Increased Ca\(^{2+}\)-sensitivity of the contractile apparatus in end-stage human heart failure results from altered phosphorylation of contractile proteins

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Abstract

**Objective:** The alterations in contractile proteins underlying enhanced Ca\(^{2+}\)-sensitivity of the contractile apparatus in end-stage failing human myocardium are still not resolved. In the present study an attempt was made to reveal to what extent protein alterations contribute to the increased Ca\(^{2+}\)-responsiveness in human heart failure. **Methods:** Isometric force and its Ca\(^{2+}\)-sensitivity were studied in single left ventricular myocytes from non-failing donor (\(n=6\)) and end-stage failing (\(n=10\)) hearts. To elucidate which protein alterations contribute to the increased Ca\(^{2+}\)-responsiveness isoform composition and phosphorylation status of contractile proteins were analysed by one- and two-dimensional gel electrophoresis and Western immunoblotting. **Results:** Maximal tension did not differ between myocytes obtained from donor and failing hearts, while Ca\(^{2+}\)-sensitivity of the contractile apparatus (pCa) was significantly higher in failing myocardium (\(D\text{pCa}=0.17\)). Protein analysis indicated that neither re-expression of atrial light chain 1 and fetal troponin T (TnT) nor degradation of myosin light chains and troponin I (TnI) are responsible for the observed increase in Ca\(^{2+}\)-responsiveness. An inverse correlation was found between pCa and percentage of phosphorylated myosin light chain 2 (MLC-2), while phosphorylation of MLC-1 and TnT did not differ between donor and failing hearts. Incubation of myocytes with protein kinase A decreased Ca\(^{2+}\)-sensitivity to a larger extent in failing (\(D\text{pCa}=0.20\)) than in donor (\(D\text{pCa}=0.03\)) myocytes, abolishing the difference in Ca\(^{2+}\)-responsiveness. An increased percentage of dephosphorylated TnI was found in failing hearts, which significantly correlated with the enhanced Ca\(^{2+}\)-responsiveness. **Conclusions:** The increased Ca\(^{2+}\)-responsiveness of the contractile apparatus in end-stage failing human hearts cannot be explained by a shift in contractile protein isoforms, but results from the complex interplay between changes in the phosphorylation status of MLC-2 and TnI.

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**Keywords:** Contractile function; Heart failure; Myocytes; Protein phosphorylation

This article is referred to in the Editorial by W. Schillinger and H. Kögler (pages 5–7) in this issue.

1. Introduction

In end-stage human heart failure the isoform composition and phosphorylation status of various cardiac
contractile proteins are altered. Changes have been observed in isofrom composition of troponin T (TnT) [1–4] and myosin [5,6], whilst others have found degradation of myosin light chain 2 (MLC-2), TnT and troponin I (TnI) [7]. In addition, cardiac overload causes changes in the hormone-mediated activation of protein kinase A (PKA) and protein kinase C (PKC) via e.g. noradrenaline, endothelin or angiotensin [8–11], which may alter the phosphorylation status of contractile proteins. Moreover, increased PKC [12] and protein phosphatase [13] activities have been observed in failing human hearts, which may phosphorylate and dephosphorylate contractile proteins, respectively. Until now, changes have been reported in the phosphorylation level of TnI [14] and MLC-2 [15] in human heart failure. The changes in contractile protein isoform expression and phosphorylation will influence cardiac contractile performance and may contribute to the decreased pump function observed in end-stage human heart failure.

From previous studies it has become clear that in end-stage human heart failure Ca$^{2+}$-sensitivity of the contractile apparatus is increased [4,5,15–17]. Alternative explanations have been given for the origin of the increased Ca$^{2+}$-responsiveness in human heart failure. According to Morano et al. [5] the increased Ca$^{2+}$-sensitivity of the contractile apparatus is due to expression of atrial light chain 1 (ALC-1) in the left ventricle. Wolff et al. [16] suggested that the increased Ca$^{2+}$-sensitivity might be due to a reduction of the β-adrenergically mediated phosphorylation of TnI via PKA. In addition, re-expression of a fetal TnT isoform was observed in end-stage failing myocardial tissue exhibiting increased Ca$^{2+}$-responsiveness of the contractile apparatus [4]. Since these previous studies concentrated on a single factor, the question remains whether the increased Ca$^{2+}$-sensitivity of the contractile apparatus is attributed to one of the above mentioned protein changes or is the complex resultant of several combined protein changes.

In the present study we focussed on the combination of contractile protein changes which occur during human heart failure. We determined to what extent contractile protein alterations contribute to the increased myocardial Ca$^{2+}$-responsiveness. To this end, isoform composition and phosphorylation status of contractile proteins were analysed in non-failing donor and end-stage failing human left ventricular myocardium. To correlate changes in contractile protein composition with changes in mechanical properties, maximal isometric force and its Ca$^{2+}$-sensitivity were measured in mechanically isolated single skinned cardiomyocytes.

Our results demonstrate that the observed increase in Ca$^{2+}$-responsiveness is not due to re-expression or degradation of contractile protein isoforms, but is the complex resultant of changes in degree of phosphorylation of MLC-2 and TnI.

### 2. Methods

#### 2.1. Biopsies

Left ventricular biopsies were taken during heart transplantation surgery from 10 explanted end-stage failing (New York Heart Association class IV) hearts and from six non-failing donor hearts. Heart failure resulted from dilated (DCM, $n=2$) or ischaemic (ICM, $n=7$) cardiomyopathy. From one patient cardiomyopathy was unknown. Characteristics of patients and donors are given in Table 1. Biopsies were transferred in cardioplegic solution and upon arrival in the laboratory, stored in liquid nitrogen. Samples were obtained after informed consent and with approval of the local Ethical Committees. The investigation conforms with the principles outlined in the Declaration of Helsinki [18].

#### 2.2. Myocyte isolation

Cardiomyocytes were mechanically isolated and mounted in the experimental set-up as described previously [4,19]. Before mechanical isolation, tissue was defrosted in relaxing solution (pH 7.0; in mmol/l: free Mg$^{2+}$ 1, KCl 145, EGTA 2, ATP 4, imidazole 10). During the isolation the tissue was kept on ice. To dissolve all membranous structures the myocytes were incubated for 5 min in relaxing solution containing 1% Triton X-100. Triton removes soluble and membrane-bound kinases and phosphatases and thereby arrests the phosphorylation status of myofibrillar proteins. To remove Triton, cells were washed twice in relaxing solution and kept at 0°C up to 24 h.

### Table 1

<table>
<thead>
<tr>
<th>Characteristics of donor and end-stage failing hearts</th>
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<td>Donor 1</td>
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<td>Patient 10</td>
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ICM, ischaemic cardiomyopathy; DCM, dilated cardiomyopathy; UCM, unknown cardiomyopathy; AC, anticoagulant; ACE, ACE-inhibitor; Ca$^{2+}$ AG, Ca$^{2+}$-antagonist; D, diuretics; Dx, digoxin; N, nitrates; PI, positive inotropic agent.

* Domino heart.
Fig. 1. Single cardiomyocyte from a failing human heart in relaxing solution glued between a force transducer and a piezoelectric motor.

Thereafter, a single myocyte was attached between a force transducer and a piezoelectric motor (Fig. 1).

2.3. Experimental protocol

Isometric force measurements were performed in 30 donor and 40 failing myocytes at 15 °C and a sarcomere length of 2.2 μm. The composition of relaxing and activating solutions (pH 7.1) was calculated as described by Fabiato [20]. The pCa, i.e. −log_{10}[Ca^{2+}], of the relaxing and activating (maximal [Ca^{2+}]) solution were, respectively, 9 and 4.5. Solutions with submaximal [Ca^{2+}] were obtained by mixing of the activating and relaxing solutions. After the first control activation at maximal [Ca^{2+}], resting sarcomere length was re-adjusted to 2.2 μm, if necessary. The second control measurement was used to calculate maximal isometric tension (i.e. force divided by cross-sectional area). The next measurements were carried out at submaximal [Ca^{2+}] (pCa>4.5) followed by a control measurement at pCa 4.5. Submaximal force values obtained at pCa>4.5 were normalised to the interpolated control values, assuming a linear decrease in maximal force with every activation. After this force–pCa series, 10 donor and 27 failing myocytes were incubated in relaxing solution containing the catalytic subunit of protein kinase A (3 μg/ml [100 units/ml]; Sigma, batch 35H9522) and 6 mmol/l dithiothreitol for 40 min at 20 °C, after which a second force–pCa series was recorded.

2.4. One-dimensional gel electrophoresis

Isoform composition of troponin T, troponin I and myosin heavy chain (MHC) were analysed using one-dimensional SDS polyacrylamide gel electrophoresis (1D-PAGE) and Western immunoblotting, as described previously [21]. The separating gel contained 12% total acrylamide (acrylamide to bisacrylamide ratio 200:1; pH 9.3), while the stacking gel contained 3.5% total acrylamide (acrylamide to bisacrylamide ratio 20:1; pH 6.8). Gels were stained with silver. After electrophoresis, proteins were transferred onto a nitrocellulose membrane [21] and stained using the Vectastain ABC–AmP Immunodetection Kit (Vector Laboratories, Burlingame, CA, USA). MHC, TnT and TnI isoforms were identified using the following monoclonal antibodies: α-MHC, 249-5A4 (diluted 1:50) [22]; β-MHC, 169-15D (diluted 1:100) [22]; TnT, JLT-12 (diluted 1:1000, Sigma); TnI, 1691 (diluted 1:1000, Chemicon) and C5 (diluted 1:1000, Research Diagnostics).

2.5. Two-dimensional gel electrophoresis

Expression of atrial light chain 1 and the ratio between myosin light chain 1 (MLC-1) and 2 (MLC-2) were analysed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) using a mini-protean II system (Bio-Rad, Hercules, CA, USA) as described previously by Morano et al. [5,23]. Samples were treated with trichloroacetic acid to fix the phosphorylation status of contractile proteins [23]. Isoelectric focusing was performed in glass capillary tubes (length 7 cm, diameter 1 mm) with a pH gradient of 4.0–6.5 (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Capillary gels contained 6.8% total acrylamide (acrylamide to bisacrylamide ratio 17.5:1). Comparable samples (300 μg dry weight) were loaded in each tube. In the second dimension the proteins were separated based on molecular mass in a slab gel using an acrylamide to bisacrylamide ratio of 37.5:1 (total acrylamide 13.5%; pH 8.8). Gels were stained with Coomassie Blue. The non-linear characteristic of the Coomassie-stained gels was determined by loading different quantities of atrial and ventricular tissue on the gels.

Phosphorylation of ventricular myosin light chain 2 (MLC-2) has been shown to increase Ca^{2+}-responsiveness of the contractile apparatus in myocardial tissue [24]. To investigate the composition of MLC-2 trichloroacetic acid treated samples (600 μg dry weight) were loaded on immobiline strips with a pH gradient of 4.5 to 5.5 (Amersham Pharmacia Biotech) [15]. In the second dimension proteins were separated by SDS–PAGE, as described in 1D-PAGE. Gels were stained with Colloid Coo massie Blue (Bio-Rad). All 2D-PAGE gels were scanned and analysed using Image Quant (Molecular Dynamics, version 1.1).

2.6. Phosphorylation of troponin I

To determine the composition of bis-, mono- and dephosphorylated troponin I, Tnl was extracted from 80 to 120 mg of tissue (five donor and seven failing hearts) and subsequently analysed by non-equilibrium isoelectric focussing (Phast System, Pharmacia), as described by Ardelt et al. [25]. Briefly, frozen tissue samples were homogenised in urea buffer (8 mol/l, ultrapure, pH 8)
containing in mmol/l: Tris/HCl 25, CaCl₂ 1, mercaptoethanol 15, benzamidine 100 and ocdalic acid 0.0005 using a teflon ball mill (dismembrator). TnI was isolated from the homogenised tissue by affinity chromatography using cardiac troponin C sepharose 4B. The affinity purified TnI was desalted and concentrated by high-performance liquid chromatography (HPLC), vacuum dried and dissolved in 10 µl sample buffer (9 mol/l urea and 2% servalyte pH 3–10, Serva), of which 4 µl was applied to the focussing gel (1.2% servalyte pH 3–10 and 4.8% servalyte pH 7–9). Bovine cTnI containing all phospho states was used as standard. Protein bands were stained with Coomassie Blue and analysed using a high-resolution scanner and ‘Whole Band Software’ (Bioimage, UK).

2.7. Data analysis

Force–pCa relations were fit to a modified Hill equation:

\[ \frac{F(Ca^{2+})}{F_0} = \frac{[Ca^{2+}]^{nH}/([Ca_{50}^{nH} + [Ca^{2+}]^{nH}]} \]

where F is steady-state force, \( F_0 \) denotes the steady force at saturating \([Ca^{2+}]\), \( nH \) reflects the steepness of the relationship, and \( Ca_{50} \) (or \( pCa_{50} \)) represents the midpoint of the relation.

Values are given as means±S.E.Ms of \( n \) experiments. Mean values for donor and failing samples were compared using an unpaired Student \( t \)-test. Paired Student \( t \)-tests were used when comparing \( Ca^{2+} \)-sensitivity of single myocytes before and after PKA treatment. Differences were tested two-sided at a 0.05 level of significance (\( P < 0.05 \)).

3. Results

3.1. Force measurements

Maximal isometric tension did not significantly differ between donor (29.6±4.5 kN/m², \( n = 6 \)) and failing (29.6±2.9 kN/m², 8 = 10) hearts. Passive force per cross-sectional area amounted to 1.8±0.3 and 1.9±0.2 kN/m² in donor and failing myocardium, respectively. In Fig. 2 recordings of isometric force development obtained at saturating (pCa 4.5) and at submaximal \([Ca^{2+}] \) (pCa 5.2) are shown. The average force–pCa relationships obtained in donor and failing hearts are shown in Fig. 3A. It can be noted that \( Ca^{2+} \)-responsiveness was substantially increased in failing hearts (pCa_{50} 5.35±0.03) compared to donor myocardium (pCa_{50} 5.16±0.02) (\( P < 0.05 \)). Moreover, \( Ca^{2+} \)-sensitivity of the contractile apparatus was significantly higher in patients with dilated cardiomyopathy (\( n = 2 \); pCa_{50} 5.47±0.03) compared to patients suffering from ischaemic heart disease (\( n = 7 \); pCa_{50} 5.32±0.03) (\( P < 0.05 \)). The steepness of the force–pCa curves, \( nH \), did not differ significantly between donor (3.74±0.18) and failing (3.28±0.17) myocardium.

3.2. Effect of PKA on \( Ca^{2+} \)-sensitivity

To investigate if phosphorylation of TnI completely or partially reverses the difference in \( Ca^{2+} \)-responsiveness between donor and failing hearts, force measurements were repeated after PKA treatment in 10 donor and 27 failing cardiomyocytes. Fig. 2 illustrates force registrations obtained in a cardiomyocyte from a failing heart before

![Fig. 2](https://academic.oup.com/cardiovascres/article-abstract/57/1/37/375624)

Fig. 2. Recordings of isometric force development in a failing cardiomyocyte before and after PKA treatment during maximal (pCa 4.5) and submaximal activation (pCa 5.2). The abrupt changes in force mark the transitions of the preparation through the interface between solution and air. The dashed horizontal lines indicate the passive force level.
in force at submaximal \([\text{Ca}^{2+}]\) reflects a decreased \(\text{Ca}^{2+}\)-sensitivity of the contractile apparatus after PKA. In donor cardiomyocytes PKA treatment caused only a minor, though significant shift in \(\text{pCa}_{50}\) (\(\Delta\text{pCa}_{50}=0.03\pm0.01\); \(P=0.01\)), while in failing myocytes PKA-mediated phosphorylation of Tn1 decreased \(\text{Ca}^{2+}\)-sensitivity by \(0.20\pm0.02\) \(\text{pCa}\) units \((P<0.0001)\) (Fig. 3B). The shift in \(\text{pCa}_{50}\) after PKA was significantly larger in cardiomyocytes \((n=7)\) from DCM patients \((\Delta\text{pCa}_{50}=0.30\pm0.02)\) compared to cells \((n=19)\) from ICM patients \((\Delta\text{pCa}_{50}=0.16\pm0.02)\) \((P=0.0002)\). Fig. 3B illustrates that the difference in \(\text{Ca}^{2+}\)-sensitivity of the contractile apparatus between donor and failing hearts is completely abolished after PKA treatment. The steepness of the force–\(\text{pCa}\) curves was not affected by PKA in both donor and failing myocardium. Values for \(\text{pCa}_{50}\) and \(nH\) before and after PKA treatment are given in Table 2.

### 3.3. Isoform composition of contractile proteins

Isoform composition of myosin heavy chain, troponin T and troponin I were analysed by 1D-PAGE and Western immunoblotting. One donor biopsy was too small for protein analysis. Fig. 4A illustrates a silver-stained gel of a donor and failing ventricular sample. The \(\beta\)-MHC was predominant in all samples. In two samples (one donor and one failing) an extra protein band was observed above \(\beta\)-MHC (Fig. 4B), indicative of \(\alpha\)-MHC. Western immunoblotting using specific antibodies against \(\alpha\)- and \(\beta\)-MHC confirmed that this additional band was indeed the \(\alpha\)-MHC isoform (Fig. 4C). In all samples only one band was observed at the level of TnT on the silver-stained gels, which was confirmed by Western immunoblotting (Fig. 4D). This band represents the adult TnT isoform (TnT3). In addition, only one TnI band could be detected in all samples using monoclonal antibody C5 (Fig. 4E) or 1691 (not shown).

Expression of atrial light chain 1 in ventricular samples was detected using 2D-PAGE with a pH gradient of 4.0–6.5. Fig. 5A shows a mini 2D-gel from failing ventricular tissue expressing a small amount of ALC-1. Comparable amounts of ALC-1 were observed in two donor samples and in four other end-stage failing ventricles, ranging from 0.3 to 7.4% of total MLC-1. No significant correlation was present between \(\text{Ca}^{2+}\)-sensitivity of the contractile apparatus (\(\text{pCa}_{50}\)) and percentage of ALC-1 (Fig. 5B).

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<thead>
<tr>
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<th>Before PKA</th>
<th>After PKA</th>
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<tr>
<td></td>
<td>(\text{pCa}_{50})</td>
<td>(nH)</td>
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<tr>
<td>Donor ((n=10))</td>
<td>5.18±0.02</td>
<td>3.27±0.10</td>
</tr>
<tr>
<td>Heart failure ((n=27))</td>
<td>5.36±0.02*</td>
<td>3.23±0.14</td>
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</table>

\(n\), number of cardiomyocytes. *\(P<0.05\) in unpaired Student’s \(t\)-test donor versus failing. **\(P<0.05\) in paired Student’s \(t\)-test before versus after PKA.

Table 2

<table>
<thead>
<tr>
<th></th>
<th>(\text{pCa}_{50})</th>
<th>(nH)</th>
<th>(\text{pCa}_{50})</th>
<th>(nH)</th>
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<tbody>
<tr>
<td>Donor ((n=10))</td>
<td>5.18±0.02</td>
<td>3.27±0.10</td>
<td>5.15±0.02**</td>
<td>3.17±0.07</td>
</tr>
<tr>
<td>Heart failure ((n=27))</td>
<td>5.36±0.02*</td>
<td>3.23±0.14</td>
<td>5.16±0.01**</td>
<td>3.37±0.10</td>
</tr>
</tbody>
</table>
To assess possible myosin light chain 2 degradation the ratio of total MLC-1 to MLC-2 was analysed. No significant difference was found in this ratio between donor (0.92±0.13) and failing (1.19±0.26) hearts.

3.4. Phosphorylation of contractile proteins

Ventricular myosin light chain 2 in the human heart consists of two isoforms (MLC-2 and MLC-2*), which
Recently, Arrell et al. [26] have shown that in addition to MLC-2, the ventricular myosin light chain 1 (MLC-1) isoform may be phosphorylated. On our mini 2D-gels (Fig. 5A) two spots were evident at the level of MLC-1, which were both recognised by a specific antibody directed against MLC-1 in Western immunoblotting (not shown). Therefore, these two MLC-1 spots may represent unphosphorylated and phosphorylated MLC-1 (respectively, MLC-1 and MLC-1* in Fig. 5A). The amount of MLC-1* as a percentage of total myosin light chain 1 did not differ between donor (11.6±2.6%) and failing (13.3±1.4%) hearts.

Troponin T phosphorylation was also visible on the large 2D-gels (Fig. 6). The amount of monophosphorylated TnT, in percentage of total TnT, did not differ between donor (66.3±2.7%) and failing (68.4±1.4%) hearts.

The different forms of troponin I (bis-, mono- and dephosphorylated) separated on an IEF-gel in a donor and a failing ventricular sample are shown in Fig. 7A. Bovine cTnI containing all phospho-states was used as standard. Note that the isoelectric points of the human cTnI forms are lower than those of bovine cTnI. It can be observed that in the failing heart sample only one band is present, which corresponds to dephosphorylated TnI, while all forms were present in the donor sample. Analysis of all samples revealed a higher level of mono- and bisphosphorylated TnI in donor ventricles, while dephosphorylated TnI was more abundant in failing hearts (Table 3). TnI phosphorylation was less in DCM than in ICM hearts, although the difference was not significant. A positive correlation was found between pCa and percentage of dephosphorylated TnI (Fig. 7B; R = 0.59; P<0.05).

4. Discussion

In accordance with previous studies, Ca\(^{2+}\)-sensitivity of the contractile apparatus was increased in end-stage failing human hearts compared to non-failing donor hearts, while maximal isometric force development was unchanged [4,5,16,17]. The present study indicates that increased Ca\(^{2+}\)-responsiveness cannot be attributed to contractile protein isoform changes, but rather is the resultant of changes in the phosphorylation status of contractile proteins.

4.1. Isoform composition of contractile proteins

A study by Metzger et al. [27] indicated a role for the MHC composition in Ca\(^{2+}\)-sensitivity of the contractile apparatus. A shift from α- to β-MHC in hypothyroid rats was associated with a decrease in Ca\(^{2+}\)-responsiveness. However, an age-dependent shift in MHC composition in guinea-pig hearts did not alter Ca\(^{2+}\)-responsiveness [20]. Although previous studies have reported predominance of β-MHC in human ventricles, in a recent study [6] a
considerable amount of α-MHC protein (7%) was found in all non-failing human ventricles, while α-MHC could only be detected in one out of 10 failing ventricles. Thus, in accordance with MHC changes in rodent hearts, a shift in MHC composition with human heart failure may be responsible for changes in myocardial pump function. We
have observed α-MHC in one donor and one failing ventricle. Therefore, it is unlikely that a shift in MHC composition is the cause of the difference in Ca^{2+}-responsiveness observed between non-failing and failing human hearts.

Proteolysis of contractile proteins may alter myocardial performance. In the present study no degradation products of TnT and TnI were observed in any of the failing hearts. Moreover, the ratio between MLC-1 and MLC-2 did not differ between donor and failing myocardium, indicating that the stoichiometry of MLC-1 versus MLC-2 was preserved in heart failure.

Morano et al. [5] have reported a positive correlation between ALC-1 expression and Ca^{2+}-sensitivity of the contractile apparatus, though ALC-1 was not present in all failing tissues. In the present study, expression of ALC-1 was small in both donor and failing hearts and did not correlate with pCa_{so}. Data concerning re-expression of fetal TnT have been conflicting. Anderson et al. [1] observed re-expression of fetal TnT4 isoform in end-stage failing human hearts, while Solaro et al. [2] found this fetal TnT isoform in one out of 10 failing heart samples. Similarly, in an extensive study we have observed fetal TnT4 in only one patient with end-stage heart failure [4]. Mesnard-Rouiller et al. [3] found expression of TnT4 in half of the failing ventricles and a decrease in TnT1 isoform expression in all failing hearts. Altogether these studies indicate that re-expression of fetal TnT isoforms is not a general feature of heart failure. In the present study we did not detect fetal TnT isoforms in the failing hearts.

Our study suggests that the observed increase in Ca^{2+}-responsiveness is not due to degradation or re-expression of contractile proteins in the failing ventricle. However, our results do not rule out the possibility that fetal TnT isoforms and ALC-1 influence Ca^{2+}-sensitivity, but they exclude the possibility that they are predominant changes involved in the alteration in Ca^{2+}-responsiveness during the transition from compensatory hypertrophy to end-stage human heart failure.

### 4.2. Phosphorylation of contractile proteins

Previously, Bodor et al. [14] found a significant decrease in the degree of TnI phosphorylation in failing compared to non-failing human hearts. In this study a shift from mono- and bisphosphorylated TnI to dephosphorylated TnI was observed in failing human hearts, resulting in a positive correlation between pCa_{so} and percentage of dephosphorylated TnI. As suggested by Wolff et al. [16] depressed β-adrenergic response in human heart failure may be responsible for decreased TnI phosphorylation and increased Ca^{2+}-responsiveness of the contractile apparatus.

In agreement with previous observations [16,18] incubation of myocytes with PKA decreased Ca^{2+}-sensitivity to a larger extent in failing than in donor cardiomyocytes, completely abolishing the difference in Ca^{2+}-responsiveness between donor and failing hearts. Moreover, the shift in Ca^{2+}-responsiveness after PKA was larger in DCM than in ICM hearts, which may be explained by different alterations in the β-adrenergic pathway in the two types of myocardial disease [28]. Our observations indeed suggest that PKA-mediated phosphorylation of TnI is impaired in

![Image](https://academic.oup.com/cardiovascres/article-abstract/57/1/37/375624/45)
end-stage human heart failure and increases Ca$^{2+}$-responsiveness of the contractile apparatus.

In addition to changes in PKA-mediated phosphorylation, alterations have been reported in PKC-mediated pathways. Elevations of endothelin-1 [9] and angiotensin [10], which may alter the level of PKC [9–11], have been found under pathological conditions in the human myocardium. An increase has been reported in the β-isofrom of PKC in failing human hearts [12]. A report on transgenic mice constitutively overexpressing high levels of PKCβ suggested a role for PKC-mediated TnI phosphorylation in depression of myofilament contractility [29]. A recent study by Huang et al. [30] reported a decrease in maximal tension and no change in Ca$^{2+}$-responsiveness in skinned fibre bundles from transgenic mice hearts conditionally expressing low levels of active PKCβ. In these animals, an increase in phosphorylation of myosin binding protein C, TnT, TnI and MLC-2 was found. In contrast, we observed a decrease in both TnI and MLC-2 phosphorylation in failing human myocardium, while TnT phosphorylation was unaltered. It is conceivable that PKC-mediated phosphorylation of contractile proteins is counterbalanced by the actions of other kinases and phosphatases in the human heart. In addition to increased PKC [12], an increase has been found in protein phosphatase 1 in failing human hearts [13], which may account for the decreased MLC-2 phosphorylation observed in the failing human samples.

A significant inverse correlation was found between pCa$_{40}$ and percentage of phosphorylated MLC-2, that is, MLC-2 phosphorylation is associated with a decrease in Ca$^{2+}$-responsiveness. However, Morano [23] has shown that phosphorylation of MLC-2 increases Ca$^{2+}$-responsiveness. Recently, we have demonstrated that dephosphorylation of MLC-2 decreases Ca$^{2+}$-responsiveness of the contractile apparatus in human hearts [31]. The effect of MLC-2 dephosphorylation may be overruled by the effect of TnI dephosphorylation, resulting in an increased Ca$^{2+}$-sensitivity of the contractile apparatus in end-stage failing human hearts.

It is noteworthy to mention that the decreased MLC-2 phosphorylation may result indirectly from β-adrenergic desensitisation, since PKA may reduce Protein Phosphatase-1 (PP-1) activity. Previously, it has shown that PKA phosphorylates and thereby activates phosphatase inhibitor 1 [32], which is known to be a potent inhibitor of PP-1. The decreased PKA activity during β-adrenergic desensitisation would reduce the inhibitory action of phosphatase inhibitor 1 on PP-1, resulting in an increased activity of PP-1. Thus, the reduction in phosphorylation of TnI and MLC-2 may both result from β-adrenergic desensitisation, either directly or indirectly coupled to a decrease in PKA.

5. Conclusion

Our data show that the increased Ca$^{2+}$-sensitivity of the contractile apparatus in end-stage human heart failure is the result of combined changes in phosphorylation status of TnI and MLC-2. The phosphorylation status of contractile proteins is determined by the action of several protein kinases and protein phosphatases, which may be altered under pathological conditions.

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References


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