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The effect of myosin light chain 2 dephosphorylation on Ca^{2+}-sensitivity of force is enhanced in failing human hearts

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Abstract

Objective: Phosphorylation of the myosin light chain 2 (MLC-2) isoform expressed as a percentage of total MLC-2 was decreased in failing (21.1±2.0%) compared to donor (31.9±4.8%) hearts. To assess the functional implications of this change, we compared the effects of MLC-2 dephosphorylation on force development in failing and non-failing (donor) human hearts. **Methods:** Cooperative effects in isometric force and rate of force redevelopment (K_{tr}) were studied in single Triton-skinned human cardiomyocytes at various [Ca^{2+}] before and after protein phosphatase-1 (PP-1) incubation. **Results:** Maximum force and K_{tr} values did not differ between failing and donor hearts, but Ca^{2+}-sensitivity of force (pCa_{50}) was significantly higher in failing myocardium ($\Delta pCa_{50}=0.17$). K_{tr} decreased with decreasing [Ca^{2+}], although this decrease was less in failing than in donor hearts. Incubation of the myocytes with PP-1 (0.5 U/ml; 60 min) decreased pCa_{50} to a larger extent in failing (0.20 pCa units) than in donor cardiomyocytes (0.10 pCa units). A decrease in absolute K_{tr} values was found after PP-1 in failing and donor myocytes, while the shape of the K_{tr}–Ca^{2+} relationships remained unaltered. **Conclusions:** Surprisingly, the contractile response to MLC-2 dephosphorylation is enhanced in failing hearts, despite the reduced level of basal MLC-2 phosphorylation. The enhanced response to MLC-2 dephosphorylation in failing myocytes might result from differences in basal phosphorylation of other thin and thick filament proteins between donor and failing hearts. Regulation of Ca^{2+}-sensitivity via MLC-2 phosphorylation may be a potential compensatory mechanism to reverse the detrimental effects of increased Ca^{2+}-sensitivity and impaired Ca^{2+}-handling on diastolic function in human heart failure.
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1. Introduction

Ca^{2+} binding to the thin filament initiates the actin–myosin interaction, the cross-bridge cycle. Phosphorylation of thin filament proteins (e.g., troponin) plays a key role in regulation of Ca^{2+}-sensitivity of this interaction [1–3]. However, the effect of myosin light chain 2 (MLC-2) phosphorylation on Ca^{2+}-responsiveness is also of interest, as it relates to a thick filament-based regulatory process, which is complementary to and possibly interfering with that of the thin filament.

Myosin is composed of two heavy chains and two pairs of light chains. Each pair of light chains consists of an essential light chain (myosin light chain 1) and a regulatory light chain (myosin light chain 2) [4]. Two isoforms of the ventricular MLC-2 are known (MLC-2 and MLC-2*) [4], which may both be phosphorylated by Ca^{2+}/

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calmodulin-dependent myosin light chain kinase (MLCK) [5] and protein kinase C (PKC) [6] and dephosphorylated by light chain phosphatase [7]. In human heart failure increased activity has been reported for PKC [8] and for Ca^{2+}/calmodulin-dependent protein kinase (CaM-kinase), which may phosphorylate and activate MLCK [9]. In addition, type 1 phosphatase activity was increased in failing human myocardium [10]. Recently a decreased MLC-2 phosphorylation was observed in end-stage failing human hearts [11,12].

During the past years several animal studies have been performed concerning the effect of MLC-2 phosphorylation on myocardial contraction [13–16]. Phosphorylation of MLC-2 has been found to increase Ca^{2+}-responsiveness of force [13–16] and the rate of force redevelopment (K_{tr}) [7]. Based on this latter finding it was proposed that the increase in Ca^{2+}-responsiveness after MLC-2 phosphorylation resulted from a change in cross-bridge cycling kinetics [7]. However, data in human myocardium concerning the effect of MLC-2 phosphorylation on Ca^{2+}-responsiveness of force are limited to the atrium [7]. Therefore, in the present study the effects of altered MLC-2 phosphorylation on Ca^{2+}-sensitivity of force and K_{tr} were determined in human ventricular myocardium.

In failing human myocardium changes have been found in both thin and thick filament proteins [11,12,17–19], which might influence the effect of MLC-2 (de)phosphorylation on cross-bridge cycling kinetics in heart failure. Since basal MLC-2 phosphorylation is decreased in end-stage human heart failure [11,12], we hypothesized that the response to MLC-2 dephosphorylation is decreased in failing compared to non-failing donor myocardium. To test this hypothesis, the effect of MLC-2 dephosphorylation on isometric force development and rate of force redevelopment were studied at various [Ca^{2+}] in single Triton-skinned myocytes from donor and end-stage failing human hearts.

Surprisingly, our results indicate that the response to MLC-2 dephosphorylation is enhanced in human heart failure, despite the decreased basal level of MLC-2 phosphorylation. Changes in the endogenous phosphorylation status of other thin and thick filament proteins in human heart failure may have altered the response to protein phosphatase-1 (PP-1).

2. Methods

2.1. Biopsies

Left ventricular biopsies were obtained during heart transplantation surgery from six explanted end-stage failing (New York Heart Association class IV) hearts (five males, one female; age range 41–61 years) and from three non-failing donor hearts (one male, two females; age range 26–46 years). Heart failure resulted from ischemic ($n=3$) or dilated ($n=3$) cardiomyopathy. All patients received angiotensin converting enzyme inhibitors and diuretics. Some patients also received anti-arrhythmic agents, anticoagulants, digitoxin and/or nitrates. The tissue was transported in cardioplegic solution (ranging from half an hour to 14 h) 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 (Cardiovascular Research 1997;35:2–3).

2.2. Two-dimensional gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) [7,13] was performed on donor and failing heart samples to determine the phosphorylation status of MLC-2 and troponin T (TnT). Tissue samples were treated with trichloroacetic acid (TCA) to fixate the phosphorylation status of contractile proteins [7,13]. Samples (600 μg dry weight) were loaded on immobiline strips with a pH gradient of 4.5 to 5.5 (Amersham Pharmacia Biotech, Uppsala, Sweden). In the second dimension proteins were separated by sodium dodecyl sulfate (SDS)–PAGE [19]. Gels were stained with Coomassie Blue, scanned and analyzed using Image Quant (Molecular Dynamics) [11]. We have checked the linearity of the Coomassie-staining of the protein spots to be analyzed. When 300 and 600 μg of tissue were loaded on the gels the density of all protein spots of interest were in the linear range.

To investigate PP-1 specificity a suspension of Triton-skinned donor cardiomyocytes was incubated in 1 ml of relaxing solution with and without PP-1 (0.5 U/ml, lot no. 16757; Upstate Biotechnology) for 60 min at room temperature. Subsequently, TCA-treated cells (75 μg dry weight) were analyzed by 2D-PAGE. These gels were silver-stained to enhance resolution.

2.3. Myocyte isolation

Cardiomyocytes were mechanically isolated and mounted in the experimental set-up as described previously [20]. 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. Isolated myocytes were immersed 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 exclude an effect of kinases/phosphatases during the isolation, we have analyzed the protein composition in a human ventricular tissue sample before and after the complete isolating procedure (mechanical isolation and skinning in Triton). The 2D-gels did not reveal differences in the phos-

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phorylation status of contractile proteins between the samples taken before and after myocyte isolation (not shown).

Triton-skinned myocytes enable the study of myofibrillar contractile properties under standardized conditions (i.e. composition of the intracellular medium, sarcomere length) without disturbing factors present in the intact heart (i.e. hormonal factors, variable $[Ca^{2+}]$). To remove Triton, cells were washed twice in relaxing solution. Thereafter, a single myocyte was attached between a force transducer and a piezoelectric motor.

2.4. Experimental protocol

Isometric force measurements were performed at 15 °C and a sarcomere length, measured in relaxing solution, of 2.2 μm. The composition of relaxing and activating solutions used during force measurements was calculated as described by Fabiato [21]. The pCa, i.e. $-\log_{10}[Ca^{2+}]$, of the relaxing and activating solution (pH 7.1) were, respectively, 9 and 4.5. Solutions with intermediate free $[Ca^{2+}]$ were obtained by mixing of the activating and relaxing solutions. After the first control activation at saturating (maximal) $[Ca^{2+}]$ (pCa=4.5), resting sarcomere length was readjusted 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 four to five measurements were carried out at submaximal $[Ca^{2+}]$ followed by a control measurement. Force values obtained in solutions with submaximal $[Ca^{2+}]$ were normalized to the interpolated control values. After the initial force–pCa series, the myocyte was incubated in relaxing solution containing 0.5 U/ml PP-1 and 6 mM dithiothreitol (DTT) for 60 min at 20 °C. Thereafter, the force–pCa series was repeated.

In addition to MLC-2, troponin I (TnI) may be dephosphorylated by PP-1. To investigate if dephosphorylation of TnI had occurred after PP-1, a number of myocytes were treated with protein kinase A (PKA), which is known to phosphorylate TnI and to decrease Ca^{2+}-sensitivity of force [3]. After the second force–pCa series, the myocytes were incubated in relaxing solution containing the catalytic subunit of PKA (3 μg/ml, batch no. 35H9522; Sigma) and 6 mM DTT for 40 min at 20 °C. This treatment was followed by a third series of force–pCa measurements.

Rate of force redevelopment (K_{tr}) was determined at pCa values ranging from 4.5 to 5.4 using the slack-test as described previously [22]. Briefly, when a steady level of force was developed in activating solution the myocyte was rapidly slackened and re-stretched by 20% of its length. Upon slackening force drops to zero and upon re-stretch force redevelopment occurs to the initial steady level. Force redevelopment was fitted to a single exponential to estimate K_{tr}. At low $[Ca^{2+}]$ (pCa>5.4) force redevelopment could not be fitted accurately due to the low signal-to-noise ratio.

2.5. Data analysis

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

$$F(Ca^{2+})/F_0 = (1-k)\,[Ca^{2+}]^{nH}/(Ca_{50}^{nH} + [Ca^{2+}]^{nH}) + k$$

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. Force–pCa relations obtained after PP-1 contained a small component approximated by an offset, k. To determine nH at high ($pCa<pCa_{50}$; n_1) and low ($pCa>pCa_{50}$; n_2) $[Ca^{2+}]$, force–pCa data were analyzed using a Hill plot transformation [3]:

$$\log(F_{rel}/(1-F_{rel})) = nH \cdot (\log[Ca^{2+}] + pCa_{50})$$

F_{rel} is the relative force, $F(Ca^{2+})/F_0$.

Values are given as means±S.E.M. of n experiments. Mean values for donor and failing samples were compared using an unpaired Student t-tests. Paired Student t-tests were used when comparing maximal force, Ca^{2+}-sensitivity and K_{tr} of single cardiomyocytes before and after PP-1 treatment. A two-tailed P-value of less than 0.05 was considered significant.

3. Results

3.1. Myosin light chain 2 phosphorylation

Fig. 1 shows Coomassie-stained 2D-gels from a donor and a failing heart illustrating the difference observed in MLC-2 phosphorylation. 2D-PAGE analysis revealed decreased phosphorylation of the MLC-2 isoform (MLC-2P) expressed as a percentage of total MLC-2 in failing compared to non-failing ventricular tissue ($P<0.05$; Table 1) from 31.9 to 21.1%. The percentage of phosphorylated and unphosphorylated MLC-2* isoform did not differ between failing and donor myocardium. Troponin T (TnT) phosphorylation was also visible on these gels. The amount of monophosphorylated TnT, in percentage of total TnT, did not differ significantly between donor (69.7±3.1%) and failing (72.3±3.0%) hearts. To investigate PP-1 specificity donor myocytes were incubated with and without PP-1 and analyzed on silver-stained 2D-gels. The densitometric scans shown in Fig. 1C indicate that MLC-2 was completely dephosphorylated by incubation of myocytes in PP-1 containing (0.5 U/ml; 60 min) relaxing solution (dashed line), while incubation in relaxing solution without PP-1 did not alter MLC-2 phosphorylation (continuous line). Since silver-staining is only linear in a narrow concentration range, these gels are not optimal for quantitative analysis. Therefore, the densitometric scans shown in panel (C) cannot be used quantitatively. Phosphorylation of TnT and MLC-1 was preserved after PP-1 treatment (not shown).

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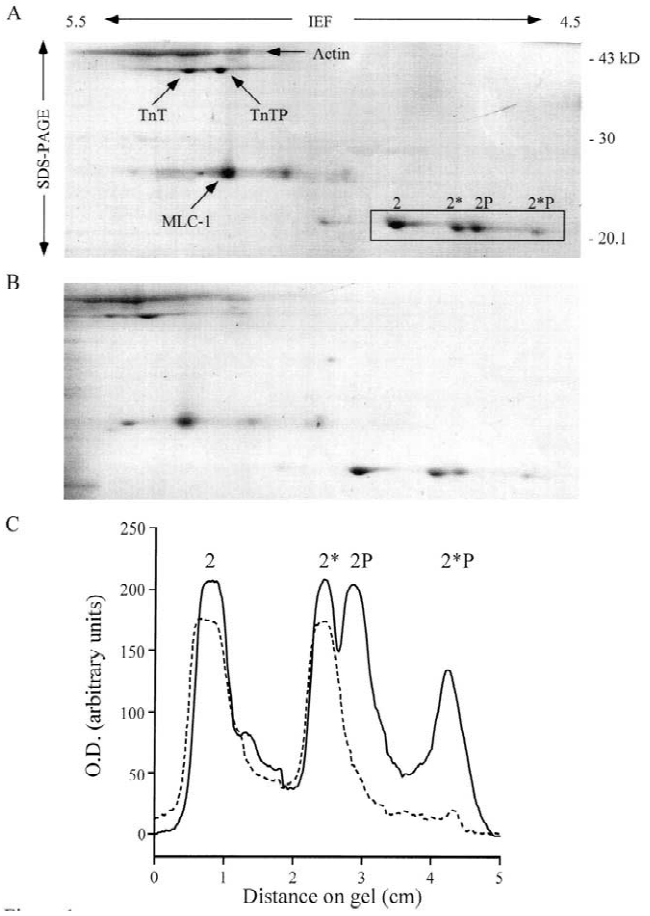

Fig. 1. 2-D gels to illustrate MLC-2 composition in a donor (A) and a failing (B) heart. MLC-2 phosphorylation was decreased in end-stage failing myocardium. (C) shows densitometric scans of MLC-2 composition in donor cardiomyocytes treated without (continuous line) and with (dashed line) PP-1. PP-1 specifically dephosphorylated MLC-2, while TnT and MLC-1 phosphorylation were preserved (not shown). IEF, isoelectric focussing; TnT and TnTP, unphosphorylated and monophosphorylated troponin T; MLC-1, myosin light chain 1; MLC-2, myosin light chain 2 composed of two isoforms (2 and 2*), which are both partly phosphorylated (respectively, 2P and 2*P). The proteins have been identified using commercially available monoclonal antibodies in Western immunoblotting.

3.2. Force measurements

Recordings of force development during maximal (pCa 4.5) and submaximal activation (pCa 5.6) before and after incubation in PP-1 are shown in Fig. 2. Maximal isometric tension amounted to 34.8±5.8 and 34.7±6.7 kN/m^2 in donor (17 myocytes) and failing (19 myocytes) hearts, respectively. Passive tension did not differ between donor (1.8±0.2 kN/m^2) and failing (2.4±0.5 kN/m^2) myocardium.

Table 1
MLC-2 phosphorylation

Group	MLC-2	MLC-2*	MLC-2P	MLC-2*P
Donor (n=3)	41.2±5.6	18.8±0.5	31.9±4.8	8.1±3.1
Heart failure (n=6)	50.0±2.7	20.9±1.3	21.1±2.0*	8.0±1.1

n, number of tissue samples. Values are given as percentage of total MLC-2. MLC-2 and MLC-2* are the unphosphorylated MLC-2 isoforms, which both may be phosphorylated (MLC-2P and MLC-2*P, respectively). *P<0.05, donor versus heart failure.

The average force–pCa relationships obtained in donor and failing hearts before PP-1 treatment are shown in Fig. 3A. It can be noted that Ca^{2+}-responsiveness of force was significantly higher in end-stage failing hearts (pCa_{50} = 5.65±0.03) than in donor myocardium (pCa_{50} = 5.48±0.01) (P<0.05). The force–pCa relation tended to be less steep in failing (nH=2.34±0.22) than in donor (nH=3.02±0.16) hearts, although the difference did not reach statistical significance. To determine steepness at high [Ca^{2+}] (n_1) and at low [Ca^{2+}] (n_2) the force–pCa data were analyzed using a Hill plot transformation (inset Fig. 3A). Statistical analysis revealed that the steepness of the force–pCa relation at high [Ca^{2+}] (n_1) was significantly lower in failing than in donor hearts. Moreover, in donor hearts n_1 was significantly higher than n_2.

The rate of force redevelopment was determined at pCa values ranging from 4.5 to 5.4. Maximal K_{tr} did not differ significantly between donor (0.77±0.02 1/s) and failing (0.86±0.05 1/s) hearts. The dependency of K_{tr} on [Ca^{2+}] in donor and failing hearts is presented in Fig. 3B. K_{tr} decreased with increasing pCa, although this decrease was less pronounced in failing myocardium. At pCa 5.2 and 5.4 a significant difference in K_{tr} was found between donor and failing hearts. In both donor and failing hearts approximately linear relations were found between K_{tr} and relative force (Fig. 3C).

3.3. Effect of PP-1 on Ca^{2+}-sensitivity of force

Fig. 2 illustrates that maximal force (pCa 4.5) decreased by approximately 10% after PP-1. This decline is attributable to the duration of the incubation period as it was also found in control experiments described below. However, PP-1 significantly decreased Ca^{2+}-sensitivity of force in donor (n=13) and failing (n=15) cardiomyocytes (Fig. 4). The shift in pCa_{50} after PP-1 treatment was significantly larger in failing (0.20 pCa units) than in donor (0.10 pCa units) cardiomyocytes. Hence PP-1 reduced the difference existing in Ca^{2+}-responsiveness between donor and failing hearts from 0.17 to 0.08 pCa units (P<0.05). It should be noted that at low [Ca^{2+}] (pCa>6) relative force values were somewhat elevated after PP-1 in both donor and failing myocytes. PP-1 did not alter the steepness of the force–pCa relationships. In Table 2 pCa_{50} and nH values before and after PP-1 are summarized.

To investigate if the decrease in Ca^{2+}-responsiveness could be attributed to PP-1 or was due to time-dependent alterations during the incubation period, two donor cardiomyocytes were incubated in relaxing solution for 60

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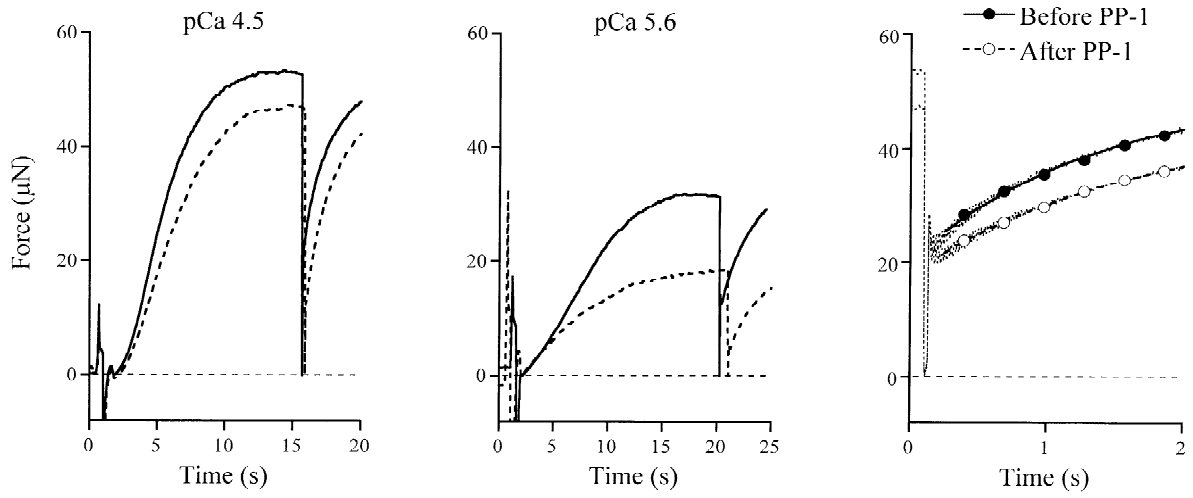

Fig. 2. Recordings of isometric force development during maximal (A) and submaximal (B) activation before (continuous recording) and after (dashed recording) PP-1 incubation in a failing myocyte. The abrupt changes in force mark the transitions of the preparation through the interface between solution and air. The dashed horizontal lines indicate the passive force level. To determine K_{tr} the myocyte was slackened when force development reached a steady level and restretched (slack-test). Registrations of force redevelopment at maximal [Ca^{2+}] before and after incubation with PP-1 are shown in (C).

min at 20 °C without addition of PP-1 (time-control). No alterations were observed in the midpoint (5.44±0.02 and 5.45±0.01) and steepness (2.71±0.29 and 2.76±0.13) of the force–pCa relationship before and after incubation in relaxing solution, respectively.

To assess whether the PP-1-induced shift in Ca^{2+}-sensitivity was maximal, two failing cardiomyocytes were incubated for 60 min with a 10-fold higher dose of PP-1 (5 U/ml). The decrease in pCa_{50} (0.21 pCa units) was similar to the decrease after incubation with 0.5 U/ml PP-1.

In addition to MLC-2, troponin I may be dephosphorylated by PP-1. TnI dephosphorylation would increase Ca^{2+}-sensitivity of force and would interfere with the effect of MLC-2 dephosphorylation on Ca^{2+}-responsiveness. To investigate if TnI dephosphorylation occurred, myocytes were incubated with PKA after PP-1 treatment. In a previous study [23], we observed a small non-significant decrease in Ca^{2+}-responsiveness of force after PKA in donor cardiomyocytes ($\Delta pCa_{50} = 0.02$), but a significant decrease in failing ($\Delta pCa_{50} = 0.24$) cells. Hence, if dephosphorylation of TnI would occur by PP-1 the effect of PKA on Ca^{2+}-sensitivity of force would be enlarged. However, after PP-1 treatment, PKA did not significantly alter Ca^{2+}-responsiveness of force in donor cardiomyocytes ($n = 4$), while the decrease in Ca^{2+}-responsiveness after PKA in failing cardiomyocytes ($n = 4$) was smaller ($\Delta pCa_{50} = 0.10$) than in the absence of PP-1 [23]. From these data we consider it likely that PP-1 did not alter the phosphorylation status of TnI. Hence, the changes in myofilament contractility after PP-1 may be fully attributed to MLC-2 dephosphorylation. These experiments also showed that the difference in Ca^{2+}-responsiveness between donor and failing myocytes was completely abolished by the combined action of PP-1 and PKA.

3.4. Effect of PP-1 on rate of force redevelopment

Fig. 2C illustrates force redevelopment after a slack-test, before and after incubation with PP-1 at maximal [Ca^{2+}]. The mean K_{tr} values as a function of pCa are summarized in Fig. 5. K_{tr} values were not significantly altered in the time-control. PP-1 treatment decreased K_{tr} values at maximal and submaximal [Ca^{2+}] in both donor and failing cardiomyocytes (Fig. 5A and B). However, relative K_{tr} values (normalized to K_{tr} at pCa 4.5) remained unaltered after PP-1 indicating that MLC-2 dephosphorylation did not alter Ca^{2+}-responsiveness of K_{tr}. The linear relationships between absolute K_{tr} and relative force shown in Fig. 6 reveal that PP-1 decreased the force-dependency of K_{tr} in both donor and failing myocytes.

4. Discussion

Although the basal level of MLC-2 phosphorylation was decreased in failing myocardium, the response to MLC-2 dephosphorylation was enhanced in human heart failure. Therefore our hypothesis ‘the response to MLC-2 dephosphorylation is decreased in failing compared to non-failing hearts’ has to be discarded.

4.1. Differences between donor and failing myocardium

The present study confirms and extends our previous observation [11,12] that the phosphorylation level of the MLC-2 isoform is decreased in end-stage human heart failure. However, no significant difference was found in phosphorylation of the less abundant MLC-2* isoform between donor and failing hearts. It was mentioned

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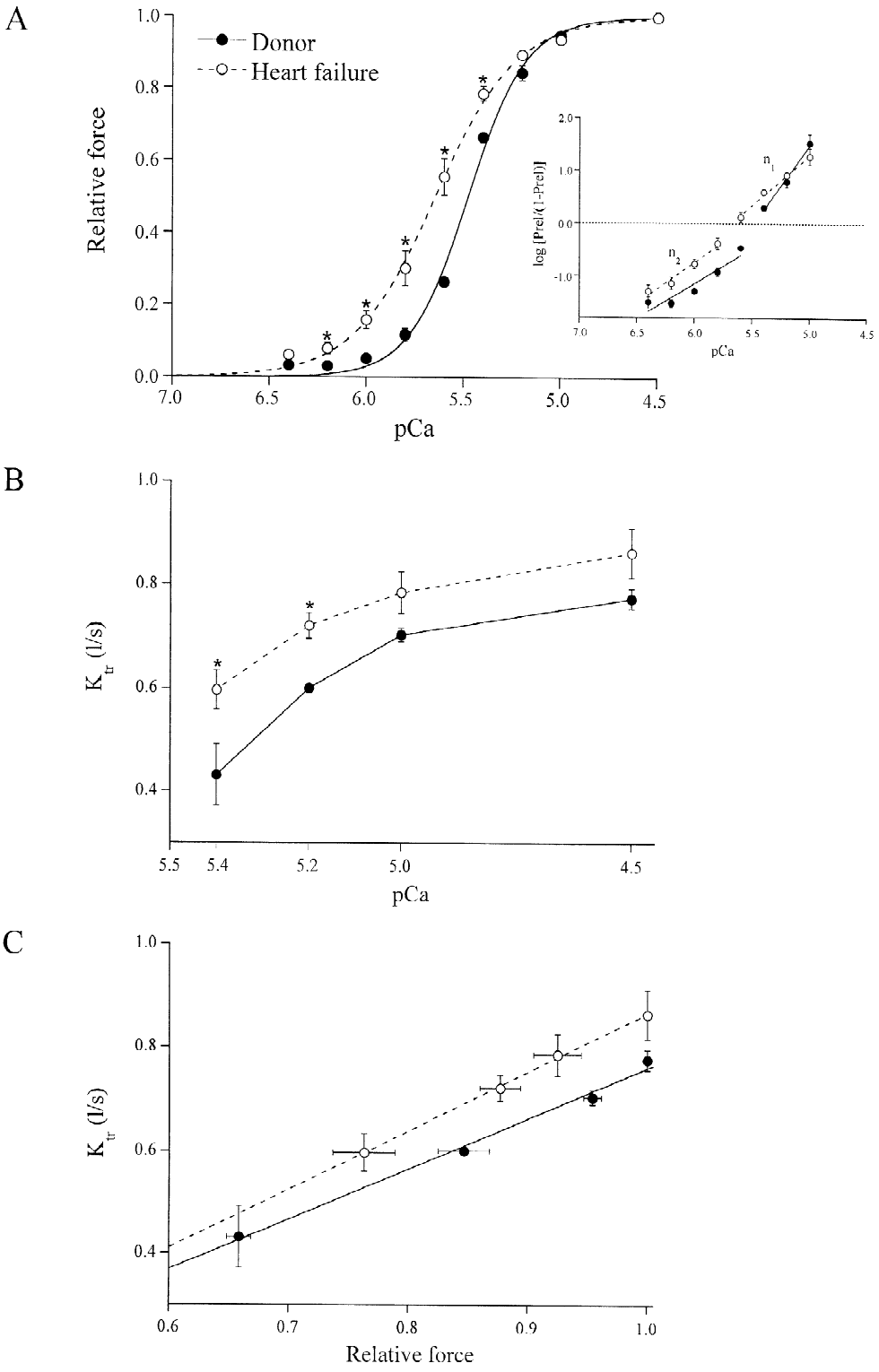

Fig. 3. Force (A) and K_{tr} (B) as a function of pCa in donor ($n=3$) and failing ($n=6$) hearts before PP-1 treatment. The myocyte values per donor/patient were averaged to obtain individual values for donor and patient hearts. Force at submaximal [Ca^{2+}] was normalized to the control force at saturating [Ca^{2+}]. Ca^{2+}-sensitivity of force was significantly increased in failing myocardium compared to donor hearts ($\Delta pCa_{50}=0.17$). K_{tr} decreased with decreasing [Ca^{2+}], although this decrease was less steep in failing than in donor hearts. A, inset: Hill plot transformations revealed that steepness was significantly different at high [Ca^{2+}] in donor ($n_1=3.26\pm0.39$) and in failing ($n_1=1.72\pm0.15$) myocardium ($P<0.05$), while at low [Ca^{2+}] values were similar ($n_2=$respectively, 1.46 ± 0.24 and 1.65 ± 0.10 in donor and failing hearts). (C) The relation between K_{tr} and relative force was approximately linear in both donor and failing hearts. *$P<0.05$, donor versus failing.

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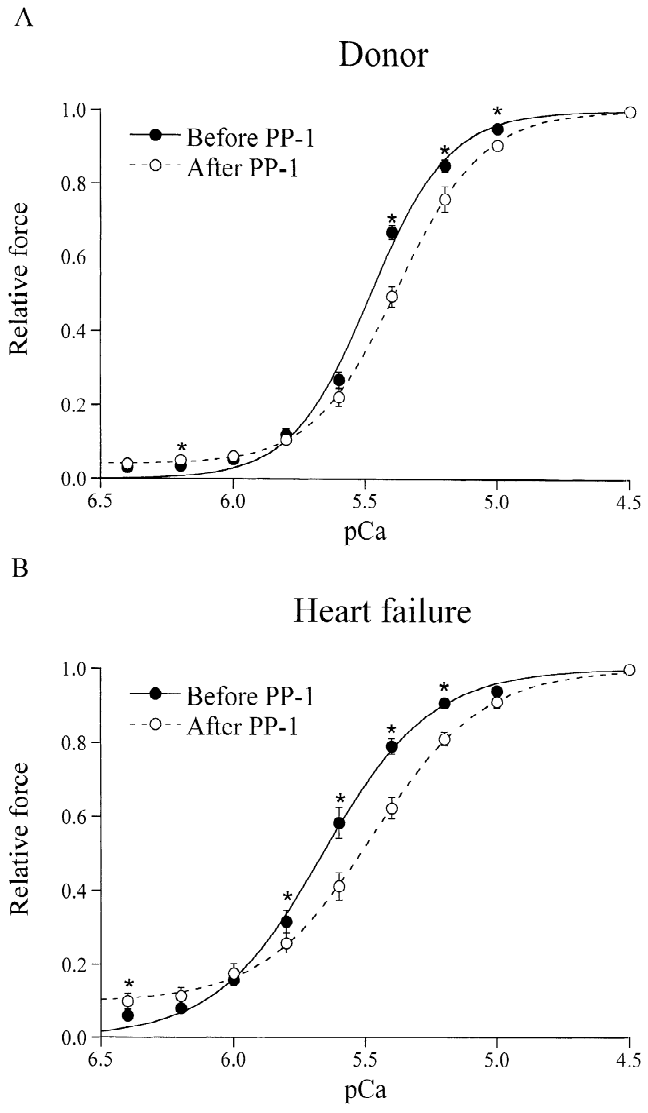

Fig. 4. Ca^{2+}-sensitivity of force before and after PP-1 in donor (A; $n=13$) and failing (B; $n=15$) cardiomyocytes. Force was measured at different $[Ca^{2+}]$ before and after PP-1 in the same myocyte. Force at submaximal $[Ca^{2+}]$ was normalized to the control force at saturating $[Ca^{2+}]$. PP-1 decreased pCa_{50} by 0.10 and 0.20 units in donor and failing cardiomyocytes, respectively. *$P<0.05$, before versus after PP-1.

previously by Morano et al. [17] that incubation in cardioplegic solution would dephosphorylate MLC-2. However, although our biopsies were transported in cardioplegic solution, we did not observe complete dephosphorylation of MLC-2. In fact, in this study and in a previous study [12] we observed that the level of TnI, TnT and MLC-2 phosphorylation was quite homogeneous in the donor and failing group.

Table 2
pCa_{50} and nH values before and after PP-1

		Donor ($n=13$)	Heart failure ($n=15$)
Before PP-1	pCa_{50}	5.48±0.01	5.66±0.02†
	nH	3.06±0.17	2.38±0.15†
After PP-1	pCa_{50}	5.38±0.02*	5.46±0.03*†
	nH	2.95±0.19	2.29±0.12†

Force was measured at different $[Ca^{2+}]$ before and after PP-1 in the same myocyte. *n*, number of cardiomyocytes; *$P<0.05$, before versus after PP-1. $^{\dagger}P<0.05$, donor versus heart failure.

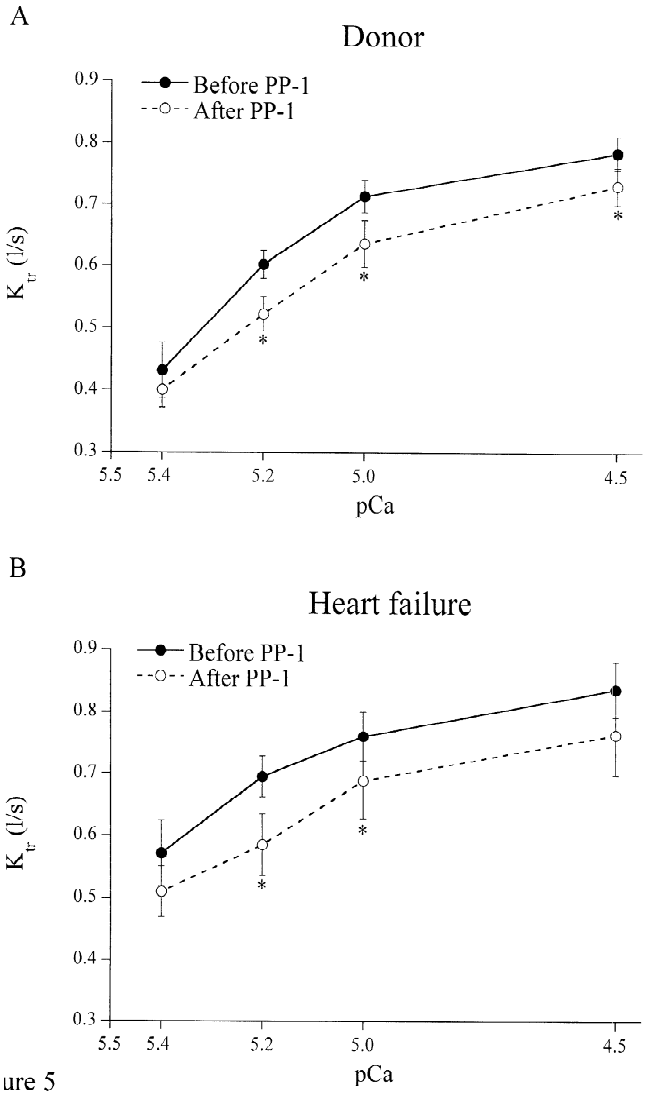

Fig. 5. Absolute K_{tr} values as a function of pCa before and after PP-1 in donor (A; $n=13$) and failing (B; $n=15$) cardiomyocytes. *$P<0.05$, before versus after incubation.

In accordance with previous studies on human hearts, no difference was observed in maximal isometric tension, while Ca^{2+}-responsiveness of force was significantly increased in end-stage failing hearts [17,18]. In a recent study [12], we have shown that the increased Ca^{2+}-responsiveness in failing human hearts most likely results from altered phosphorylation of myosin light chain 2 (MLC-2) and troponin I (TnI).

In previous studies on rabbit skeletal and rat cardiac muscle [24–26], the force–pCa relationship was found to be biphasic, with the strongest cooperativity present at low $[Ca^{2+}]$ ($n_1<n_2$), i.e. the force–pCa relation is steeper at

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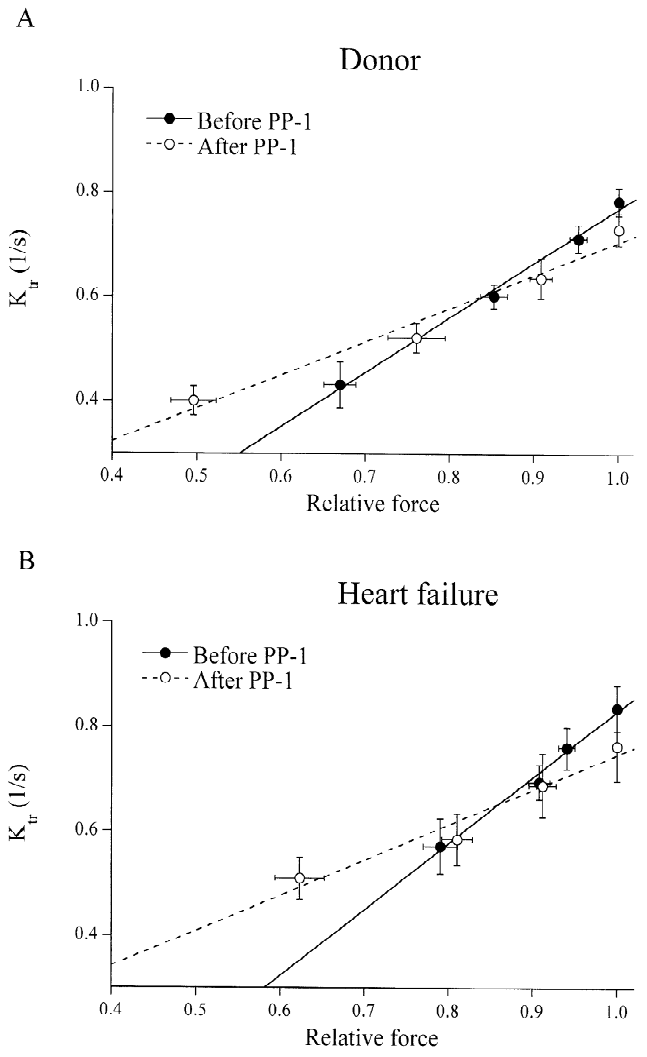

Fig. 6. K_{tr} as a function of relative force before and after PP-1 in donor (A, $n=13$) and failing (B, $n=15$) cardiomyocytes. Force at submaximal [Ca^{2+}] was normalized to values at saturating [Ca^{2+}]. Force-dependence of K_{tr} decreased after PP-1 in both donor and failing myocytes.

low [Ca^{2+}] than at high [Ca^{2+}]. It should be noted that albeit our data at low Ca^{2+} were quite noisy, the opposite ($n_1 > n_2$) was found in donor hearts (inset Fig. 3A). In failing human hearts no difference in n_1 and n_2 was found, but n_1 was significantly less than in donor hearts, which indicates that the cooperativity at high [Ca^{2+}] is somewhat depressed in human heart failure.

In addition to increased force levels at submaximal [Ca^{2+}], K_{tr} tended to be higher in failing than in donor hearts, with a significant difference at pCa≥5.2 (Fig. 3B). These findings indicate that, like an enhanced Ca^{2+}-sensitivity of force, the responsiveness of K_{tr} to Ca^{2+} is also increased in human heart failure.

K_{tr} values in human ventricular myocytes were 10- to 20-fold lower than K_{tr} values found previously in rat ventricular myocardium [22,27,28], but similar to those found in pig ventricular tissue [29]. The differences in K_{tr} may be attributed to differences in myosin heavy chain (MHC) composition which is a determinant of the rate of tension recovery [30]. In adult rats almost exclusively the fast α-MHC is present, while the slow β-MHC isoform predominates in human [19,31] and pig [31] ventricular myocardium.

In agreement with previous studies on myocardial tissue [25,27,28], K_{tr} decreased with increasing pCa in both donor and failing hearts, indicating that redevelopment of force gets slower with decreasing [Ca^{2+}]. When K_{tr} values were plotted against relative force at different [Ca^{2+}] (Fig. 3C), an approximately linear relationship was obtained in donor and failing hearts. In skeletal muscle a curvilinear relationship between K_{tr} and force has been observed [26,32]. Data concerning the K_{tr}–force relationship in cardiac tissue have been conflicting. Wolff et al. [27] observed a linear relationship between K_{tr} and force in rat trabeculae, while others [25,28] found a curvilinear relationship in rat and guinea-pig myocardium. Since we could not accurately determine K_{tr} at low [Ca^{2+}], it remains to be established if the relation between K_{tr} and force in human myocardium is linear or becomes curvilinear at high pCa values.

4.2. Effect of PP-1

Surprisingly, PP-1 decreased Ca^{2+}-responsiveness of force to a larger extent in failing than in donor cardiomyocytes, although the endogenous level of MLC-2 phosphorylation was significantly lower in failing than in donor hearts. After PP-1 the difference in Ca^{2+}-responsiveness between failing and donor myocardium was diminished, while it was completely abolished after subsequent treatment with PKA. These results suggest that the difference in Ca^{2+}-responsiveness does not reflect intrinsic differences in contractile protein isoform composition, but rather differences in endogenous phosphorylation status of thin and thick filament proteins. The enhanced response to MLC-2 dephosphorylation in failing myocytes may result from differences in TnI phosphorylation levels between donor and failing hearts [12]. Since in non-failing donor hearts Ca^{2+}-responsiveness is lower compared to failing hearts, the desensitizing effect of PP-1 may already be saturated, while failing hearts may be more susceptible to MLC-2 dephosphorylation due to the enhanced Ca^{2+}-sensitivity of force.

An alternative explanation for the difference in responsiveness to MLC-2 dephosphorylation between donor and failing hearts could be differences in the phosphorylation level of myosin binding protein-C (MyBP-C). It has been suggested that phosphorylation of MyBP-C performs a permissive role in contraction of the heart muscle [33,34]. Phosphorylation of MyBP-C would decrease the restriction of myosin and would thereby facilitate changes in the actin–myosin interaction upon (de)phosphorylation of

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MLC-2 [33]. Thus, a difference in MyBP-C phosphorylation between donor and failing hearts could alter the response to MLC-2 (de)phosphorylation. An increased activity of Ca^{2+}/calmodulin-dependent protein kinase, which may phosphorylate MyBP-C [35], has been found in idiopathic dilated cardiomyopathy [9]. Knowledge on the phosphorylation status of MyBP-C in healthy and failing myocardium is of great interest and warrants further study, particular in human tissue.

The decrease in Ca^{2+}-sensitivity of force and in absolute K_{tr} values after dephosphorylation of MLC-2 are in agreement with the results obtained after phosphorylation of MLC-2 in cardiac and skeletal muscle [7,13]. Huiting-Hollander et al. [16] reported no difference in Ca^{2+}-responsiveness of K_{tr} between control mice expressing endogenous MLC-2, in which 39% of the total MLC-2 was phosphorylated, and knock-in mice expressing a non-phosphorylable form of MLC-2. In accordance, we did not observe an effect of PP-1 on Ca^{2+}-dependency of relative K_{tr} values. Since Ca^{2+}-sensitivity of force development was decreased, while Ca^{2+}-dependency of K_{tr} remained unaffected, the force-dependency of K_{tr} in both donor and failing myocytes was decreased after PP-1. The increase in K_{tr} upon MLC-2 phosphorylation in rabbit psoas fibers might be due to an increase in f_{app} [7]. The effect of MLC-2 dephosphorylation on force development and K_{tr} in human cardiomyocytes observed in the present study both may be explained by a decrease in f_{app}. It has been proposed that upon MLC-2 dephosphorylation myosin heads move toward the backbone of the thick filament away from the thin filament, diminishing the probability of actin to myosin binding [36]. Hence, the decrease in cross-bridge attachment may originate from a structural change in the thick filament.

4.3. Clinical relevance

Our results indicate that the enhanced response to MLC-2 dephosphorylation in failing myocytes, despite the decreased basal level of MLC-2 phosphorylation, might result from differences in endogenous phosphorylation of thin and thick filament proteins between donor and failing hearts. By and large, decreased MLC-2 phosphorylation in end-stage human heart failure may be a compensatory mechanism in order to improve myocardial contractility by opposing the detrimental effects of increased Ca^{2+}-responsiveness of force and impaired Ca^{2+}-handling on diastolic function.

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