aetiology

There are many underlying causes for heart failure. In general, heart failure results when there is a loss of cardiomyocytes from the heart (Olivetti et al. 1995), usually with alterations in their mechanical properties (Beuckelmann et al. 1992) to a degree that adequate cardiac output cannot be maintained. It has been suggested that cardiomyocytes are lost at a background rate in normal individuals, and that the rate of loss increases with age (Olivetti et al. 1995). Certain pathological conditions may accelerate this process, either acutely or chronically. There is no doubt that myocardial infarctions cause a substantial loss of cardiomyocytes acutely. However, coronary artery disease can impair the performance of the ventricle in the long term, even without acute infarctions (Cowie et al. 1999).

In patients with long-term hypertension, the development of hypertrophy is well recognized. In addition, it has been postulated that apoptotic cell loss may be exacerbated in such cases (Bing 1994). In Western societies including the United Kingdom myocardial infarction and hypertension are thought to be the two leading causes of the increasing incidence of heart failure. In developing countries, other causes such as rheumatic heart disease are also a common cause (Amoah & Kallen 2000).
In addition to these factors, there are numerous other pathologies that are recognized as causes of heart failure (Oakley 1997, Lip et al. 2000, Jessup & Brozena 2003, Cowie et al. 1999). These are summarized in Table 1.1 on page 24. It is worth noting that in many patients these factors often co-exist. For example, many patients who have suffered a myocardial infarction often also have hypertension. Some of the identified risk-factors for coronary artery disease are now also recognized as being independent risk factors for heart failure. These include, for example, diabetes mellitus, cigarette smoking, and obesity (the Framingham Heart Study, Kenchaiah et al. 2002). The recent recognition from this study that obese people had double the risk of developing heart failure compared to those with a normal body mass index is of particular importance, given the endemic and increasing prevalence of obesity in Western countries.
<table>
<thead>
<tr>
<th>Table 1.1: The causes of heart failure</th>
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<tbody>
<tr>
<td>Ischaemic heart disease</td>
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<tr>
<td>Hypertension</td>
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<td>Rheumatic heart disease</td>
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<tr>
<td>Valvular heart disease</td>
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<tr>
<td>Arrhythmias</td>
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<td>Excessive consumption of alcohol</td>
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<tr>
<td>Connective tissue disorders (e.g. SLE, scleroderma)</td>
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<tr>
<td>Structural abnormalities within the heart (septal defects)</td>
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<tr>
<td>Cardiomyopathies</td>
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<tr>
<td>- Familial dilated</td>
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<tr>
<td>- Familial hypertrophic</td>
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<tr>
<td>- Restrictive (e.g. amyloidosis, sarcoidosis)</td>
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<td>- Obstructive</td>
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<tr>
<td>Infection</td>
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<tr>
<td>- Viral (e.g. coxsackie B, cytomegalovirus, HIV)</td>
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<td>- Rickettsia</td>
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<td>- Bacteria (e.g. diphtheria)</td>
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<td>- Mycobacteria</td>
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<td>- Fungus</td>
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<td>- Parasites (e.g. Chagas’ disease, toxoplasmosis)</td>
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<td>Muscular dystrophy</td>
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<td>Cardiotoxic drugs (e.g. adriamycin, doxorubicin)</td>
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<td>Endocrine disease (e.g. myxoedema, thyrotoxicosis, acromegaly)</td>
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<td>Idiopathic</td>
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**Abbreviations:** SLE systemic lupus erythematosus. HIV human immunodeficiency virus.
1.1.4 Ventricular remodeling

The tissue necrosis and inflammation that occurs during the first few hours of a myocardial infarction is followed by a phase of scar formation that is completed over several weeks. During the period of resorption of necrotic tissue, but before there has been extensive deposition of collagen, the transient decrease in tensile strength can result in the infarcted region becoming thinner and longer. This phenomenon is called infarct expansion (Hutchins & Bulkley 1978, Jugdutt & Michorowski 1987), and is not accompanied by additional cardiomyocyte death. As can be seen in Fig. 1.1, infarct expansion directly increases the LV dimensions.

In addition to infarct expansion, the non-infarcted region also undergoes gradual lengthening, even at sites remote to the infarcted area. This was first noted in canine models of myocardial infarction (Theroux et al. 1977) and subsequently confirmed in patients monitored after myocardial infarction using echocardiography (Erlebacher et al. 1982). These two changes both contribute towards LV dilation, and can initially enable the LV to maintain a normal stroke volume despite a decreased ejection fraction (Pfeffer & Braunwald 1990). However, this dilation substantially increases...
the LV wall stress throughout the cardiac cycle (by the Law of LaPlace\(^2\)), which then stimulates further ventricular enlargement (Grossman et al. 1975). A vicious cycle can be entered in which initial dilatation begets dilatation. The ventricle also often becomes more spherical in its shape as it dilates, and the collective pattern of alterations to the ventricular architecture is often referred to as ventricular remodeling (Braunwald & Pfeffer 1991).

Remodeling has been found to be associated with hypertrophy of cardiomyocytes (Olivetti et al. 1991). Although cardiomyocyte hypertrophy in such circumstances can help maintain wall tension, it is associated with maladaptive changes in excitation-contraction coupling (EC coupling) and dynamics of cardiomyocyte contraction and relaxation (these are discussed further in section 1.3). Consequently, remodeling is correlated with deterioration of ventricular function (Cohn 1995, Mann & Bristow 2005), and adversely affects patient prognosis. White et al. (1987) found that survivors of myocardial infarction with LV end-systolic volumes of 75 and 125 ml had their relative risks of death increased by approximately 2.5 times and five times, respectively, compared to survivors who had their LV end-systolic volumes maintained within normal limits. In fact, the study identified LV end-systolic volumes as the single most powerful predictor of survival in patients after myocardial infarction. There is increasing recognition of the importance of myocardial remodeling in the pathophysiology of heart failure (Cohn et al. 2000). Consequently, myocardial remodeling is increasingly becoming identified as a major therapeutic target (Mann et al. 2002).

\(^2\)LaPlace’s Law describes the relationship between the transmural pressure difference and the tension, radius, and thickness of a vessel wall. Where \(T\) is the tension in the walls, \(P\) is the pressure difference across the wall, \(R\) is the radius of the cylinder, and \(M\) is the thickness of the wall, the relationship between these variables is \(T = \frac{P \times R}{M}\).
1.1.5 Treatment

Modification of lifestyle

In all patients with cardiovascular disease, including heart failure, control of the modifiable risk factors has been shown to decrease the incidence and hinder the progression of disease, ultimately improving prognosis (Jessup & Brozena 2003). The major risk factors such as hypertension and diabetes must be adequately managed. Current guidelines indicate a target diastolic blood pressure below 80 mmHg (Mosterd et al. 1999). Modifications of lifestyle, particularly those leading to reduction of body weight in obese patients should be encouraged by doctors.

In addition dietary modifications should be recommended specifically to reduce the intake of salt and alcohol. The intake of alcohol in patients should be limited to less than two units per day, whilst those who have developed heart failure as a direct consequence of alcohol abuse must abstain completely (Gould et al. 1971).

Medication therapy

A variety of classes of drugs are indicated for improving the symptoms and prognoses of heart failure patients (Fig.1.2). Since heart failure is a syndrome of intravascular volume overload diuretics are commonly used for controlling circulatory congestion and peripheral œdema in symptomatic patients (Brater 1998). By reducing preload, diuretics such as thiazides and the loop diuretics can reduce the diastolic stress on the atrial and ventricular walls, inhibiting the progression of ventricular dysfunction.

A class of diuretics, the aldosterone antagonists, are especially indicated in the advanced stages of heart failure, when patients show New York Heart Association (NYHA) class III or IV symptoms (i.e. marked limitation of activity: patients are comfortable only at rest or suffer even when resting.). In these patients circulating levels of aldosterone are elevated due to increased stimulation from the angiotensin-II pathway. Furthermore, impaired liver function due to venous congestion may lead to inhibited hepatic clearance. The elevation of aldosterone can be measured even
When angiotensin-converting enzyme (ACE) inhibitors are administered, via an “escape” phenomenon of aldosterone production (Pitt 1995). Aldosterone stimulates the retention of salt and excretion of potassium, and exacerbates myocardial hypertrophy. Administration of an aldosterone antagonist such as spironolactone counteracts these responses (Pitt et al. 1999, The RALES Investigators 1996). Despite these advantages, and the fact that spironolactone decreases myocardial fibrosis, the use of spironolactone is limited to patients with advanced disease due to unwanted anti-androgenic side effects.

ACE inhibitors such as enalapril and ramipril inhibit the renin-angiotensin system of the body at the level of conversion of angiotensin-I to angiotensin-II. They therefore minimize the multiple pathophysiological effects of angiotensin II, and decrease the degradation of bradykinin. Bradykinin promotes vasodilatation and causes natriuresis in the kidney. The notion that LV remodeling after myo-
cardiac infarction might be attenuated by reducing the pressure load has led to the study of ACE inhibitors in animal models, which demonstrated smaller LV volumes along with improved cardiac output and survival (Pfeffer & Pfeffer 1987). The beneficial effects of ACE inhibitors in patients after myocardial infarction include improvements in cardiac performance and survival (Levine et al. 1984, The SOLVD Investigators 1991). In addition, there is reverse remodeling and a decrease in hospitalization (Garg & Yusuf 1995). ACE inhibitors are currently recommended for many patients with heart failure, even during early disease.

As an alternative to ACE inhibitors the renin-angiotensin system can be blocked at the level of angiotensin-II binding to its receptor (subtype 1) by the use of angiotensin receptor antagonists, such as valsartan or candesartan. These should not be used as first-line therapy for heart failure of any stage but are a valuable alternative in patients who cannot tolerate ACE inhibitors because of severe cough or angio-oedema (Hunt et al. 2005). Several trials involving patients with heart failure have shown that angiotensin receptor antagonists have efficacy similar to that of ACE inhibitors but are not superior (Pitt et al. 2000). On the other hand, in a randomized trial of patients with symptomatic left ventricular systolic dysfunction, the addition of valsartan to ACE inhibitor treatment reduced the rate of death and development of cardiovascular events as well as improving the symptoms of heart failure (Cohn & Tognoni 2001).

Digoxin is a drug that inhibits the Na\(^+\)/K\(^+\) ATPase in cardiomyocytes, increasing their contractility. Its therapeutic efficacy in heart failure patients with normal sinus rhythm has been disputed for many years, and it was only in 1997 that a large, randomized, placebo-controlled study of digoxin for symptomatic patients with a low ejection fraction was completed (The Digitalis Investigation Group 1997). There was no difference in mortality between patients receiving digoxin and patients receiving placebo, but there were decreases in the digoxin group in the rates of worsening heart failure and hospitalization.

Drugs such as atenolol and carvedilol inhibit the \(\beta\)-adrenergic receptors in the
heart (Foody et al. 2002). They have long been used for the treatment of hypertension, angina, and arrhythmias as well as for prophylaxis in patients who have had a myocardial infarction. By inhibiting the harmful effects of the sympathetic nervous system that are activated during heart failure $\beta$-blockers confer remarkable benefits, as demonstrated in trials involving patients with heart failure from various causes and of all stages (Farrell et al. 2002). These include improvements in quality of life, survival, and left ventricular ejection fraction and remodeling as well as decreased incidences of hospitalization and sudden death. $\beta$-blockers are now recommended to be used in all stable patients without substantial fluid retention and without recent exacerbations of heart failure requiring inotropic therapy (Mann et al. 2002).

Re-synchronization therapy

Approximately one third of heart failure patients show widened QRS complexes on 12-lead electrocardiography (ECG) in addition to LV dilation (Wilensky et al. 1988). The underlying ventricular dyssynchrony resulting from intra-ventricular conduction defects puts the already failing LV at further disadvantage. In such patients cardiac re-synchronization therapy using a percutaneous, three-lead, biventricular pacemaker system can be of benefit. It can reduce the pathological mechanical interventricular dyssynchrony between the right and the left ventricle and the dyssynchrony within the left ventricle, improving global LV function and cardiac output without increasing myocardial oxygen consumption (Kass et al. 1999). These acute mechanical effects of cardiac re-synchronization therapy result in immediate symptomatic improvements and can be accompanied by more chronic adaptations that lead to long-term benefit. NYHA class, exercise capacity, and quality of life of patients have been demonstrated to increase (Linde et al. 2002, Young et al. 2003). There is measurable reverse-remodeling of the LV with re-synchronization therapy (Stellbrink et al. 2001), ultimately leading to improved patient survival (Cleland et al. 2005, Bristow et al. 2004).
Surgical therapy

Despite the significant advances in a variety of drugs and interventions such as resynchronization therapy for the failing heart as described above, mortality remains high and the disease progresses. There is a growing understanding that, in addition to new and evolving surgical approaches old surgical procedures formerly contraindicated for the failing heart can combat ventricular remodeling and improve cardiac function (for a review, see Zeltsman & Acker 2002).

Cardiac transplantation was first performed in 1967 in South Africa by Christiaan Barnard. This was followed two months later by Norman Shumway in the United States (Hunt 2006). However, poor survival became evident soon after, with patient survival rates usually measured in days or weeks. This was not because of poor surgical technique, but an inadequate understanding of the type of postoperative complications that would affect heart recipients. The main problems affecting patients, such as immunological rejection of the donor heart and opportunistic infections were the subject of much research during the 1970s and 80s. During the 1970s improved methods for the cold preservation of donor hearts became available, enabling the procurement of hearts from distant donors. In addition, the introduction of endomyocardial biopsy to confirm a clinical diagnosis of acute allograft rejection (Caves et al. 1974) and of a new immunosuppressant, cyclosporine (Reitz et al. 1980), led to a new resurgence in cardiac transplantation. Most cardiac transplant centres around the world now employ a combination of immunosuppressants, usually consisting of corticosteroids (such as prednisolone), a calcineurin inhibitor such as cyclosporine, and an anti-proliferative agent such as azathioprine (Al Khaldi & Robbins 2006).

The one-year survival after heart transplantation is approximately 85% and at five years survival is 68.5% (Fig. 1.3). Patients can generally expect a very good quality of life (Grady et al. 2005, Taylor et al. 2005) and cardiac transplantation remains the gold standard of surgical therapies for advanced and end-stage heart failure refractory to medication therapy.
Despite the cumulative technological advances over the last 40 years, however, there is a severe and worsening shortage of organ donors worldwide. There has been a gradual decline in the number of heart transplants (Fig. 1.4), and compared to the number of patients who are suffering from severe heart failure transplantation is offered to only a tiny proportion of individuals.

Where there is an underlying structural cause to the heart failure, surgical correction is increasingly considered an option, where it may have been previously contraindicated. As illustrated in Table 1.1 the commonest cause of heart failure is ischaemic heart disease, and coronary artery bypass grafting for patients with LV ejection fractions < 20% to recruit the poorly perfused myocardium that is viable
but not contracting optimally ("hibernating" myocardium) is becoming commonplace. In such patients peri-operative survival is in the region of 85%, and one-year survival $\sim 72\%$ (Dreyfus et al. 1994). Surgical repair is also increasingly performed for severe mitral valve regurgitation in patients with dilated left ventricles and ejection fractions of $< 25\%$. Mitral valve annuloplasty can be performed in patients suffering from severe mitral valve regurgitation (either regurgitant fraction $> 60\%$ or regurgitant orifice area $> 0.3$ cm$^2$) causing NYHA class IV symptoms, with symptomatic improvements and operative mortality of approximately 5% (Bolling et al. 1998).

Surgical procedures have been performed with the aim of reversing the remodeling process directly, particularly in idiopathic dilated cardiomyopathy patients. Batista et al. (1996) described an operation in which normal myocardium between the anterior and posterior papillary muscles is resected to restore the left ventricle to a more normal volume. The reduction in ventricular diameter, according to LaPlace’s Law, results in decreased ventricular wall tension and improved systolic performance. Surgical ventricular restoration has also been performed in patients
who have suffered large anterior myocardial infarctions using endoventricular circular patch plasty (Dor et al. 1998). The akinetic portion of the left ventricle is excluded by deployment of an endoventricular polyester fibre (Dacron™) patch to restore a more normal ventricular geometry and improved overall systolic performance. The enthusiasm for these procedures have waned recently (Zeltsman & Acker 2002). It appears that carefully controlled studies are needed to determine if there are haemodynamic improvements without diastolic compromise, if the reported increases in ejection fraction combined with decreased ventricular volumes translate to an increase in stroke volume, and most importantly, the degree of long-term clinical benefit. An additional recent approach for reversal of cardiac remodeling is implantation of external constraint devices which girdle the heart from its outside and limit ventricular remodeling (e.g. CardioCor® “Acorn Jacket”). Initial pre-clinical studies in canine models of dilated cardiomyopathy demonstrated their safety and a halting of the remodeling process together with preservation of ventricular performance. The latest three-year follow-up data in heart failure patients reveal decreased left ventricular end-diastolic and end-systolic volumes (Starling et al. 2007) along with improvement in NYHA class and quality of life (Mann et al. 2007). However, there is no data showing a survival benefit of these external constraint devices.

Ventricular assist devices (VADs) can unload the failing heart mechanically by drawing blood from the left ventricle and delivering it into a major artery, usually the aorta. Their use as a “bridge to transplantation” is now an established therapy. VADs are most often inserted into patients in extreme cardiogenic shock with multiple organ failure. Operations are associated with high rates of mortality due to bleeding, decreased vascular resistance or multiple organ failure in the early postoperative phase, and infection and thromboembolism later. Nonetheless, surviving patients can recover enough to become mobile and rehabilitate whilst waiting for their transplant, sometimes even at home. Of patients surviving the first 30 days of support, 84% survive to either transplantation or explantation of the device (Mancini & Burkhoff 2005).
Patients other than those with severe, chronic heart failure waiting for a transplant but might benefit from device placement are those with sudden cardiac failure. These patients include those unable to be weaned from cardiopulmonary bypass during heart surgery and patients with heart failure due to acute myocarditis. Some of these patients, particularly those with a short but aggressive history of failure or myocarditis, sometimes recover so that the VAD can be removed and heart transplantation avoided (“bridge to recovery”, Muller et al. 1997). The presence of a VAD can often cause marked atrophy of the ventricle, which then prevents its explant. To minimize this effect a combination therapy including clenbuterol, a β2-receptor agonist, together with high dose ACE inhibitors and spironolactone has been introduced (Yacoub 2001). A number of patients who had received such combination therapy together with an implanted LVAD showed high rates of recovery to the degree of VAD explantation with nearly normal quality of life at three years thereafter (Birks et al. 2006).

The success of VADs as a bridge to transplantation has also led to its consideration as a permanent device, particularly in patients ineligible for transplantation. Such chronic support is termed “destination therapy”. REMATCH was a trial study of the efficacy of destination therapy in patients who were not candidates for transplantation (Rose et al. 2001). Patient survival with a VAD was significantly better than maximum medical treatment at both one and two years. Following these results, use of LVADs as destination therapy is now approved for some patients ineligible for transplantation. However, problems such as infection, stroke, bleeding, and device failure remain areas requiring further progress.

Although there have been many advances in the medical management of heart failure over the last 50 years, transplantation is the only available treatment of the underlying cause. Despite its success, however, transplantation carries disadvantages including operative mortality and those relating to immunosuppression. Furthermore, the number of patients who can be helped with transplantation worldwide
remains extremely small, and continues to decrease. There is therefore, an urgent need for additional alternative forms of therapy, especially those aiming to correct the underlying defect.
1.2 Cardiac excitation-contraction (EC) coupling

*The physiological process of converting an electrical stimulus to a mechanical response.*

– Sandow (1952)

The process of excitation-contraction (EC) coupling occurs in individual cells, and is fundamental to the physiology of muscle including myocardium. The electrical stimulus is an action potential and the mechanical response is contraction during systole, followed by relaxation during diastole. Although EC coupling has been known for over fifty years, it continues to be an active area of research (Bers 2002, Bers 2003). The Ca$^{2+}$ ion has been identified as the ubiquitous second messenger in EC coupling. Of the various ions involved in cardiac EC coupling the dynamics of Ca$^{2+}$ is considered perhaps the most important. In this chapter the details of processes involved in EC coupling in cardiomyocytes will be discussed.

1.2.1 The importance of single cell studies

Cardiomyocytes are the sole generators of contractile force in the myocardium. Their main role is to contract during systole and relax during diastole in a coordinated manner. Although they constitute only 30 ∼ 35% of the cells in the heart by number, cardiomyocytes are the dominant cell type in the normal heart with respect to volume, making up approximately 75% (Nag 1980, Vliegen et al. 1991).

In the myocardium cardiomyocytes are strongly bound to each other by the existence of intercalated discs. The intercalating discs contain membrane junctions (desmosomes and fascia adherens) which form anchoring sites to the cytoskeletal elements within cardiomyocytes (Green & Jones 1996). The arrangement of cardiomyocytes attached via intercalated discs leads to the formation of a mechanical syncytium out of unitary contractile elements. The intercalating discs also contain gap junctions, which enable efficient electrical conduction between cardiomyocytes (Davis et al. 1995). This feature, together with the ability of cardiomyocytes to transmit action potentials along their membranes, allows the formation of an electr-
cal syncytium. The fact that cardiac muscle functions as a mechanical and electrical syncytium is in contrast to skeletal muscle, where a variable number of fibres can be recruited to meet mechanical load. In addition, cardiac muscle must relax regularly with each heart beat and is therefore unable to utilize tetany. Thus, in cardiac muscle adaptation to dynamic mechanical loads occurs within individual myocytes, in large part by changes in the dynamics of Ca\(^{2+}\) handling and the arrangements of myofilaments.

The isolation of contracting, morphologically intact cardiomyocytes from adult rat hearts became available in 1970. Initial preparations involved incubation of minced muscle in digestive enzyme solutions (Kono 1969), but have been replaced by methods whereby low-Ca\(^{2+}\) solutions containing digestive enzymes are perfused through the coronary arteries (Berry et al. 1970). Today, it is possible to isolate cardiomyocytes which can be used for physiological experiments from many other animal species including mouse, human, bovine, and guinea-pig (for a review see Tytgat 1994).

The availability of intact isolated cardiomyocytes enabled detailed studies of physiological mechanisms underlying the contraction of the heart. The effects of various ionic and physical conditions as well as pharmacological agents could be examined without the need for consideration of their effects on other non-cardiomyocyte elements that are normally present in the myocardium. As described in the following sections, sophisticated methods are available for detailed characterization of cellular processes underlying EC coupling in isolated normal cardiomyocytes. Furthermore, characterizing the properties of isolated cardiomyocytes of diseased hearts have provided much valuable insight into pathological mechanisms.

1.2.2 EC coupling in the normal cardiomyocyte

During the cardiac action potential (see below), Ca\(^{2+}\) enters the cardiomyocyte through voltage-gated Ca\(^{2+}\) channels as inward Ca\(^{2+}\) current (\(I_{Ca}\)). The Ca\(^{2+}\) that has entered the cardiomyocyte triggers the release of additional Ca\(^{2+}\) from the sar-
Coplasmic reticulum (SR) via the ryanodine receptors (RyR). The combination of Ca$^{2+}$ influx and release causes a rapid rise in the free intracellular Ca$^{2+}$ concentration ([Ca$^{2+}]_i$), allowing Ca$^{2+}$ to bind to the myofilament protein troponin-C. The binding of Ca$^{2+}$ to troponin-C leads to the activation of the myofilaments (Fig. 1.5). The waveform describing the rise and fall of [Ca$^{2+}]_i$ with each heartbeat is termed the Ca$^{2+}$ transient.

For relaxation of the cardiomyocyte to occur the entry and release of Ca$^{2+}$ must be terminated and [Ca$^{2+}]_i$ must decline, allowing Ca$^{2+}$ to dissociate from troponin. Ca$^{2+}$ is removed from the cytosol by four pathways: i) sarcoplasmic and endoplasmic reticulum Ca$^{2+}$ ATPase (SERCA), ii) sarcolemmal Na$^+$/Ca$^{2+}$ exchange (NCX), iii) sarcolemmal Ca$^{2+}$-ATPase, and iv) mitochondrial Ca$^{2+}$ uniport. These mechanisms are discussed in further detail in later sections.
The action potential

The action potential, originally described in the giant squid axon by Hodgkin & Huxley (1952), is an event consisting of a series of membrane potential changes. It is the result of a complex interplay of many ion channels and transporters (DiFrancesco & Noble 1985, Luo & Rudy 1994), and forms a waveform of electrical activity that travels along the membranes of electrically excitable cells. In cardiomyocytes the action potential is the initiating event in EC coupling, and also forms the driving membrane potential that eventually generates the Ca$^{2+}$ transient. Cardiomyocytes within the heart are well-connected electrically via gap junctions and the action potential of one cardiomyocyte causes depolarization of its adjacent cardiomyocytes above its threshold for action potential generation. Thus, the action potential is the electrical signal that is transmitted between cardiomyocytes within the heart.

The waveform of the action potential varies considerably not only between animal species, but also between different parts of the heart (Nerbonne 2000). These various waveforms within the heart (Fig.1.7) underlie phenomena such as the initiating pacemaker activity in the sino-atrial node and also explain why T-waves in a normal human electrocardiogram are in the same direction as the QRS complex. The ventricular action potential in many animal species, including human, demonstrates a long plateau at a positive potential before the late repolarization phase. The formation of the plateau is the result of a delicate balance between inward currents such as the L-type Ca$^{2+}$ current ($I_{Ca,L}$) and outward currents such as the delayed rectifier K$^+$ currents ($I_{Kr}$, see Luo & Rudy 1994). The presence of a long plateau at a positive $E_m$ increases the inward flow of Ca$^{2+}$ via the L-type Ca$^{2+}$ channel.

The L-type Ca$^{2+}$ current

The importance of extracellular Ca$^{2+}$ in cardiac contraction was first demonstrated by Ringer (1883), but it was only in 1967 when the inward current carried by Ca$^{2+}$ was described (Reuter 1967). Cardiomyocytes express two types of voltage-gated
Fig. 1.6: An example action potential in a guinea-pig ventricular myocyte (A). The fast kinetics and large amplitude of the fast Na⁺ current ($I_{Na}$, B) result in the upstroke of the AP. The $I_{Ca,L}$ (C) is also activated quickly to support the action potential plateau against the repolarizing K⁺ current ($I_K$, D) and the total time-independent current ($I_V$, E). Finally, the large increase of $I_K$ and the late peak of $I_V$ during its negative slope phase repolarize the membrane to the resting potential. During the late repolarization and early postrepolarization phases, the Na⁺-Ca²⁺ exchanger current ($I_{NaCa}$, F) is activated to extrude Ca²⁺ and contributes an additional component of inward current. Currents shown are for 1 µF of membrane capacitance. Original figure taken from Luo & Rudy (1994).

Ca²⁺ channels on their surface membranes, L-type (‘Long lasting’, also known as the dihydropyridine receptor (DHPR)) and T-type (‘Transient’) (Hagiwara et al. 1975, Bean 1985). Of these, the T-type Ca²⁺ channel is present in low density in normal ventricular myocytes, and it is the L-type that are functionally dominant.

Cardiomyocyte L-type Ca²⁺ currents ($I_{Ca,L}$) are rapidly opened by depolarization of the membrane. Although the opening threshold potential depends on physical parameters such as temperature and [Ca²⁺]o, under most physiological conditions it is approximately −40 mV (Rosenberg et al. 1988, Hess 1988). This is notably higher than that for sodium currents. Although $I_{Ca,L}$ was initially termed “slow inward
current” (Reuter 1967) subsequent single-cell studies in isolated cardiomyocytes have revealed that peak $I_{Ca,L}$ is reached in $2 \sim 3$ ms (Bean 1985).

Inactivation of Ca$^{2+}$ channels is dependent on time, $E_m$, and [Ca$^{2+}$]$_i$ (Lee et al. 1985). The dependence on [Ca$^{2+}$]$_i$ can be demonstrated by experiments in which Ca$^{2+}$-free extracellular solutions are used, with alternative divalent cations being the charge carriers: when Ca$^{2+}$ is substituted with Ba$^{2+}$ or Sr$^{2+}$ $I_{Ca,L}$ is prolonged. The inactivation of Ca$^{2+}$ channels by a rise in [Ca$^{2+}$]$_i$ limits the amount of Ca$^{2+}$ influx during the action potential. One notable feature of the Ca$^{2+}$-dependent inactivation of $I_{Ca,L}$ is that it can be observed even when the intracellular Ca$^{2+}$ is heavily buffered using a Ca$^{2+}$ chelator such as ethylene glycol bis(2-aminoethyl ether)-N,N,N′N′-tetraacetic acid (EGTA). This suggests that Ca$^{2+}$ entering via $I_{Ca,L}$ can exert an inactivating effect locally (Höfer et al. 1997). On the other hand, increasing the amount of Ca$^{2+}$ released from the SR during a normal twitch results in faster inhibition of $I_{Ca,L}$ (Sipido et al. 1995), indicating that Ca$^{2+}$ released from the SR also
Fig. 1.8: The relationship between L-type Ca$^{2+}$ current and membrane voltage. The upper panel illustrates the dependence of the Ca$^{2+}$ transient amplitude on the membrane voltage. The lower panel illustrates the peak L-type Ca$^{2+}$ current on the membrane voltage. Figure taken from Beuckelmann & Wier (1988).

 contributes to this inactivation process. It is thought that $I_{Ca,L}$ inhibition by Ca$^{2+}$ released from the SR can occur because the Ca$^{2+}$ channels are located near the SR Ca$^{2+}$ release channels, the RyRs (Scriven et al. 2000).

One of the hallmarks of Ca$^{2+}$ channels is their sensitivity to dihydropyridines (DHP), and this property has been key to understanding their function and in identifying the existence of multiple subtypes (Fleckenstein 1983). DHPs bind specifically to the $\alpha_{1C}$ subunit of the L-type Ca$^{2+}$ channel to stabilize their closed state, and for this reason are often called “Ca$^{2+}$ antagonists”. The various classes of Ca$^{2+}$ antagonists were found to block $I_{Ca,L}$ with differential selectivity in cardiac and smooth muscle, and this forms the basis of their therapeutic roles in the treatment of conditions such as hypertension and angina.
**Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release**

The Ca\textsuperscript{2+} that enters the cardiomyocyte via the L-type Ca\textsuperscript{2+} channels is not sufficient to constitute a Ca\textsuperscript{2+} transient and activate myofilaments. Instead, the release of a larger amount of Ca\textsuperscript{2+} from the SR is triggered. The mechanisms by which the Ca\textsuperscript{2+} is released from the SR continues to be an area of active research but there is much evidence to support the “Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release” (CICR) model, described by Fabiato (1983) following a series of experiments using mechanically skinned canine Purkinje fibres. Ca\textsuperscript{2+} entry via $I_{Ca,L}$ was simulated by rapid application of Ca\textsuperscript{2+}, which induced the release of Ca\textsuperscript{2+} from the SR. Although one may anticipate such a mechanism to result in the formation of a positive feedback loop, always proceeding to complete emptying of the SR, Fabiato described a graded response: the amount of Ca\textsuperscript{2+} released from the SR is graded towards \(i\) the level of Ca\textsuperscript{2+} preloading of the SR, \(ii\) level of $[Ca^{2+}]_i$, and also \(iii\) the rate of change of $[Ca^{2+}]_i$, (Fig.1.9).

Based on his experimental observations, Fabiato proposed that the SR Ca\textsuperscript{2+} release channel has two Ca\textsuperscript{2+} binding sites: an activating site with modest affinity, and also an inactivating site with a higher affinity but a slower rate of association. At low $[Ca^{2+}]_i$, rapid application leads to channel opening by the preferential binding on the activation sites. When the same $[Ca^{2+}]_i$ is applied slowly, the inactivation sites can compete for the available Ca\textsuperscript{2+}, and the channels do not open. When the SR channels are subjected to very high $[Ca^{2+}]_i$, even rapid application does not result in channel opening because the very high $[Ca^{2+}]_i$ can overcome the limitation of the slow association with inactivating sites.

CICR also exhibits a refractory period, during which further CICR events do not occur. However, this is not due to the SR being empty, since Ca\textsuperscript{2+} can still be released during this period by the acute application of caffeine (Fabiato 1985).

Further support for Fabiato’s model of CICR comes from experiments using intact cardiomyocytes where the extracellular Ca\textsuperscript{2+} is substituted with other divalent cations such as Ba\textsuperscript{2+}. In such cases, release of Ca\textsuperscript{2+} from the SR does not occur,
Fig. 1.9: Relationship between trigger $[\text{Ca}^{2+}]_i$ and SR $\text{Ca}^{2+}$ release. The amplitude of the $\text{Ca}^{2+}$ transient and developed tension depends on the trigger $[\text{Ca}^{2+}]_i$, and also the time taken to reach that $[\text{Ca}^{2+}]_i$. With high trigger $[\text{Ca}^{2+}]_i$ inhibition is seen. Figure taken from Fabiato (1985).

despite a substantial flow of charge through the L-type $\text{Ca}^{2+}$ channel (Näbauer et al. 1989). This experiment showed that EC coupling does not occur by the exposure of the SR $\text{Ca}^{2+}$ release channels to positive charge or current, but that the presence of $\text{Ca}^{2+}$ ions is an absolute requirement. This interpretation is offered further support from the experiments in which CICR is triggered by $I_{\text{Ca,L}}$ tail currents (Cannell et al. 1987): when the $E_m$ of a cardiomyocyte is voltage-clamped (see section 2.12.2 on page 107 for a more detailed description of voltage-clamping experiments) and acutely increased from resting levels to $+100$ mV (which is more positive than the equilibrium potential of $\text{Ca}^{2+}$) the L-type $\text{Ca}^{2+}$ channels open but no CICR occurs because there is no flow of $\text{Ca}^{2+}$. When the cardiomyocyte is subsequently allowed to repolarize, a brief tail current is elicited which triggers CICR leading to a full $\text{Ca}^{2+}$ transient and contraction.
Termination of Ca\(^{2+}\) release

For the cardiomyocyte to relax SR Ca\(^{2+}\) release must be terminated, and the cytosolic Ca\(^{2+}\) extruded. So although CICR is inherently a positive-feedback process additional mechanisms are in place for Ca\(^{2+}\) release termination in a controlled manner, whilst maintaining a graded, and highly amplifying release of SR Ca\(^{2+}\).

Until the late 1980s all mathematical models of cardiac EC coupling assumed the cytosol of a cardiomyocyte to be a uniform space wherein the trigger Ca\(^{2+}\) reaches the SR via the same cytosolic calcium pool into which SR calcium is released: the “common pool” model (e.g. Hilgemann & Noble 1987). However, Stern (1992) reasoned, following extensive mathematical analysis of numerous models of CICR, that “common pool” models are incompatible with graded high amplification. He proposed that the SR Ca\(^{2+}\) release channels are arranged in clusters, in close proximity to the L-type Ca\(^{2+}\) channels, where they have initial exposure to the Ca\(^{2+}\): “local control theory; cluster-bomb model”. Evidence for the existence of such clusters of SR Ca\(^{2+}\) release channels has subsequently become available with the introduction of line-scan confocal microscopy. Cheng et al. (1993) visualized focal Ca\(^{2+}\) release events in rat cardiomyocytes using this tool, and named them “Ca\(^{2+}\) sparks”. Ca\(^{2+}\) sparks are elementary Ca\(^{2+}\) release events that are restricted in space (see Fig.2.19 and Fig.2.20 for illustrative examples of visualized Ca\(^{2+}\) sparks). They raise the local [Ca\(^{2+}\)] to a peak of \(\sim 200\) nM, have a time-to-peak of \(\sim 10\) ms, and decline with a decay time constant (\(\tau\)) of \(\sim 25\) ms (Cheng et al. 1993).

With the local-control model arrangement of L-type Ca\(^{2+}\) channels and RyRs it is possible to suggest three mechanisms, which are not necessarily mutually exclusive, to explain the termination of Ca\(^{2+}\) release: stochastic attrition, local depletion of SR Ca\(^{2+}\), and inactivation of the RyR. Stochastic attrition describes simultaneous closure of all L-type Ca\(^{2+}\) channels and RyRs in a junction occurring as a random event at a given time. On its own, with the numbers of L-type Ca\(^{2+}\) channels and RyRs involved, the stochastic attrition model can not adequately explain the effective, consistent termination of Ca\(^{2+}\) release that occurs with every heartbeat.
Local depletion of SR Ca\textsuperscript{2+} also does not offer an adequate model of termination of Ca\textsuperscript{2+} release, because it is known that diffusion of Ca\textsuperscript{2+} can occur within the SR lumen that enables Ca\textsuperscript{2+} sparks that last > 200 ms (Cheng et al. 1993).

It is also conceivable that the RyR, after opening for a period of time, closes automatically and becomes inactivated for reopening, in a manner similar to that seen with Na\textsuperscript{+} channels: the absorbing inactivation model (Sitsapesan et al. 1995, Sham et al. 1998). RyRs, instead of becoming inactivated, could alternatively become desensitized to Ca\textsuperscript{2+}, so that their probability of opening upon exposure to Ca\textsuperscript{2+} decreases: (Győrke & Fill 1993, Valdivia et al. 1995). While the opening of RyRs shows a refractory period, the precise molecular mechanisms underlying the refractory period continue to be an interesting area of research.

**Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange**

Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange (NCX) is the sarcolemmal ion transport process by which three Na\textsuperscript{+} ions are exchanged for one Ca\textsuperscript{2+} ion in opposite directions (Philipson & Nicoll 2000). NCX is a reversible secondary active transport process - no ATP is utilized, but the electrochemical gradients of the ions involved drive the transport process. The stoichiometry of NCX implies electrogenicity: the direction and size of the NCX current (\(I_{\text{NCX}}\)) depend not only on the concentration gradients of Na\textsuperscript{+} and Ca\textsuperscript{2+}, but also on \(E_m\). Therefore, NCX provides a mechanism by which Ca\textsuperscript{2+} is transported through the sarcolemma in a manner dependant on \(E_m\). \(I_{\text{NCX}}\) exhibits a reversal potential, \(E_{\text{NCX}}\), where \(E_{\text{NCX}} = 3 \cdot E_{\text{Na}^+} - 2 \cdot E_{\text{Ca}^{2+}}\).

Entry of Ca\textsuperscript{2+} through the L-type Ca\textsuperscript{2+} channel that occurs with each contraction requires an equal amount of sarcolemmal Ca\textsuperscript{2+} extrusion within a single heartbeat to maintain steady-state Ca\textsuperscript{2+} homeostasis. NCX provides the principal pathway for Ca\textsuperscript{2+} removal through the sarcolemma during diastole. In the presence of caffeine (the rapid application of which empties the SR of Ca\textsuperscript{2+} and prevents Ca\textsuperscript{2+} re-accumulation), a ventricular cardiomyocyte would not relax if Ca\textsuperscript{2+} extrusion via NCX is also disabled by the removal of external Na\textsuperscript{+} (Bridge et al. 1990).
The direction of NCX can be reversed: Na\(^+\) out, and Ca\(^{2+}\) in. LeBlanc & Hume (1990) provided evidence that Na\(^+\) entering myocytes through Na\(^+\) channels during the action potential could accumulate and drive reverse-mode exchange. The amount of Ca\(^{2+}\) entering the cardiomyocyte by reverse-mode NCX could elicit SR Ca\(^{2+}\) release in a manner dependent on \([\text{Ca}^{2+}]_o\). Since \(E_m\) during the plateau of the cardiac action potential is far higher than the normal physiological \(E_{NCX}\) of approximately \(-40\) mV, it might also add a substantial driving force for reverse mode NCX. However, the importance of such reverse mode NCX \textit{in vivo}, both in normal and pathological conditions, remains a topic of ongoing discussion. Matters are complicated by \(i\) the dynamically changing \([\text{Ca}^{2+}]_i\) (and hence \(E_{\text{Ca}^{2+}}\)) with the Ca\(^{2+}\) transient, and \(ii\) the difficulty in accurately measuring local \([\text{Na}^{+}]\) and \([\text{Ca}^{2+}]\) near the internal surface of the exchanger, which are likely to be affected by restricted diffusion given the complex internal architecture of cardiomyocytes. Under experimental conditions, reverse mode NCX can be of sufficient magnitude even to trigger CICR (Wasserstrom & Vites 1996, Litwin et al. 1996), but the physiological significance of such events remain controversial (Sipido et al. 1997).

The removal of Ca\(^{2+}\) following release

During diastole Ca\(^{2+}\) is removed from the cytosol by \(i\) SERCA, \(ii\) NCX, \(iii\) sarcolemmal Ca\(^{2+}\)-ATPase, and \(iv\) mitochondrial Ca\(^{2+}\) uniport. The last two of these are often collectively called ‘slow systems’, and contribute only 1 \(\sim\) 2\% towards Ca\(^{2+}\) removal (Bassani et al. 1994). SERCA and NCX compete effectively for removal of the majority of the Ca\(^{2+}\), and their relative quantitative importance varies between animal species (Bassani et al. 1994). For example, NCX removes approximately 30\% of the Ca\(^{2+}\) in guinea-pig (Terracciano & MacLeod 1994), and 28\% in rabbit (Bassani et al. 1994), but only 7\% in rat (Bassani et al. 1994). In humans, this figure is reported to be approximately 37\% (Pieske et al. 1999).

NCX is influenced by \(E_m\), and more Ca\(^{2+}\) extrusion occurs by NCX at lower \(E_m\) (Bridge et al. 1988). Thus, the fraction of Ca\(^{2+}\) removal by NCX depends on
Fig. 1.10: Relative contributions of various processes towards Ca$^{2+}$ removal from the cell. Integrated Ca$^{2+}$ fluxes during twitch relaxation in rabbit and rat ventricular myocytes. Curves are based on $[Ca^{2+}]_i$ and the $[Ca^{2+}]_i$ dependence of transport rates measured for each system. Percentages are relative contributions to Ca$^{2+}$ removal. Figure adapted from Bassani et al. (1994).

the profile of the action potential: a long action potential will result in more of the Ca$^{2+}$ being removed by SERCA rather than NCX. Other variables that affect the relative contributions of SERCA vs NCX include $[Na^+]_i$ and resting $[Ca^{2+}]_i$, and the states of the SR and Na$^+$-Ca$^{2+}$ exchanger. The importance of $[Na^+]_i$ was studied by Armoundas et al. (2003) who demonstrated shortening of APD with increasing $[Na^+]_i$ in canine ventricular myocytes. In failing cardiomyocytes with $[Na^+]_i \geq 10$ mM outward $I_{NCX}$ during the action potential plateau facilitated repolarization, whereas when $[Na^+]_i = 5$ mM $I_{NCX}$ had a depolarizing effect.
Role of the released Ca\textsuperscript{2+} in activating myofilament activity

The intracellular myofilaments are the contractile machinery of the cell, responsible for transducing chemical energy into mechanical force. The myofilaments are composed of myosin, actin, the troponins, and titin. In the mammalian ventricle, they occupy 45 $\sim$ 60\% of the intracellular volume, although this fraction is smaller in atrial cardiomyocytes and Purkinje fibres.

Ca\textsuperscript{2+} is the direct activator of the myofilaments, which effect cardiomyocyte contraction (Fig.1.11). Contractile force develops in a graded but non-linear manner with increasing [Ca\textsuperscript{2+}]\textsubscript{i}, mainly because of strong myofilament cooperativity with respect to [Ca\textsuperscript{2+}]\textsubscript{i} (Solaro 1999). The sensitivity of the myofilaments to [Ca\textsuperscript{2+}]\textsubscript{i} is also enhanced by stretching the myocardium (Allen & Kentish 1985), because the number of cross bridges formed depend on the sarcomere length\textsuperscript{3}. Other factors that affect the sensitivity of myofilament to Ca\textsuperscript{2+} include the intracellular concentrations of H\textsuperscript{+} (Fabiato & Fabiato 1978), PO\textsubscript{4}\textsuperscript{3-}, and Mg\textsuperscript{2+} (Fabiato & Fabiato 1975). Drugs such as $\beta$-adrenergic agonists, caffeine, and other phosphodiesterase inhibitors (such as milrinone, a cardiac inotrope) can also alter the sensitivity of myofilament to Ca\textsuperscript{2+}. Thus, factors that influence the dynamics of intracellular Ca\textsuperscript{2+} have direct consequences on cardiomyocyte contraction, but how the myofilaments sense the intracellular Ca\textsuperscript{2+} are important additional considerations.

\textsuperscript{3}This is the dominant theory for the cellular basis of Frank-Starling’s Law, which states that the more the ventricle is filled with blood during diastole, the greater the volume of ejected blood will be during systolic contraction.
**Fig. 1.11:** Activation of contractile myofilaments by intracellular Ca$^{2+}$.  

**A.)** The organization of the sarcomere.  
**B.)** During systole Ca$^{2+}$ binds to and activates troponin-C. The binding signal is transmitted to tropomyosin through troponin-T (the tropomyosin-binding unit of the troponin complex) and also to troponin-I. Troponin-I is then released from its tether on actin, and the tropomyosin becomes free of steric hindrance of the actin-cross bridge reaction. Strong binding of a cross bridge promotes the reaction of a near neighbour cross bridge by actively pushing tropomyosin away from its blocking position. This mechanism results in strong cooperative activation. Figure adapted from Solaro (1999).
1.3 Excitation-contraction coupling in the failing heart

In section 1.1 the aetiology and clinical features of heart failure were discussed, and in section 1.2 the cellular mechanisms underlying EC coupling in a normal cardiomyocyte were described. During the last 20 years there has been an accumulation of substantial evidence that altered cardiomyocyte EC coupling is of significant relevance for the pathophysiology of heart failure (Hasenfuss & Pieske 2002, Tomaselli & Marban 1999). Studies of the properties of cardiomyocytes isolated from human patients (Beuckelmann et al. 1992) as well as numerous animal models of heart failure (Hasenfuss 1998) have been performed.

A number of experimental animal models of chronic heart failure have consistently demonstrated depressed cardiomyocyte function (Capasso & Anversa 1992, Spinale et al. 1997). Studies that employed tissue strips (Pieske et al. 1996) or isolated cardiomyocytes (Davies et al. 1995, del Monte et al. 1995) from failing human myocardium obtained in the setting of cardiac transplantation have also demonstrated depressed contractility. Impairment of Ca$^{2+}$ handling are also more apparent with increasing frequencies (Pieske et al. 1996). Accordingly, an altered force-frequency relationship of myocardial function is considered as one of the most characteristic alterations related to pathological Ca$^{2+}$ homeostasis in the failing heart.

1.3.1 Changes to the action potential profile in heart failure

A common finding in failing hearts, regardless of species or mode of failure is prolongation of the action potential (Beuckelmann et al. (1992), Näbauer & Kääb (1998). See Fig.1.13A). The prolongation is usually most apparent at low frequencies and becomes smaller with increasing frequency, as the action potential duration shortens. The delay in repolarization is due to alterations in several currents that contribute towards the action potential profile. When measured directly, reductions in the tran-
Fig. 1.12: Cardiomyocytes isolated from heart failure patients have impaired contractions. Figure taken from Davies et al. (1995).

Transient outward current ($I_{to}$) has been commonly seen (Nabauer et al. 1993). Another $K^+$ current that has been closely studied is the inward rectifier $K^+$ current ($I_{K1}$), which sets the resting membrane potential and contributes to the terminal phase of repolarization. Reduced $I_{K1}$ has been reported in heart failure, including patients with dilated cardiomyopathy, but not with the same consistency as with $I_{to}$ (for a general review of $K^+$ current changes in heart failure see Tomaselli & Marban 1999).

1.3.2 Ca$^{2+}$ handling in heart failure

The impairment of mechanical properties of cardiomyocytes in heart failure is reflected in changes in Ca$^{2+}$ transients (Gwathmey et al. 1987, Beuckelmann et al. 1992). Where changes have been detected, the Ca$^{2+}$ transients of failing cardiomyocytes show increased diastolic baseline levels, reduced amplitudes, prolonged times-to-peak, and slower decay rates compared to normal counterparts (Fig.1.13B).

There are several mechanisms that could underlie the reduced Ca$^{2+}$ transient, which are not necessarily mutually exclusive. Defects in EC coupling can occur, for example, in $i)$ the $I_{Ca,L}$, $ii)$ the storage of Ca$^{2+}$ in the SR, or $iii)$ the release of Ca$^{2+}$
Fig. 1.13: Action potential (A) and Ca\textsuperscript{2+} transient (B) traces from cardiomyocytes from human hearts with dilated cardiomyopathy. The action potential duration is longer in cardiomyocytes from failing hearts compared to control. The Ca\textsuperscript{2+} transients of cardiomyocytes from failing hearts show higher baseline, smaller amplitude, and a slower rate of decline. Figure adapted from Beuckelmann et al. (1992).

stored in the SR via RyRs. There have been some studies reporting reduced peak $I_{Ca,L}$ density in heart failure patients (Ouadid et al. 1995) as well as in animal models of heart failure (Santos et al. 1995). On the other hand, a number of other studies have reported no significant change (Beuckelmann et al. 1992, Gomez et al. 1997). The discrepancy may be due to differences in animal species, mode of heart failure induction, and duration and severity of disease. In any case, since numerous models of heart failure show clearly reduced Ca\textsuperscript{2+} transients without reduced $I_{Ca,L}$, it appears that other EC coupling defects are important.

The SR Ca\textsuperscript{2+} load in failing human myocardium has been assessed using rapid cooling contractures in muscle strips (Pieske et al. 1999) or rapid application of caffeine onto isolated cardiomyocytes (Lindner et al. 1998). These reveal that SR Ca\textsuperscript{2+} load in failing human hearts is decreased. This reduction may, at least in part,
be due to reduced SERCA activity, since an additional characteristic feature of failing cardiomyocytes is slower relaxation and Ca$^{2+}$ transient decay. The SERCA activity measured in crude membrane preparations of SR is lower in failing cardiomyocytes compared to normal cardiomyocytes (Schwinger et al. 1995). Further evidence for the involvement of SERCA in heart failure comes from experiments where SERCA2 is overexpressed in cardiomyocytes from failing human hearts by adenoviral gene transfer: this not only increases the rate of Ca$^{2+}$ transient decay, but also increases the amplitude of the transient and improves overall myocyte contractility (del Monte et al. 1999).

The RyR is a 600 kDa protein which forms a homotetrameric complex through which Ca$^{2+}$ is released from the SR during CICR (Nakai et al. 1990). Studies comparing expression levels of RyR in failing hearts with normal hearts have reported mixed results. Subsequently, the modulation of the RyR function through its phosphorylation state and association with other proteins such as FK506-binding protein 12.6 (FKBP12.6) has been a focus of much research. In particular, Marx et al. (2000) showed that the RyR can become hyperphosphorylated by protein kinase A (PKA) and become dissociated with FKBP12.6. This dissociation increases the open probability of the channel and disturbs the functional coupling of the RyRs with each other, resulting in increased leak of Ca$^{2+}$ during diastole and a reduced SR Ca$^{2+}$ load. Consistent with this theory, overexpression of FKBP12.6 in rabbit cardiomyocytes reduces spontaneous SR Ca$^{2+}$ leak with increased SR Ca$^{2+}$ content and myocyte contractility (Prestle et al. 2001).

As well as decreasing the amount of Ca$^{2+}$ in the SR available for release, it has been proposed that abnormal regulation of the RyR by FKBP12.6 may decrease the RyR threshold for spontaneous Ca$^{2+}$ release (Jiang et al. 2004). It is conceivable that Ca$^{2+}$ released in such manner, if it becomes substantial, may cause acute depolarization of the cardiomyocyte by reverse-mode NCX - a plausible explanation for the arrhythmias seen in failing hearts.

In addition to the maladapative changes occurring in the RyRs, it has been
suggested that disruptions in the spatial relationships between the L-type Ca\(^{2+}\) channels and RyRs might impair CICR (Gomez et al. 1997). As reasoned by Stern (1992) the activation of a RyR is sensitive to its geometric relation to the L-type Ca\(^{2+}\) channel. In cardiomyocytes isolated from rats with spontaneously hypertensive heart failure, the Ca\(^{2+}\) transient is reduced despite a normal \(I_{Ca,L}\), normal SR Ca\(^{2+}\) content, RyR open probability, and Ca\(^{2+}\) spark morphology. Following these observations Gomez et al. (1997) reasoned that the reduced ability of the \(I_{Ca,L}\) to trigger SR Ca\(^{2+}\) release comes from its further physical separation from the RyRs.

The importance of SR Ca\(^{2+}\) regulation has been further highlighted by experiments in which the SERCA2A gene was over-expressed in cultured isolated failing cardiomyocytes (del Monte et al. 1999). Compared to control failing cardiomyocytes, those which were virally transfected with the SERCA2A gene showed lower diastolic [Ca\(^{2+}\)]\(_i\) and higher peak systolic [Ca\(^{2+}\)]\(_i\). In addition, contractility was improved and a positive force-frequency response was maintained. In corresponding in vivo experiments, viral delivery of the SERCA2A gene in rat hearts subjected to aortic banding resulted in maintenance of smaller ventricular volumes, superior myocardial contractility, and improved survival (del Monte et al. 2001). These studies not only confirm the importance of the identified Ca\(^{2+}\) handling mechanisms in cardiac function, but also suggest that correction of Ca\(^{2+}\) handling defects can reverse the adverse remodeling process.

### 1.3.3 Na\(^{+}/Ca^{2+}\) exchange in heart failure

The expression of the Na\(^{+}/Ca^{2+}\) exchanger protein is increased in heart failure (Studer et al. 1994, Quinn et al. 2003). However, the net effect of such an increase on cytoplasmic [Ca\(^{2+}\)] is difficult to predict because of the bidirectional nature of exchange and its dependence on multiple dynamic factors including \(E_m\) and [Na\(^{+}\)]\(_i\). An increase in forward mode exchange would remove more Ca\(^{2+}\) during diastole over SERCA and this might contribute towards the reduction of the SR Ca\(^{2+}\) load that is seen (Pogwizd et al. 1999). On the other hand, an increase in reverse mode
exchange might increase Ca$_{2+}$ influx during systole, especially given the background of the prolonged action potentials and increased [Na$^+$]$_i$ that are characteristic of heart failure. Sipido et al. (2002) summarized the published studies of NCX in heart failure and found that, out of 29 animal studies of heart failure, 14 reported an increase in NCX expression and/or function, 10 a decrease, and 5 no change. The heterogeneity of results appears to be due to a variety of factors, such as mode, severity, and duration of pressure overload to the heart as well as animal species.

The cellular changes that occur in failing cardiomyocytes are complex, depend on many variables, and continue to be an active area of study. Some cellular features, such as depressed contractility and relaxation, decreased amplitude and slower decay of the Ca$_{2+}$ transients, and prolongation of the action potential are common findings. Other important aspects of EC coupling, for example the regulation of the RyR, continue to be debated, whilst mixed results have also been reported for $I_{Ca,L}$ and $I_{NCX}$. The seemingly inconsistent and sometimes contradictory findings highlight the important species differences in EC coupling and, more importantly, the complexity of the pathological process. Increasingly, it is becoming apparent that EC coupling in failing cardiomyocytes are characterized by a number of compensatory processes superimposed on central defects.
1.4 Cell transplantation for heart failure

The understanding that a common mechanism of development of heart failure is the loss of ventricular cardiomyocytes (Narula et al. 1996) naturally leads to the notion of supplementing those losses by delivering cells directly into the diseased ventricle as a mode of treatment. During the last 10 years there have been numerous studies performed in the field of cardiac cell transplantation involving a wide range of animal models and also in large clinical trials. Some of the reported data have stimulated much interest, and the field continues to be a highly active area of ongoing research. On the other hand, there has also been considerable controversy over some of the key points, and some important questions remain unanswered. In this section the major recent developments will be summarized, and the outstanding issues discussed.

1.4.1 Animal studies of cardiac cell transplantation

The majority of the animal studies of cardiac cell transplantation have employed the strategy of directly injecting cells which were labeled using genetic or fluorescent markers into normal or diseased hearts (for comprehensive reviews see Dowell et al. 2003, Laflamme et al. 2007). Such a study model enabled tracking of the phenotypic fate of the transplanted cells along with monitoring of the function of the diseased recipient ventricle.

Fœtal & neonatal cardiomyocytes

Cardiomyocytes would constitute the ideal donor cell for transplantation into diseased hearts, as they already possess the necessary structural and physiological attributes to integrate with the recipient myocardium. Some of the early animal studies injected committed cardiomyocytes from fœtal and neonatal sources into rat hearts which had previously undergone myocardial infarction. The implanted myocytes formed stable grafts within the myocardium and resulted in improved left ventricular function (Li et al. 1996, Scorsin et al. 2000, Rubart et al. 2003).
As expected for differentiated cardiomyocytes, the implanted cells retained their contractile phenotype and even expressed the necessary gap junction connexins for intercellular electrical communication. Further direct evidence of electrical integration of injected foetal cardiomyocytes were reported by Rubart et al. (2003). They injected cardiomyocytes from transgenic mice overexpressing enhanced green fluorescent protein (eGFP) into recipients, and used two-photon laser scanning microscopy for simultaneous visualization of Ca\(^{2+}\) transients in the transplanted cells and the recipient cells within intact myocardium. These studies demonstrated synchronous Ca\(^{2+}\) transients in adjacent cardiomyocytes of different origin, indicating that the grafted cells had integrated and were capable of appropriate activation. Clearly, delivery of committed cardiomyocytes can form nascent myocardium in the normal heart, with appropriate electrical integration.

The studies utilizing committed cardiomyocytes can only be considered proof-of-principle studies, since there is no accessible source of foetal or neonatal cardiomyocytes for transplantation into patients. The other major potential obstacle that needs to be overcome with foetal and neonatal cardiomyocytes is the likely immune rejection. For these reasons, the majority of animal studies of cardiac cell transplantation utilized types of cells which are available from autologous sources, as discussed below.

**Skeletal myoblasts**

The first cardiac cell transplantation experiments utilized skeletal myoblasts derived from skeletal muscle satellite cells (Chiu et al. 1995, Murry et al. 1996, Taylor et al. 1998). The advantages of skeletal myoblasts that lead to their selection include their \(^i\) availability from autologous sources (which bypasses the immunological and ethical considerations associated with some of the other cell types that become prohibitive when applied to humans), \(^ii\) ability to proliferate and be expanded \textit{ex vivo}, and \(^iii\) superior resistance to ischaemia compared to cardiomyocytes. Skeletal myoblasts are also well-known to be committed to a contractile tissue phenotype.
Initially, it was hoped that these cells might transdifferentiate into cardiomyocytes following injection, or at least be genetically manipulated into acquiring more of the features of cardiomyocytes. However, it has become apparent that skeletal myoblasts remain committed to forming mature skeletal myotubes in the heart. Furthermore, mature myotubes as well as skeletal myoblasts that remain unfused appear to remain mechanically and electrically isolated from the recipient myocardium (Leobon et al. 2003, Rubart et al. 2004). Another important consideration is the limited survival of the skeletal myoblasts following injection. Labeling the injected skeletal myoblasts with $^{14}$C-thymidine and monitoring $^{14}$C radioactivity after injection revealed that only $\sim 7.4\%$ of the injected cells survived at 72 hours (Suzuki et al. 2004). Clearly, the small numbers of surviving cells cannot contribute a substantial contractile force to the myocardium. However, despite these disappointing findings from characterization of the injected cells, there appears to be a modest improvement in ventricular performance (Scorsin et al. 2000, Taylor et al. 1998). This important paradox remains unexplained. A variety of interpretations have been put forward, some of which are discussed later.

**Bone marrow-derived cells**

In 2001, Orlic et al. reported that widespread myocardial regeneration could be induced in mice that had undergone myocardial infarction by injecting the $\text{Lin}^-, \text{c-kit}^+$ subset of bone marrow cells. The authors described developing tissue consisting of proliferating myocytes and vascular structures, and suggested that locally delivered bone marrow-derived cells could generate new myocardium through transdifferentiation. Their report sparked tremendous interest not only amongst scientists, but also in the clinical community since methods for obtaining and sorting large numbers of bone marrow-derived cells from autologous sources were already well established and there was a good degree of clinical familiarity of transplanting such cells into patients for treating haematopoietic disorders.

Unfortunately, despite exhaustive attempts by several research groups the results
of Orlic et al. could not be reproduced (Murry et al. 2004, Deten et al. 2005). Instead, the subsequent studies concluded that bone marrow-derived cells adopt mature haematopoietic fates (Balsam et al. 2004, Nygren et al. 2004). The studies identified only very small numbers of cardiomyocytes from the recipient myocardium that also expressed the genetic markers of cells that were injected. These cells, as well as occurring only in extremely low frequency, invariably contained genetic material from both injected and recipient cells, suggesting that they were the result of cell fusion rather than transdifferentiation. Further evidence that bone marrow-derived cells fuse with recipient cardiomyocytes came from experiments utilizing Cre/lox recombination technology (for a description of this DNA manipulation tool see Sauer 1998). Bone marrow-derived cells harvested from mice constitutively expressing Cre recombinase in addition to eGFP were injected into the hearts of R26R reporter mice (Alvarez-Dolado et al. 2003). The R26R reporter cardiomyocytes expressed the LacZ reporter gene only after a loxP-flanked stop cassette was excised by Cre recombinase, supplied by the donor bone marrow-derived cells. \( \beta \)-galactosidase\(^+\) cardiomyocytes were morphologically indistinguishable from other recipient cardiomyocytes, and expressed troponin-I and gap junction connexins. Their karyotypes were either tetraploid or hexaploid. On the other hand, eGFP\(^+\)/\( \beta \)-galactosidase\(^-\) cells had appearances of small mononuclear cells.

A subset of bone marrow-derived cells, termed mesenchymal stem cells (MSC) have attracted special interest. MSCs compose the stromal compartment of bone marrow, and are not haematopoietic. In culture they adhere to polystyrene surfaces and proliferate indefinitely. They express CD29 and CD90, and are negative for the surface markers found on other bone marrow-derived cells, including CD31, CD34, and CD45, which enables their separation using fluorescence-activated cell sorting (FACS). MSCs have been shown to be able to differentiate into a variety of tissue cell types in vitro, including adipocytes, chondrocytes, osteoblasts, and skeletal myoblasts. In 1999 Makino et al. demonstrated that exposure of MSCs to 5-azacytidine, which methylates DNA and can alter the expression of genes that
regulate differentiation, can yield proliferating cells similar to those of fetal cardiomyocytes. Their described cells beat spontaneously, fused into tube-like structures that exhibited sarcomeres, stained positive for myocardial proteins such as myosin, desmin, and actinin, and also had measurable action potentials.

Despite these special properties of MSCs, the results of their injection into hearts appear to be no different from that of other bone marrow-derived cells. The overall survival of cells after injection is low, and those few that do survive do not form mature cardiomyocytes which integrate with their native neighbours (Dai et al. 2005). Mangi et al. (2003), Toma et al. (2002), and Nagaya et al. (2005) all demonstrated cells which exhibited the markers of donor cells along with some of the proteins which are only expressed in cardiomyocytes, but none specifically addressed the possibility of cell fusion. It is interesting however, that all three groups measured improvement in whole heart function.

To summarize, the large number of animal model studies involving various types of cells have not succeeded in generating large numbers of new cardiomyocytes. Although injected fetal cardiomyocytes can provide additional myocardium, the formation of new cardiomyocytes by transdifferentiation of skeletal myoblasts or bone marrow-derived cells has not been convincingly demonstrated. However, several studies have surprisingly reported moderate improvements in whole heart function in post-infarct rat or mice hearts following transplantation of skeletal myoblasts or bone marrow-derived cells. This paradox remains unaccounted for.

1.4.2 Clinical trials of cardiac cell transplantation

Skeletal myoblasts

After approximately ten years of pre-clinical testing resulting in more than 40 studies skeletal myoblasts were the first to enter the clinical arena (Menasché et al. 2001). Because autologous skeletal myoblasts need to be expanded ex vivo over several
weeks these trials were performed in patients with chronic ischaemic heart disease with severe heart failure, rather than acute myocardial infarction. Autologous skeletal myoblasts were prepared from muscle biopsies, and expanded using foetal bovine serum. Four of the trials that have been published are summarized in Table 1.2. Additional feasibility studies are ongoing. As can be seen, all of these studies entailed coronary artery bypass graft (CABG) surgery. Further trials in which skeletal myoblasts are administered via percutaneous catheters are in progress.

These trials have demonstrated that hundreds of millions of skeletal myoblasts can be grown from muscle biopsies and subsequently injected into the heart without early procedural complications. Long-term engraftment of skeletal myoblasts, featuring clusters of myotubes aligned parallel to host cardiomyocytes, has been visualized by microscopy of explanted hearts up to 18 months after transplantation (Pagani et al. 2003, Hagège et al. 2003, Dib et al. 2005). However, the myotube grafts were only very small compared to the left ventricles, and given the large numbers of cells injected this implies that the vast majority of cells were lost, either to inefficient seeding or high rates of cell death after injection.

No meaningful conclusions can be drawn regarding the efficacy in augmentation of function in the injected areas from these phase I clinical trials. The large randomized controlled trial by Menasché et al. (2008) found no improvement in ejection fraction, but measured decreased ventricular chamber dimensions 6 months after skeletal myoblast transplantation. Other studies, such as that by Gavira et al. (2006) and Dib et al. (2005) found clear improvements in ejection fraction. The main limitation of these trials is that their interpretation has been made difficult by concomitant coronary artery bypass surgery, which sometimes included the region receiving myoblast injections. Furthermore, there are differences among studies that make direct comparisons difficult, including differences in cell culture processes (which may influence myoblast viability and differentiation), the variable end points used to judge efficacy (including tool of cardiac function assessment), and the variable baseline function of the engrafted regions.
Patient safety has been a major concern, especially following observations of ventricular tachycardia in 4 out of 10 patients in the early study carried out by Menasché et al. (2001). As a safety precaution and also to assess the incidence and timing of graft related arrhythmias, this group has implanted internal cardiac defibrillators in all subsequent study patients. In the study published in 2008, Menasché et al. detected ventricular arrhythmias in 12 ~ 17% of patients who received skeletal myoblast transplantation, compared to 6% in control patients ($p = ns$).
<table>
<thead>
<tr>
<th>Study</th>
<th>n</th>
<th>Control</th>
<th>Cell dose</th>
<th>CABG</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menasché et al. (2008)</td>
<td>97</td>
<td>placebo injection</td>
<td>$4 \sim 8 \times 10^8$</td>
<td>yes</td>
<td>• No change in EF at 6 months</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• EDV and ESV ↓ at 6 months</td>
</tr>
<tr>
<td>Hagège et al. (2006)</td>
<td>10</td>
<td>none</td>
<td>$8.7 \times 10^8$</td>
<td>yes (remote)</td>
<td>• Improved symptoms</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• EF ↑ by 6.7% at 4 years.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• VT arrhythmias in 3 patients</td>
</tr>
<tr>
<td>Gavira et al. (2006)</td>
<td>12</td>
<td>none</td>
<td>$2.2 \times 10^8$</td>
<td>yes</td>
<td>• EF ↑ by 20% at 1 year</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Improved viability of injected segments</td>
</tr>
<tr>
<td>Dib et al. (2005)</td>
<td>30</td>
<td>none</td>
<td>$5 \times 10^7$</td>
<td>yes</td>
<td>• EF ↑ by 8% at 2 years</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• VT arrhythmias in 2 patients</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Skeletal myotubes were visualized in hearts</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>which were explanted later.</td>
</tr>
<tr>
<td>Siminiak et al. (2004)</td>
<td>10</td>
<td>none</td>
<td>$1.1 \times 10^7$</td>
<td>yes</td>
<td>• EF ↑ by 6.8% at 1 year</td>
</tr>
</tbody>
</table>

**Abbreviations:** CABG: coronary artery bypass graft. EF: ejection fraction. EDV: end-diastolic volume. ESV: end-systolic volume. VT: ventricular tachycardia.
Bone marrow cells

In marked contrast to the ten years of preparation which preceded initiation of the skeletal myoblast transplantation clinical trials phase I, non-randomized clinical trials of bone marrow-derived cell transplantation were reported within only six months of the publication by Orlic et al. (2001). Subsequently, there have been many randomized clinical trials published.

Unlike skeletal myoblasts, hundreds of millions of autologous bone marrow-derived cells can be obtained without the need for \textit{ex vivo} expansion. This has allowed most of the clinical studies to be aimed towards treatment of patients who suffered recent acute myocardial infarction (summarized in Table 1.3), although others have performed bone marrow-derived cell transplantation in patients with chronic ischaemic heart failure (Table 1.4). Another difference between the trials using bone marrow-derived cells compared to skeletal myoblasts is that most of those injecting bone marrow-derived cells have done so using percutaneous approaches. The commonest method has been intracoronary perfusion, where pressures up to ten times atmosphere have been applied. All of these studies have been carried out on patients who received coronary revascularization. There has been one study of direct intramuscular cell injection during coronary artery bypass surgery (Hendrikx et al. 2006). Other trials have employed percutaneous endocardial injection (Perin et al. 2004), which enables electromechanical mapping to help identify viable myocardium for appropriate delivery.

As can be seen in Tables 1.3 and 1.4 the results for both acute myocardial infarction and chronic disease have been mixed. This is despite careful performance of prospectively randomized trials using controls with well-matched baseline characteristics. Although only the studies by Ge et al. (2006), Janssens et al. (2006), and Schächinger et al. (2006) have carried out blinded sham-infusions (placebo treatment) into their recruited controls, most of the studies outlined in Tables 1.3 and 1.4 were at least appropriately blinded during follow-up. The inconsistency of results, together with the modest improvements measured, has prompted a variety of
interpretations. Some have commented that variables such as different protocols of cell preparation, timing (relative to onset of myocardial infarction) of injection, baseline patient characteristics, and method of evaluation of cardiac function might account for the variation on the results seen (Rosenzweig 2006). For example, Chen et al. (2004) used purified MSCs whereas most others injected unfractionated bone marrow mononuclear cells. Several investigators, including Kang et al. (2006) mobilized bone marrow-derived cells by administering systemic granulocyte colony-stimulating factor (G–CSF) prior to cell harvest, which may have had additional effects. Subgroup analysis of the data obtained by Schächinger et al. (2006) suggested that bone marrow-derived cell transplantation is more beneficial when performed more than five days after the onset of myocardial infarction. Finally, Penicka et al. (2007) commented that the negative finding from their trial (which was terminated early) may be related to the fact that they recruited only patients with severe heart failure (those with ejection fraction < 40%).

With increasing skepticism over the collective results, two systematic meta-analyses of bone marrow-derived cell transplantation trials have been published (Abdel-Latif et al. 2007, Lipinski et al. 2007). Both have concluded that modest yet statistically significant benefits exist compared to control, in terms of left ventricular dimensions, ejection fraction and infarct size. However, no new insight into the underlying physiological mechanisms of action were revealed. The meta-analysis of 18 studies including 999 patients by Abdel-Latif et al. found no correlation between the number of cells injected and beneficial effect. Many researchers in the field, including the authors of these meta-analyses, have expressed doubt over the clinical significance of the small benefit (Lipinski et al. 2007, Nadal-Ginard & Fuster 2007, Laflamme et al. 2007). With regard to future directions, opinions vary widely. Some have suggested that larger randomized, double-blind, controlled trials, perhaps even with repeated treatment, are warranted for further sub-group analysis (The task force of the European Society of Cardiology concerning the clinical investigation of the use of autologous adult stem cells for repair of the heart: Bartunek
et al. (2006)). On the other hand, others (Nadal-Ginard & Fuster 2007, Arnesen et al. 2007) have proposed a moratorium on new clinical trials until additional insight is gained from further animal studies on key points such as optimum cell type, timing and method of delivery, and perhaps most crucially, the underlying mechanisms of action.
Table 1.3: Randomized controlled trials of bone marrow cell transplantation following acute myocardial infarction

<table>
<thead>
<tr>
<th>Study</th>
<th>n</th>
<th>Follow up</th>
<th>Cell dose</th>
<th>Assessment method</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chen et al. (2004)</td>
<td>69</td>
<td>6 months</td>
<td>$6 \times 10^{10}$</td>
<td>Echo</td>
<td>EF ↑ 18%.</td>
</tr>
<tr>
<td>Ge et al. (2006) (TCT-STAMI)</td>
<td>20</td>
<td>6 months</td>
<td>$4 \times 10^{7}$</td>
<td>SPECT/Echo</td>
<td>EF ↑ 6.7%.</td>
</tr>
<tr>
<td>Janssens et al. (2006)</td>
<td>67</td>
<td>4 months</td>
<td>$1.7 \times 10^{8}$</td>
<td>MRI</td>
<td>No effect.</td>
</tr>
<tr>
<td>Kang et al. (2006) (MAGIC Cell-3-DES)</td>
<td>56</td>
<td>6 months</td>
<td>$1.4 \times 10^{8}$</td>
<td>MRI</td>
<td>EF ↑ 5.2%.</td>
</tr>
<tr>
<td>Lunde et al. (2006) (ASTAMI)</td>
<td>100</td>
<td>6 months</td>
<td>$8.7 \times 10^{7}$</td>
<td>SPECT/Echo/MRI</td>
<td>No effect.</td>
</tr>
<tr>
<td>Meyer et al. (2006) (BOOST)</td>
<td>60</td>
<td>18 months</td>
<td>$2.5 \times 10^{9}$</td>
<td>MRI</td>
<td>EF ↑ 6.7% at 6 mo. ns at 18 mo†.</td>
</tr>
<tr>
<td>Schächinger et al. (2006) (REPAIR-AMI)</td>
<td>204</td>
<td>4 months</td>
<td>$2.4 \times 10^{8}$</td>
<td>LV Angiography</td>
<td>EF ↑ 2.5%.</td>
</tr>
<tr>
<td>Meluzin et al. (2006)</td>
<td>66</td>
<td>3 months</td>
<td>$10^{8}$</td>
<td>SPECT/Echo</td>
<td>EF ↑ 3%. ns when $10^{7}$ cells injected.</td>
</tr>
<tr>
<td>Li et al. (2007)</td>
<td>70</td>
<td>6 months</td>
<td>$7.3 \times 10^{7}$</td>
<td>Echo</td>
<td>EF ↑ 5.5%.</td>
</tr>
<tr>
<td>Penicka et al. (2007)</td>
<td>24</td>
<td>4 months</td>
<td>$2.6 \times 10^{9}$</td>
<td>SPECT/Echo</td>
<td>No effect.†</td>
</tr>
</tbody>
</table>

All studies used the intracoronary injection route. All studies demonstrated satisfactory patient matching.

†EF at 18 months was higher than at baseline, but showed no statistically significant difference compared to control.

‡Patients with severe heart failure recruited. Trial terminated early because of adverse events and no significant benefit.

**Abbreviations:** SPECT: single-photon-emission computed tomography. MRI: magnetic resonance imaging. ns: not significant.
<table>
<thead>
<tr>
<th>Study</th>
<th>n</th>
<th>Follow up</th>
<th>Cell dose</th>
<th>Function assessment</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erbs et al. (2005)</td>
<td>26</td>
<td>3 months</td>
<td>$6.9 \times 10^6$</td>
<td>MRI</td>
<td>EF $\uparrow$ 7.2%.</td>
</tr>
<tr>
<td>Assmus et al. (2006) (TOPCARE-CHD)</td>
<td>75</td>
<td>3 months</td>
<td>$2.1 \times 10^8$</td>
<td>LV Angiography</td>
<td>EF $\uparrow$ 2.9%.</td>
</tr>
<tr>
<td>Hendrikx et al. (2006)</td>
<td>20</td>
<td>4 months</td>
<td>$6 \times 10^7$</td>
<td>MRI</td>
<td>No effect</td>
</tr>
<tr>
<td>Kang et al. (2006) (MAGIC Cell-3-DES)</td>
<td>40</td>
<td>6 months</td>
<td>$1.4 \times 10^8$</td>
<td>MRI</td>
<td>No effect</td>
</tr>
</tbody>
</table>

All studies demonstrated satisfactory patient matching.

† In this trial cells were injected intramuscularly during CABG.

**Abbreviations:** SPECT: single-photon-emission computed tomography. MRI: magnetic resonance imaging.
1.4.3 Putative mechanisms mediating the benefits of cell transplantation

“Do these distinctions matter? . . . patients benefited from many established therapies, including aspirin, before we understood the underlying mechanisms. There is no doubt that the ultimate success or failure of cell therapy will rest on its ability to show clinical efficacy rather than on the imputed mechanism.”


“The results reported so far are modest at best, inconsistent and unconvincing . . . . . . Even if the reported improvements are real, their clinical significance is highly doubtful. This messy situation has been caused, at least partly, by the lack of meaningful pre-clinical data.”


Although therapies did not always need to be fully understood before gaining clinical acceptance, elucidation of their underlying cellular mechanisms provide the most promising path towards their optimization, whilst maintaining safety. It remains impossible to predict the ideal cell type, number, and delivery protocol for cardiac cell therapy in the absence of knowledge of their physiological mechanisms of action. To obtain such information by trial and error would not only take many years and valuable resources, but also expose patients to substantial avoidable risk.

The clinical trials outlined in the previous pages and the pre-clinical experiments using animal models of heart failure were originally undertaken with the aims of true cardiac regeneration. It was hypothesized that supplementation of the diseased heart with the correct type of cells would correct the deficiency in numbers (Orlic et al. 2001). However, as discussed in section 1.4.1 the animal studies, whilst often
demonstrating clear functional improvements, have shown that substantial primary remuscularization does not happen. Apart from foetal cardiomyocytes, no type of cell has formed adequately integrated, additional myocardial tissue following injection. The numerous clinical trials have suggested between them that a modest functional benefit might exist, but the lack of human cardiac tissue for histological analysis has meant that, apart from some exceptions (e.g. Dib et al. 2005), clinical studies have not been able to follow the phenotypic fate of the cells injected into human hearts. The demonstration that the function of a diseased myocardium can be enhanced by cell therapy without any remuscularization has resulted in a search for alternative mechanisms of action. Some of these mechanisms, which are not mutually exclusive, are discussed below.

**Neoangiogenesis**

This hypothesis relates to both acute myocardial infarction and chronic ischaemia, and states that injected cells contribute towards increased development of collateral blood vessels. In the case of acute myocardial infarction, there is ongoing loss of cells at the border region (Abbate et al. 2003). It is proposed that cell transplantation attenuates the ongoing cell loss by increasing perfusion to these tenuously supplied areas (Kinnaird et al. 2004, Kocher et al. 2001).

Direct evidence that cell transplantation might benefit the recipient myocardium in such manner is provided by the study by Reffelmann et al. (2003). Using radioactive microspheres they demonstrated an increase in regional myocardial blood flow after transplantation of neonatal cardiomyocytes. In addition, the capillary densities were higher when histological ventricular sections were examined.

In the case of bone marrow-derived cell transplantation it has been suggested that the endothelial progenitor cells (EPC) might play a central role in neoangiogenesis. EPCs are CD34+ and were originally described by Asahara et al. (1997). As their name implies they are thought to be the bone marrow cells in the circulation with a chief role in formation of new collateral vessels. Kocher et al. (2001)
carried out intravenous injections of human EPCs into athymic rats which had undergone myocardial infarction and later found them incorporated in newly formed coronary vessels. Controls which had received injections of other types of human bone marrow cells did not show this effect. The injection of EPCs also reduced the extent of myocardial infarction, resulting in relatively preserved LV function. Interestingly, it has subsequently been demonstrated that injection of MSCs into animal models of ischaemic hearts and hindlimbs might also increase collateral perfusion, albeit without direct integration into the newly formed vessels (Kinnaird et al. 2004). This suggested that transplantation of bone marrow-derived cells may augment neoangiogenesis via indirect as well as direct mechanisms.

**Modulation of the extracellular matrix**

In body tissues extracellular matrix (ECM) formation and digestion, and maintenance of optimal tissue structure, are related to the fine balance between the activities of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinase (TIMPs). Pathological ventricular remodeling involves maladaptive changes in the ECM, which leads to further myocardial deterioration (Lindsey et al. 2003). A net increase in ECM leads to interstitial fibrosis. Although this can be useful in limiting ventricular enlargement, it also decreases tissue compliance and adversely affects systolic and diastolic performance.

Murtuza et al. (2004) first showed that transplantation of skeletal myoblasts results in decreased levels of collagen in the ECM, correlating with attenuated up-regulation of MMP. Xu et al. (2005) injected MSCs into infarcted rat hearts and found that the increases in cardiac expression of collagen and TIMP–1 were attenuated, whilst there was no effect on the levels of MMP–1. Correspondingly, the left ventricular enlargement was reduced and whole ventricular function improved. Thus, there is limited, but interesting experimental data to support the hypothesis that cell transplantation might modulate the ECM to augment mechanical performance of the myocardium.
Paracrine effects on the recipient myocardium

As briefly mentioned above, Kinnaird et al. (2004) demonstrated that MSCs improve the collateral circulation without directly integrating into new vessels. This effect was demonstrated in a rat model of hindlimb ischæmia. In the same study the authors found that growth media that were conditioned by 24 hour exposure to MSCs enhanced the proliferation of cultured endothelial cells and smooth muscle cells in a dose-dependent manner. Analysis of the growth media revealed increased levels of various cytokines including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), placental growth factor (PIGF), and monocyte chemoattractant protein-1 (MCP–1). Inhibition of VEGF and bFGF using antibodies only partly attenuated these effects, implying that multiple cytokines had exerted their influence in combination. From these experiments the authors concluded that MSCs increased collateral circulation through paracrine mechanisms.

A similar study supporting the presence of a paracrine effect was performed by Takahashi et al. (2006), but using unfractionated bone marrow mononuclear cells rather than only MSCs. The growth media that were conditioned by 24 hour exposure to bone marrow mononuclear cells contained higher concentrations of interleukin-1α (IL–1α), platelet-derived growth factor (PDGF), and insulin-like growth factor-1 (IGF–1), and protected cardiomyocytes isolated from normal rat hearts from hypoxia. When injected into rat hearts undergoing acute myocardial infarction the conditioned media decreased the sizes of infarcts and increased blood vessel densities, with corresponding improvements in cardiac function.

The long-term in vivo benefit demonstrated by Takahashi et al. is unexpected, considering that soluble factors remain in body tissues for only minutes to hours. However, similar effects were demonstrated when the same experiments were performed using MSCs overexpressing the pro-survival gene Akt1 (Gnecci et al. 2006). Growth media conditioned by 12 hour exposure to AKT-overexpressing MSCs protected normal isolated rat cardiomyocytes exposed to hypoxia in vitro. When injected into rat hearts undergoing myocardial infarction, the resulting infarct sizes
were reduced and ventricular function relatively preserved.

Following the demonstrations of cardioprotective effects of cell-free conditioned media the *paracrine theory* of cell transplantation has increasingly become the subject of discussion amongst scientists and also those performing clinical trials (Dimmeler et al. 2005, Srivastava & Ivey 2006, Rosenzweig 2006, Laflamme et al. 2007). However, although commonly invoked the *paracrine theory* remains undefined. At its minimum, it postulates that locally secreted substances from the injected cells benefit their surrounding myocardium, but the cellular targets and the precise physiological effects resulting in the potential benefit have not been described. Furthermore, there is only a vague consensus on the disease process that is targeted by cell transplantation. The few published studies mentioned above that have led to the *paracrine theory* have all focussed on protection of cardiomyocytes from death during an acute myocardial infarction, but the vast majority of reports in the field have put their main emphases on the function of the whole heart in the long term. Therefore, studies in cardiac cell transplantation attempting to elucidate their underlying mechanisms need to clarify their goal as an initial step – limitation of cardiomyocyte death, or attenuation of the ensuing maladaptation of the surviving cardiomyocytes?

As discussed in section 1.3 individual cardiomyocytes that have survived a myocardial infarction subsequently undergo substantial long-term maladaptive changes as the ventricle becomes remodeled. These changes contribute directly to further deterioration of whole heart performance. Whilst the potential effects of any type of transplanted cell on these chronically failing cardiomyocytes would be of major interest, they have never been studied.
1.5 Hypotheses

The collective evidence suggests that the beneficial effects of adult progenitor cell transplantation to the heart do not result from myocardial regeneration. It has been suggested that transplanted cells might improve performance by exerting indirect effects on the diseased recipient myocardium, possibly involving paracrine secretions (Dai et al. 2005, Kinnaird et al. 2004, Murry et al. 2005). The aim of this study was to address this hypothesis using a rat model of post-infarct chronic heart failure. In particular, the following hypotheses were tested.

1. The properties of individual failing recipient cardiomyocytes are affected by adult progenitor cell transplantation.

2. Paracrine mechanisms can improve the function of failing cardiomyocytes.

In Chapter 3 the first hypothesis is tested by studying the morphological and functional changes in the recipient ventricular cardiomyocyte population of a chronically failing rat heart following skeletal myoblast or bone marrow-derived cell transplantation. In Chapters 4 and 5 explanations for those observations made in Chapter 3 are sought by experiments utilizing cell co-culture systems.
Chapter 2

General Methods

The choice of animal model of heart failure to use for this study took many considerations into account. Animals in which heart failure could be induced reliably and in a consistent, reproducible manner were considered to be useful candidates. The availability of a transgenic model constitutively expressing green fluorescent protein in all organs (GFP\(^+\)) was a necessity for distinguishing transplanted cells from native cardiomyocytes. The technical feasibility with which GFP\(^+\) cells could be transplanted into failing hearts several weeks after an initial operation was important. Taken together, these three main factors led to the decision to use adult rats. Other considerations included the availability of robust methods to monitor \textit{in vivo} heart function and isolate single ventricular cardiomyocytes. The rat was also a good candidate from practical points such as cost and the need for a significant number of housed animals over many weeks.

The adult rat has been the subject of the largest number of previous studies of cell transplantation into the heart (Dowell et al. 2003). Such familiarity gave reassurance that measurable physiological effects would be induced following cell transplantation, allowing the hypotheses of this study to be tested. Furthermore, it was also felt that it would be technically feasible to inject cells with sufficient consistency to allow subsequent comparison between control and various treatment groups.
The features of cardiac EC coupling in normal hearts of many different animal species has been described by others previously (for reviews, see Hasenfuss 1998, Bers 2002). Various parameters of cardiomyocyte function and electrophysiological properties are known to differ between animal species, which is perhaps unsurprising considering differences in functional demand, whole organ size, and normal beating rate (Bers 2003). The action potential profile and gain of CICR, for example, differ significantly between mouse, rat, guinea-pig, and human (Wettwer et al. 1993, Bassani et al. 1994, Terracciano & MacLeod 1994, Pieske et al. 1999). In the rat heart the force of contraction shows a negative correlation with increasing frequency (Banijamali et al. 1991), which is a relationship opposite to that seen in guinea-pigs (Kurihara & Sakai 1985) and humans (Pieske et al. 1999). That this study was performed ultimately to increase an understanding of cell transplantation in patients with heart failure meant it would have been most useful if an animal with cardiac EC coupling similar to that of human were available for study. However, the various considerations laid out above led to the decision to use adult rats. The relevance of experiment data obtained in this study for humans is discussed later.

Previous studies of animal models of heart failure have employed various methods to apply chronic wall stress to the ventricle in several animal species (Sipido et al. 2002). Myocardial infarction caused by permanent left coronary artery ligation (Dixon et al. 1992) was the preferred mode of heart failure induction in this study, rather than alternative methods employed by others previously such as rapid electrical pacing (O’Rourke et al. 1999), chronic administration of cardiotoxic drugs, or constrictive banding of the ascending aorta (Weinberg et al. 1999). This was because coronary ligation would mimic the commonest clinical scenario (i.e. patients developing chronic heart failure following myocardial infarction and ventricular remodeling). Animal models of heart failure resulting from genetic manipulation (Gomez et al. 1997) or chronic cardiotoxic drug administration (Siveski-Iliskovic et al. 1995) were not considered appropriate, due to the possibility of interference of any physiological effects of cell transplantation. Other advantages of coronary artery ligation
included the high rate of development of heart failure as well as technical simplicity and low cost.

To date many different types of cells have been injected into failing hearts, both in animal studies (Dowell et al. 2003) and in clinical trials (Abdel-Latif et al. 2007, Lipinski et al. 2007). This study focused on the two types of cell most widely employed by others previously: skeletal myoblasts and bone marrow mononuclear cells. These two cell types are also the subjects of the most active ongoing research, probably due to their large availabilities from autologous sources. Bone marrow mononuclear cells comprise of a complex assortment of cells, including HSCs, MSCs, and the so-called side-population (SP) cells. To date no study has identified a sub-population of bone marrow mononuclear cells which exert a greater effect on whole myocardial function following injection (Laflamme et al. 2007). The majority of studies demonstrating beneficial effects of bone marrow mononuclear cells have utilized freshly prepared cells without further purification or ex vivo expansion, and this study sought to reproduce such findings and elucidate the physiological mechanisms accounting for the observations.

2.1 Induction of myocardial infarction and heart failure

All animal procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. Appropriate project and personal licenses were acquired from the United Kingdom Home Office for the duration of the study.

Myocardial infarction was induced by ligation of the left coronary artery using standard surgical methods previously described by Johns & Olson (1954) and later modified by Selye et al. (1960). Adult female Sprague-Dawley rats weighing 200 g (Harlan, UK) were anaesthetized with 1~2% isoflurane (Schering-Plough Animal Health, UK) in 100% oxygen and intubated with a 16G plastic cannula. They were then ventilated using a volume-controlled mechanical ventilator (Harvard Appara-
tus, Kent, UK) at 2.5 ml tidal volume and 70 breaths per minute. Operations were performed using aseptic conditions and autoclave-sterilized instruments to prevent infections. The skin was shaved using an electric shaver. Povidone-iodine antiseptic solution (Videne®) was applied on the operation site. After incising the skin with a scalpel, the left pectoralis major and latissimus dorsi muscles were parted using blunt dissection and retracted to reveal the ribs and intercostal muscles. Thoracotomy at the fourth intercostal space was performed using pointed-tip scissors, taking care to avoid injury to the left lung, intercostal neurovascular bundle and left internal mammary artery. Pericardiectomy then provided direct access to the heart. The left coronary artery was identified and permanently ligated at the level of the left atrial appendage using a round-needle 6–0 polypropylene suture to cause myocardial infarction and heart failure (Fig.2.1). Left coronary artery occlusion was confirmed by observing discolouration of the left ventricle (LV), including the apex, which occurred within a few seconds.

After re-inflating the lungs by positive-pressure ventilation, the chest wall was closed using a continuous 4–0 polypropylene intercostal suture. Antiseptic solution was applied again to the inner operation site and the skin was closed using a continuous 3–0 silk suture.

Once established, it became possible to complete the entire operation from skin incision to skin closure within 20 minutes. Bleeding was minimal. The 24 hour mortality following coronary artery ligation was 26/132 (∼20%).

Sham operated animals were also generated for use as normal heart controls. These were subjected to thoracotomy and pericardiectomy in an identical manner, but received no LAD ligation. Mortality in these animals was 0%.

2.2 Preparation of bone marrow mononuclear cells

Bone marrow mononuclear cells were freshly prepared on the day of use. They were harvested from adult male Sprague-Dawley transgenic rats constitutively expressing
Fig. 2.1: A) The left coronary artery was identified and permanently ligated at the level of the left atrial appendage. B) Discolouration of the left ventricle, including the apex, confirmed acute myocardial ischaemia and the onset of infarction.
green fluorescent protein (GFP+) in all tissues (from Rat Resource and Research Centre, MO, USA).

GFP is a fluorescent chromophore originally discovered in the jellyfish species *Aequorea victoria* (Johnson et al. 1962, Morise et al. 1974). GFP consists of 238 amino-acids and is a stable protein within cells. Its formation from its encoding gene is species independent, and does not require other factors. Its maximum emission is at wavelength 509 nm (green) following maximum excitation at 395 nm without the need for additional substrates (Chalfie et al. 1994). Together, these properties of GFP make it a useful *in vivo* reporter system, and it has been used in many studies of gene expression patterns and tracking of transplanted cells (for reviews, see Prasher 1995, Tsien 1998).

Rats were anaesthetized and then sacrificed by cervical dislocation. The femurs and tibiæ were removed and all soft tissue excised. The marrow cavity of each bone was flushed using 5 ml of Hank’s balanced salt solution (HBSS, Sigma-Aldrich, Poole, UK) containing 10% foetal bovine serum (FBS, Gibco, Paisley, UK) to obtain whole bone marrow aspirate. The 20 ml of whole marrow aspirate was then spun at 1200 rpm for 10 minutes, and the supernatant layer was removed, leaving only 3 ml. After trituration 1 ml of this concentrated whole marrow aspirate was then spun through a *Percoll™* gradient formed by 5 ml of 55% *Percoll™* (Density 1.130 g/ml, Amersham, Little Chalfont, UK), diluted with HBSS, layered above 5 ml of 70% *Percoll™* (also diluted with HBSS) in a 15 ml test tube at $\times 1600$ g for 25 minutes ([Fig.2.2](#)). Using a glass Pasteur pipette, the mononuclear cell fraction was then extracted from the border between the two layers, washed twice, and stored in HBSS containing 10% FBS.

Before use, the viability of these cells was checked using 0.4% trypan blue solution. Using this method, it was possible to obtain bone marrow mononuclear cells with $> 99\%$ viability consistently. Cells prepared for injection into hearts were washed once again using PBS and stored on ice.
**Fig. 2.2:** Separation of the mononuclear fraction of bone marrow cells from whole cell aspirate was done using a Percoll™ gradient. Whole cell aspirate was placed on top of 55% Percoll™. After centrifugation, the mononuclear cell fraction was extracted from the border between the 55% and 70% layers.

### 2.3 Preparation of primary skeletal myoblasts

#### 2.3.1 Harvest and passage of primary skeletal myoblasts

Primary skeletal myoblasts were cultured using the *single fibre method*, as described by Rosenblatt et al. (1995). This is a well-established method of obtaining large numbers of expanding primary skeletal myoblasts whilst avoiding contamination by other cell types such as fibroblasts.

GFP⁺ male Sprague-Dawley rats were anaesthetized and then sacrificed by cervical dislocation. The skin of the lower limbs and abdomen was shaved and prepped with antiseptic solution. Using aseptic technique, the *extensor digitorum longus* muscles were removed from the hindlimbs. Care was taken to include the tendons at both ends, and also to minimize trauma to the muscles. The excised muscles
were then shaken for 90 minutes at 35 °C in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, UK) with 2 mg/ml added collagenase (type I, Sigma-Aldrich, UK). The partially digested muscle tissue was triturated in DMEM in Petri dishes using a wide-bore (4 mm) glass Pasteur pipette to separate the single myofibres. Intact individual myofibres with healthy appearance were identified under light microscopy using a ×4 objective and plated in individual droplets of 20% Matrigel™ (Becton Dickinson, Oxford, UK) in DMEM in standard 6-well polystyrene culture plates. They were then bathed in *plating medium*, consisting of DMEM with 10% added horse serum (Gibco, UK), 0.5% chick embryo extract (Sera Labs, Bolney, UK), and 1% penicillin/streptomycin (Sigma-Aldrich) and incubated at 37 °C. At 37 °C 20% Matrigel™ sets from liquid into gel, mechanically supporting the myofibre. By 24 ∼ 48 hours after plating, the proliferating satellite cells had started to migrate away from myofibres and adhered onto the culture plate floor. The myofibres were then removed and the bathing medium changed to *proliferation medium*, which was similar to that above, but also with 20% FBS and 5 ng/ml basic fibroblast growth factor (bFGF, Invitrogen, Paisley, UK) added. Thereafter, primary skeletal myoblasts were passaged in 75 ml culture flasks every 2 ∼ 3 days and used within two weeks.

Although the *single fibre method* has been known to yield primary skeletal myoblasts of high purity consistently, measures were taken to confirm that the proliferating cells were primary skeletal myoblasts rather than contaminating cell types such as fibroblasts. Approximately 5% of the passaged cells were separated and allowed to proliferate until a confluent layer was formed. The growth medium was then changed to *differentiation medium*, consisting of DMEM with only 1% added horse serum and 1% penicillin/streptomycin. Under such conditions skeletal myoblasts were seen to fuse and form uniformly-aligned myotubes within 48 hours, confirming their skeletal tissue phenotype. However, such formed myotubes were not used for any further experimentation.

Immediately before injection skeletal myoblasts were checked using 0.4% trypan
blue. The average viability was > 98%. Cells prepared for injection into hearts were washed once again using PBS and stored on ice.

2.4 Cell injection into the heart

Three weeks after coronary artery ligation rats were anæsthetized using 1 ~ 2% isoflurane and mechanically ventilated. Thoracotomy via the fifth intercostal space was carried out, and blunt dissection was performed to free the heart from adhesions and allow access. Using a 25G needle mounted on a standard 1 ml syringe, either $10^7$ bone marrow mononuclear cells or $5 \times 10^6$ cultured skeletal myoblasts were directly injected into the myocardium. These numbers were chosen following earlier studies performed within our research group, and those reported by others (Pouzet et al. 2001, Tambara et al. 2003, Nagaya et al. 2005, Dai et al. 2005), which have demonstrated improvements in LV ejection fraction. However, it should be noted that there has been no report establishing the optimal injection numbers in any animal model or patients. Cells were suspended in 150 µl PBS. Half of this volume was injected into viable myocardial tissue at the apex of the LV. The remaining half of the cells were injected into the viable portion of the LV lateral wall. By the time of cell injection the infarcted portion of the ventricle had formed mature scar tissue which was distinguishable from the viable myocardium by visual inspection. This portion of the ventricle received no cell injection. The needle was inserted obliquely, and care was taken such that the tip would not reach the ventricular cavity (this was considered important in order to avoid injecting cells into the circulating blood as well as to minimize bleeding). Successful injection in such manner was confirmed by visualization of the needle tip within the ventricle wall throughout the procedure, blanching of the ventricle upon injection and lack of bleeding from the ventricle cavity after withdrawal of the needle.

In control heart failure animals, and in the sham-operated group, 150 µl of PBS only was injected in the same manner. After injection the lungs were re-inflated and
the chest wall was closed using a 4–0 polypropylene suture. Antiseptic solution was applied to the operation site, and the skin was closed using a 3–0 silk suture.

2.5 Monitoring heart function using echocardiography

Animals were anaesthetized by administering 1 ~ 1.5% isoflurane in 100% oxygen via a nose-cone and allowed to self-ventilate whilst in a supine position. Attempts were made to keep the level of anaesthesia minimal and constant throughout image acquisition, to avoid the haemodynamic artefacts induced by the myocardial depression and vasodilation effects of isoflurane (Davies et al. 2000, Hanouz et al. 1998, Sprague et al. 1974). Animals were shaved over the anterior chest wall and ultrasound gel was applied. Using a 15 MHz probe (model “15L8”) on an Acuson Sequoia™ 256 system (Siemens Medical Systems, Germany) transthoracic echocardiography was performed to obtain parasternal short-axis views at the level of the mid-papillary muscle (Fig.2.3). Ejection fraction (EF) was calculated from the systolic and diastolic 2-dimensional cross-sectional LV areas, using the formula below, as previously described by Yang et al. (1999):

$$\text{EF}(\%) = \left( \frac{\text{End Diastolic Area} - \text{End Systolic Area}}{\text{End Diastolic Area}} \right) \times 100 \quad (2.1)$$

Although it was also possible to calculate EF using data acquired from the M-mode studies, only the EF calculated from cross-sectional LV areas was used. A small disparity was sometimes noted between the figures obtained by the two different methods, and because the anterior wall of the LV was sometimes tethered to the inner chest wall by inflammatory adhesions, the method utilizing 2-dimensional cross-sectional LV areas was considered to be more accurate. Studies were performed immediately before cell injection and also immediately before the harvesting of hearts.
2.6 Cell isolation

Ventricular cardiomyocytes were isolated using a modification of standard enzymatic digestion protocols described previously (Hering et al. 1983, Tytgat 1994). The main strategy was to perfuse the excised whole heart via its aorta and coronary arteries with solutions containing digestive enzymes and low concentrations of Ca\(^{2+}\) to break the structural bonds between the cardiomyocytes.

Rats were sacrificed and the hearts removed quickly. The excised heart was promptly attached to a Langendorff perfusion system at the aorta via a blunted 16G needle (Fig. 2.4). The heart was then perfused via its coronary arteries for 2 minutes at 37 °C with oxygenated normal Tyrode solution containing (in mM) NaCl 140; KCl 6; glucose 10; MgCl \(_2\) 1; CaCl\(_2\) 1; \(N\)-2-hydroxyethylpiperazine-\(N\)\(^\prime\)-2-ethansulphonic acid (HEPES) 10; pH 7.4. Perfusion was carried out at a rate of approximately 12 ml per minute. Care was taken to minimize myocardial hypoxia. The perfusing solution was then changed to a low-Ca\(^{2+}\) solution containing (in mM) NaCl 120; KCl 5.4; CaCl\(_2\) 0.045; MgSO\(_4\) 5; Na\(^+\)-pyruvate 5; glucose 20; taurine 20; HEPES 10; nitrilotriacetic acid 5; pH 6.96. The change was made with the use
Fig. 2.4: Heart attached on Langendorff apparatus for retrograde perfusion via the coronary arteries

of a three-way tap, enabling continuous perfusion of the heart throughout. The heart was perfused with low-Ca\(^{2+}\) solution for 5 minutes, before changing to an enzyme solution containing (in mM) NaCl 120; KCl 5.4; CaCl\(_2\) 0.2; MgSO\(_4\) 5; Na\(^+\)-pyruvate 5; glucose 20; taurine 20; HEPES 10; pH 7.4. In addition, this solution contained 1 mg/ml collagenase (Type-2, 280 u/mg, Worthington Biochemical Corp, Lakewood, NJ, USA) and 0.6 mg/ml hyaluronidase (999 u/mg, Sigma-Aldrich). Perfusion with this enzyme solution continued for 9 minutes, after which the heart was removed from the Langendorff setup, and both atria and the right ventricle were discarded. The infarcted scar tissue was visually assessed and removed from the LV. The viable LV tissue was cut into smaller pieces and shaken in the solution containing enzymes for a further 5 minutes before gentle trituration to yield a single-cell suspension. This was then filtered through a 300 \(\mu\)m nylon mesh and stored in enzyme-free buffer at room temperature until use.

Using this method it was possible to reliably obtain sufficient amounts of Ca\(^{2+}\)-tolerant left ventricular cardiomyocytes for physiological experiments. Approxi-
mately 70% of the resulting cardiomyocytes were rod-shaped, and there was no noticeable difference in yield between the different heart groups. Isolated cardiomyocytes were used within 6 hours.

2.7 Cell area planimetry

The dimensions of individual isolated cardiomyocytes were measured and compared between groups for assessment of degrees of hypertrophy. Cardiomyocytes were examined using an Olympus IX-71™ inverted microscope with a ×60 oil-immersion objective. Digital images of rod-shaped cardiomyocytes of healthy appearance were acquired using an IonOptix Myocam™ camera (IonOptix Corp, Milton, MA, USA) attached to the microscope light path, and a standard personal computer (PC) running Wisepixel Handysnap® software (Wisepixel Multimedia, freeware). The magnification scale was calibrated by the use of a standard stage micrometer, and the projected 2-dimensional area for each cell was measured offline using ImageJ software (Rasband 1997, Abramoff et al. 2004).

![An example photograph of an isolated cardiomyocyte (left). A line was drawn around the cardiomyocyte (right) to measure the 2-dimensional area for each cell. Scale bar represents 10 μm.](image-url)
Wild Type

Bright Field | Fluorescence
---|---

GFP+

Bright Field | Fluorescence
---|---

Fig. 2.6: Example photographs of cardiomyocytes isolated from wild-type rats (left) and also from GFP$^+$ rats (right). For both examples, normal bright-field photomicrographs and fluorescence micrographs are shown. Scale bar represents 10 $\mu$m.

2.8 Examination for GFP fluorescence

To determine whether the isolated cardiomyocytes studied had originated from host or injected cells their level of green fluorescence was assessed. Cardiomyocytes were placed in a bath on the stage of a Nikon TE2000-U$^\text{TM}$ inverted microscope and initially studied by light microscopy via a $\times40$ oil-immersion objective. Using a DeltaRAM–V$^\text{TM}$ Monochromator (Photon Technology International, NJ, USA) cardiomyocytes were subjected to fluorescent excitation at 488 nm. Fluorescence emissions were passed through a 545 nm band-pass optical filter (model XF3074, Omega optical Inc. VT, USA) for visual assessment (Fig. 2.6).

2.9 Assessment of cardiomyocyte contraction by measurement of sarcomere shortening

Freshly isolated cardiomyocytes were initially studied for their contractile characteristics. The main parameters analyzed for comparison between groups were magni-
tude of contraction, speed of contraction, and speed of relaxation. These parameters were measured in field-stimulated cardiomyocytes which were not individually mechanically stretched.

The dynamics of cardiomyocyte contraction were studied by measuring the average length of the sarcomere. In this method the average distance between striations within a highlighted area of interest is calculated. A fast Fourier transform is then performed and the peak within the power spectrum represents the average sarcomere spacing, measured in real time. Compared to the traditional method of measuring whole cell length changes, this method was considered to be more sensitive to length changes, and less susceptible to artefacts from sideways movements of the cardiomyocyte.

Cardiomyocytes were placed onto a bath held within the stage of an Olympus IX-71™ inverted microscope. Approximately 3 µl of 1 mg/ml laminin solution (Sigma-Aldrich) was applied to the bath before plating cardiomyocytes to aid their adhesion to the bath floor. Cardiomyocytes were superfused with normal Tyrode solution containing 1 mM Ca²⁺. The flow of normal Tyrode solution through the bath was by gravity, and controlled to ensure laminar flow and maintain a bath depth of approximately 1 mm. The normal Tyrode solution was heated by a custom-made heating jacket covering the solution tubing. The temperature of the heating jacket was controlled to maintain a bath temperature of 36 ∼ 38 °C, and was checked regularly.

The cardiomyocytes in the bath were electrically field-stimulated at 1 ∼ 5 Hz using platinum electrodes connected to an IonOptix Myopacer™ bipolar field stimulator (IonOptix Corp.). The stimulation pulse duration was 5 ms. The output voltage was adjusted to stimulate over 80% of the rod-shaped cardiomyocytes and was usually in the range of 10 ∼ 20 V. The output signal from the field-stimulator was also connected to the IonOptix Fluorescence System Interface™ (the image data processor, IonOptix Corp.), enabling its recording during data acquisition.

Images of contracting cardiomyocytes were acquired at a rate of 240 frames per
second using an IonOptix Myocam™ video camera (model CCD100M, IonOptix Corp.) attached to the microscope light-path. The signal was processed using an IonOptix Video Power Controller™ and IonOptix Fluorescence System Interface™ connected to a standard personal computer (PC) running IonOptix IonWizard™ data acquisition software (Version 5.0. IonOptix Corp.). The captured images underwent real-time Fourier analysis of the striations in a selected area of approximately 50% of the contracting cardiomyocyte under study. This yielded sarcomere length shortening data averaged for the cardiomyocyte.

Offline analysis of the acquired sarcomere shortening patterns were carried out using IonWizard™ software (Fig. 2.7). Between 10 and 20 representative steady-state contractions were averaged with reference to the field-stimulation signal. The baseline sarcomere length, peak contraction amplitude, and time-to-peak ($T_{peak}$) of the averaged contraction were calculated using the field-stimulation signal as a reference point. The time-to-50% relaxation ($T_{50}$) and time-to-90% relaxation ($T_{90}$) of the averaged contraction were calculated from $T_{peak}$.
2.10 Measurement of Ca\textsuperscript{2+} transients using indo-1

The dynamics of intracellular Ca\textsuperscript{2+} is a major determinant of EC coupling. Cytoplasmic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) and its pattern of change with each contraction was monitored using the Ca\textsuperscript{2+}-sensitive fluorescent indicator indo-1. Indo-1 is a widely used intracellular Ca\textsuperscript{2+} indicator first introduced by Grynkiewicz et al. in 1985 and has several useful characteristics:

- High selectivity for Ca\textsuperscript{2+} than for other divalent cations
- High fluorescence quantum efficiency and low affinity for Ca\textsuperscript{2+}, leading to less buffering of cellular Ca\textsuperscript{2+} and easier observation of intracellular Ca\textsuperscript{2+} dynamics
- Ratiometry. The absorption maximum of indo-1 is in the region of 360 nm. The maximum emission shifts from 495 nm in zero Ca\textsuperscript{2+} towards 405 nm when indo-1 combines with Ca\textsuperscript{2+}. This means the fluorescence emission at 405 nm increases whilst that at 495 nm decreases with increasing [Ca\textsuperscript{2+}] (Fig. 2.8A). Thus, the ratio of the fluorescence emissions (i.e. \(\frac{F_{405}}{F_{495}}\)) allows accurate estimation of [Ca\textsuperscript{2+}]\textsubscript{i}, changing only with alterations in [Ca\textsuperscript{2+}]\textsubscript{i} and independent of other factors (Tsien & Poenie 1986). This ratiometric property of indo-1 is useful in that it corrects for uneven loading between cells, dye leakage, different cell sizes (including thickness), and photobleaching during the course of an experiment.

**Loading cardiomyocytes with indo-1:** Cardiomyocytes were loaded with indo-1 (Molecular Probes, UK) using its acetoxyemale (AM) ester form, as originally described by Tsien (1981). In this preparation the indo-1 is made membrane-permeable temporarily by masking its charged groups with esterifying groups. Once in the cytoplasm, the esterifying groups are hydrolyzed and the indo-1 becomes trapped within the cell. The only alternative method of using pure indo-1 salts necessitates direct microinjection into cells. Not only is this cumbersome, but the membrane integrity and cytoplasmic composition
Fig. 2.8:  A. Fluorescence emission spectra of indo-1 in solutions containing various concentrations of free Ca$^{2+}$. The fluorescence emission at 405 nm increases and that at 495 nm decreases when indo-1 combines with Ca$^{2+}$ (Taken from “Fura and Indo Ratiometric Calcium Indicators”, Molecular Probes® Product information.). B. Schematic illustration of the microscope setup for simultaneous visual assessment, cardiomyocyte sarcomere length measurement, and indo-1 fluorescence studies.

are disturbed. The method using the AM preparation enables simultaneous loading of many cells without disrupting their cell membranes.

Indo-1 AM was dissolved in enzyme buffer at 10 µM concentration. Cardiomyocytes were incubated in this solution at room temperature for 20 minutes in a dark environment. They were then allowed to de-esterify in enzyme buffer for at least 1 hour before study.

Hardware setup: Cardiomyocytes loaded with indo-1 were placed onto a bath held within an inverted microscope stage and superfused, as above. They
were then subjected to visible illumination by light of wavelength $> 700$ nm. At the same time, they underwent fluorescence excitation at a wavelength of 355 nm by means of a xenon lamp light source and a narrow band-pass optical filter (Type 355HT15, Omega Optical Inc. VT, USA) held within an IonOptix Microstepper™ device (IonOptix Corp.). The 355 nm illumination was limited to the duration of fluorescence acquisition only, minimizing the photobleaching of indo-1.

**Acquisition of fluorescence emissions:** This setup enabled simultaneous visual assessment via the microscope eyepiece, measurement of cardiomyocyte sarcomere length dynamics, and indo-1 fluorescence detection (Fig.2.8B). A dichroic mirror (type 585DCXR, Chroma Technology®, VT, USA) separated visible light within the light path of the microscope such that light of wavelength $> 585$ nm was directed to the video camera whilst that $< 585$ nm was directed to the photomultiplier tubes (PMTs). Fluorescence emissions from cardiomyocytes (light $< 585$ nm) were split into two light paths via another dichroic mirror (type 455DCLPO2, Omega Optical Inc.). The emissions with wavelength $< 455$ nm were allowed to pass through a 405 nm narrow band-pass optical filter and those $> 455$ nm through a 495 nm optical filter (Types 405DF40 and 495DF20, respectively, both from Omega Optical Inc.) before detection by two separate PMTs (Type P30CWAD5-62, from Electron Tubes Ltd, Ruislip, UK.).

Analogue output signals from the two photomultiplier tubes were digitized at 500 Hz and recorded by the IonOptix Fluorescence System Interface™ connected to a PC running IonOptix IonWizard™ data acquisition software, as above. The indo-1 fluorescence ratio ($F_{405/495}$) was calculated and taken as a measure of $[\text{Ca}^{2+}]_i$ for comparison between groups.

**Background subtraction:** Immediately before fluorescence acquisition the field of view was moved to a part of the bath containing no cells and the fluorescence
levels recorded. After setting the fluorescence levels at this background to zero the field of view was moved back to the cardiomyocyte under study for the experiment.

**Protocol for field-stimulation transients:** Cardiomyocytes were studied whilst being field-stimulated at 1, 2, or 3 Hz. Once steady-state contractions were established fluorescence emissions were acquired. Offline analysis of the acquired indo-1 transients was carried out using Ionwizard™ software, in a manner similar to the analysis of sarcomere shortening. Between 10 and 20 transients were averaged with reference to the field-stimulation signal. The baseline levels, peak amplitudes, and times-to-peak ($T_{\text{peak}}$) of the averaged transient were calculated using the field-stimulation signal as a reference point. The times-to-50% decay ($T_{\text{50}}$) and times-to-90% decay ($T_{\text{90}}$) of the transients were calculated from $T_{\text{peak}}$ (Fig.2.9).

**Protocol for caffeine-induced transients** Caffeine application studies were performed to assess the sarcoplasmic reticulum (SR) Ca$^{2+}$ content, fractional release, and the relative contribution of Na$^+$/Ca$^{2+}$ exchange (NCX) towards Ca$^{2+}$ removal during diastole (Callewaert et al. 1989). The protocol relies on the ability of rapidly applied caffeine to release Ca$^{2+}$ from the SR completely,
Fig. 2.10: Protocol for studying SR Ca\textsuperscript{2+} handling. Following a train of 1 Hz field stimulation in normal Tyrode solution, the stimulator was stopped, and the solution was rapidly changed to that containing 0 Na\textsuperscript{+} and 0 Ca\textsuperscript{2+} for 100 ms. This was then followed by a 1 s application of 20 mM caffeine diluted in 0 Na\textsuperscript{+}/0 Ca\textsuperscript{2+} solution. Finally, 20 mM caffeine in normal Tyrode was applied for 5 s to determine the rate of Ca\textsuperscript{2+} extrusion mediated by NCX.

whilst inhibiting its re-uptake. Field-stimulation was stopped and then 20 mM caffeine was rapidly applied for 5 seconds using a solenoid-controlled solution switching device, as described previously by Terracciano & MacLeod (2001). The protocol was as follows: the superfusate was switched to a 0 Na\textsuperscript{+}/0 Ca\textsuperscript{2+} solution containing (in mM) LiCl 140; glucose 10; HEPES 10; EGTA 0.75; MgCl\textsubscript{2} 1; KOH 6; pH 7.40 for 100 ms. This prevented Ca\textsuperscript{2+} extrusion by NCX. This was followed by a 1 second application of 20 mM caffeine dissolved in the same 0 Na\textsuperscript{+}/0 Ca\textsuperscript{2+} solution to open the ryanodine receptors, leading to complete Ca\textsuperscript{2+} release from the SR (see Fig.2.10). Finally, 20 mM caffeine in normal Tyrode was then applied for 5 seconds to determine the rate of Ca\textsuperscript{2+} extrusion mediated by NCX.
The peak amplitude of the caffeine-induced transient (Amp_{Caffeine}) was taken as a measure of the total SR Ca^{2+} content. The amplitude of the immediately preceding 1 Hz transient (Amp_{Twitch}) divided by the caffeine-induced transient was taken as the fractional release. Monoexponential curves were fitted to the decay phases of the caffeine-induced transient and the preceding 1 Hz twitch transient. Once the decay rate constants (\(\kappa\)) of these monoexponential curves were calculated the relative contribution of NCX towards Ca^{2+} removal during diastole was calculated by dividing the decay time constant (\(\tau\)) of the caffeine-induced transient by that of the 1 Hz twitch transient, as described by Maier et al. (2003):

\[
\text{Fractional release} = \frac{Amp_{Twitch}}{Amp_{Caffeine}} \tag{2.2}
\]

\[
\text{Contribution of NCX} = \frac{\tau_{Twitch}}{\tau_{Caffeine}} \tag{2.3}
\]

Offline analysis of the caffeine-induced transients was also carried out using Ionwizard™ software. Only caffeine-induced transients with complete, singular release were analyzed.

### 2.11 Measurement of intracellular pH and sodium-hydrogen exchanger activity

#### 2.11.1 Measurement of intracellular pH

The intracellular pH (pH_{i}) of cardiomyocytes was measured using the pH-sensitive fluorescent indicator 5-(and-6)-Carboxy-SNARF®-1 (SNARF). Similar to the Ca^{2+}-sensitivity of indo-1, SNARF shows pH-dependent emission wavelength shift. This enables the fluorescence intensities at two different wavelengths (typically 580 nm and 640 nm) to be measured for accurate pH determination, with the ratiometric property correcting between uneven loading, different cell sizes, and bleaching during an experiment.
**Fig. 2.11**: Fluorescence emission spectra of 5-(and-6)-Carboxy-SNARF-1 following excitation at 488 nm in solutions of varying pH. The fluorescence emission at 640 nm increases whilst that at 580 nm decreases with increasing pH (Taken from “SNARF® pH Indicators”, Molecular Probes® Product information.)

The AM form of SNARF (Molecular Probes, UK) was dissolved in enzyme solution at 10 µM concentration. Cardiomyocytes were immersed in this solution at room temperature for 8 minutes, and then allowed to de-esterify for at least 1 hour before study. They were then plated on a perfusion bath attached to the stage of a Nikon TE200™ inverted microscope. They were studied with a ×40 oil immersion objective whilst being superfused with normal Tyrode solution containing 1 mM Ca²⁺ at 37 °C. Light from a Nikon® xenon lamp (Model No LH-M100CB-1) powered by a Hamamatsu® power supply (Model No C6979, Hamamatsu Photonics®, Japan) was passed through a 480 nm narrow band-pass optical filter for fluorescent excitation.

Fluorescent light emitted was split within the microscope light path by means of a 610 nm dichroic mirror (Model No 610DRLP). The light < 610 nm was then passed through a 580 nm narrow band-pass optical filter (Model No 580DF30) and that > 610 nm was passed through a 640 nm filter (Model No 640DF35; both filters and the dichroic mirror were from Omega Optical Inc.) before detection by two separate PMTs (Type 9789B, Electron Tubes Ltd.).
The outputs from the two PMTs were directed to a custom-made analogue signal ratio amplifier. Immediately before fluorescence acquisition the field of view was moved to a part of the bath containing no cells (the background). The fluorescence levels at this background was set to zero by the analogue signal ratio amplifier. The field of view was then moved back to the cardiomyocyte under study for data acquisition.

The ratio of the two background-subtracted output signals \( \frac{F_{580}}{F_{640}} \) which was given out by the ratio amplifier was digitized at 250 Hz by an Axon MiniDigi™ analogue-to-digital converter. The digitized signal was acquired and stored by a standard PC running Axon pClamp™ software (version 9.2).

### 2.11.2 Calibration of SNARF in situ

A standard curve plotting the relationship between \( \frac{F_{580}}{F_{640}} \) and \( \text{pH}_i \) was generated using calibration superfusates, as described previously (Lagadic-Gossmann et al. 1992, Sun et al. 1996). Calibration superfusates of pH 5.5, 7.5, and 9.5, containing 10 \( \mu \text{M} \) nigericin (Sigma-Aldrich, UK) were used (see Table 2.1 for compositions). The nigericin permeabilized the cardiomyocytes to allow intracellular pH to equilibrate with the superfusate, but not to allow the intracellular SNARF to diffuse away. Readings of \( \frac{F_{580}}{F_{640}} \) at pH 5.5, 7.5, and 9.5 were used to plot the standard curve, allowing subsequent readings to be translated directly to \( \text{pH}_i \).

### 2.11.3 Measurement of intracellular buffering capacity and sodium-hydrogen exchanger activity

Because the changes in pH in response to an intracellular acid load depend on intracellular buffering capacity (\( \beta_i \)) as well as the ability of the cardiomyocyte to extrude acid through the sodium-hydrogen exchanger (NHE), \( \beta_i \) was derived from a separate series of experiments. SNARF-loaded cardiomyocytes were superfused in normal Tyrode solution as above, with added 5-(N-ethyl-N-isopropyl)amiloride
Table 2.1: Calibration superfusates used for generating the standard curve plotting the relationship between $F_{580/640}$ and pH$_i$.

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<th>5.5</th>
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<tr>
<td>KCl</td>
<td>140 mM</td>
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<td>MgCl$_2$</td>
<td>1 mM</td>
<td>1 mM</td>
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<tr>
<td>nigericin</td>
<td>10 µM</td>
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<tr>
<td>MES</td>
<td>20 mM</td>
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<td>HEPES</td>
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<td>CAPSO</td>
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<tr>
<td>pH (using NaOH)</td>
<td>5.5</td>
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Abbreviations: MES: 2-((N-morpholino)ethanesulphonic acid. 
HEPES: N-2-hydroxyethylpiperazine-N’-2-ethansulphonic acid. 
CAPSO: 3-cyclohexylamino-2-hydroxy-1-propane-sulphonic acid.

(EIPA, Sigma-Aldrich, UK), an inhibitor of NHE ($n=3$). 0, 5, 10, and 20 mM NH$_4$Cl was applied to exert varying loads of intracellular acid. The resulting shifts in pH$_i$ measured allowed the deduction of $\beta_i$, which was estimated to be

$$\beta_i = -15.6 \times \text{pH}_i + 85$$

### 2.11.4 Protocol for measurement of resting pH$_i$ and NHE activity

Cardiomyocytes were studied for their resting pH$_i$ and NHE activity. For assessing NHE activity, pH$_i$ recovery was measured after subjecting the cardiomyocyte to an intracellular acid load using the NH$_4$Cl removal technique (Fig.2.12B-E.). This technique allows delivery of a consistent acid load to the cytoplasm. The acid load can be varied by changing the extracellular NH$_4$Cl concentrations and/or exposure time. Furthermore, the extracellular pH remains unaffected during the experiment, including the recovery phase. For these reasons, the NH$_4$Cl removal technique has been widely adopted by others in studies of acidosis (Bountra & Vaughan-Jones).
The technique utilizes the difference between the permeability of cell membranes to \( \text{NH}_3 \) and \( \text{NH}_4^+ \). When a cardiomyocyte is subjected to \( \text{NH}_4 \text{Cl} \) solution for a few minutes, \( \text{NH}_3 \), which is uncharged and therefore more permeable to the cell membrane, enters the cell faster than \( \text{NH}_4^+ \). Once intracellular, \( \text{NH}_3 \) buffers protons to cause pH to rise. Subsequently, as \( \text{NH}_4^+ \) eventually also enters the cell in similar quantities, pH returns to normal values. Upon changing the superfusate to one containing no \( \text{NH}_4 \text{Cl} \) a large intracellular acidosis is then induced because this time \( \text{NH}_3 \) leaves the cell faster, leaving \( \text{NH}_4^+ \) behind which dissociates from protons (for a review of this mechanism, see Roos & Boron 1981).

Throughout this study cardiomyocytes were superfused with normal Tyrode solution. A bath depth of less than 1 mm was maintained, and free gas exchange was allowed between cardiomyocytes and the atmosphere. There was no added \( \text{HCO}_3^- \) in the normal Tyrode solution, minimizing the effect of the \( \text{CO}_2/\text{HCO}_3^- \) buffer system on pH. The baseline \( F_{580/640} \), representing resting pH, was measured during the initial period of superfusion with normal Tyrode solution. The superfusate was then changed to that containing 15 nM \( \text{NH}_4 \text{Cl} \) for 5 minutes (Fig.2.12B,C). This was followed by a recovery phase in normal Tyrode solution, during which the cardiomyocyte was allowed to extrude protons via NHE (Fig.2.12D,E). \( F_{580/640} \) was translated to pH directly using the standard curve obtained using the method above. Having identified \( \beta_i \) it was also possible to measure NHE activity at various pH points.
**Fig. 2.12:** A. An example trace of SNARF $F_{580/640}$ ratiometric signal during the NH$_4$Cl removal protocol. Following measurement of the baseline value for $F_{580/640}$ 15 nM NH$_4$Cl was applied for 5 minutes. During this time pH$_i$ initially increases as NH$_3$ enters the cardiomyocyte and binds to the free protons (B). As NH$_4^+$ then follows the NH$_3$ into the cell pH$_i$ returns to baseline levels (C). During washout with normal Tyrode solution, NH$_3$ leaves the cardiomyocyte first. This applies an intracellular acid load as NH$_4^+$ dissociates into NH$_3$andH$^+$ (D). Finally, as protons are removed by NHE, pH$_i$ recovers to baseline levels (E).
2.12 Electrophysiological parameters

EC coupling in cardiomyocytes isolated from hearts were further studied using electrophysiological measurements. Whole cell clamping was employed for direct measurements of membrane potentials and currents in intact cardiomyocytes. High-resistance microelectrodes were used for action potentials (AP) and L-type Ca\(^{2+}\) currents (\(I_{\text{Ca,L}}\)). The Na\(^{+}\)/Ca\(^{2+}\) exchanger current (\(I_{\text{NCX}}\)) was measured using the whole cell patch clamping method, employing low-resistance patch pipettes.

As part of these experiments the whole cell membrane capacitance data were also obtained. Membrane capacitance is a useful measurement of cell surface area, and the collated data obtained were compared to the cell surface area estimation made using planimetry (section 2.7).

2.12.1 Measurement of action potentials

The APs of isolated cardiomyocytes were measured using the current clamping method using high-resistance electrode micropipettes (Fig.2.13). This method enables measurement of fast changes in membrane potentials in intact cells whilst causing minimal disruption to the composition of the cytoplasm.

**Hardware Setup:** Cardiomyocytes were superfused with normal Tyrode solution containing 1 mM Ca\(^{2+}\) at 37 °C. The bath was attached to a Nikon Diaphot 200™ inverted microscope which was supported by an air-inflated vibration isolation table (Technical Manufacturing Corp®. Peabody, MA, USA). A Type-2 Faraday cage (Technical Manufacturing Corp®) was attached to the table, shielding the microscope setup in order to minimize interference from external static electrical fields.

**Electrode pipettes:** High-resistance electrode micropipettes were fabricated from borosilicate glass tubes of 0.86 mm inner diameter (Harvard Apparatus, UK) using a microprocessor-controlled Flaming/Brown pipette-pulling machine (Model P97, Sutter Instruments, CA, USA). Once filled with the pipette-filling
solution, the pipette resistances were $20 \sim 30$ M$\Omega$. The pipette-filling solution contained (in mM) KCl 2000, HEPES 5, EGTA 0.1; pH 7.2 using KOH.

**Circuit**: Pipettes were mounted onto an Axon HS-2A headstage (Axon Instruments) which was manoeuvred by a Burleigh PCS-5000™ piezoelectric micromanipulator (EXFO Lifesciences, Ontario, Canada). An AgCl pellet ground electrode was placed in the same bath and connected to the headstage to complete the circuit. The headstage was controlled by an Axon Axoclamp 2B™ amplifier (Axon Instruments) which communicated with a standard PC via an Axon Digidata 1200™ analogue-to-digital conversion board. The PC utilized pClamp™ software (version 8.2) for running current-clamp protocols whilst acquiring and recording data.

**Entry into the cardiomyocyte**: Once the configuration above was prepared the micropipette tip was inserted into the bath superfusate whilst applying a 100 Hz, 10 mV square wave. The potential difference between the electrodes
Fig. 2.14: Example trace of a rat ventricular myocyte action potential elicited from a 1 ms pulse. \( \text{APD}_{50} \) and \( \text{APD}_{90} \) were measured from the upstroke of the stimulation pulse.

(pipette offset) was then calibrated to zero. The pipette resistance and pipette capacitance were compensated. The pipette tip was then inserted into the cardiomyocyte by the micromanipulator under visual guidance.

**Amplifier configuration:** Action potentials (AP) were measured in *discontinuous current-clamp (DCC) mode*. In DCC mode the clamping current is passed cyclically through the microelectrode. After all transient voltages due to this current passing have decayed completely, the microelectrode tip records the membrane voltage, which is memorized by a “sample-and-hold” circuit. A new cycle of clamping current application is then started again (Brennecke & Lindemann 1974a, Finkel & Redman 1984). Compared to alternative modes of sampling DCC mode may increase the measurement noise but offers the advantage of recording membrane potentials independently of voltage drops across the microelectrode. Furthermore, DCC mode is also more tolerant of small changes in pipette resistance that may occur during experiments. In order to maintain the fastest dynamic response and smallest steady-state error, the highest cycling frequency and highest open-loop gain that would allow stable maintenance of clamp were chosen. In these AP measurement experiments
the frequency of cycling between current clamping and voltage sampling was usually 6 ∼ 7 kHz.

**Acquisition protocol and data analysis**: Cardiomyocytes were stimulated at 1, 3, and 5 Hz using a 1 ms, 1.2 ∼ 1.4 nA pulse. Data were sampled at 10 kHz for recording. The recorded APs were analyzed offline using pClamp™ software (version 9.0). Approximately 20 ∼ 30 representative traces were averaged with reference to the stimulation pulse, and the time to 50% repolarization (APD$_{50}$) and time to 90% repolarization (APD$_{90}$) from the stimulation pulse were taken as measures of the AP duration for comparison between groups (Fig.2.14).

### 2.12.2 Measurement of the L-type Ca$^{2+}$ current

**Hardware setup and amplifier configuration**: L-type Ca$^{2+}$ currents ($I_{Ca,L}$) were measured using the identical hardware configuration as for AP measurement, but the amplifier was set to discontinuous single electrode voltage-clamp mode (dSEVC). In this mode the amplifier continuously cycles between passing the clamping currents and measuring voltages. The amount of pulsed clamping current is proportional to the measured deviation of the membrane voltage from the command voltage (Brennecke & Lindemann 1974a, Brennecke & Lindemann 1974b). Within each cycle, the voltage drop across the electrode is allowed to decay completely before each membrane potential measurement. This gives dSEVC its main advantage, that actual membrane voltages are measured rather than command voltage. As well as eliminating errors arising due to electrode series resistance dSEVC enables accurate control of membrane voltages when large clamping currents are required. The main disadvantage of dSEVC is the inherently greater noise, making dSEVC only suitable for measuring large membrane currents, such as $I_{Ca,L}$. In this study the cycling frequency was generally 6 ∼ 7 kHz.

**Cell membrane capacitance measurement**: The cell membrane acts like an
electrical insulator between two conducting media, the superfusate and the cytoplasm. This constitutes an electrical capacitor, whereby the two ionic media are the two conducting plates and the cell membrane is the dielectric. The capacitance of the membrane, $C_m$, can be approximated by the following equation:

$$C_m \simeq \varepsilon \cdot \frac{A}{d}$$

(2.4)

where $A = \text{Cell Membrane Area}$, $d = \text{Cell Membrane Thickness}$ and $\varepsilon = \text{permittivity of the membrane}$. The equation is a good approximation when $A \gg d$. Because $\varepsilon$ and $d$ can be assumed to be constant under most physiological conditions, $C_m$ is directly proportional to the membrane area.

In these experiments $C_m$ was measured after establishing the stable whole cell configuration by means of a capacitative surge technique (Fenwick et al. 1982): a 10 mV step was applied at $\sim 100$ Hz and a monoexponential curve fitted to the decay phase of the resulting membrane current trace. Once the decay time constant ($\tau$) of this curve was determined $C_m$ was calculated by:

$$C_m = \frac{I_0 \cdot \tau}{V_{step}}$$

(2.5)

where $I_0$ is the membrane current at time $= 0$, and $V_{step}$ is 10 mV (Fig. 2.15).

The $C_m$ values obtained were used to normalize currents measured from cardiomyocytes of different sizes (i.e. the densities of measured currents were compared between heart groups). The value of $C_m$ itself was also compared between heart groups, as a measure of hypertrophy.

**Protocol for measuring current density** : Current-voltage (I–V) relationships were built using 450 ms depolarization steps from a holding potential of $-40$ mV (range $-45$ mV to $+50$ mV, in 5 mV increments, Fig. 2.16A). Holding the membrane potential at $-40$ mV, rather than at the normal resting membrane potential of $-75$ mV enabled activation of $I_{Ca,L}$ without contamination by the voltage-gated sodium channel current. The protocol steps were
Fig. 2.15: Measurement of cell membrane capacitance using the capacitative surge technique. **Abbreviations:** $V_m$ membrane voltage; $V_{step}$ voltage step, which was usually 10 mV; $I_m$ membrane current; $I_0$ initial membrane current, at $t = 0$; $Q_m$ charge held by membrane, which is represented by the area under the curve; $\tau$ time constant of the monoeponential curve fitted to the decay phase of the current trace.

applied at 1 Hz. $I_{Ca,L}$ was taken to be the peak current elicited following depolarization minus the maintained current (Fig. 2.16B), and was normalized to the whole cell capacitance for comparison between groups.

In previous experiments conducted in the laboratory using this technique it was possible to abolish the current measured in Fig. 2.16B by acute application of 10 mM nifedipine, a specific antagonist of the L-type Ca$^{2+}$ channel. On the other hand application of 4-aminopyridine (4–AP), an antagonist of the K$^+$ channel, had no effect. These observations confirmed that the measured currents were attributable to $I_{Ca,L}$.

**Protocol for measuring time- and Ca$^{2+}$-dependent inactivation of $I_{Ca,L}$:**

To study time- and Ca$^{2+}$-dependent inactivation of $I_{Ca,L}$ a two-exponential decay of the current at 0 mV was calculated (protocol step shown in Fig. 2.16C). This particular step was used because it usually activated the largest current. The fast and slow components of $I_{Ca,L}$ inactivation were then identified for calculation of their respective decay rate constants ($\tau_{fast}$ and $\tau_{slow}$).
Protocol for voltage-dependent activation and inactivation of $I_{Ca,L}$: The voltage-dependent steady-state activation profile ($G/G_{MAX}$) was derived from the same multiple depolarization step protocol used for obtaining the I–V relationships (Fig. 2.16A). The command voltage step which elicited the maximum $I_{Ca,L}$ was initially identified. The fraction of this maximum $I_{Ca,L}$ elicited by the preceding command steps was then calculated.

The steady-state inactivation ($F/F_{MAX}$) profile of the $I_{Ca,L}$ was studied using a double-pulse protocol, as previously described (Keung et al. 1991, Lee et al. 1985). Inactivating prepulses of 200 ms duration were applied from a holding potential of $-50$ mV, after which the membrane potential was held at $-40$ mV for 5 ms, and then the test pulse applied (Fig. 2.16E). As the initial pulse was gradually increased the $I_{Ca,L}$ elicited by the second (test) pulse was seen to gradually decrease, until it was completely inhibited, usually at 0 or $+5$ mV. Boltzmann curves were fitted to the mean data points to obtain the profiles of $G/G_{MAX}$ and $F/F_{MAX}$. All $I_{Ca,L}$ data were analyzed offline using pClamp™ software (version 9.0).

2.12.3 Measurement of the Na$^+/Ca^{2+}$ exchanger current density

The Na$^+/Ca^{2+}$ exchanger currents ($I_{NCX}$) of cardiomyocytes were measured with the whole-cell patch-clamping method, originally described by Hamill et al. (1981). Compared to the method employing high-resistance microelectrodes which was used to measure APs and $I_{Ca,L}$, this method has the advantage of lower electrical access resistance. The signals recorded are less affected by interference noise, enabling measurements of small currents (for a review on whole-cell patch-clamping see Sakmann & Neher 1984). Unlike the high-resistance microelectrodes the large bore of the
Fig. 2.16: A. Protocol for measurement of $I_{Ca,L}$ density, $I_{Ca,L}$ time-dependent inactivation, and $G/G_{MAX}$. Voltage steps of 450 ms duration were applied from a holding potential of $-40$ mV. B. The resulting current trace from A. $I_{Ca,L}$ was taken to be the difference between the peak inward current and the steady-state current at the end of the 450 ms depolarization step. C. Command step from $-40$ mV to 0 mV was used to activate the maximum $I_{Ca,L}$, shown in (D). The two-exponential decay of this current was studied for identification of $\tau_{fast}$ and $\tau_{slow}$. E. Double-pulse protocol for measurement of $F/F_{MAX}$. Variable inactivating prepulse steps of 200 ms duration were applied from $-50$ mV. The membrane potential was then held at $-40$ mV for 5 ms, and before applying the test pulse. F. The resulting current trace from E.
Fig. 2.17: Schematic view of whole-cell patch clamping configuration. The electrode pipette is first “patched” onto the cardiomyocyte membrane, forming a high-resistance (> 1 GΩ) seal. The portion of cell membrane enclosed within the pipette bore is then carefully ruptured by a combination of suction and application of a brief voltage pulse to allow access. The large bore of patch electrodes (∼ 1 µm) allows full dialysis between the pipette-filling solution and the cytoplasm (coloured in grey). One AgCl electrode wire is placed inside the pipette and an AgCl pellet electrode is placed in the superfusate bath to complete the circuit.

patch pipette tip (∼ 1 µm) enables the pipette-filling solution to dialyze the cytoplasm of the cell under study within a few minutes. Because the volume of the pipette is many orders of magnitude larger than that of the cardiomyocyte, the composition of the pipette-filling solution effectively determines the intracellular environment conditions (Fig. 2.17).

Hardware Setup: Cardiomyocytes were placed in a bath on the stage of a Nikon TE2000-U™ inverted microscope. They were then superfused at 37 °C with K⁺-free solution containing (in mM) NaCl 140; HEPES 10; glucose 10; MgCl₂ 1; CaCl₂ 1; CsCl 6; pH 7.4. In addition, the solution contained 0.01 mM strophanthidin (Sigma-Aldrich, UK). Together with the lack of K⁺ in the superfusate, this eliminated the current attributable to Na⁺/K⁺ ATPase. The superfusate also contained 0.01 mM nifedipine (Sigma-Aldrich, UK) to eliminate contaminating ICa,L.
Electrode pipettes: The low-resistance electrode pipettes were fabricated from borosilicate glass tubes of 0.86 mm inner diameter. The same Flaming-Brown pipette-pulling machine was used as for the high-resistance pipettes, but glass tubes were heated less and pulled in five stages to give pipettes with much wider, fire-polished tips. Once filled with the pipette-filling solution, the pipette resistances were 2.3 ± 0.1 MΩ. The pipette-filling solution contained (in mM) CsCl 45; HEPES 20; MgCl₂ 11; Na₂ATP 10; CsOH 100; EGTA 50; CaCO₃ 25; pH 7.2 with CsOH. The high concentrations of Ca²⁺-EGTA were added to buffer the [Ca²⁺]ᵢ during application of the protocol. [Ca²⁺]ᵢ was calculated to be ~ 250 nM, as previously described by Quinn et al. (2003).

Circuit: Pipettes were held by an Axon pipette holder (model HL-U) which had a side-vent connected to a 1 ml syringe via a three-way tap for application of air suction as required during the patching process. The pipette holder was attached to an Axon CV-7A headstage, which was manoeuvred by a Burleigh PCS-5000™ micromanipulator. A silver chloride pellet ground electrode was placed in the same bath and connected to the headstage.

Entry into the cardiomyocyte: The patch pipette tip was inserted into the superfusate and the pipette offset was nulled. A 100 Hz, 10 mV square wave was continuously applied and the current induced was monitored continuously on an oscilloscope. The pipette tip was manoeuvred onto the cardiomyocyte membrane whilst monitoring visually and also via the oscilloscope. A small amount of suction was applied to the side-vent of the the pipette holder to establish a high-resistance (> 1 GΩ) seal. The pipette was then voltage clamped at −40 mV, which approximates the anticipated resting membrane potential, before rupturing the portion of membrane within the pipette tip using a combination of suction and application of a brief voltage transient (“zap”) to allow access into the cytoplasm. After achieving the whole-cell configuration, a period of at least 4 minutes was allowed for complete dialysis of the
pipette-filling solution into the cardiomyocyte cytoplasm. Following dialysis, the resting membrane potential was usually in the region of $-35$ mV, and the access resistance was $3 \sim 6$ MΩ.

**Amplifier configuration:** With the low-resistance access obtained above, it was possible to use continuous single electrode voltage-clamp (cSEVC) mode on a Multiclamp 700A™ amplifier (Axon Instruments). Here, the membrane current is continuously recorded whilst the voltage at the top of the patch pipette is simply clamped to dynamically changing command voltages by means of a feedback amplifier. Voltages acquired using this mode become meaningful only when the current-induced voltage drop across the pipette is small (i.e. when the pipette resistance is low). Compared to dSEVC, cSEVC of a whole-cell patch gives superior signal-to-noise ratio, making it ideal for recording small whole cell currents.

**Acquisition protocol and data analysis:** From a holding potential of $-40$ mV, a 3 second descending ramp was applied from $+80$ mV to $-120$ mV (see Fig.2.18A). The ramp was applied repeatedly once every 10 seconds until a steady-state was reached, and then acquired 5 times to yield an average current trace for analysis. The superfusate was then acutely changed to that with $5$ mM Ni$^{2+}$ added by means of a custom made solenoid-controlled solution switcher device. Ni$^{2+}$ at this concentration is known to block all NCX activity (Brommundt & Kavaler 1987, Kimura et al. 1987). Further current traces were acquired once a new steady state was reached (Fig.2.18B). $I_{NCX}$ was taken as the $5$ mM Ni$^{2+}$-sensitive component of the active current (Fig.2.18C), which was normalized to the whole cell capacitance (measured using the method described in section 2.12.2). $I_{NCX}$ was calculated for various command voltage points to obtain the current-voltage (I–V) relationship, which was subsequently used for cross-group comparisons. Data were analyzed offline using pClamp™ software (version 9.0).
Fig. 2.18: An example NCX current trace. A. The 3 second descending ramp protocol, from +80 mV to −120 mV. B. Whole-cell currents measured in the presence and absence of 5 mM Ni\textsuperscript{2+}. C. Ni\textsuperscript{2+}-subtracted current, which is taken as $I_{NCX}$.

### 2.13 Ca\textsuperscript{2+} sparks study

The Ca\textsuperscript{2+}-sensitive fluorescent dye, fluo-4 (Molecular Probes, UK) was used to monitor localized changes in [Ca\textsuperscript{2+}]\textsubscript{i}. The most important properties of fluo-4 include an absorption spectrum compatible with excitation at 488 nm by an argon-ion laser source and a large fluorescence intensity increase in response to Ca\textsuperscript{2+} binding (i.e. a wide dynamic range). For this reason, fluo-4 has been widely used in Ca\textsuperscript{2+} sparks detection studies. On the other hand, fluo-4 is a single excitation, single emission dye. It does not have the ratiometric property of indo-1 or fura-2, so self-correction between cells of different sizes and uneven loading is unavailable.

**fluo-4 loading:** Aliquots of cardiomyocytes were incubated with the AM preparation of fluo-4 (10 µM in enzyme solution) at room temperature for 20 mins. They were then allowed to de-esterify for at least 30 mins prior to use. Cardiomyocytes were kept in the dark throughout to minimize photobleaching.
Hardware setup: Cardiomyocytes were placed on a bath mounted on the stage of a Zeiss Axiovert™ microscope with an LSM 510 confocal attachment and superfused with normal Tyrode solution containing 1 mM Ca\(^{2+}\) at 37 °C. The bath contents were field-stimulated when required using platinum wires and a custom-made bipolar stimulator. Cardiomyocytes were observed through a Zeiss EC Plan-NeoFluar™ ×40 oil-immersion objective (numerical aperture = 1.3). Fluo-4 was excited using the 488 nm line of an argon laser (Zeiss LSM 510 Laser module) and the emitted fluorescence was collected through a 505 nm long-pass filter.

Protocol: After a period of steady-state 1 Hz field-stimulation a period of 30 s quiescence was allowed to pass before line scans (x-t scans) were collected. Up to 30,000 lines ranging from 50 ∼ 173 µm were scanned at speeds between 546 ∼ 964 µs. Acquired x-t scans were exported as high-resolution tagged image file format (TIFF) digital files.

Analysis: Analysis of the collected TIFF image files was performed offline using custom scripts written by Dr. Mark A. Stagg (Laboratory of Cell Electrophysiology, Harefield Heart Science Centre) to run Matlab® R2006b (Math-Works Inc. MA, USA) following the algorithm for automatic Ca\(^{2+}\) spark detection described by Cheng et al. (1999). Detection criteria for Ca\(^{2+}\) sparks were set at 3.8 times the standard deviation above the mean background noise level (baseline, \(F_0\)). The frequency of Ca\(^{2+}\) spark occurrence (CaSpF) was obtained from the line scans. The amplitude of Ca\(^{2+}\) sparks was defined as the peak fluorescence divided by the background fluorescence (\(F/F_0\)). Further morphometric analysis of Ca\(^{2+}\) sparks elucidated the full-width at half-maximal (FWHM) and full-duration at half-maximal (FDHM) parameters (Fig.2.20).

From these parameters, the leak of Ca\(^{2+}\) from the SR was calculated using the following formula, as previously described by Maier et al. (2003):

\[
SR \text{ Ca}^{2+} \text{ Leak} = \text{CaSpF} \times \text{Amplitude} \times \text{FWHM} \times \text{FDHM} \tag{2.6}
\]
From the same line scans it was also possible to determine the frequency of
dyssynchronous Ca\textsuperscript{2+} cycling (i.e. Ca\textsuperscript{2+} ‘waves’, \textbf{Fig.2.21}).
Fig. 2.19: Example line scan image acquired from a cardiomyocyte showing Ca$^{2+}$ sparks. In some instances the analysis algorithm recognized a single spark as two or more. In this example sparks N° 2 and 3 are actually one spark. Because of cases such as these, human verification of every line scan image acquired was necessary.
Fig. 2.20: Surface plot image (above) derived from a line scan image (below) of a typical Ca$^{2+}$ spark. A cross section of the Ca$^{2+}$ spark in this surface plot was taken at half of its peak $F/F_0$ (represented as the ellipse). The width and duration of this cross section were taken as the FWHM and FDHM of the Ca$^{2+}$ spark, respectively.
Fig. 2.21: Example line scan image acquired from a cardiomyocyte showing $\text{Ca}^{2+}$ waves in addition to numerous $\text{Ca}^{2+}$ sparks.
2.14 Cell culture on glass-bottom dishes

Cardiomyocytes were isolated from rat hearts using methods described in section 2.6 on page 87. They were then rinsed with Dulbecco’s modified Eagle’s medium (DMEM) and counted using a standard haemocytometer. Cardiomyocytes were then plated onto sterile 35 mm glass-bottom culture dishes (Fig. 2.22A, glass thickness, No 1.5. MatTek Corp, Ashland, MA, USA). The glass-bottom dishes were pre-coated with 10 µl of 1 mg/ml laminin solution to aid cardiomyocyte adhesion. Plating density was 5,000 rod-shaped cells per dish. This provided adequate numbers of cardiomyocytes at the end of the culture period, but at the same time individual cardiomyocytes were sufficiently separated from one another to enable fluorescence measurement studies without nearby interference. The glass-bottom dishes were incubated at 37 °C, 100% humidity, 5% CO₂ for 48 hours. Each glass-bottom culture dish contained 2 ml of rat ventricular myocyte culture medium, which contained DMEM 67%, Medium-199 16%, horse serum 10%, foetal bovine serum 4%, 1 M HEPES 2%, penicillin/streptomycin 1%.

In some experiments cardiomyocytes were physically separated from other cell types by means of a porous polyester membrane (“Transwell™”, Corning Incorporated, NY, USA. Pore size 0.4 µm, Fig. 2.22C). This setup allowed diffusion of any soluble substances secreted between the different cell populations whilst preventing direct physical contact.

2.15 Analysis of co-culture supernates using the rat cytokine antibody array

The culture media that were bathing cardiomyocytes were analyzed for active paracrine substances. At the end of the 48 hour culture period, the resulting culture media were collected and spun at 1000 rpm. The supernates were stored in 2 ml Eppendorf® tubes at −80 °C. At the time of analysis the samples were thawed
at room temperature and gently agitated. They were then individually filtered using syringe-filters (Pore size 0.2 μm, “Minisart plus”, Vivascience®, Hannover, Germany).

Supernate samples were initially analyzed using the rat cytokine antibody array (“Type-I”, RayBiotech Inc. Norcross, GA, USA). The array provides a sensitive method for simultaneous detection of multiple cytokine expression levels in culture media, sera or cell lysates (Huang et al. 2001, Huang 2003). Details of the cytokines that could be detected using the array are illustrated in Table. 2.2. The basic working principle of the array and experimental protocol are illustrated in Fig.2.23 and can be described as follows:

1. The arrays are available with pre-determined quantities of primary antibodies adhered onto nitrocellulose sheets. These were initially blocked using the
supplied blocking buffer to prevent non-specific protein binding.

2. Blocked arrays were rinsed using the wash buffer provided and then exposed to undiluted culture media samples for 1 hour at room temperature. The arrays were gently rocked using a rocking table. During this period, any cytokines present in the culture media bound onto the array via their specific primary antibodies.

3. Arrays were rinsed again and then immersed in the supplied biotinylated secondary antibody mixture for 2 hours. During this period, the biotinylated secondary antibodies bound onto the array via their specific cytokines.

4. Arrays were rinsed and then streptavidin conjugated to horse radish peroxidase (HRP) was applied for 2 hours, allowing its binding onto the biotin tag.

5. Arrays were rinsed and laid out on a glass sheet. Enhanced chemiluminescence (ECL) substrate mixture was applied onto the arrays, which were then covered with clear polyvinylidene chloride film (SaranWrap™). ECL is a commonly used, highly sensitive technique for a variety of detection assays in biology. In the presence of HRP and hydrogen peroxide a luminol-based substrate is oxidized to produce an excited (“triplet”) carbonyl. As this compound decays to the singlet carbonyl it emits light.

   Emitted chemiluminescence was detected by direct exposure to high performance autoradiography film (Hyperfilm™, Amersham Biosciences, Little Chalfont, UK), for variable periods of 1 ∼ 60 seconds.

6. Developed radiography film signals were analyzed using optical densitometry. Films were scanned at 600 dots per inch resolution using an Agfa Arius-II scanner connected to a standard PC running Quantity-One™ software (version 4.6.1. Bio-Rad Laboratories, Hercules, CA, USA.). Optical densities of the scanned signals were measured using the same Quantity-One™ software. The
optical density signal level of each dot was normalized to the local background level.
Table 2.2: RayBio rat antibody array I.

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**Abbreviations:**  
Pos: positive control. Neg: negative control. CINC: cytokine-induced neutrophil chemoattractant.  
VEGF: vascular endothelial growth factor.
Fig. 2.23: Detection of cytokines in culture media using the rat cytokine antibody array. 

A. The array of primary antibodies adhered on nitrocellulose sheets is blocked and then immersed in the undiluted culture medium sample. 
B. The array is rinsed and the mixture of biotinylated secondary antibodies is applied. 
C. Application of streptavidin conjugated to horse radish peroxidase for 2 hours. 
D. Enhanced chemiluminescence (ECL) reagent mixture is added to the array. 
E. Chemiluminescence is detected using autoradiography film.
2.16 Enzyme-linked immunosorbent assay of insulin-like growth factor-1 levels in co-culture supernates

Concentrations of insulin-like growth factor-1 (IGF–1) in the culture media were measured using enzyme-linked immunosorbent assay (ELISA). ELISA is a biochemical technique where a specific antibody immobilized on a solid support is used to detect antigen in a fluid sample (Engvall & Perlman 1971, van Weemen & Schuurs 1971). The basic principle of ELISA and details of experiment protocol can be described as follows:

1. Samples were prepared as for the rat cytokine antibody array (previous section) and used without further processing or dilution. The assay was performed using a Quantikine® ELISA kit (type “MG100”, R & D Systems, Abingdon, UK). An IGF–1 standard was prepared by serial dilution of the provided sample. The standard concentration range was 0 ~ 2000 pg/ml. A positive control was also prepared from the separately provided sample. The culture media samples were thawed and syringe-filtered, as described for the cytokine antibody array.

2. A monoclonal capture antibody for IGF–1 was pre-coated onto a 96-well polystyrene microplate. The samples and IGF–1 standard were added into the plate wells. The plate was then shaken using an orbital shaker for 2 hours at room temperature. During this period any IGF–1 present in the culture media were captured onto the well surface via the antibody in a concentration-dependent manner.

3. After rinsing with the provided rinse buffer HRP-conjugated polyclonal antibodies specific for IGF–1 was added to the wells and the plate was shaken again for 2 hours. During this period the HRP conjugated to the secondary antibody bound onto the wells via the immobilized IGF–1 to saturation.
4. The wells were rinsed again. The substrate mixture of hydrogen peroxide added to 3,3′,5,5′-Tetramethylbenzidine (TMB, the chromagen) was pipetted into the wells. The plate was incubated in the dark at room temperature for 30 minutes. During this period the HRP catalyzes the oxidation of TMB from a colourless compound into one that emits yellow light.

5. The oxidation reaction was stopped and TMB stabilized by adding 1 M hydrochloric acid solution. The optical density of each well was then measured using a μQuant™ microplate reader (Bio-Tek® Instruments Inc. Vermont, USA.) operated by a standard PC running KC-Junior™ software (version 1.4.1. Bio-Tek® Instruments Inc.). The reading wavelength was 450 nm with wavelength correction set to 570 nm. The KC-Junior™ software was able to fit a line to the standard, against which concentrations of IGF–1 in the culture media samples were read.

### 2.17 Statistical analyses

The results in the following chapters are expressed as mean ± standard error of the mean (S.E.M.) of n observations. Statistical analyses within groups, such as before-after comparisons of parameters within the same subject, were performed using paired Student’s t-tests. Statistical comparisons between groups were performed using one-way analysis of variance (ANOVA) and post hoc testing using Newman-Keul’s multiple comparison test on Prism™ software (Version 4, Graphpad Software Inc. CA, USA).

### 2.18 Solutions

0 Na⁺/0 Ca²⁺ solution : (in Milli-Q™ water, in mM) LiCl 140; glucose 10; HEPES 10; EGTA 0.75; MgCl₂ 1; KOH 6; pH 7.40 using LiOH

55% percoll : 55% percoll (1.130 g/ml), 45% HBSS
70% percoll : 70% percoll (1.130 g/ml), 30% HBSS

enzyme buffer : (in AnalaR water, in mM) NaCl 120; KCl 5.4; CaCl$_2$ 0.2; MgSO$_4$ 5; Na$^+$-pyruvate 5; glucose 20; taurine 20; HEPES 10; pH 7.4 using NaOH

enzyme solution : collagenase (Worthington, USA. 1 mg/ml of Type-I, 280 u/mg) and hyaluronidase (Sigma-Aldrich, UK. 0.6 mg/ml of 999 u/mg) dissolved in enzyme buffer, above

K$^+$-free solution for measurement of $I_{NCX}$ : (in Milli-Q™ water, in mM) NaCl 140; HEPES 10; glucose 10; MgCl$_2$ 1; CaCl$_2$ 1; CsCl 6; strophanthidin 0.01, nifedipine 0.01, pH 7.4 using NaOH

low-Ca$^{2+}$ solution : (in AnalaR water, in mM) NaCl 120; KCl 5.4; CaCl$_2$ 0.045; MgSO$_4$ 5; Na$^+$-pyruvate 5; glucose 20; taurine 20; HEPES 10; nitrilotriacetic acid 5; pH 6.96 using NaOH

normal Tyrode solution : (in Milli-Q™ water, in mM) NaCl 140; KCl 6; glucose 10; MgCl 1; CaCl$_2$ 1; HEPES 10; pH 7.4 using NaOH

pipette-filling solution for measurement of AP and $I_{Ca,L}$ : (in Milli-Q™ water, in mM) KCl 2000, HEPES 5, EGTA 0.1; pH 7.2 using KOH

pipette-filling solution for measurement of $I_{NCX}$ : (in Milli-Q™ water, in mM) CsCl 45; HEPES 20; MgCl$_2$ 11; Na$_2$ATP 10; CsOH 100; EGTA 50; CaCO$_3$ 25; pH 7.2 using CsOH ([Ca$^{2+}$], calculated to be ~ 250 nM)

rat ventricular myocyte culture medium : DMEM 67%; Medium-199 16%; horse serum 10%; fœtal bovine serum 4%; 1 M HEPES 2%; penicillin/streptomycin 1%

skeletal myoblast differation medium : 98% DMEM, 1% horse serum, 1% penicillin/streptomycin

skeletal myoblast plating medium : 88.5% DMEM, 10% horse serum, 0.5% chick embryo extract, 1% penicillin/streptomycin
skeletal myoblast proliferation medium: 68.5% DMEM, 20% fœtal bovine serum, 10% horse serum, 0.5% chick embryo extract, 1% penicillin/streptomycin, 5 ng/ml bFGF

2.19 Suppliers

Animals, equipment, and materials were purchased from the following suppliers. Chemicals were of the highest grade purity commercially available.

adult female Sprague-Dawley rats: Harlan, UK

adult male GFP+ Sprague-Dawley rats: Rat Resource & Research Center, MO, USA

bFGF: Invitrogen, UK

CGP–20712A: Sigma-Aldrich, UK

chick embryo extract: Sera Labs, UK

clenbuterol: Sigma-Aldrich, UK

collagenase (type 1): Sigma-Aldrich, UK

collagenase (type-2): Worthington, USA

CsCl: Sigma-Aldrich, UK

DMEM: Gibco, UK

EIPA: Sigma-Aldrich, UK

EGTA: Sigma-Aldrich, UK

fenoterol: Sigma-Aldrich, UK

fœtal bovine serum (heat inactivated): Gibco, UK
HBSS (×10 stock) : Sigma-Aldrich, UK

horse serum (heat inactivated) : Gibco, UK

hyaluronidase : Sigma-Aldrich, UK

isoflurane : Schering-Plough Animal Health, UK

isoproterenol : Sigma-Aldrich, UK

laminin : Sigma-Aldrich, UK

Matrigel™ : Becton-Dickinson, UK

medical oxygen (100%) : BOC, UK

NiCl₂ : Sigma-Aldrich, UK

nifedipine : Sigma-Aldrich, UK

nigericin : Sigma-Aldrich, UK

nitrilotriacetic acid : Sigma-Aldrich, UK

penicillin/streptomycin : Sigma-Aldrich, UK

Percoll™ : GE Healthcare, UK

salbutamol : Sigma-Aldrich, UK

strophanthidin : Sigma-Aldrich, UK

taurine : Sigma-Aldrich, UK

all other chemicals : VWR international, UK
Chapter 3

Effects of cell transplantation on the properties of recipient cardiomyocytes in vivo

In this chapter the effects of transplantation of either bone marrow mononuclear cells or skeletal myoblasts on the properties of recipient ventricular myocytes in vivo are described. Myocardial infarction was induced in the rat heart. Once chronic heart failure had established, cell transplantation was performed. After the transplanted cells were allowed a period of time to exert their effects, the whole heart function was assessed and cardiomyocytes were isolated from the left ventricle to be characterized in detail.

3.1 Materials & Methods

Myocardial infarction was induced by coronary artery ligation, as described in section 2.1 on page 79. A period of three weeks was allowed to pass for ventricular remodeling to occur and the severity of left ventricular dysfunction was assessed using echocardiography. Animals were then divided into four groups for cell injection, as below (Fig.3.1):
• **Normal**: Normal heart controls. These animals were subjected to SHAM operation, and subsequently received PBS injection

• **Heart Failure (HF)**: those that received coronary ligation, and subsequently received PBS injection

• **Bone marrow (BM)**: those that received coronary ligation, and subsequently received bone marrow mononuclear cell injection

• **Skeletal Myoblast (SK)**: those that received coronary ligation, and subsequently received skeletal myoblast injection

The animals in the bottom three groups were *randomized by minimization* following echocardiography at three weeks. This was to ensure that there was no significant difference between the heart function of the rats in these three groups *before* any injection. After randomization cell injections were performed using methods described in section 2.4 on page 85.
Fig. 3.1: The main protocol for studying the effects of cell transplantation in vivo. After either SHAM operation or coronary ligation, three weeks were allowed to pass before animals were studied using echocardiography. Animals were then randomized to receive PBS, bone marrow mononuclear cells, or skeletal myoblasts. After a further four weeks, the animals were studied by echocardiography again and cell isolation was performed.
3.2 Results: Development of heart failure following coronary artery ligation

Following the protocol described in section 2.1 it was possible to induce myocardial infarction in a consistent manner. The appearance of the LV changed within a few seconds after ligation, confirming correct application of suture. The 24 hour mortality following ligation was 26/132 (∼20%). Despite the use of sterilized instruments and aseptic techniques 2/106 animals (∼1.9%) developed deep infection within the operative site. These animals were sacrificed and their hearts were discarded without study.

During injection of either PBS or cells at three weeks, the maturation of the infarct scars was clearly apparent. Echocardiography revealed akinetic anterior LV segments (seen in M-Mode in Fig.3.2). The presence of full-thickness infarcts was further confirmed at a later time, when the hearts were harvested for cell isolation: after perfusion of the coronary arteries with enzyme solution the infarct scar was inspected from both outside and inside the ventricle and discarded (as described on page 88). The remaining ventricular tissue was then cut into small pieces before shaking in enzyme solution for 5 minutes to isolate cardiomyocytes.

Echocardiography revealed that the LV end-diastolic dimensions of failing hearts were dilated compared to normal hearts (70.1±2.2 mm² vs 46.9±1.9 mm², p < 0.001 by unpaired t-test). The mean ejection fraction (EF) of failing hearts, also derived from echocardiography, was 35.3 ± 0.8%. This is in contrast to the EF of normal hearts, which was 76.2 ± 1.2% (p < 0.001). Despite having dilated left ventricles with low EF, the rats which had infarcted hearts showed no symptoms of heart failure. Although 20% of the animals died during the first 24 hours, the surviving animals showed no peripheral œdema, behavioural withdrawal, shortness of breath, or failure to thrive. During harvest of the hearts abdominal cavities were opened and the livers were also examined macroscopically. No significant pigmentation about the central veins of the liver lobules (“nutmeg liver”) was noted. There was also no
significant abnormal accumulation of ascites within the peritoneum.
Fig. 3.2: Echocardiography of a normal (left) and a failing (right) rat heart. In each window the short-axis cross-sectional (2D) views are in the windows above, whilst the M-mode views are below. Anterior akinesia and LV cavity dilation is apparent in the failing heart.
3.3 Results: Effect of cell transplantation on ventricular function

Echocardiography studies were performed again four weeks after injection of either PBS or cells to examine the effect of cell transplantation on global heart performance. As shown in Fig.3.3 there was no significant difference between the HF, BM, and SK groups with respect to LV end-diastolic area or EF before any injection. There was no significant change in the EF of failing hearts in the four weeks following PBS injection. In comparison, injection of bone marrow mononuclear cells increased the EF from 34.3 ± 1.1% to 42.9 ± 2.2% over this same period. Injection of skeletal myoblasts also increased EF, from 36.5 ± 1.3% to 44.4 ± 2.3%. Collectively, these results agree with previous reports that intramuscular injection of either cell type improves global ventricular function.

As mentioned earlier, the degree of failure in these hearts were seen as modest, given the lack of symptoms in the animals. The improvements following cell transplantation were also moderate, especially when compared to the EF of a normal, healthy heart of ~ 76%. The degrees of impairment pre-cell transplantation and improvement after transplantation in these hearts are similar to those seen in the clinical trials (Assmus et al. 2006), making them suitable subjects for the cellular studies described in the following sections.

3.4 Results: Green fluorescence of cardiomyocytes obtained from hearts following cell transplantation

Following echocardiography studies cardiomyocytes were isolated from the LV for characterization using methods described on page 87. The isolated cardiomyocytes were initially studied for their levels of green fluorescence to determine whether they
Fig. 3.3: Ventricular end-diastolic area (A) and ejection fraction (B) measured using echocardiography before and after cell transplantation. Failing hearts were dilated and showed reduced EF compared to Normal. There was no significant difference between the HF, BM, and SK groups before injection. Following injection of PBS HF showed further LV dilation. There was no effect of PBS on EF over four weeks, whereas both BM and SK increased EF over this period. N° of animals: HF 18; BM 18; SK 21. ANOVA was used for comparisons between different heart groups. Paired Student’s t-test was used within groups for before-after comparisons.

had derived from the host (native cardiomyocytes) or from the injected cells. To test whether epifluorescence was sufficient to detect GFP, ventricular myocytes were isolated from the hearts of GFP
+ Sprague-Dawley rats in separate experiments for comparison. The levels of green fluorescence was examined using methods described in section 2.8 on page 90. Cardiomyocytes isolated from wild-type Sprague-Dawley rats emitted low levels of background green autofluorescence. Cardiomyocytes isolated from GFP
+ Sprague-Dawley rats showed a notably higher level of green fluorescence compared to cardiomyocytes isolated from wild type rat hearts (Fig.3.4). Cardiomyocytes isolated from hearts which had received cell transplantation showed low levels of green fluorescence (i.e. not above the level of background autofluorescence, lower panels of Fig.3.4). These observations indicated that the isolated cardiomyocytes under investigation were of recipient origin, and not derived from the injected GFP
+ bone marrow or skeletal cells.

Although the frequency of occurrence of GFP
+ cardiomyocytes was not systematically measured, these observations are consistent with previous reports that very
small numbers of transplanted cells, if any, survive and adopt a cardiomyocyte-like morphology (Fukushima et al. 2007, Suzuki et al. 2004). Furthermore, recent evidence suggests that the few that do had arisen from fusion of transplanted GFP+ cell with recipient cardiomyocytes (Balsam et al. 2004, Murry et al. 2004). Following these findings, the current study focused more on characterization of cardiomyocytes assumed to have originated from the recipient population, all of which emitted only background levels of green fluorescence.
Fig. 3.4: Microscopic examination of green fluorescence emitted from cardiomyocytes. Wild type cardiomyocytes (above left) showed a low degree of green autofluorescence. Cardiomyocytes were isolated from rats constitutively expressing GFP for comparison (above right). These emitted notably increased green fluorescence. Cardiomyocytes isolated from hearts after bone marrow mononuclear cell (below left) or skeletal myoblast transplantation (below right) showed only background levels of green fluorescence, similar to wild type cardiomyocytes.
3.5 Results: Cardiomyocyte size

The dimensions of the freshly isolated cardiomyocytes from the four heart groups were estimated using planimetry. The methods are described in section 2.7 on page 89. Only cardiomyocytes of healthy, rod-shaped appearance were characterized using this approach.

As shown in Fig. 3.5 the 2-dimensional cell areas of cardiomyocytes isolated from failing hearts were larger than of those isolated from normal (SHAM-operated) hearts, indicating hypertrophy. Cardiomyocytes isolated from hearts which had received bone marrow mononuclear cell transplantation were smaller than those from failing hearts. Similarly, cardiomyocytes isolated from hearts which had received skeletal myoblast transplantation also showed reduction in size. These data suggest that cell transplantation caused either attenuation or possibly even regression of the hypertrophy that developed during the seven weeks following myocardial infarction. Cardiac hypertrophy in humans is recognized as a maladaptive process which is associated with an unfavorable outcome, and its reduction is thought to improve prognosis (Frey & Olson 2003).

These data agree well with results obtained from cell membrane capacitance measurements, which represent an alternative method of assessing cell size. The cell membrane capacitance measurement data are described later, in section 3.10.
Fig. 3.5: Cell area measured using planimetry. A. Left ventricular myocytes isolated from failing hearts were larger than those from normal hearts. After transplantation of either BM or SK, this hypertrophy was reduced. B. Example photomicrographs of cardiomyocytes isolated from normal hearts. C. Example photomicrographs of hypertrophied cardiomyocytes isolated from failing hearts. Scale bars represent 10 μm. No of animals/cells studied: Normal 6/220; HF 5/225; BM 5/229; SK 5/328. ANOVA was used for statistical comparisons.
3.6 Results: Contractile properties of cardiomyocytes

Freshly isolated cardiomyocytes were studied for their contractile properties by measurement of sarcomere length changes, using methods described in section 2.9 (page 90). The cardiomyocytes used for these particular studies were not loaded with indo-1 AM. Field-stimulation frequency was varied between 1, 2, 3, 4, and 5 Hz.

Fig.3.6 illustrates the results from sarcomere length studies. There was no difference between the four heart groups in terms of baseline (diastolic) sarcomere length (Fig.3.6B). Cardiomyocytes from failing hearts showed markedly reduced amplitude of contraction. This was true both in terms of absolute sarcomere length (expressed in µm, Fig.3.6C) as well as in terms of contraction percentage (Fig.3.6D). Transplantation of either bone marrow mononuclear cells or skeletal myoblasts lead to full restoration of this contraction amplitude.

The duration of cardiomyocyte contraction and relaxation were also determined from the same acquisition traces, using methods illustrated in Fig.2.7. As shown in Fig.3.6E, there was no difference between the four heart groups in terms of their contraction times-to-peak. Similarly, there was no difference measured between the times-to-50% relaxation (Fig.3.6F).

Collectively, these results demonstrate that the contractile performance of individual cardiomyocytes from the model of heart failure used in this study are impaired. Furthermore, it is clear that injection of either bone marrow mononuclear cells or skeletal myoblasts restore the contractile performance back towards that of normal cardiomyocytes.
Fig. 3.6: Contractile properties of cardiomyocytes obtained from the four heart groups. 
A. Representative example sarcomere length measurement traces at 1 Hz contraction. 
B. Baseline sarcomere length. C. Amplitude of cardiomyocyte contraction expressed as sarcomere shortening, in µm. D. Amplitude of cardiomyocyte contraction expressed as shortening percentage. By either parameter, failing cardiomyocytes showed impaired contraction, which was restored following transplantation of either cell type. E. Time-to-peak contraction and F. Time-to-50% of relaxation showed no difference between groups. N° of animals/cells: Normal 6/66; HF 6/60; BM 5/53; SK 6/57. *** p < 0.001 vs Normal, BM, SK.
3.7 Results: Indo-1 transients - twitch transients

To elucidate the physiological mechanisms responsible for the changes in contractile properties described in the previous section, a number of the same cardiomyocytes were studied for their Ca\(^{2+}\) handling properties using the Ca\(^{2+}\) sensitive fluorescent indicator indo-1. Detailed methods are described in section 2.10 on page 93. The field-stimulation frequency was 1, 2, and 3 Hz.

Representative 1 Hz indo-1 transient traces acquired from cardiomyocytes can be seen in Fig.3.7A. There was no significant difference in the diastolic baseline levels (Fig.3.7B). No significant difference was measured in the amplitudes of the transients (Fig.3.7D). The time-to-peak (T\(_{peak}\)) of the transient was prolonged in cardiomyocytes from failing hearts (Fig.3.7D), indicating impaired release of Ca\(^{2+}\) during systole. Interestingly, this prolongation was corrected following transplantation of either cell type. The time-to-50% decay, a measure of Ca\(^{2+}\) re-uptake during diastole, was also prolonged in failing cardiomyocytes when compared to normal (Fig.3.7E). In contrast to the T\(_{peak}\), this defect in Ca\(^{2+}\) handling was not corrected by cell transplantation. Similar data were seen following 2 Hz and 3 Hz field-stimulation (data not shown). No change in data pattern was seen when other parameters were assessed, such as time-to-50% of peak, time-to-90% of peak, or time-to-90% decay (data not shown).

The increased contraction amplitudes of cardiomyocytes, in the absence of change in the amplitude of the Ca\(^{2+}\) transients suggest that the sensitivity of the myofilaments to intracellular Ca\(^{2+}\) might be altered. In the current series of experiments this aspect of cardiomyocyte function was not formally assessed. The sensitivity of cardiomyocyte myofilaments to Ca\(^{2+}\) can be estimated and compared between cell groups by permeabilizing cardiomyocytes (by using saponin, Lukyanenko & Györke 1999) and measuring their contractions whilst applying varying concentrations of Ca\(^{2+}\).

The two main aspects of Ca\(^{2+}\) regulation, Ca\(^{2+}\) entry and Ca\(^{2+}\) extrusion, have both been implicated in the pathophysiology of heart failure with varying relative
contributions that depend on the animal species, mode of stress induction, and severity (Hasenfuss 1998). The model of heart failure in this study showed impairment in both aspects of Ca\textsuperscript{2+} handling. The speed of Ca\textsuperscript{2+} release during systole was normalized by cell transplantation, which might help explain the improved mechanical function of the cardiomyocytes. On the other hand, Ca\textsuperscript{2+} re-uptake during diastole remained unaffected, implying persistent defects in some of the aspects of Ca\textsuperscript{2+} handling.

3.8 Results : Indo-1 transients - caffeine application studies

As well as studying the twitch transients to characterize Ca\textsuperscript{2+} release and re-uptake, the Ca\textsuperscript{2+} handling and storage properties of cardiomyocytes were further assessed using rapid application of 20 mM caffeine. Detailed experiment protocol and methods of data analysis are described in section 2.10 on page 96.

The results obtained from these caffeine experiments are shown in Fig.3.8. The SR Ca\textsuperscript{2+} content, measured as the amplitude of the caffeine-induced transient, showed no significant difference seen between the four heart groups (Fig.3.8A). From the same experiments, fractional release (Fig.3.8B), \( \kappa_{\text{Caffeine}} \) (Fig.3.8C), and relative contribution of NCX towards clearance of Ca\textsuperscript{2+} during diastole (Fig.3.8D) were calculated. There were no significant differences seen for these measured parameters.

These data suggest that the amount of Ca\textsuperscript{2+} stored in the SR of these cardiomyocytes are not different from each other. In all heart groups, NCX removed approximately 6\% of the Ca\textsuperscript{2+} that was released. This is in agreement with the measured values from rat cardiomyocytes previously reported by others (Bassani et al. 1994), and suggests that the impaired Ca\textsuperscript{2+} re-uptake measured in cardiomyocytes from HF, BM, and SK groups is due to impairment in both NCX and SERCA function by similar degrees.
Fig. 3.7: A. Representative traces obtained following 1 Hz field stimulation. B. Baseline (diastolic) levels. C. Amplitude of transient. There was no significant difference between groups. D. $T_{\text{peak}}$ of the transient was prolonged in failing cardiomyocytes when compared to normal cardiomyocytes. The prolongation was completely restored to normal following transplantation of either type of cell. E. Time-to-50% decay of the indo-1 transient was also prolonged in failing cardiomyocytes compared to normal. This prolongation remained unaffected by cell transplantation. N° of animals/cells: Normal 6/53; HF 5/46; BM 5/43; SK 5/41. Similar data were seen from 2 Hz and 3 Hz field stimulation (data not shown).
Fig. 3.8: Indo-1 traces from rapid caffeine application studies. Example twitch transients and a caffeine transient elicited from a normal (A) and a failing (B) cardiomyocyte. C. SR Ca\(^{2+}\) content, measured as the amplitude of the caffeine-induced transient. D. Fractional release. E. NCX rate constant. F. Relative contribution of NCX to clearance of Ca\(^{2+}\) during diastole. There were no significant differences between the four heart groups for any of these measured parameters. N\(^\circ\) of animals/cells: Normal 6/32; HF 5/14; BM 5/24; SK 5/13.
3.9 Results: Intracellular pH and sodium-hydrogen exchanger activity

It has been suggested previously that disturbances in [Na$^+$], and intracellular pH (pH$_i$) via altered Na$^+/H^+$ exchanger activity occur during heart failure leading to dysfunctional intracellular Ca$^{2+}$ homeostasis (Baartscheer et al. 2003). For this reason pH$_i$ and acid-extrusion ability was compared between groups using the pH-sensitive fluorescent indicator 5-(and-6)-Carboxy-SNARF®-1. Details of experiment methods are described in section 2.11 on page 98.

Fig.3.9 summarizes the data obtained. There was no disturbance in resting pH$_i$ in cardiomyocytes isolated from failing hearts (Fig.3.9A). There was no major effect on pH$_i$ resulting from transplantation of either cell type. Acid extrusion was measured between every 0.1 pH$_i$ unit between pH$_i$ 6.8 and 7.3. These measurements also revealed no significant difference between groups (example data from pH 6.9 shown in Fig. 3.9B, Data for other pH$_i$ points not shown). From these data it appears that effects on pH$_i$ are not related to the benefits to the recipient cardiomyocytes resulting from cell transplantation.

3.10 Results: Cell capacitance

The surface electrical capacitances of isolated cardiomyocytes were measured as part of the electrophysiological studies (i.e. AP, $I_{Ca,L}$ and $I_{NCX}$ measurements, which are described in the following sections). Cell capacitance represents an estimation of the total surface area of the sarcolemma. Data were collated from experiments utilizing both high-resistance and low-resistance pipettes and used for comparison of cell size between the heart groups.

Ventricular cardiomyocytes from normal hearts had an average electrical capacitance of 178.4 ± 8.4 pF. This value is consistent with those measured by others using similar methods (Keung et al. 1991, Benitah et al. 1993, Tomita et al. 1994).
Fig. 3.9: A) Resting pH\textsubscript{i}, measured using SNARF fluorescence. B) Acid efflux capabilities of cardiomyocytes at pH 6.9. Similar data were seen at pH\textsubscript{i} 6.8, 7.0, 7.1, 7.2, and 7.3 (not shown). No\textsuperscript{o} of animals/cells studied: Normal 6/28; HF 5/25; BM 5/25; SK 5/25.

In comparison, cardiomyocytes isolated from failing hearts had larger capacitances (Fig. 3.10), suggesting hypertrophy. The increase (44.4\%) was larger than that estimated by planimetry (34.8\%, Fig.3.5). Cell capacitance is a representation of the entire cell surface area, including that in the T-tubules. On the other hand, planimetry estimates only the 2-dimensional, projected area of the cell. The cell area differences that may exist in the z-axis of visualization and those arising from differences in T-tubule density are not incorporated in planimetry. These differences may account for the discrepancy seen.

Following transplantation of either bone marrow mononuclear cells or skeletal myoblasts, there was a reduced cell capacitance compared to heart failure. The pattern of change was similar to that estimated by planimetry measurements. Both data sets show the occurrence of hypertrophy in the hearts following coronary artery ligation. Reduction of this hypertrophy was seen following cell transplantation, suggesting two possibilities: either the development of hypertrophy was limited, or regression of hypertrophy had occurred.
Fig. 3.10: Electrical capacitance of cardiomyocytes isolated from HF were significantly higher than that of Normal. This increase was significantly reduced following transplantation of either BM or SK. N° of animals/cells studied: Normal 6/45; HF 5/57; BM 5/39; SK 5/45.

3.11 Results: Action potential duration (APD)

To further elucidate the physiological effects of cell transplantation on the recipient cardiomyocytes, electrophysiological measurement studies were performed. Action potentials were measured directly in individual cardiomyocytes using whole cell clamping methods as described in section 2.12.1 on page 104. Representative example traces from 1 Hz stimulation and APD₉₀ comparison data can be seen in Fig.3.11. APD was prolonged in failing cardiomyocytes compared to normal by up to 40% at 1, 3, and 5 Hz stimulation. The prolongation was most marked at 1 Hz. At higher frequencies the difference between normal and failing cardiomyocytes was smaller, but detectable nonetheless. These observations are consistent with those made by others in previous studies of failing cardiomyocytes from various species (Bassett & Gelband (1973), Brooksby et al. (1993), Keung et al. (1982). For a review, see Tomaselli & Marban (1999)).

Transplantation of bone marrow mononuclear cells or skeletal myoblasts had no significant effect on the prolonged APD. This suggests persistent impairment of
the main components of electrophysiological remodeling contributing towards APD prolongation. For example, reductions in outward currents such as the transient outward current ($I_{to}$), inward rectifier current ($I_{K1}$), and Na$^+$/K$^+$-ATPase current ($I_p$) are all known to contribute (Näbauer & Kääb 1998). However, whether this is the case in the current model of heart failure would require verification with further experiments. Increases in inward currents such as $I_{Ca,L}$ or forward mode $I_{NCX}$ could also prolong the APD. These possibilities are discussed in the following sections.
Fig. 3.11: Action potentials measured from failing cardiomyocytes were prolonged when compared to normal. The prolonged AP was not significantly affected by transplantation either cell type. **A.** Representative 1 Hz stimulation action potential traces. **B.** \( \text{APD}_{90} \) following 1 Hz stimulation. **C.** \( \text{APD}_{90} \) following 3 Hz stimulation. **D.** \( \text{APD}_{90} \) following 5 Hz stimulation. No of animals/cells: Normal 6/30; HF 5/38; BM 5/40; SK 5/40. ANOVA was used for statistical comparisons. Similar patterns of data were observed when \( \text{APD}_{50} \) were compared (data not shown).
3.12 Results: L-Type Ca$^{2+}$ currents

The L-type Ca$^{2+}$ current ($I_{Ca,L}$) represents the main route of entry of Ca$^{2+}$ into the cardiomyocyte to trigger Ca$^{2+}$-induced Ca$^{2+}$ release (CICR, Fabiato 1983). $I_{Ca,L}$ was studied in isolated cardiomyocytes using whole cell voltage-clamping methods. Details of protocols are described in section 2.12.2 on page 107. The parameters measured were $I_{Ca,L}$ peak density, time- and Ca$^{2+}$-dependent inactivation, and voltage-dependent activation and inactivation.

The peak $I_{Ca,L}$ density (Fig.3.12) was significantly reduced in failing cardiomyocytes compared to normal ($p < 0.05$ for $-10$ to $+5$ mV). This reduction was normalized by transplantation of skeletal myoblasts ($p < 0.05$ vs HF for $-20$ to $+5$ mV). Interestingly, this was in contrast to transplantation of bone marrow mononuclear cells, which had no effect on the reduced $I_{Ca,L}$.

The fast and slow components of time-dependent inactivation of $I_{Ca,L}$ were identified for comparison, using methods illustrated in Fig.2.16D. Comparison of $\tau_{fast}$.
Fig. 3.13: Inactivation time constants of $I_{Ca,L}$. Both $\tau_{fast}$ (A) and $\tau_{slow}$ (B) were significantly reduced in HF compared to Normal, indicating further impairment of $I_{Ca,L}$. With both parameters this prolongation was corrected following SK, but not BM, transplantation. No of animal/cells: Normal 6/17; HF 5/22; BM 5/15; SK 5/22. ANOVA was used for statistical comparisons.

As well as current density and inactivation time constants, voltage-dependent activation ($G/G_{MAX}$) and inactivation ($F/F_{MAX}$) profiles of $I_{Ca,L}$ were characterized. Boltzmann curves were fitted to mean data points of the I–V plots obtained from application of protocols illustrated in Fig.2.16A and Fig.2.16E on page 111. There was no difference between the groups in $G/G_{MAX}$ (Fig.3.14A). Heart failure resulted in a leftward shift in $F/F_{MAX}$ ($* p < 0.05$ vs Normal for $-25$ to $+15$ mV). This implies further limitation of $I_{Ca,L}$. As with other parameters of $I_{Ca,L}$, this defect was normalized following transplantation of skeletal myoblasts ($§, p < 0.05$ vs HF for $-25$ to $+15$ mV), but not bone marrow cells (Fig.3.14B).

Overall, these data show that the $I_{Ca,L}$ is impaired in this model of heart failure. Previously, many groups have measured $I_{Ca,L}$ density in cardiomyocytes from heart
failure patients and animal models of heart failure, reporting varied results (for
reviews summarizing the findings, see Mukherjee & Spinale (1998) and also Richard
et al. (1998)). The general trend appears to be that, while the effects of compensated
ventricular hypertrophy range from no change to increases in $I_{Ca,L}$, the effects of
heart failure range from no change to significant increases. The heterogeneity prob-
able arises from a variety of factors, which may include animal species, age, mode
and severity of heart failure. With humans, additional variability is introduced by
co-morbidities, sex, and drug therapy.

The decreased $I_{Ca,L}$ density seen in heart failure is normalized by skeletal myo-
blast, but not bone marrow cell, transplantation. $I_{Ca,L}$ represents the main trigger
for CICR and changes in this current influence the rate of Ca$^{2+}$ release from the SR
(Wier et al. 1994). Thus, the faster Ca$^{2+}$ transient development seen from the indo-1
studies after skeletal myoblast transplantation may, at least in part, be explained by
improvements in $I_{Ca,L}$. Nonetheless, alterations in other components of EC coupling
cannot be excluded. As mentioned earlier, a large number of studies have reported
unchanged $I_{Ca,L}$ density in various models of heart failure, indicating the presence
of other defects in EC coupling. Furthermore, the results from bone marrow mono-
nuclear cell transplantation in the current study show normalized indo-1 transient
$T_{peak}$ with persistently decreased $I_{Ca,L}$, indicating changes in other components that
may have become defective in heart failure, most notably the RyR.

The prolongation of $\tau$ values in failing cardiomyocytes was an observation consist-
tent with those made previously by others (Gomez et al. 1997, Gomez et al. 2001).
The $\tau$ values represent speed of L-type Ca$^{2+}$ channel closure in a stochastic manner
and by Ca$^{2+}$ released from the neighbouring RyRs (Kass & Sanguinetti 1984, Lee
et al. 1985, Yue et al. 1990). It has been suggested that their prolongation is re-
lated to physical separation of the two type of Ca$^{2+}$ channels: the increased distance
between the RyRs and L-type Ca$^{2+}$ channels delays the inactivation of the L-type
Ca$^{2+}$ channels by Ca$^{2+}$ released from RyRs (Gomez et al. 1997). The normalization
of the $\tau$ values suggests that skeletal myoblast transplantation may restore some of
the structural alterations between the L-type Ca\(^{2+}\) channels and their juxtaposed RyRs that occur in heart failure.

In the previous section, the possible currents contributing to the prolongation of APD were discussed (page 153). That \(I_{\text{Ca,L}}\) was decreased in this particular model of heart failure suggests that the prolongation of APD is not likely to be due to alterations in \(I_{\text{Ca,L}}\).
Fig. 3.14: A. Voltage-dependent activation profile of $I_{Ca,L}$. B. Voltage-dependent inactivation profile. * $p < 0.05$ vs Normal; § $p < 0.05$ vs HF. No° of animal/cells: Normal 6/17; HF 5/22; BM 5/15; SK 5/22. ANOVA was used for statistical comparisons.
3.13 Results : \( \text{Na}^+/\text{Ca}^{2+} \) exchanger currents

It has been suggested previously that changes in NCX may be relevant to the abnormal \( \text{Ca}^{2+} \) homeostasis in heart failure. For this reason, \( I_{\text{NCX}} \) density was measured using the whole-cell patch clamping method described in section 2.12.3 on page 110.

**Fig.3.15** shows the current-voltage (I–V) relationship curves for \( I_{\text{NCX}} \) from the four groups of hearts. \( I_{\text{NCX}} \) at +80 mV was +1.6 ± 0.3 pA/pF. At −120 mV it was −1.4 ± 0.4 pA/pF. These are values in agreement with those previously measured by others using whole-cell patch clamping in rat ventricular myocytes (Chorvatova et al. 2004, Wasserstrom et al. 2000, Zhang et al. 1999). Compared to \( I_{\text{NCX}} \) density measured in other mammal species such as guinea-pig (Perchet et al. 2000), rabbit (Quinn et al. 2003) or human (Ginsburg & Bers 2005), these are relatively small values. No significant difference was measured between failing and normal hearts in terms of average current density. This also remained unchanged following cell transplantation. The reversal potentials (range: −17 to −20 mV) were also not significantly different between heart groups.

\( I_{\text{NCX}} \) has been previously studied in various animal models of heart failure by other research groups (for a review, see Sipido et al. 2002). In the model employing coronary artery ligation in the rat some investigators reported decreased \( I_{\text{NCX}} \) density along with decreased protein expression levels (Dixon et al. 1992, Zhang et al. 1996), whereas others reported opposite findings (Wasserstrom et al. 2000). Although these discrepancies are not easily explained, they may be the result of various factors such as age of animals, severity of infarcts and period over which remodeling was allowed to take place.

The findings in the current study can be considered consistent with the data obtained from the rapid caffeine application studies described in section 3.8, where no difference was seen between the four heart groups in terms of SR \( \text{Ca}^{2+} \) content and relative contributions of NCX towards \( \text{Ca}^{2+} \) removal during diastole. Since there was neither an increase in inward \( I_{\text{NCX}} \) nor a decrease in outward \( I_{\text{NCX}} \) in
the the current model of heart failure it appears that $I_{NCX}$, similar to $I_{Ca,L}$, does not contribute significantly to the prolongation of the APD discussed on page 153.
Fig. 3.15: $I_{NCX}$ I-V relationships obtained using whole cell patch clamping. There were no differences between any of the four heart groups. $N^o$ of animal/cells: Normal 6/18; HF 5/22; BM 5/17; SK 5/17. ANOVA was used for statistical comparisons.
3.14 Results : Ca\textsuperscript{2+} sparks studies

The RyR and its relationship with other components of EC coupling has become a major subject of interest in the field of heart failure. It is suggested that the defective Ca\textsuperscript{2+} release from the SR during systole in failing cardiomyocytes results from alterations in the gating properties of RyRs (Xiao et al. 2005, Wehrens et al. 2003), or their physical locations with respect to their neighbouring L-type Ca\textsuperscript{2+} channels (Gomez et al. 1997). To study these possibilities, and to characterize the unitary Ca\textsuperscript{2+} release patterns from the SR, cardiomyocytes were loaded with the Ca\textsuperscript{2+} sensitive fluorescent indicator fluo-4 and Ca\textsuperscript{2+} sparks were studied. Detailed methods are described in section 2.13 on page 115.

Fig.3.16 summarizes the results of these studies. In the absence of field-stimulation (i.e. during quiescence) Ca\textsuperscript{2+} sparks were observed at a higher frequency in failing cardiomyocytes (Fig.3.16A). Although there appeared to be a tendency for this increase to become normalized following cell transplantation statistical testing with ANOVA revealed no significant difference (p = ns).

Morphometric analysis was carried out on the Ca\textsuperscript{2+} sparks. When measured as $F/F_0$, there was no difference in spark amplitude between groups (Fig.3.16B). The width of Ca\textsuperscript{2+} sparks, expressed as FWHM, was greater in failing cardiomyocytes compared to normal (Fig.3.16C), suggesting wider diffusion of Ca\textsuperscript{2+}. This increase was reduced following transplantation of either cell type. There was no difference seen in decay rate of the sparks, measured as FDHM, between normal and failing cardiomyocytes (Fig.3.16D). Interestingly, transplantation of skeletal myoblasts reduced the FDHM compared to both normal and failing cells, indicating faster decay.

The overall rate of leak of Ca\textsuperscript{2+} from the SR during diastole should be related to the product of these variables, $CaSpF \times Amplitude \times FWHM \times FDHM$ (Maier et al. 2003). Using this product for comparison indicated that SR Ca\textsuperscript{2+} leak increased in failing cardiomyocytes, but was normalized by skeletal myoblast trans-
In a proportion of cardiomyocytes which exhibited a high frequency of Ca\(^{2+}\) sparks Ca\(^{2+}\) waves were also observed (example illustration of a Ca\(^{2+}\) wave in Fig.2.21 on page 120). The frequency with which Ca\(^{2+}\) waves were observed are compared in Fig.3.16F. Failing cardiomyocytes had a higher frequency of Ca\(^{2+}\) waves, suggesting increased aberrant leakage of Ca\(^{2+}\) from the SR. This increase was significantly reduced after transplantation of either bone marrow mononuclear cells or skeletal myoblasts.

To summarize, the Ca\(^{2+}\) sparks studies reveal that the gates controlling the release of Ca\(^{2+}\) from the SR are defective in failing cardiomyocytes. Some parameters studied are restored following cell transplantation whereas others, such as Ca\(^{2+}\) spark frequency, are not. Overall, the increased rate of Ca\(^{2+}\) leak was reduced back towards normal following transplantation of skeletal myoblasts.
Fig. 3.16: Parameters of Ca\(^{2+}\) sparks. A. Ca\(^{2+}\) sparks occurred at higher frequency in HF compared to Normal. This increase remained unaffected following transplantation. B. The peak amplitude of Ca\(^{2+}\) sparks did not differ between the heart groups. C. The width of the Ca\(^{2+}\) sparks (FWHM), was larger in HF compared to Normal. This change was normalized with transplantation of either cell type. D. There was no difference between HF and Normal cardiomyocytes in terms of Ca\(^{2+}\) spark duration (FDHM). The FDHM of SK cardiomyocytes were shortened when compared to HF. E. The total SR Ca\(^{2+}\) leak product was increased in HF, and reduced back towards normal following transplantation of SK, but not BM. F. Ca\(^{2+}\) waves were observed at a much higher frequency in HF compared to Normal. This change was normalized following transplantation of either BM or SK. No of animal/cells: Normal 5/60; HF 5/53; BM 4/46; SK 5/44. ANOVA was used for statistical comparisons.
3.15 Discussion

The results presented in this chapter confirm the development of heart failure following coronary artery ligation, and show that some of the resulting changes in the myocardium were further altered following cell transplantation. The main important finding is the identification of recipient cardiomyocytes as a target of the physiological effects of the transplanted cells. The recipient ventricular cardiomyocytes, which had undergone chronic pathological changes following myocardial infarction, showed reduced hypertrophy and superior contractile characteristics with improved Ca\(^{2+}\) handling following cell transplantation. In the case of skeletal myoblast transplantation, the parameters in \(I_{Ca,L}\) in heart failure was normalized.

Validation of the animal model of heart failure. The left ventricles of the rats that underwent myocardial infarction in this study showed markedly reduced ejection fraction. Although the rats showed no symptoms or signs of congestive cardiac failure the animal model was considered suitable for this study of cell transplantation because ejection fraction of \(\sim 40\%\) approximates the cardiac function of patients receiving cell transplantation in the current clinical trials (Assmus et al. 2006, Abdel-Latif et al. 2007). Characterization of isolated left ventricular cardiomyocytes revealed marked hypertrophy, impaired contractile properties and Ca\(^{2+}\)-handling, prolonged action potentials, and reduced \(I_{Ca,L}\). The frequency of Ca\(^{2+}\) sparks during quiescence was also increased.

Some of the cellular parameters measured showed no significant change. These include amplitude of Ca\(^{2+}\) transients, SR Ca\(^{2+}\) load, and parameters relating to NCX. NCX showed no alterations in terms of current density, or its relative contribution towards Ca\(^{2+}\) removal during diastole. These findings do not necessarily mean that the post-infarction hearts are not a valid model of chronic heart failure. However, it is tempting to speculate what further cellular changes, if any, might have manifested if more severe infarcts were induced (for example, by ligating the left coronary artery closer towards its left main stem), or if chronic heart failure was allowed to develop for longer than the three weeks given before cells were
transplanted. Further experiments may reveal novel important findings but on the other hand operative mortality may have become unacceptably higher, imposing a practical limitation.

**Comparison of bone marrow mononuclear cells vs skeletal myoblasts.**

From the results presented in this chapter, it is not possible to compare directly the efficacy of bone marrow mononuclear cells versus skeletal myoblasts. For such a comparison to be made, the dose-response relationships for each cell type need to be measured, which was not feasible in this study. The number of cells that were injected (5 × 10⁶ skeletal myoblasts or 10⁷ bone marrow mononuclear cells per heart) were chosen following earlier studies performed within our research group (Fukushima et al. 2007), as well as by others (Pouzet et al. 2001). To date there has been no report establishing the optimal injection numbers in any animal model or patients. This remains a crucial point in tailoring cell therapy for patients not only for treatment efficacy, but also from a safety standpoint (Nadal-Ginard & Fuster 2007).

Given these limitations, the $I_{Ca,L}$ results from this study are particularly interesting because they suggest that the mediated effects depend on the transplanted cell type. The observation that a wide variety of cell types seem to benefit the performance of failing hearts has led some researchers to suspect that the main effect of cell transplantation is simply to cause an inflammatory response triggered by the death of the transplanted cells - the so-called “dying stem cell hypothesis” (Thum et al. 2005). This hypothesis proposes that alterations of the activities of macrophages and T-cells following the death of large numbers of transplanted cells ultimately leads to attenuation of the infarction process with reduced scar formation and a favourable outcome. Although the data from this current study do not rule out that possibility, the clear difference between skeletal myoblasts and bone marrow mononuclear cells in terms of their effects of $I_{Ca,L}$ implies that more complex mechanisms are likely to be involved, and that overall cardiomyocyte function might be improved in more ways than one.
What happens to the transplanted cells? The experiments in this chapter focused on characterization of the recipient cardiomyocytes. There have been numerous previous studies tracking the phenotypes of transplanted cells, in the anticipation that they might adopt cardiomyocyte-like characteristics (Orlic et al. 2001). Similar studies were performed and published by our research group (Fukushima et al. 2007). In these experiments, male bone marrow mononuclear cells were injected into female hearts, and real-time polymerase chain reaction (RT–PCR) assay identifying the sex-determining region of the Y-chromosome \((sry)\) was employed to measure the proportion of cells that were surviving. These experiments revealed that, after injection of \(10^7\) bone marrow mononuclear cells only 0.07% are remaining in the entire left ventricle by 7 days. The survival of bone marrow mononuclear cells then remained stable until 84 days after transplantation. When slice preparations of the left ventricles were studied using immuno-labeling of GFP, the majority of the surviving cells visualized assumed small, round morphology. A small fraction of the cells were spindle-shaped. There were no GFP\(^+\) cells similar in appearance to mature cardiomyocytes.

These observations, together with the results presented in this chapter suggest the presence of a physiological effect of transplanted cells towards recipient cardiomyocytes. One can postulate that such physiological effects may be mediated by a variety of mechanisms (Laflamme et al. 2007, Dimmeler et al. 2005), such as neoangiogenesis and modulation of the extracellular matrix. The results are also consistent with “the paracrine theory”, although they do not provide conclusive proof. The possibility that the physiological effects of transplanted cells are mediated by mechanisms other than by paracrine secretions must be considered. For example direct cell-to-cell contact or the recruitment of other circulating cell types may be necessary. To narrow down the possibilities it became necessary to determine whether bone marrow mononuclear cells or skeletal myoblasts could directly affect the properties of nearby cardiomyocytes in the absence of other factors that are present \textit{in vivo} by means of further experiments. These experiments were carried out using
cell culture systems, and the results are discussed in the next chapter.
Chapter 4

Effects of bone marrow mononuclear cells and skeletal myoblasts on the properties of nearby cardiomyocytes \textit{in vitro}

In the previous chapter, it was demonstrated that transplantation of either bone marrow mononuclear cells or skeletal myoblasts influenced the contractile properties and Ca$^{2+}$ handling of individual cardiomyocytes of chronically failing hearts. This may be due to the actions of paracrine substances secreted by the transplanted cells, which then act directly on the myocardium, as previously proposed by Kinnaird et al. (2004) and Uemura et al. (2006). Although such secretions may act upon the cardiomyocytes, other possibilities also need to be considered. For example, they may target the microcirculation and/or extracellular matrix rather than cardiomyocytes. This is especially plausible given that the microcirculation and extracellular matrix are already known to undergo pathological changes in ventricular remodeling (Fazel et al. 2006, Murtuza et al. 2004). Another consideration is that, although the recipient cardiomyocytes may be the primary target of the effects of the transplanted cells, additional factors or cells might be required. For example,
soluble factors present in the serum or other types of cells in the circulation may need to be recruited to the heart to enable the transplanted cells to exert their effect (Dimmeler et al. 2005).

Thus, to determine whether the nearby presence of either bone marrow mononuclear cells or skeletal myoblasts is sufficient to influence the contractile and Ca$^{2+}$ handling properties of the failing cardiomyocytes, further experiments were performed where cardiomyocytes were co-cultured with the donor cells. Failing cardiomyocytes were isolated from remodeled hearts and then cultured for 48 hours in the presence of either of the two types of cell. At the end of this period alterations to the contractile performances and Ca$^{2+}$ handling characteristics of cardiomyocytes were studied. In such an environment there is no microcirculation, extracellular matrix, or other types of cells to be affected by the addition of the bone marrow mononuclear cells or skeletal myoblasts. There is also no “inflammatory response” that is commonly initiated in vivo when tissue is damaged, and likely to occur following cell transplantation (Thum et al. 2005). Therefore, any change that might be effected onto the failing cardiomyocytes during co-culture can be said to be due to the direct effects of the cells that were present in the vicinity.
4.1 Materials & Methods

4.1.1 Cell culture
Cardiomyocytes were isolated and prepared for culture for 48 hours using methods outlined in section 2.14. Cardiomyocytes were cultured at a density of 5,000 cells per 35 mm dish -

- on their own (control) or
- together with 5,000 cultured skeletal myoblasts per dish or
- together with 50,000 freshly prepared bone marrow mononuclear cells per dish.

These plating densities were chosen after initial pilot studies. Plating 5,000 skeletal myoblasts per dish yielded large numbers of proliferating cells after 48 hours, but left sufficient space between cells to enable indo-1 fluorescence measurement studies (Fig. 4.3). The bone marrow mononuclear cells were smaller than skeletal myoblasts and did not proliferate significantly or exhibit morphological changes. Therefore, 50,000 cells were plated in each dish for co-culture.

4.1.2 Study of contractile properties and indo-1 transients of cultured cardiomyocytes
After 48 hours of culture, cardiomyocytes were studied for their contractile characteristics using sarcomere length measurements and Ca^{2+} handling using indo-1. Whereas in the previous chapter the contractions of freshly isolated cardiomyocytes were studied without indo-1 loading, the cultured cardiomyocytes discussed in this chapter were studied for their contractions and indo-1 transients simultaneously. The hardware configuration which enabled this is described in section 2.10 on page 93.

Loading cardiomyocytes with indo-1: Freshly isolated cardiomyocytes were loaded with indo-1 AM in the standard manner, as described on page 93. In com-
parison, the cultured cardiomyocytes were loaded with indo-1 AM whilst still attached to the glass-bottom dishes. First, the culture media were removed from the glass-bottom dishes using a standard 1 ml micropipette. These samples were then centrifuged at 1000 rpm for 5 minutes. The pellets were discarded and supernates were immediately frozen and stored at $-80^\circ$C for later analysis.

The cultured cardiomyocytes were then bathed in 2 $\mu$M indo-1 AM, diluted in DMEM for 10 minutes. Finally, the indo-1 solution was changed to DMEM only to allow de-esterification for at least 1 hour.

**Hardware setup:** The same hardware setup used for studying cardiomyocyte contractions and indo-1 transients described in section 2.9 on page 90 was employed. Cardiomyocytes were studied whilst being superfused in their glass-bottom dishes with normal Tyrode solution containing 1 mM Ca$^{2+}$. A 35 mm Petri-dish insert (Fig.2.22B, Bioscience Tools Inc. San Diego, CA, USA) was used to maintain laminar flow of superfusate, a constant bath depth of approximately 1 mm, and a constant temperature of 37 $\pm$ 1 $^\circ$C around the superfused cardiomyocytes.

**Protocol for field stimulation transients:** The bath contents were field-stimulated at 0.2 or 0.5 Hz using a pair of custom-made platinum wire electrodes. These were adapted to fit within the Petri-dish insert at its periphery, minimizing impedance to the flow of superfusate. Sarcomere length shortening data and indo-1 signals were simultaneously acquired. Only cardiomyocytes contracting upon field-stimulation and showing healthy appearance with clear striations were included in the study.
4.2 Results : Effects of 48 hour culture on the properties of normal and failing cardiomyocytes

Cardiomyocytes cultured for 48 hours showed morphological changes which were apparent under light microscopy. Their corners and edges became rounded and there was some loss of striations, as described in previous reports by others (Volz et al. 1991, Nag & Cheng 1986). Normal and failing cardiomyocytes were further characterized by comparing their contractile properties and indo-1 transients before and after 48 hours culture. As illustrated below, cultured cardiomyocytes loaded with indo-1 showed markedly slower relaxation, leading to reduced contractions with increasing rate of field-stimulation (Zhou et al. 2000). Contractions were small at field-stimulation frequencies > 0.5 Hz. Following this observation, experiments were performed using field-stimulation frequencies of 0.2 and 0.5 Hz only.

Fig.4.1 shows the results obtained from measuring cardiomyocyte sarcomere length at 0.2 Hz field stimulation. Consistent with the results seen in the previous chapter, the contractions of freshly isolated failing cardiomyocytes were smaller than those of normal cardiomyocytes. In contrast to the results seen in the previous chapter (section 3.6 on page 144) failing cardiomyocytes showed a prolongation of the $T_{peak}$ and $T_{50}$ in this series of experiments compared to normal cardiomyocytes.

Following culture for 48 hours normal cardiomyocytes showed reduced amplitude of contraction, whereas failing cardiomyocytes showed no further impairment. The degree was such that there was no measurable difference between normal and failing cardiomyocytes. In addition, both types of cardiomyocytes showed marked further prolongation of $T_{peak}$ and $T_{50}$, such that there was no longer any significant difference between failing and normal cardiomyocytes.

Fig.4.2 shows the results for indo-1 transients, also acquired at 0.2 Hz. There was no change in the (diastolic) baseline level of fluorescence. The transient amplitudes of freshly isolated failing cardiomyocytes were slightly greater compared to
normal, although this difference was not seen at 0.5 Hz (data not shown). The $T_{\text{peak}}$ and $T_{50}$ were both prolonged in failing cardiomyocytes, as seen with the sarcomere length data.

Following culture for 48 hours the transient amplitudes of failing cardiomyocytes were reduced, such that there was no longer any difference compared with normal (Fig. 4.2C). As with sarcomere length changes, the transient $T_{\text{peak}}$ and $T_{50}$ were markedly prolonged in both cell types. After 48 hours, there was no longer any significant difference between normal and failing cardiomyocytes.

Collectively, these results show that the contractile properties and EC coupling of cardiomyocytes are markedly affected by culturing for 48 hours, as well as their morphology. Following culture both normal and failing cardiomyocytes showed slower contraction and relaxation, which correlated with slower Ca$^{2+}$ release and re-uptake. These changes were considered unsurprising, since cardiomyocytes were cultured in an environment different to that in vivo: they were isolated, adhered onto a glass surface, mechanically unloaded, and not electrically stimulated to contract. In addition, the bathing culture medium, whilst maintained at 37 °C and pH 7.4, was most probably devoid of many of the substances that may have been present in the extracellular fluid of the intact heart.
**Fig. 4.1:** Sarcomere length shortening profiles of normal and failing cardiomyocytes, before and after 48 hours culture.  
**A.** Example sarcomere length traces from failing cardiomyocytes before and after 48 hours culture.  
**B.** There was no difference between freshly isolated normal and failing cardiomyocytes in terms of sarcomere length baseline, and culture had no effect.  
**C.** Freshly isolated failing cardiomyocytes contracted less than normal. Culture for 48 hours reduced the contraction amplitude of normal cardiomyocytes, but had no effect on failing cardiomyocytes.  
**D.** Freshly isolated failing cardiomyocytes had longer $T_{\text{peak}}$ than normal. Following culture, $T_{\text{peak}}$ of both normal and failing cardiomyocytes were significantly prolonged.  
**E.** Cardiomyocyte relaxation, measured as $T_{50}$, was prolonged in freshly isolated failing cardiomyocytes when compared to normal. Culture also caused prolongation of $T_{50}$ in both normal and failing cardiomyocytes. Similar data were seen following 0.5 Hz field stimulation.  

$N$ of hearts/cells: Normal Day 0 8/78; HF Day 0 7/41; Normal 48 hours 6/48; HF 48 hours 5/24. Unpaired Student’s t-test was used for comparison between the two groups.
Fig. 4.2: Indo-1 transients from normal and failing cardiomyocytes, before and after 48 hours culture. A. Example indo-1 traces from failing cardiomyocytes before and after 48 hours culture. B. There was no difference between freshly isolated normal and failing cardiomyocytes in terms ofindo-1 baseline, and culture had no effect. C. Freshly isolated failing cardiomyocytes had slightly larger indo-1 transient amplitudes compared to normal. Following culture, the transient amplitude was reduced. D. Freshly isolated failing cardiomyocytes had prolonged indo-1 $T_{\text{peak}}$ compared to normal. Following culture, $T_{\text{peak}}$ of both normal and failing cardiomyocytes were significantly prolonged. E. Indo-1 transient decay, measured as $T_{50}$, was prolonged in freshly isolated failing cardiomyocytes when compared to normal. Culture also caused prolongation of $T_{50}$ in both normal and failing cardiomyocytes. No of hearts/cells: Normal Day 0 8/78; HF Day 0 7/41; Normal 48 hours 6/48; HF 48 hours 5/24. Unpaired Student’s $t$-test was used for comparison between the two groups.
4.3 Results: Effects of bone marrow mononuclear cells and skeletal myoblasts on the properties of nearby failing cardiomyocytes in culture

The results outlined in the previous chapter showed that bone marrow mononuclear cells and skeletal myoblasts influenced the properties of failing cardiomyocytes in vivo. One possible explanation for this is that they alter the properties of neighbouring cardiomyocytes directly. This possibility was tested using a 48 hour co-culture system.

The morphological changes of cardiomyocytes following 48 hours culture can be seen in Fig.4.3. There were no apparent changes to the morphology or density of bone marrow mononuclear cells (Fig.4.3A). Furthermore, bone marrow mononuclear cells did not appear to adhere to the culture dish floor, and did not remain in the bath during superfusion with normal Tyrode solution. In contrast, skeletal myoblasts proliferated markedly and also adhered onto the culture dish. Their morphology, initially round upon plating, became stellate or spindle-shaped (Fig.4.3B). When an initial plating density of 5,000 cells per dish was used, their proliferation did not reach confluence after 48 hours, and there were still many cardiomyocytes which were sufficiently far away from skeletal myoblasts to enable acquisition of their indo-1 fluorescence emissions without interference.

Fig.4.4 shows the results from measuring sarcomere length changes in the cardiomyocytes at 0.2 Hz. There were no changes in the baseline (diastolic) sarcomere length. The contraction amplitude was markedly increased following culture together with skeletal myoblasts (Fig.4.4C), whereas the presence of bone marrow mononuclear cells had no significant effect. The $T_{peak}$ were not significantly different between groups. The relaxation of cardiomyocytes, measured as $T_{50}$, were significantly hastened by the presence of either cell type (Fig.4.4E).
The Ca\textsuperscript{2+} handling properties of these cardiomyocytes were studied simultaneously using indo-1. The baseline (diastolic) level was slightly lower when cardiomyocytes were cultured in the presence of skeletal myoblasts, but remained unchanged with bone marrow mononuclear cells (Fig. 4.5B). There was no measurable effect on the amplitude (Fig. 4.5C) or T\textsubscript{peak} (Fig. 4.5D) of the indo-1 transients with either bone marrow mononuclear cells or skeletal myoblasts. The decay of the transient, expressed as T\textsubscript{50}, was significantly enhanced following culture with skeletal myoblasts (Fig. 4.5E). In contrast, the presence of bone marrow mononuclear cells had no measurable effect. As with the results obtained from the in vivo model presented in the previous chapter, the relatively large changes in myocyte contractility compared to the Ca\textsuperscript{2+} transient point towards effects on the sensitivity of the myofilaments to intracellular Ca\textsuperscript{2+}. However, direct experimental assessment of myofilament Ca\textsuperscript{2+} sensitivity or molecular characterization of the myofilaments is required to clarify this point.

Overall, these data illustrate that the contractile properties and Ca\textsuperscript{2+} handling of failing cardiomyocytes are enhanced when they are cultured for 48 hours in the presence of skeletal myoblasts compared to when they are cultured alone. This effect needs to be interpreted in the context of changes in these failing cardiomyocytes brought about by their culture, described in the previous section. Compared to skeletal myoblasts, bone marrow mononuclear cells exerted much smaller effects (with only T\textsubscript{50} of relaxation showing a significant reduction). This was true even though ten times more bone marrow mononuclear cells were plated compared to cardiomyocytes. The results of the previous chapter raised the possibility that the physiological effects exerted onto failing cardiomyocytes by the two transplanted cell types may differ. The difference between skeletal myoblasts and bone marrow mononuclear cells observed here may be considered consistent with such an interpretation.
Fig. 4.3: Appearances of cardiomyocytes cultured with either bone marrow mononuclear cells (A) or skeletal myoblasts (B) for 48 hours. Cardiomyocytes became rounded in morphology, with some loss of striation. There was no change in the morphology of bone marrow mononuclear cells, whilst skeletal myoblasts proliferated and attached to the dish to assume stellate appearances.
Fig. 4.4: Effects of nearby bone marrow mononuclear cells or skeletal myoblasts on the contractile properties of failing cardiomyocytes in co-culture. A. Representative example sarcomere length traces from failing cardiomyocytes which were field stimulated at 0.2 Hz after 48 hours culture on their own (HF), or with either bone marrow mononuclear cells (BM) or skeletal myoblasts (SK). B. Baseline sarcomere lengths were not different between groups. C. The presence of skeletal myoblasts resulted in an increased contraction amplitude, whereas the presence of bone marrow mononuclear cells had no effect. D. $T_{\text{peak}}$ was not different between groups. E. Cardiomyocyte relaxation, measured as $T_{50}$, was hastened by the presence of either cell type. N° of hearts/cells: HF 6/29; BM 6/17; SK 6/19. Similar data were seen at 0.5 Hz. ANOVA was used for statistical comparisons.
Fig. 4.5: Effects of nearby bone marrow mononuclear cells or skeletal myoblasts on the indo-1 transients of failing cardiomyocytes in co-culture. **A.** Representative 0.2 Hz indo-1 transients from failing cardiomyocytes which were cultured on their own (HF), or with either bone marrow mononuclear cells (BM) or skeletal myoblasts (SK). **B.** The indo-1 baseline levels was reduced after co-culture with skeletal myoblasts but not bone marrow mononuclear cells. Indo-1 transient amplitudes (C), and $T_{peak}$ (D) showed no difference between groups. **E.** Indo-1 transient decay, measured as $T_{50}$, was hastened by the presence of skeletal myoblasts, but not bone marrow mononuclear cells. N° of hearts/cells: HF 6/29; BM 6/17; SK 6/19. Similar data were seen at 0.5 Hz. ANOVA was used for statistical comparisons.
4.4 Results: Effects of skeletal myoblasts which are physically separated from the failing cardiomyocytes during culture

Following the observation in the previous section that the presence of skeletal myoblasts enhances the contractile performance of failing cardiomyocytes in co-culture, the question arose: “Do the skeletal myoblasts require physical contact with the cardiomyocytes to exert their influence?”. Previous studies had demonstrated that skeletal myoblasts contacting adult rat cardiomyocytes in culture are able to form electrical connections (Stagg et al. 2006). During image and fluorescence acquisition, only solitary cardiomyocytes which were not in direct physical contact with other cells were selected for functional assessment. This was in order to avoid fluorescence interference. However, because the cardiomyocytes under study were superfused with normal Tyrode solution, the possibility that they might have been in contact with other types of cells during the culture period could not be eliminated.

The co-culture experiments using skeletal myoblasts were therefore repeated, but with the two cell populations physically separated by means of a porous membrane (“Transwell®”, see Fig.2.22 on page 122). This preparation prevented any direct physical contact between the skeletal myoblasts and cardiomyocytes, whilst allowing diffusion of any secreted substances between them. After the culture period cardiomyocytes were studied as before, and visual inspection confirmed the presence of cardiomyocytes and the absence of skeletal myoblasts in the glass-bottom dish.

The results from sarcomere length measurements are illustrated in Fig.4.6. The contraction profile of failing cardiomyocytes cultured on their own were similar to that seen in section 4.3. The contractions of these cardiomyocytes were enhanced when skeletal myoblasts were present in the culture dish, even though they were separated by the porous membrane. The amplitude of contraction (Fig.4.6C) and speed of relaxation (measured as T_{50}, Fig.4.6E) were increased, as seen before. The indo-1 transients of these cardiomyocytes showed corresponding decreased T_{50}, as
before (Fig.4.7).

These results suggested that direct physical contact between skeletal myoblasts and cardiomyocytes was not necessary for the beneficial effects to be mediated, and that the influence of skeletal myoblasts were most likely mediated via soluble paracrine substances secreted by the skeletal myoblasts.
Fig. 4.6: Effects of skeletal myoblasts on the contractile properties of failing cardiomyocytes in co-culture, physically separated by a porous membrane.  

A. Example sarcomere length traces at 0.2 Hz.  
B. Baseline sarcomere length was not different between the two groups.  
C. Amplitude of contraction was increased by co-culture with skeletal myoblasts.  
D. There was no difference in $T_{\text{peak}}$.  
E. The relaxation of cardiomyocytes, measured as $T_{50}$, was hastened by the presence of skeletal myoblasts.  

No of hearts/cells: HF 6/28; SK 6/19. Similar data were seen at 0.5 Hz (results not shown).  
Unpaired Student’s $t$-test was used for statistical comparisons.
Fig. 4.7: Effects of skeletal myoblasts on the contractile properties of failing cardiomyocytes in co-culture, physically separated by a porous membrane. A. Example indo-1 transients at 0.2 Hz. B. Indo-1 baseline level was not different between the two groups. There was no difference between CM and CM+SK in terms of transient amplitude (C) or T_peak (D). The transient decay rate, measured as T_{50} (E), was hastened by the presence of skeletal myoblasts. No of hearts/cells: HF 6/28; SK 6/19. Similar data were seen at 0.5 Hz (results not shown). Unpaired Student’s t-test was used for statistical comparisons.
4.5 Results: Effects of skeletal myoblasts on the properties of normal cardiomyocytes in culture

The experiments described in section 4.2 showed that both normal and failing cardiomyocytes were affected by a 48 hour period of culture. By the end of this period, the cardiomyocyte contractions were slower and decreased in amplitude, and the relaxations were slower. Results from the subsequent experiments (section 4.3) demonstrated that the contractility and Ca\textsuperscript{2+} handling of hypertrophied, failing cardiomyocytes improved in the presence of skeletal myoblasts in the same culture dish, and that direct physical contact between the two cell populations was not required for the mediated effect (section 4.4). Further experiments were carried out to determine the effects on normal cardiomyocytes in similar conditions.

Left ventricular myocytes were isolated from normal adult Sprague-Dawley rat hearts and cultured for 48 hours, as described in the previous sections. In some glass-bottom dishes, skeletal myoblasts were also allowed to grow within a Transwell\textsuperscript{®}. Plating densities were identical to those of previous experiments (i.e. 5,000 cardiomyocytes with or without 5,000 added skeletal myoblasts per dish). After 48 hours, the contraction dynamics and indo-1 transients of cardiomyocytes were simultaneously assessed using the same protocols as in previous sections.

The results from sarcomere length measurements at 0.2 Hz are illustrated in Fig. 4.8. Consistent with results seen in section 4.3, the contractions of normal cardiomyocytes cultured on their own were decreased in amplitude and slower compared to freshly isolated normal cardiomyocytes. There was no significant effect of culturing with skeletal myoblasts on the contraction and relaxation of these normal cardiomyocytes. Correspondingly, indo-1 transients showed no difference in any of the measured parameters between the two groups (Fig. 4.9).

These observations, that the presence of skeletal myoblasts have no effect on the properties of normal cardiomyocytes in co-culture, are in contrast to those seen
when failing cardiomyocytes were cultured with skeletal myoblasts.
Fig. 4.8: Effects of skeletal myoblasts on the contractile properties of normal cardiomyocytes in co-culture, physically separated by a porous membrane. A. Example sarcomere length trace at 0.2 Hz of a normal cardiomyocyte after 48 hours culture (left), and a normal cardiomyocyte cultured in the presence of skeletal myoblasts (right). B. Baseline sarcomere length. C. Amplitude of contraction. D. T_{peak}. E. The relaxation of cardiomyocytes, measured as T_{50}. There was no significant difference between the two groups in any of these parameters. N° of hearts/cells: Normal 7/27; Normal + SK 5/12. Similar data were seen at 0.5 Hz (results not shown). Unpaired Student’s t-test was used for statistical comparisons.
**Fig. 4.9:** Effects of skeletal myoblasts on the indo-1 transients of normal cardiomyocytes in co-culture, physically separated by a porous membrane. A. Example indo-1 transient at 0.2 Hz of a normal cardiomyocyte after 48 hours culture (left), and a normal cardiomyocyte cultured in the presence of skeletal myoblasts (right). B. Baseline indo-1 level. C. Amplitude of transient. D. $T_{\text{peak}}$. E. Indo-1 transient decay, measured as $T_{50}$. There was no significant difference between the two groups in any of these parameters. No of hearts/cells: Normal 7/27; Normal + SK 5/12. Similar data were seen at 0.5 Hz (results not shown). Unpaired Student’s $t$-test was used for statistical comparisons.
4.6 Discussion

4.6.1 Skeletal myoblasts and bone marrow mononuclear cells influence the Ca$^{2+}$ handling and contractile properties of nearby cardiomyocytes via paracrine secretions.

The experiments described in this chapter were performed after the observations made in chapter 3, that transplantation of bone marrow mononuclear cells or skeletal myoblasts altered the properties of the recipient cardiomyocytes in a failing heart. A number of possible explanations could be considered as to how these changes might be mediated in vivo. Although it has been previously proposed that transplanted cells influence the myocardium (Dimmeler et al. 2005, Dai et al. 2005), such “paracrine theory” has not yet been supported by experimental evidence. The cellular targets of such paracrine effects remain undefined: as well as the cardiomyocytes, other components such as blood capillaries or the extracellular matrix may be important. Furthermore, other indirect physiological mechanisms warrant consideration. For example, it is conceivable that the effects of the transplanted cells are mediated via by direct cell-to-cell contact rather than secreted substances. Additional types of cells may need recruitment from the circulation.

To eliminate a number of these possibilities a similar experiment was performed in a simplified environment. In the culture conditions employed in the experiments in this chapter there were no blood vessels, extracellular matrix, or cell types other than those plated. Although the culture media contained animal sera (for detailed composition, see page 129), there was no active inflammatory process. The results seen in this chapter after elimination of such factors show that skeletal myoblasts and bone marrow mononuclear cells exert direct effects on failing, hypertrophied cardiomyocytes in their proximity. Following co-culture the contractile properties and Ca$^{2+}$ extrusion dynamics of cardiomyocytes were enhanced. That these effects were maintained even when the different cell populations were physically separated
using a porous membrane showed that the effect could be mediated purely by soluble factors, without the need for direct cell-to-cell contact.

Previously, Takahashi et al. (2006) performed similar experiments. Left ventricular myocytes isolated from normal adult rat hearts were subjected to culture medium which was conditioned by the growth of bone marrow mononuclear cells for 24 hours previously. After culture for 72 hours, cardiomyocytes exposed to growth medium conditioned by bone marrow mononuclear cells showed increased contraction amplitudes at 1 Hz. Contraction $T_{\text{peak}}$ and relaxation times such as $T_{50}$ or $T_{90}$ were not reported. There was no change in the $Ca^{2+}$ transients, as measured using the $Ca^{2+}$-sensitive fluorescent indicator fura-2. A number of differences exist in the experimental details between the results of this chapter and those of Takahashi et al. These include the use of normal vs failing cardiomyocytes; different initial composition of the culture media; different frequency of field-stimulation of cardiomyocytes; and the use of conditioned media vs a co-culture system. The final of these points deserves particular attention: in the studies reported by Takahashi et al. the growth medium was conditioned by bone marrow mononuclear cells at a density of $5 \times 10^6$ cells per ml. In comparison, the bone marrow mononuclear cells were plated in the co-culture system used in this chapter at a density of $5 \times 10^4$ cells per dish (equal to $2.5 \times 10^4$ cells per ml). Despite these differences, both studies agree in suggesting that co-culture with bone marrow mononuclear cells result in small improvements in the mechanical properties of cardiomyocytes.

### 4.6.2 Comparison of bone marrow mononuclear cells vs skeletal myoblasts

In the previous chapter the results from $I_{Ca,L}$ measurement studies suggested that skeletal myoblasts and bone marrow mononuclear cells influenced the recipient myocardium in different ways. The results from the results in this chapter also show differences between the two cell types. Compared to skeletal myoblasts, bone marrow mononuclear cells exerted a smaller influence on the properties of nearby cardiomyo-
cytes.

Given the similar degree of improvement measured after transplantation of skeletal myoblasts and bone marrow mononuclear cells in vivo, it appears that the bone marrow mononuclear cells in the present co-culture experiments were unable to exert their full effect. Given that there were ten times more bone marrow mononuclear cells than cardiomyocytes, and also considering the findings reported by Takahashi et al (who used approximately 200 times more bone marrow mononuclear cells compared to this current study), the plating density of bone marrow mononuclear cells is unlikely to have been a limiting factor. It is possible that bone marrow mononuclear cells need to be in the presence of cardiomyocytes for longer durations. One can also speculate that further factors that were present in vivo, but absent in the culture environment, may have also been required. These include, for example, endothelial cells, other types of circulating cells, and mechanical or electrical stimulation. The identification of additional factors that may enable bone marrow mononuclear cells to influence their neighbouring cardiomyocytes requires further investigation.

4.6.3 Why are normal cardiomyocytes not affected by skeletal myoblasts in a manner similar to failing cardiomyocytes?

Having demonstrated the paracrine effects on skeletal myoblasts on failing cardiomyocytes, the experiment was repeated using normal cardiomyocytes. The contractile and Ca\(^{2+}\)-handling characteristics of these were not enhanced by co-culture with skeletal myoblasts. A direct retrospective comparison cannot be made between normal cardiomyocytes cultured with skeletal myoblasts and failing cardiomyocytes cultured with skeletal myoblasts, because the experiments were performed over different time periods.

The lack of an effect is perhaps not surprising, since normal cardiomyocytes did
not possess the same defects in EC coupling as the failing cardiomyocytes. Both normal and failing cardiomyocytes were affected by culture and by 48 hours there was no significant difference between them in terms of their contractile properties or Ca$^{2+}$ transients (section 4.2, page 174). It is plausible that different cellular mechanisms were underlying the changes in contraction and relaxation during the culture period for the two different cardiomyocyte populations.

4.6.4 The limitation of experiments

Although bone marrow mononuclear cells and skeletal myoblasts enhanced cardiomyocyte contractility both in vivo and in culture, the patterns of change were different. For example, the main effect of skeletal myoblasts on Ca$^{2+}$ cycling in vivo was normalization of Ca$^{2+}$ release, whereas following co-culture it was the rate of Ca$^{2+}$ re-uptake during diastole that was enhanced. Such differences may have been due, at least in part, to the culture environment being non-physiological. In other words, because cardiomyocytes were isolated, adhered onto a glass surface, mechanically unloaded, and not electrically stimulated regularly to contract their morphology and dynamics of contraction and Ca$^{2+}$ handling had changed over 48 hours. It would be useful to see if an improved culture system could be established that enabled i) incubation of small blocks of adult cardiomyocytes from failing hearts which are devoid of blood vessels and other cell types, and ii) regular electrical stimulation. One would anticipate that such a culture environment may mimic the in vivo environment more closely and help minimize the alterations in cardiomyocytes that are observed. This in turn would enable better elucidation of the influence of neighbouring skeletal myoblasts and bone marrow mononuclear cells.
Chapter 5

Analysis of culture media composition

The experiments performed in the previous chapter demonstrated that skeletal myoblasts influenced the properties of failing cardiomyocytes via paracrine secretions. During these experiments the culture media supernates were collected at the end of the 48 hour culture period and frozen for storage. These samples were analyzed in an attempt to identify the possible key mediators of the effects of skeletal myoblasts. Initially, the supernates resulting from co-culture of failing cardiomyocytes and skeletal myoblasts were analyzed using the *rat cytokine antibody array*. This offers a simple and effective method for simultaneously screening the presence of several cytokines in a variety of fluid samples, including serum and culture media.

Further to this experiment, the same supernate samples were analyzed for insulin-like growth factor-1 (IGF–1) using a separate enzyme-linked immunosorbent assay (ELISA). Measurement of IGF–1 concentrations was of particular interest because IGF–1 is a secretory protein that is known to have important regulatory roles in cell metabolism and survival (Cohick & Clemmons 1993). IGF–1 has also been shown previously to influence the contractility of perfused hearts directly (Freestone et al. 1996, von Lewinski et al. 2003).
5.1 Materials & Methods

Details of supernate sample processing and storage, usage of the cytokine antibody array, and data analyses are described in section 2.15 on page 121. Four arrays were used for analysis of supernates from culturing cardiomyocytes only, and four arrays were used to analyze supernates from culturing cardiomyocytes with skeletal myoblasts. Samples were syringe-filtered, but not diluted or processed further after thawing. The standard experimental protocol as recommended by the manufacturer was used throughout.

With the IGF–1 ELISA, a 96-well plate was provided, allowing simultaneous analysis of up to 70 fluid aliquots. Pairs of fluid aliquots from seven samples were used from each group. Details of the methods are described in section 2.16 on page 127. Standard experimental protocol as recommended by the manufacturer was used. Supernate samples were diluted using the provided diluent (1:1), as recommended by the manufacturer.
5.2 Results: Cytokines secreted by skeletal myoblasts detected using the rat cytokine antibody array

The results from studies using the cytokine antibody arrays are illustrated in Fig.5.1. The culture media supernates which contained ventricular cardiomyocytes only contained negligible trace amounts of cytokines. This was considered unsurprising, since cardiomyocytes are highly differentiated and specialized cells whose primary function is mechanical. In comparison the culture media supernates which were bathing skeletal myoblasts in addition to cardiomyocytes contained notably increased levels of three cytokines: monocyte chemoattractant protein (MCP–1), tissue inhibitor of metalloproteinase (TIMP–1), and vascular endothelial growth factor (VEGF).

5.3 Results: Levels of IGF–1 present in the coculture supernates

The rat cytokine antibody array (used in the section above) enables comparison between fluid samples for relative levels of multiple cytokines. It does not provide measurements of actual concentrations. In comparison, ELISA, which was used for measurement of IGF–1, is able to yield actual concentration measurements against a concentration standard. As shown in Fig.5.2 IGF–1 was absent in the culture media supernates that contained ventricular cardiomyocytes only. In comparison, culture media supernates that grew skeletal myoblasts in addition to the cardiomyocytes contained $720 \pm 126$ pg/ml IGF–1.
Fig. 5.1: Cytokines present in culture media supernates detected using rat cytokine antibody arrays. Representative example rat cytokine antibody arrays developed after exposure to media supernate obtained from culturing cardiomyocytes only (A), and from culturing cardiomyocytes with skeletal myoblasts (B). The levels of MCP–1 (C), TIMP–1 (D), and VEGF (E) were increased following culture with skeletal myoblasts. There was no significant change in the levels of other cytokines detected by the array. Full details of these cytokines are illustrated in Table 2.2. 

No. of arrays: cardiomyocytes only 4; cardiomyocytes + skeletal myoblasts 4. Unpaired Student’s t-tests were used for comparison between these two groups.

Abbreviations: “O. D. Units” = Optical Density Units.
Fig. 5.2: Concentrations of IGF–1 in culture media supernates measured using ELISA. A. Photograph of the developed ELISA microplate comparing levels of IGF–1 in culture media supernates. Serial dilutions of the provided IGF–1 were used to generate a concentration standard (the two left columns of wells) from 0 to 2000 pg/ml. B. Results of compiled unpaired Student’s t-tests between cardiomyocytes only and cardiomyocytes + skeletal myoblasts. No. of supernate samples: cardiomyocytes only 7; cardiomyocytes + skeletal myoblasts 7.
5.4 Discussion

The results described in this chapter reveal the increased presence of four cytokines: MCP–1, TIMP–1, VEGF, and IGF–1. These findings provide further support to the notion that skeletal myoblasts influence the function of failing cardiomyocytes in co-culture by paracrine mechanisms.

One important consideration in the interpretation of these results is that the growth medium already contained horse serum (10%) and foetal bovine serum (4%). Although animal sera are known to contain all of the above-mentioned cytokines in abundance, they were not detected in significant levels in the control samples with either assay. This can be interpreted in a number of ways. First, there are clearly identified species differences in the molecular composition of these cytokines. For example, the primary amino-acid sequence of IGF–1 are different between bovine and rat at four residues (Humbel 1990). The IGF–1 ELISA assay used is highly specific for mouse and rat IGF–1, but not other animal species. Another consideration is the possible consumptive depletion of the cytokines during the 48 hour culture period. It is likely that even if the concentrations of these cytokines were high in both groups of culture samples initially, early consumption of cytokines by cardiomyocytes may have led to depletion in the wells that contained only cardiomyocytes, but not in those that contained skeletal myoblasts in addition.

In section 4.5, it was observed that cultured normal cardiomyocytes were no affected by the nearby presence of skeletal myoblasts in the same manner as failing cardiomyocytes. One possible explanation for this is that skeletal myoblasts did not secrete cytokine in a similar pattern in the presence of normal cardiomyocytes. To exclude this possibility requires further direct measurement of cytokines using identical assays.
5.4.1 IGF–1

IGF–1, also known as somatomedin–C, is a single-chain polypeptide consisting of 70 residues, and shares structural similarities with pro-insulin. Its structure is relatively highly conserved amongst animal species, with 100% sequence homology between human, bovine, equine, porcine, and canine proteins (Humbel 1990). IGF–1 is a progression factor in the cell cycle of many cell types, and has regulatory roles in the proliferation and differentiation of a variety of tissues (Cohick & Clemmons 1993). In the body it is produced by many tissue types and is abundant in the circulation. However, local production is thought to be important for its regulatory roles. For example, autocrine secretion by skeletal myoblasts stimulates their differentiation in vitro upon serum withdrawal (Florini et al. 1991).

The identification of IGF–1 as a candidate mediator of the effects of skeletal myoblasts on failing cardiomyocytes is of particular interest, especially since it has been previously identified to affect EC coupling and contractility of cardiomyocytes directly (Duerr et al. 1995, Freestone et al. 1996, von Lewinski et al. 2003). In addition to the effects on the contractile performance of cardiomyocytes, IGF–1 may increase their rate of survival under stress. Experiments using transgenic mice overexpressing IGF–1 within cardiomyocytes (under the control of the α-myosin heavy chain promoter) have also shown that increased expression of IGF–1 can limit tissue loss and remodeling after myocardial infarction (Li et al. 1997).

5.4.2 VEGF

VEGF is a highly specific mitogen for vascular endothelial cells. It exists as 5 different isoforms, which are generated by alternative splicing. Regarding its physiological importance in vivo, its role in the regulation of angiogenesis has been the subject of most research (Ferrara 2001, Seko et al. 1999). VEGF is expressed in a spatially and temporally coordinated manner during development of the normal cardiovascular system. Animals that lack either of the two VEGF alleles and animals lacking
VEGF receptors die in utero due to severe abnormalities in blood vessel formation (Neufeld et al. 1999). In normal tissues, exposure to hypoxia potentiates VEGF production and release (Shweiki et al. 1992). Furthermore, inhibition of the binding of VEGF to its receptors in solid tumours results in tumour regression. VEGF acts via its receptors FLT–1 and FLK–1 to activate intracellular phospholipase–Cγ1 (PLCγ1). Both FLT–1 and PLCγ1 are expressed in cardiomyocytes (Seko et al. 1999). Interestingly, interference with the VEGF ⇒ FLT–1 ⇒ PLCγ1 signaling pathway using Morpholino oligonucleotides results in significant impairment of cardiac contractility (Rottbauer et al. 2005). Conversely, incubation of neonatal rat cardiomyocytes with 100 ng/ml VEGF164 increases the amplitudes of their Ca2+ transients.

5.4.3 TIMP–1

TIMPs are two-domain molecules made up of about 190 amino-acids (Brew et al. 2000). In humans four TIMPs have been recognized. Their main function in the body is the inhibitory regulation of matrix metalloproteinases (MMPs), which in turn are the chief proteinases that degrade the extracellular matrix. The fine balance between MMP and TIMP activity is largely responsible for controlling extracellular matrix formation and digestion, thus maintaining optimal tissue structure. In the heart deletion of MMP–9 has been found to affect cardiac remodeling after myocardial infarction favorably (DuCharme et al. 2000). Conversely, MMP-overexpression or TIMP–1 deficiency result in increased LV remodeling following myocardial infarction in animal models (Creemers et al. 2003). There have been several studies describing growth promoting activity of TIMP–1 affecting many cell types, which appears to be independent of its MMP–inhibitory activity (Hayakawa et al. 1992). When TIMP–1 was first cloned, it was found to be identical to a factor called erythroid potentiating activity (EPA). Other cell types which have been identified to be affected directly by TIMP–1 include B-cells and keratinocytes. Thus, the TIMPs appear to be multifunctional proteins, but the mechanisms underlying cellular func-
tions remain poorly understood. Most relevant to this study the direct effects, if any, of TIMP–1 on adult cardiomyocytes remain unclear.

5.4.4 MCP–1

MCP–1, also known as CC-motif chemokine ligand (CCL)–2, is a 76-residue profibrotic substance with a role in mononuclear cell recruitment and activation during inflammation (Yoshimura et al. 1989). Its role in post-ischaemic heart injury and myocardial remodeling has been studied using both knockout and overexpression models. MCP–1 knockout mice show less monocyte recruitment during myocardial ischaemia with reduced subsequent interstitial fibrosis and remodeling (Frangogiannis et al. 2007). On the other hand, mice overexpressing MCP–1 in the heart (under control of the α-myosin heavy chain promoter) showed markedly increased macrophage recruitment and progressive hypertrophic remodeling of the ventricle even in the absence of injury (Kolattukudy et al. 1998). In these mice cardiac contractile performance gradually deteriorated until death at around 6 months (Zhou et al. 2006). In culture, exposure of human embryonic kidney (HEK)–293 cells or the H9C2 (a neonatal rat heart cell line) cells to MCP–1 increased the presence of a transcription factor (MCP-induced protein, or MCPIP) and cell death. Using reverse-transcription polymerase chain reaction (RT-PCR) the expression of the natural receptor for MCP–1 (CCR–2) in the cardiomyocytes of 2 month-old rats has been demonstrated (Zhou et al. 2006). However, the direct effect of MCP–1 on the survival and contractile properties of isolated adult cardiomyocytes remains untested. Given the available data one may anticipate the overall effect to be adverse, but direct experimental evidence is required.

To summarize, of the four substances which were found in increased concentrations when cardiomyocytes were cultured together with skeletal myoblasts there is evidence to hypothesize that IGF–1 and VEGF mediate beneficial effects directly. Less is known about TIMP–1, the main function of which is the inhibitory regulation
of MMP by complex formation, to suggest that it may have direct effects on cardiomyocytes. Given that MCP–1 has adverse overall effects on myocardium further study of its direct effects on cardiomyocytes and elucidation of intracellular pathways may be of equal importance as the potentially beneficial ones exerted by others. To verify the roles of these substances future experiments involving corresponding antagonists of these substances or skeletal myoblasts from gene knock-out animals would be useful.
Chapter 6

Acute effects of $\beta$-agonists on the Na$^+$-Ca$^{2+}$ exchanger current

In Chapter 3 whole-cell patch-clamping experiments were carried out to measure Na$^+$-Ca$^{2+}$ exchanger currents in freshly isolated cardiomyocytes (page 160). During the development phase of these experiments additional, separate experiments were performed. The purpose of this study was partly to validate the configuration of the whole-cell patch-clamping system used to measure $I_{NCX}$ densities in cardiomyocytes isolated from the various rat hearts. The background to this separate study and experimental findings are described in this chapter.

6.1 Introduction

6.1.1 Limitation of unloading-induced myocardial atrophy by clenbuterol

As discussed in section 1.1.5 left ventricular assist devices (VADs) are primarily used as a “bridge to transplantation”, where the failing heart of a patient is mechanically assisted until a suitable donor organ becomes available (Clegg et al. 2006). However, in a small number of patients VADs have served as “bridge to recovery”, since they induced substantial functional improvement, enabling their explantation without
the need for subsequent heart transplantation (Muller et al. 1997). Clearly, this is an ideal outcome for the patient, but such “bridge to recovery” episodes have only been observed to occur at low rates of $5 \sim 10\%$ (Mancini et al. 1998, Maybaum et al. 2007). The main reasons for this low frequency include the extremely severe disease of the hearts receiving VAD support, and also the limited regenerating capacity of the heart. Another important reason for the low frequency of recovery is that complete mechanical unloading of a heart ventricle leads to functional, structural, and molecular changes in its own right (Wohlschlaeger et al. 2005). Unloading results in myocardial atrophy, often with adverse consequences. Animal studies of ventricular unloading have demonstrated time-dependent changes in cardiomyocyte contractile function and Ca$^{2+}$ handling, which correlate with impaired cardiac performance (Ito et al. 2003).

Clenbuterol is a substance classified as a $\beta_2$-AR agonist, based on its molecular structure (MacLennan & Edwards 1989). It shares structural similarities with salbutamol and was previously used to treat patients with asthma (Salorinne et al. 1975). However, in addition to its bronchodilator effects clenbuterol has been found to induce hypertrophy in skeletal muscle (George et al. 2006) and myocardium (Petrou et al. 1995). In normal rat hearts its chronic administration induces cardiomyocyte hypertrophy (Wong et al. 1997) and increases oxidative carbohydrate utilization. The SR Ca$^{2+}$ content and the amplitude of the Ca$^{2+}$ transient are increased (Soppa et al. 2005). In a recent study, Soppa et al. (2008) tested the chronic effect of clenbuterol on mechanically unloaded failing hearts. Rats underwent myocardial infarction and were allowed to developed chronic heart failure. The hearts were then mechanically unloaded for one week by means of heterotopic transplantation. Administration of clenbuterol during this period limited the unloading-induced atrophy. The Ca$^{2+}$ handling and contractile performance of individual cardiomyocytes isolated from these hearts were superior compared to cardiomyocytes from control unloaded hearts, which received only saline during the same period.

The results from these animal model experiments, which suggest that clenbu-
terol limits the atrophy induced by mechanical unloading, are consistent with data from clinical studies performed at Harefield Hospital (Birks et al. 2006). Patients with severe dilated cardiomyopathy were treated with a combination of mechanical unloading and pharmacological therapy. Clenbuterol was included in the treatment protocol after VAD implantation with the specific aim of preventing unloading-induced atrophy and dysfunction of the myocardium. Out of 15 patients sufficient myocardial recovery had occurred in 11 cases, enabling VAD explantation. The cumulative rate of freedom from recurrent heart failure among the surviving patients was 88.9% at four years after explantation, with near-normal quality of life.

6.1.2 β-adrenergic signaling in the heart

The stimulation of β-adrenoreceptors by the sympathetic nervous system and circulating catecholamines plays a pivotal role in regulating heart function. The physiological properties which are affected include pacemaker activity (and heart rate) and myocardial contractility. Since the classification of β-receptors into β₁ and β₂ subtypes by Lands et al. (1967), there has been a substantial amount of research on β-adrenoreceptors, the pharmacological properties of various agonists and antagonists, and the intracellular signaling pathways activated by the receptors.

The differences between the β₁- and β₂-adrenoreceptors were mostly revealed by studies using selective agonists and antagonists, in particular CGP–20712A (a β₁-antagonist, Dooley et al. 1986) and ICI–118,551 (a β₂-antagonist, O’Donnell & Wanstall 1980). In the human ventricle, the β₁-subtype constitutes 70 ~ 80% of the total β-adrenoreceptor complement, while the β₂-subtype constitutes the remaining 20 ~ 30% (Brodde 1991). The two subtypes of receptor initially appear to have similar functions. For example, administration of either a β₁- or a β₂-agonist acutely increases myocardial contractility. However, more detailed studies reveal different, sometimes even opposite, physiological roles between the two receptors, involving diverging intra-cellular signaling pathways. Cardiac-specific overexpression of the β₂-adrenoreceptors in mice results in enhanced myocardial contractility, even in the
absence of an agonist, without obvious pathology (Milano et al. 1994). In contrast, β1-adrenoreceptor overexpression increases cardiomyocyte apoptosis and leads to severe dilated cardiomyopathy and premature death (Engelhardt et al. 1999). Mice in which the β1-adrenoreceptor gene has been deleted show no inotropic response to acute stimulation by catecholamines (Rohrer et al. 1996), whereas β2-adrenoreceptor knock-out mice show full responses (Chruscinski et al. 1999).

Valuable insight into the long-term effects of β-adrenoreceptor stimulation is also provided by clinical data. In heart failure patients β1-agonists are useful in the acute phase as cardiac inotropes, but their long-term administration increases mortality (and conversely, β-blockers are well known to improve heart function and prognosis, as discussed on page 30). However, in β1-adrenoreceptor knock-out mice, the chronic administration of isoproterenol yields the opposite: a protective effect (Zhu et al. 2001).

That β1- and β2-adrenoreceptors mediate different physiological effects by activating different intracellular pathways is further supported by the subtle differences that can be seen in the Ca²⁺ handling and mechanical properties of cardiomyocytes following acute exposure to selective agonists. For example, the positive inotropic effect induced via β2-adrenoreceptor activation is generally less than that via β1 (Kaumann et al. 1996). Also, whereas β1-stimulation increases heart rate (chronotropy), myocardial contractility (inotropy), and speed of relaxation (lusitropy), in some animal species the lusitropic effect is not seen (Borea et al. 1992). The differences in the cellular effects of β1- and β2-activation can be explained by their coupling to guanosine triphosphate binding proteins (G proteins). Stimulation of β2-γ, but not β1-adrenoreceptors, activates an inhibitory G protein (Gi) in adult rat cardiomyocytes, while both adrenoreceptor subtypes are able to stimulate the classic adenylyl cyclase (AC) ⇒ 3',5'-adenosine monophosphate (cAMP) ⇒ protein kinase-A (PKA) pathway via the stimulatory G protein (Gs, Fig. 6.1).

The modulatory effect of Gi ⇒ phosphoinositide 3-kinase (PI3K) pathway can be revealed using pertussis toxin (PTX), which disables Gi through ribosylation (Brehler...
et al. 1992). Cardiomyocytes with disrupted $G_i$ signaling after exposure to PTX exhibit markedly enhanced $\beta_2$-mediated positive inotropy, whilst the $\beta_1$-mediated effects show no change (Xiao et al. 1995, Xiao 2001).

### 6.1.3 Pharmacological properties of various $\beta$-agonists

A variety of $\beta$-agonists and antagonists have been identified, with varying degrees of selectivity for the subtypes of receptor, and even different profiles of $G_i$ and $G_s$ activation (for reviews of $\beta$-adrenoreceptors and agonists, see Brodde (1991) and also Brodde & Michel (1999)). For example, isoproterenol is a well-characterized non-selective $\beta$-agonist. It acutely increases cardiomyocyte contractility and $\text{Ca}^{2+}$
transient amplitude, as well as speed of relaxation (Endoh & Blinks 1988, Spurgeon et al. 1990). Salbutamol is a well-characterized β-agonist, which is commonly used as an inhaled drug to treat patients with asthma (Xiao et al. 2003). Compared to isoproterenol, it is relatively selective for the stimulation of β2 subtype of receptors.

Fenoterol is also a well-studied agonist which causes positive inotropy following acute exposure. Compared to isoproterenol, it is more selective for the stimulation of β2 subtype (Xiao et al. 2003, Harding & Gong 2004). It has attracted some interest, with the hope that selective β2-stimulation using fenoterol might be beneficial in the treatment of heart failure. Indeed, β2-stimulation by fenoterol restores the contractility of cardiomyocytes isolated from the hearts of spontaneous hypertensive rat (SHR, Xiao et al. 2003) and markedly protects myocardium from ischæmia-induced hypertrophy and apoptosis (Ahmet et al. 2004, Ahmet et al. 2008). However, the effects of fenoterol are mediated in a manner insensitive to PTX, and are therefore thought to be independent of the Gi pathway. Unlike other β2-agonists such as salbutamol, fenoterol might be considered to be a selective agonist of the β2-Gs pathway (Xiao et al. 2003), but the precise mechanisms underlying its effects require elucidation in further detail.

Despite the interesting observations made regarding clenbuterol (described on page 205) and potentially important clinical implications, its direct on cardiomyocytes has not been extensively studied. How clenbuterol compares to other well-established β-agonists, such as those above, in terms of β1- and β2-adrenoreceptor stimulation, and its profile of Gs and Gi activation has not been elucidated. Previous experimental work carried out in our laboratory showed that acute application of 30 µM clenbuterol onto normal rat cardiomyocytes reversibly reduced their I_{Ca,L}, Ca^2+ transients, and contractions (Dr. Urszula Siedlecka, personal communication). The effects of clenbuterol on other parameters of EC coupling, such as the action potential profile or I_{NCX}, have not been described.
6.1.4 Experiment design

In this chapter the acute effect of clenbuterol on the $I_{NCX}$ density of normal rat cardiomyocytes was assessed, and compared to other β-active compounds, including the following:

- Isoproterenol
- Fenoterol
- Salbutamol
- **Isoproterenol plus CGP–20712A:** The possible effect of selective β$_2$-stimulation was investigated more extensively by using a mixture of isoproterenol plus the selective β$_1$-antagonist, CGP–20712A.

As mentioned earlier, these studies were performed during the preparation phase for the experiments outlined in earlier chapters, partly to test the configuration of the whole-cell patch-clamping system used to measure $I_{NCX}$ densities in cardiomyocytes isolated from the various rat hearts.

6.2 Methods

Cardiomyocytes were isolated from normal adult Sprague-Dawley rats using the standard enzymatic digestion protocol, outlined in section 2.12.3. Isolated cardiomyocytes were used within six hours. The $I_{NCX}$ densities of cardiomyocytes were measured using the whole cell patch-clamping method, as described in section 2.12.3.

Initially, steady state currents were recorded during application of the ramp protocol in K$^+$-free superfusate (see Fig.6.2). The effects of acute administration of various β-active substances were tested by rapidly switching the K$^+$-free Tyrode superfusate to one containing the added compound by means of a solenoid controlled solution switcher device. Membrane currents were recorded once steady state was
established. The superfusate was then switched to one containing the β-active compound plus 5 mM Ni\(^{2+}\). Finally, the active current measured during superfusion with 5 mM Ni\(^{2+}\) only added was recorded. The baseline \(I_{NCX}\) was taken to be the Ni\(^{2+}\)-sensitive component of the baseline current (i.e. current 4 subtracted from current 1, from Fig.6.2), whilst the \(I_{NCX}\) during superfusion of the β-active substance was taken to be the Ni\(^{2+}\)-sensitive component of the corresponding currents (i.e. current 3 subtracted from current 2).

The concentrations of the various β-active compounds tested using this setup were as follows.

- 1 µM clenbuterol
- 2 µM isoproterenol
- 10 µM fenoterol
- 50 µM salbutamol
- 300 nM isoproterenol + 300 nM CGP–20712A

All of these β-active compounds were purchased from Sigma-Aldrich, UK. They were diluted in K\(^{+}\)-free Tyrode superfusate on the day of use, either from fresh powder or from frozen concentrated aliquots which were stored at −20 °C. Manufacturer’s recommendations for preparation and storage were followed.

Statistical analyses were performed using paired Student’s \(t\)-tests to compare the current densities measured at various membrane voltages from the same cardiomyocyte before and after application of the β-agonist. Results are expressed as mean ± S.E.M. and were considered statistically significant when \(p < 0.05\).
Fig. 6.2: Protocol for measuring the effect of acute application of a $\beta$-agonist on $I_{NCX}$. 
6.3 Results

In K\textsuperscript{+}-free Tyrode solution the $I_{NCX}$ density measured $2.17 \pm 0.20$ pA/pF at $+80$ mV and $-0.85 \pm 0.32$ pA/pF at $-120$ mV (Fig.6.3, closed circles). The reversal potential was approximately $-32$ mV. These values are similar to those previously measured by others using whole-cell patch clamping in normal adult rat ventricular myocytes (Chorvatova et al. 2004, Wasserman et al. 2000, Zhang et al. 1999).

The acute application of 1 µM clenbuterol decreased the $I_{NCX}$ density by approximately 37% (Fig.6.3). The change in $I_{NCX}$ magnitude was acute, with steady state currents in clenbuterol being established within 1 ~ 2 minutes. The $I_{NCX}$ density at $-120$ mV was not significantly affected. The reversal potential also remained unchanged.

The effect of clenbuterol was compared to that of other β-active compounds. The application of isoproterenol causes substantial increases in cardiomyocyte contraction and Ca\textsuperscript{2+} transient amplitude at concentrations above 0.5 µM (Spurgeon et al. 1990). Similar results were also observed in our laboratory, data not shown). However, as can be seen on Fig.6.4 application of 2 µM isoproterenol had no measurable effect on $I_{NCX}$. Previous studies by others reported variation in the response of $I_{NCX}$ to isoproterenol, depending on animal species. For example, Ginsburg & Bers (2005) measured no effect of 1 µM isoproterenol on $I_{NCX}$ in rabbit cardiomyocytes, which agrees with the current results seen in rat. On the other hand, Perchenet et al. (2000) found that 1 µM isoproterenol increased $I_{NCX}$ density in guinea-pig cardiomyocytes at 37°C.

The possibility that clenbuterol affects $I_{NCX}$ via β-receptors was investigated further by comparing against the effects of additional β-agonists. The effect of acute application of 10 µM fenoterol, a β-agonist relatively more selective for the β\textsubscript{2} subtype of receptors, was assessed. As illustrated in Fig.6.5 there was no significant effect.

Fig.6.6 shows the effect on $I_{NCX}$ density observed following application of 50 µM salbutamol, another β\textsubscript{2}-selective agonist. In contrast to fenoterol, the $I_{NCX}$ density
at +80 mV was reduced by approximately 17%. This effect took place within 1 ~ 2 minutes. The $I_{NCX}$ density at −120 mV and the reversal potential were not significantly affected.

The effect of selective β2 stimulation was also investigated by applying a solution mixture of 300 nM isoproterenol plus 300 nM CGP–20712A. CGP–20712A is a selective inhibitor of β1 receptors (Dooley et al. 1986). Although a lower concentration of isoproterenol was present in this mixture than in the previous experiment shown in Fig.6.4, there was a decrease of $I_{NCX}$ density, by up to 37%.

These results from testing various β-active compounds are collectively summarized in Table 6.1 on page 218.
**Fig. 6.4:** Acute effect of isoproterenol on $I_{NCX}$ density. 2 µM isoproterenol had no significant effect on $I_{NCX}$ density in normal rat cardiomyocytes. $N_o$ of cells = 11. Paired Student’s $t$-test was used for the statistical comparison.

**Fig. 6.5:** Acute effect of fenoterol on $I_{NCX}$ density. 10 µM fenoterol had no significant effect on $I_{NCX}$ density in normal rat cardiomyocytes. $N_o$ of cells = 15. Paired Student’s $t$-test was used for the statistical comparison.
Fig. 6.6: Acute effect of salbutamol on $I_{NCX}$ density. 50 µM salbutamol reduced $I_{NCX}$ density in normal rat cardiomyocytes by up to 17%. N° of cells = 8. ** $p < 0.01$. * $p < 0.05$. Paired Student’s $t$-test was used for the statistical comparison.

Fig. 6.7: Acute effect of isoproterenol plus CGP–20712A on $I_{NCX}$ density. 300 nM isoproterenol + 300 nM CGP reduced $I_{NCX}$ density in normal rat cardiomyocytes by up to 37%. N° of cells = 15. ** $p < 0.01$. * $p < 0.05$. Paired Student’s $t$-test was used for the statistical comparison.
Table 6.1: The effects of various β-agonists on $I_{NCX}$ density

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Concentration</th>
<th>Effect</th>
<th>Receptor</th>
<th>G-Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clenbuterol</td>
<td>1 µM</td>
<td>↓</td>
<td>$\beta_2$</td>
<td>$G_i^\dagger$</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>2 µM</td>
<td>↔</td>
<td>$\beta_1$ and $\beta_2$</td>
<td>$G_i$ and $G_s$</td>
</tr>
<tr>
<td>Fenoterol</td>
<td>10 µM</td>
<td>↔</td>
<td>$\beta_2$</td>
<td>$G_s$ only</td>
</tr>
<tr>
<td>Salbutamol</td>
<td>50 µM</td>
<td>↓</td>
<td>$\beta_2$</td>
<td>$G_i$ and $G_s$</td>
</tr>
<tr>
<td>Iso plus CGP–20712A</td>
<td>300 + 300 nM</td>
<td>↓</td>
<td>$\beta_2$</td>
<td>$G_i$ and $G_s$</td>
</tr>
</tbody>
</table>

$^\dagger$ The contribution of $G_s$ remains unassessed.

**Abbreviations:** Iso: isoproterenol. ↓ decreased. ↔ no change.

### 6.4 Discussion

The acute application of clenbuterol resulted in a decrease in $I_{NCX}$ density. Similar effects were seen following application of selective $\beta_2$ stimulation by salbutamol, and also by the mixture of isoproterenol plus CGP–20712A. In comparison, simultaneous stimulation of both $\beta_1$ and $\beta_2$ receptors by application of isoproterenol had no overall effect. Application of an alternative $\beta_2$-agonist, fenoterol, also had no significant effect on $I_{NCX}$.

These results can be interpreted in the context of differential coupling of the $\beta$-receptors to intracellular signaling pathways. $\beta$-receptors are members of the G protein-coupled receptor superfamily, and their main effect on ventricular cardiomyocytes is to increase the amplitudes of $I_{Ca,L}$, $Ca^{2+}$ transient, and contraction. However, whereas both $\beta_1$ and $\beta_2$ stimulation result in these changes, only $\beta_1$ stimulation accelerates the decay of the $Ca^{2+}$ transient and cardiomyocyte relaxation (Borea et al. 1992, Xiao & Lakatta 1993). In addition, it has been demonstrated that selective inhibition of the $G_i$ pathway by pre-treatment of the cardiomyocytes by PTX potentiates the effects of $\beta_2$, but not $\beta_1$ stimulation (Xiao et al. 1995).
These observations suggest that activation of the G\(_i\) pathway following β\(_2\) receptor stimulation may partially antagonize the G\(_s\)-mediated effects on cardiomyocyte EC coupling (for a review on functional coupling of the β-receptor to G\(_s\) and G\(_i\) proteins see Xiao 2001).

With the background of this understanding, it appears that simultaneous stimulation of both G\(_i\) and G\(_s\) via β\(_2\) receptors causes a decrease in rat \(I_{NCX}\) density. These are seen following application of salbutamol and also the mixture of isoproterenol plus CGP–20712A. β\(_2\) receptor stimulation by fenoterol has no effect on \(I_{NCX}\), which is most likely because it selectively activates G\(_s\) only. This interpretation is consistent with previous assessment of its effect on contractility, where the effect of fenoterol was not further augmented after disrupting the G\(_i\) pathway by pre-incubating cardiomyocytes in PTX (Xiao et al. 2003).

Compared with these agonists, the effect of clenbuterol suggests that it may activate the β\(_2\)-G\(_i\) pathway. This interpretation would be supported by future experiments measuring the effect of clenbuterol on \(I_{NCX}\) in cardiomyocytes pre-treated with PTX. Abolishment of the inhibitory effect would support the conclusion above.

Additional experiments are required to help understand the potential effects of β\(_1\)-stimulation on \(I_{NCX}\). Whereas activation of both G\(_s\) and G\(_i\) pathways following β\(_2\) receptor stimulation causes a decrease in \(I_{NCX}\), simultaneous β\(_1\) activation, as when isoproterenol only was applied, appears to negate this effect. Assessment of the effect of a selective β\(_1\)-agonist, such as dobutamine, or studying the effect of isoproterenol in the presence of ICI–118,551, which is a selective β\(_2\) inhibitor, would be interesting experiments that might provide further insight into this phenomenon.
Chapter 7

Concluding remarks

Injection of skeletal myoblasts or bone marrow mononuclear cells into chronically failing rat hearts improved ejection fraction at 4 weeks. Characterization of the recipient cardiomyocytes isolated from these hearts revealed changes in their morphology and EC coupling, and enhanced contractile properties. To test the hypothesis that these effects observed in vivo are mediated by paracrine substances secreted from the transplanted cells further experiments were performed. Cardiomyocytes isolated from failing rat hearts were cultured for 48 hours. Co-culturing with skeletal myoblasts or bone marrow mononuclear cells affected Ca$^{2+}$ handling and improved the contractility of nearby failing cardiomyocytes. The effect was maintained when the different cell populations were physically separated using a porous membrane, indicating that paracrine secretions can mediate the inter-cellular effects. The supernate samples from these co-culture experiments were analyzed using ELISA and cytokine antibody arrays. These assays detected increased levels of IGF–1, VEGF, MCP–1 and TIMP–1, identifying them as secreted substances that might mediate the paracrine effects.

Together, these results provide the first direct evidence to support the hypothesis that cells transplanted into a failing heart affects recipient cardiomyocyte EC coupling through paracrine mechanisms, thereby enhancing myocardial performance.
What are the main limitations of the thesis? Although the results from Chapter 3 are supported by those described in Chapters 4 and 5, the necessity of performing the latter experiments *in vitro* has meant that the exact contribution of the demonstrated paracrine effects towards the functional benefit *in vivo* remains to be assessed. In other words, following the demonstration of a particular effect *in vitro* one can not safely assume that the same effect is the only mechanism responsible for the physiological changes occurring *in vivo*. However, as discussed in section 1.4 of Chapter 1, the *paracrine theory* has been proposed by other researchers in the field as an explanation following the exclusion of other possibilities, most notably the *de novo* regeneration of a substantial volume of myocardium. Other mechanisms have also been proposed by others to explain the functional benefits resulting from cell transplantation, such as neoangiogenesis and modulation of the extracellular matrix. These were discussed on page 72. Whilst they are potentially also important, they have not been the main subject of this thesis.

What are the future experiments that might be useful? It is possible to design further experiments that might yield data to support the findings currently presented. The identification of IGF–1, VEGF, TIMP–1 and MCP–1 as candidate paracrine mediators of the effects exerted by skeletal myoblasts leads to further study of the effects of skeletal myoblasts and bone marrow mononuclear cells obtained from animals which are deficient in these genes. Such cells, which were not available during the presented study, would be most useful for confirming the physiological involvement of these substances, as they could be studied *in vivo* as well as in co-culture systems. An alternative approach, at least for assessment using co-culture systems, would be to utilize selective inhibitors of these signaling molecules.

Should cell transplantation for heart failure be performed in humans? Large-scale clinical trials are already being performed world-wide, although cell transplantation is far from being established treatment for heart failure. The large volume of animal model studies published by numerous research groups, and also the work presented in this thesis, demonstrate that cell transplantation is capable
of delivering a clear functional benefit. Thus, it appears that cell transplantation holds considerable promise for the treatment of heart failure, and further research is warranted. However, clinical trials have shown mixed results and meta-analyses have demonstrated only small benefits. Subsequently, many researchers in the field of cardiac cell transplantation are increasingly promoting a “return from bedside to bench” (Arnesen et al. 2007, Nadal-Ginard & Fuster 2007). In this regard, it appears that current research should aim to identify the similarities between the animal and human studies and address the disparities between animal and human studies as a priority. Substantial differences in cardiomyocyte EC coupling are known to exist between different animal species, including human, under both normal and pathological conditions. Furthermore, cardiomyocytes isolated from different models of heart failure vary substantially in their properties depending on mode of induction and disease duration and severity. Thus, further characterization of the pathophysiology of heart failure and elucidation of the physiological mechanisms responsible for the beneficial effects mediated by transplanted cells under specific disease states would hold the best promise towards optimizing the future results of cell therapy.
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Appendix A

MatLab® script for detection and morphometric analysis of Ca$^{2+}$ sparks

Below is the computer script, written by Dr. Mark A. Stagg, to be run in the computer programme MatLab® R2006b. This script processed the exported TIFF digital image files containing the Ca$^{2+}$ spark data. The output data include Ca$^{2+}$ spark frequency, amplitude, FWHM, and FDHM. For details of applications, see section 2.13 on page 115.

%--------------- 26-Apr-07 -----------------%
%----------- Dr Mark A. Stagg -----------%
%----------- Zeiss tiff Files -----------% 

% INSTRUCTIONS:
% You need to cut the lsm file to the width of the cell and
% then save as a tiff file format. This can be done by exporting
% with the lsm browser software to imageJ for cropping. Open the
% mdb file for the experiments saved and extract the size of scan
% data (pixel size and line scan speed). Set the current directory
% in matlab to the exported tiff files, and then run this script.
%
IMPORTANT - clear command window and close any open images before running.

-----< BEGIN >-----

% open file and get x and y dimensions

[fileName, pathName] = uigetfile('*.tif', 'Select tiff file');
imageName = strcat(pathName,fileName);
imA = imread(imageName);
imA = double(imA);
fileInfo = imfinfo(imageName);
[xSize] = fileInfo.Width;
[ySize] = fileInfo.Height;

% user input of pixel size and time for a linescan

pixelInfo = struct( ... 'dlg_title', 'Input information about pixel acquisition', ...
'prompt', {'Enter pixel size (m):', 'Enter scan speed (ms):',
'Enter criterion (S.D.):'},...
'num_lines', 1, ...
'def', {'0.2222', '0.76364', '3.8'}, ...
'input', 0 ...);
pInfo = inputdlg({pixelInfo(1,1).prompt,pixelInfo(1,2).prompt,
pixelInfo(1,3).prompt},pixelInfo(1,1).dlg_title,pixelInfo(1,1).num_lines,
{pixelInfo(1,1).def,pixelInfo(1,2).def,pixelInfo(1,3).def},'on');
pixelInfo(1,1).input = pInfo(1);
pixelInfo(1,2).input = pInfo(2);
pixelInfo(1,3).input = pInfo(3);
pSize = str2double(pixelInfo(1,1).input);
tScan = str2double(pixelInfo(1,2).input);
cri = str2double(pixelInfo(1,3).input);
clear pInfo;

% % display imA
% figure('Name', 'imA','Position', [32 128 256 768]); % Fig 1
% get(imagesc(imA));
% colormap jet;
% colorbar('location','EastOutside');

% calculate variance
imA = double(medfilt2(imA,[3 3]));
% minimal median filter to remove data points at extremes
% imA = double(medfilt2(imA,[5 5]));
% minimal median filter to remove data points at extremes
imMedian = imA;

% % display imMedian
% figure('Name', 'imMedian','Position', [344 128 256 768]); % Fig 2
% get(imagesc(imMedian));
% colormap jet;
% colorbar('location','EastOutside');

% 0.8 m and 10 ms spatio-temporal filter
ss = (0.8/pSize); % ss = no. of pixels in 0.8 m
st = (10/tScan); % st = no. of pixels in 10 ms
imB = zeros(ySize,xSize,'double'); % set up image array Fig 3
shiftX = round(ss/2);
shiftY = round(st/2);
ct = 0; % ct = iteration counter
for ia = - shiftX : shiftX; % for groups of 2 m
    imB = imB + circshift(imA,[0,ia]); % shift image 2 m
    ct = ct + 1;
end
for ib = - shiftY : shiftY; % for groups of 10 ms
    imB = imB + circshift(imA,[ib,0]); % shift image 10 ms
    ct = ct + 1;

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end
imA = imB./ct;
% imB = imA;

% normalise to background
imMean = mean(imA); % find mean value for % each x (column)
imBx = zeros(ySize,xSize,'double'); % Preallocate matrix Fig 4
for m = 1:ySize;
    imBx(m,:) = imMean(1,:); % fill each row with mean
end
clear m;
imA = imA./imBx; % initial normalization -

sd = std(imA(:)); % divide image by mean at each x
ave = mean(imA(:));
imB = imA;
%clear shiftX;
%clear shiftY;

% display imB
figure('Name', 'imB','Position',[656 128 256 768]); % Fig 3
get(imagesc(imB));
colormap jet;
colorbar('location','EastOutside');

% % display imBx
% figure('Name', 'imBx','Position',[968 128 256 768]); % Fig 4
% get(imagesc(imBx));
% colormap jet;
% colorbar('location','EastOutside');

% % re-draw imA
% figure(1); % select the figure
% get(imagesc(imA)); % redraw the updated data
% colormap jet;
% colorbar('location','EastOutside');

% mask for potential spark regions > m+2*SD
thresh = ave+2*sd;
mask = logical(imA > thresh); % set everything above 2*SD in imA
mask = medfilt2(mask,[5 5]); % median filter (need toolbox) blur edges
imA = imB.*(not(mask)); % excise possible spark regions

% % display mask
% figure('Name', 'mask','Position',[56 96 256 768]); % Fig 5
% get(imagesc(mask));
% colormap gray;
% colorbar('YLim',[0;1],'YTick',[0;1],'YTickLabel',{'0','1'},'location',
% 'EastOutside');

% % re-draw imA
% figure(1); % select the figure
% get(imagesc(imA)); % redraw the updated data
% colormap jet;
% colorbar('location','EastOutside');

% % re-draw imB
% figure(3); % select the figure
% get(imagesc(imB)); % redraw the updated data
% colormap jet;
% colorbar('location','EastOutside');

% calculate baseline spatial profile with potential spark regions excised
% ** this is also used later for spark detection
imBack = imA;
imBase = imA;

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% imBase = zeros(ySize,xSize,'double'); % Preallocate matrix Fig 6
for m = 1:ySize
    imBase(m,:) = imBack(1,:); % fill each row with mean
end
% clear m;
% aveFilter = fspecial('average',[3 3]); % smooth 3x3 boxcar average
% imBase = imfilter(imA,aveFilter,'replicate');
% imB = imB./imBase;
% imBase = double(imB.*(1-mask));
imIndex = find(imBase > 0);
aveLessSp = mean(imBase(imIndex)); % find index of array
% with non-zero values
sdLessSp = std(imBase(imIndex)); % and calculate mean and sd
ave = aveLessSp; % update mean and sd
sd = sdLessSp;
imIndex = find(imBack == 0);
imBase(imIndex) = ave;
% sd = std(imBase(:));
% ave = mean(imBase(:));

% % display imBase
% figure('Name', 'imBase','Position', [368 96 256 768]); % Fig 6
% get(imagesc(imBase));
% colormap jet;
% colorbar('location','EastOutside');

% % display imBack
% figure('Name', 'imBack','Position', [682 96 256 768]); % Fig 7
% get(imagesc(imBack));
% colormap jet;
% colorbar('location','EastOutside');

% % re-draw imB
% figure(3); % select the figure
% get(imagesc(imB)); % redraw the updated data
% colormap jet;
% colorbar('location','EastOutside');

% % re-draw imA
% figure(1); % select the figure
% get(imagesc(imA)); % redraw the updated data
% colormap jet;
% colorbar('location','EastOutside');

% spark detection bit:
% make a binary image of cri*SD i.e. spark sites
% cri = criteria for spark region folds of SD above mean
thresh = ave+cri*sd;
    im = logical(imB > thresh); % set everything above cri*SD
im = medfilt2(im,[3 3]); % need image processing toolbox
im = medfilt2(im,[5 5]); % need image processing toolbox

% **Use the median filtered image imageMedian (with sparks) and
% baseline image (spark areas removed - imBase).
imBack = imB./imBase; % imB = new image (normalised with minimal filtering on sparks)

% re-draw imB
% %figure(3); % select the figure
% figure(1); % select the figure if all others commented out
get(imagesc(imB)); % redraw the updated data
% colormap jet;
% colorbar('location','EastOutside');

% % re-draw imBack
% figure(7); % select the figure
% get(imagesc(imBack)); % redraw the updated data
% colormap jet;
% colorbar('location','EastOutside');
% % display im
% figure('Name', 'im','Position',[992 96 256 768]); % Fig 8
% get(imagesc(im));
% colormap gray;
% colorbar('YLim',[0;1],'YTick',[0;1],'YTickLabel',{'0','1'},'location',
% 'EastOutside');

% assess possible sparks regions in imB

% define search area
xlOrR = round(6/pSize); % 3 m from xCoord
yUpOrDown = round(60/tScan); % 30 ms from tScan

% locate possible sparks from im (cri*SD)
[events,numPossSp] = bwlabeln(im,8);
possSp = regionprops(events);

% % display events as im
% figure(8); % select the figure
% get(imagesc(events)); % redraw the updated data
% colormap jet;
% colorbar('location','EastOutside');

clear imBack;
clear imBase;
clear imBx;
clear imIndex;
clear imA;
clear imMedian;
clear imMean;
clear mask;
% set up structure to hold possible sparks
% include file name, date etc as a header
sparkCell = cell([(numPossSp+1),15]);
    sparkCell{1,1} = ('file');
    sparkCell{1,2} = ('date');
    sparkCell{1,3} = ('cellWidth (m)');
    sparkCell{1,4} = ('scanTime (s)');
    sparkCell{1,5} = ('spFreq (sparks/100m/s)');
    sparkCell{1,6} = ('SpCounted');
    sparkCell{1,7} = ('possSp');
    sparkCell{1,8} = ('xCoord');
    sparkCell{1,9} = ('yCoord');
    sparkCell{1,10} = ('spBound');
    sparkCell{1,11} = ('peak (F/Fo)');
    sparkCell{1,12} = ('xPeak');
    sparkCell{1,13} = ('yPeak');
    sparkCell{1,14} = ('fWhm (m)');
    sparkCell{1,15} = ('fDhm (ms)');

%    sparkCell{16,1} = ('aveP'); % peak with 3x3 filter
%    sparkCell{17,1} = ('xAveP');
%    sparkCell{18,1} = ('yAveP');
%    sparkCell{19,1} = ('avefWhm (m)');
%    sparkCell{20,1} = ('avefDhm (ms)');

    sparkCell{2,1} = fileInfo.Filename;
    sparkCell{2,2} = datestr(now,'yyyy-mm-dd');
    sparkCell{2,3} = (xSize*pSize);
    sparkCell{2,4} = ((ySize*tScan)/1000);

% assess possible sparks in im and imB
for i = 1:numPossSp;
    xy = fix(possSp(i).Centroid); % find x and y from im (cri*SD)
    yCoord = xy(2);
    xCoord = xy(1);
end
xCoord = xy(1);
spBound = [xCoord-xLOrR,xCoord+xLOrR,yCoord-yUpOrDown,
yCoord+yUpOrDown];
sparkCell{(1+i),7} = i;
sparkCell{(1+i),8} = xCoord(1);
sparkCell{(1+i),9} = yCoord(1);
sparkCell{(1+i),10} = (spBound);

% find possible spark in imB and assess parameter
rROI = [spBound(3),spBound(4),spBound(4),spBound(3)];
cROI = [spBound(1),spBound(1),spBound(2),spBound(2)];
spROI = roipoly(imB,cROI,rROI);
imSpark = imB.*spROI;
aveFilter = fspecial('average',[3 3]); % smooth 3x3 boxcar average
imSpark = imfilter(imSpark,aveFilter,'replicate');

peak = max(max(imSpark)); % find peak of possible spark
[yPeak,xPeak] = find(imSpark >= peak);

% re-align centre of spROI to peak
rROI = [(yPeak - yUpOrDown),(yPeak + yUpOrDown),
(yPeak + yUpOrDown),(yPeak - yUpOrDown)];
cROI = [(xPeak - xLOrR),(xPeak - xLOrR),(xPeak + xLOrR),
(xPeak + xLOrR)];
spROI = roipoly(imB,cROI,rROI);
imSpark = imB.*spROI;
aveFilter = fspecial('average',[3 3]); % smooth 3x3 boxcar average
imSpark = imfilter(imSpark,aveFilter,'replicate');

thresh = ave+((peak-ave)/2); % calculate local F/Fo threshold for half-maximal
maskHM = logical(imSpark >= thresh); % set everything above HM
[probEvent,numProbSp] = bwlabel(maskHM,8);
probSp = regionprops(probEvent);
for j = 1:numProbSp;
% get spark bounding box with peak, fDhm
checkSp = probSp(j).BoundingBox;
if (xPeak(1) > checkSp(1)) …
    && (xPeak(1) < (checkSp(1)+checkSp(3)))…
    && (yPeak(1) > checkSp(2))…
    && (yPeak(1) < (checkSp(2)+checkSp(4)));

% calculate fDhm and fWhm
% find duration at peak  (calculate fDhm)
yLimBottom = ySize;
if yLimBottom > spBound(4);
yLimBottom = spBound(4);
end
yLimTop = 1;
if spBound(3) > yLimTop;
yLimTop = spBound(3);
end;
dTest = maskHM(yLimTop:yLimBottom,xPeak(1));
[dTest,dTestNum] = bwlabel(dTest);
dTest = regionprops(dTest);
fDhm = 0;
for k=1:dTestNum;
dTestChk = dTest(k).BoundingBox;
    if yPeak(1) > (dTestChk(2)+spBound(3))…
        && yPeak(1) < (dTestChk(2)+dTestChk(4)+spBound(3));
        fDhm = dTestChk(4);
    end
end
% at each y assess maximum x  (calculate fWhm)
fWhm = 0;
xLimRight = xSize;
if xLimRight > spBound(2);
xLimRight = spBound(2);
end;
xLimLeft = 1;
if spBound(1) > xLimLeft;
    xLimLeft = spBound(1);
end;
for k = yLimTop:yLimBottom;
    dTest = maskHM(k,xLimLeft:xLimRight);
    [dTest,dTestNum] = bwlabel(dTest);
    dTest = regionprops(dTest);
    kk = 0;
    for kk=1:dTestNum;
        dTestChk = dTest(kk).BoundingBox;
        if (dTestChk(3) > fWhm);
            fWhm = dTestChk(3);
        end
    end
end
%
% write spark parameters
spCell{(1+i),11} = peak;
spCell{(1+i),12} = xPeak(1);
spCell{(1+i),13} = yPeak(1);
spCell{(1+i),14} = (fWhm*pSize);
spCell{(1+i),15} = (fDhm*tScan);
end
end
%
% fig of possible caSp?
% display CaSpPNG
caSpBox = [(xPeak - xLOrR) (yPeak - yUpOrDown) (xLOrR*2) (yUpOrDown*2)];
caSpFig = imcrop(imB,caSpBox);
figure('Name', 'CaSpPNG','Position',[384 256 512 512]);
% Fig 9
get(surf(caSpFig));
% colormap jet;
shading interp;
% axis square;
%
saveas(9,(strcat((datestr(now,'yyyymmdd')),
',_CaSp_',num2str(i),'.'),png')),'png');
%
saveas(9,(strcat(pathName,(datestr(now,'yyyymmdd')),
',\',strrep(fileName,'.tif',''),'_CaSp_',num2str(i),
'.jpg')),'jpg');
%
close('CaSpPNG');

end;

% remove duplicate peaks from spark ROI overlap and fWhm < 2 m
xyDuplSpCell = sparkCell;
xyDuplTest = zeros(2,i);
for i = 1:numPossSp;
    xyDuplTest(i,1) = sparkCell{(1+i),12};
    xyDuplTest(i,2) = sparkCell{(1+i),13};
end;
% check each i in cell...
for i = 1:numPossSp;
    for j = 1:numPossSp;
        if (xyDuplTest(j,1) == xyDuplTest(i,1)) && (xyDuplTest(j,2)
        == xyDuplTest(i,2))...
            && i ~= j;
            xyDuplTest(j,1) = 0;
            xyDuplTest(j,2) = 0;
            for k = 1:15;
                xyDuplSpCell{(1+j),k} = [];
            end;
        end;
    end;
    if (xyDuplSpCell{(1+j),14} < 2)
        for k = 1:15;
            xyDuplSpCell{(1+j),k} = [];
        end;
    end;
end;

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end;
end;

sparkCellEnd = xyDuplSpCell;

% select figure imB and draw on sparks and labels
% figure(3);
figure(1);  % if all other figures not rendered

% freq of sparks
spCount = 0;
for j = 1:numPossSp;
    if find(sparkCellEnd{(1+j),7} ~= (j-1));
        spCount = spCount + 1;
        sparkCellEnd{(1+j),6} = spCount;
        rectangle('Position',[x,y,w,h])
        rectangle('Position',[sparkCellEnd{(1+j),10}(1),...
            sparkCellEnd{(1+j),10}(3),...
            (sparkCellEnd{(1+j),10}(2)-sparkCellEnd{(1+j),10}(1)),...
            (sparkCellEnd{(1+j),10}(4)-sparkCellEnd{(1+j),10}(3)))];
        %text(x,y,key);
        key = num2str(spCount);
        text((sparkCellEnd{(1+j),10}(1)+3),(sparkCellEnd{(1+j),10}(3)+30),key);
    end;
end;

spFreq = spCount/(100/xSize/pSize)/(1000/ySize/tScan);
sparkCellEnd(2,5) = spFreq;
sparkCellEnd(2,1) = fileInfo.Filename;
sparkCellEnd(2,2) = datestr(now,'yyyy-mm-dd');
sparkCellEnd(2,3) = (xSize*pSize);
sparkCellEnd(2,4) = ((ySize*tScan)/1000);

[pathstr, fName, ext, versn] = fileparts(fileName);
xlsName = strcat(pathName,fName,'.xls');
pngName = strcat(pathName,fName,'.png');
xlsWrite(xlsName, sparkCellEnd); % this works but is slow
saveas(1, pngName, 'png');

% -----< ALL DONE >----- %