Frequency-dependent myofilament Ca^{2+} desensitization in failing rat myocardium

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The positive force–frequency relation, one of the key factors modulating performance of healthy myocardium, has been attributed to an increased Ca^{2+} influx per unit of time. In failing hearts, a blunted, flat or negative force–frequency relation has been found. In healthy and failing hearts frequency-dependent alterations in Ca^{2+} sensitivity of the myofilaments, related to different phosphorylation levels of contractile proteins, could contribute to this process. Therefore, the frequency dependency of force, intracellular free Ca^{2+} ($[Ca^{2+}]_i$), Ca^{2+} sensitivity and contractile protein phosphorylation were determined in control and monocrotaline-treated, failing rat hearts. An increase in frequency from 0.5 to 6 Hz resulted in an increase in force in control (14.3 ± 3.0 mN mm^{-2}) and a decrease in force in failing trabeculae (9.4 ± 3.2 mN mm^{-2}), whereas in both groups the amplitude of $[Ca^{2+}]_i$ transient increased. In permeabilized cardiomyocytes, isolated from control hearts paced at 0 and 9 Hz, Ca^{2+} sensitivity remained constant with frequency (pCa_{50}: 5.55 ± 0.02 and 5.58 ± 0.01, respectively, $P > 0.05$), whereas in cardiomyocytes from failing hearts Ca^{2+} sensitivity decreased with frequency (pCa_{50}: 5.62 ± 0.01 and 5.57 ± 0.01, respectively, $P < 0.05$). After incubation of the cardiomyocytes with protein kinase A (PKA) this frequency dependency of Ca^{2+} sensitivity was abolished. Troponin I (TnI) and myosin light chain 2 (MLC2) phosphorylation remained constant in control hearts but both increased with frequency in failing hearts. In conclusion, in heart failure frequency-dependent myofilament Ca^{2+} desensitization, through increased TnI phosphorylation, contributes to the negative force–frequency relation and is counteracted by a frequency-dependent MLC2 phosphorylation. We propose a novel role for PKC-mediated TnI phosphorylation in modulating the force–frequency relation.

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Heart rate is a key factor modulating cardiac performance. Under physiological conditions an increase in stimulation frequency results in enhanced systolic function (Lewartowski & Pytkowski, 1987; Gao *et al.* 1998; Layland & Kentish, 1999; Stuyvers *et al.* 2002). This positive force–frequency relation has been attributed to an increased Ca^{2+} influx in the cardiomyocytes per unit of time via the L-type Ca^{2+} channels, which increases SR Ca^{2+} content and promotes Ca^{2+}-induced Ca^{2+} release (Gao *et al.* 1998; Layland & Kentish, 1999; Pieske *et al.* 1999; Stuyvers *et al.* 2002). However, because of the highly non-linear force–Ca^{2+} relation, a possible change in myofilament Ca^{2+} sensitivity, through phosphorylation of contractile proteins, may be present as a contributing factor. For instance, frequency-dependent alterations in intracellular $[Ca^{2+}]_i$ could change the Ca^{2+} sensitivity of the myofilaments by activation of Ca^{2+}-dependent kinases or phosphatases: Ca^{2+}–calmodulin kinase II (CaMK-II) (DeSantiago *et al.* 2002), the classical Ca^{2+}-dependent protein kinases C (PKCs) (Bowling *et al.* 1999; Braz *et al.* 2004), myosin light chain kinase (MLCK) (Tong *et al.* 2004) and calcineurin (PP2B) (Lim & Molkentin, 1999). Detailed phase-plane analysis of myocardial force and $[Ca^{2+}]_i$ in trabeculae of rat (Janssen *et al.* 2002), rabbit (Varian & Janssen, 2007) and mouse (Gao *et al.* 1998; Tong *et al.* 2004) and a study in mouse

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cardiomyocytes (Antoons *et al.* 2002) indeed suggested the presence of a frequency-dependent sensitization of the myofilaments.

In failing hearts a blunted, flat or negative force–frequency relation has been observed (Gwathmey *et al.* 1990; Mulieri *et al.* 1992; Schwinger *et al.* 1993; Eising *et al.* 1994; Pieske *et al.* 1995; Pieske *et al.* 1999; Janssen *et al.* 2000; Brixius *et al.* 2002; Kogler *et al.* 2003) and little is known about the impact of frequency-dependent phosphorylation on Ca^{2+} sensitivity. In several animal models and in human, heart failure is associated with a decrease in the amplitude of the Ca^{2+} transient (Gwathmey *et al.* 1990; Pieske *et al.* 1995) but an increase in Ca^{2+} sensitivity of the myofilaments (Wolff *et al.* 1996; Morano *et al.* 1997; van der Velden *et al.* 1999, 2001; Kogler *et al.* 2003). These changes in Ca^{2+} sensitivity could arise from altered isoform expression but could also be related to different phosphorylation levels of the thin and thick myofilament proteins such as troponin I (TnI) (Bodor *et al.* 1997; van der Velden *et al.* 2003*b*; Tong *et al.* 2004), troponin T (TnT) (Kameyama *et al.* 1998), myosin binding protein C (MyBP-C) (Tong *et al.* 2004) and MLC2 (van der Velden *et al.* 1999, 2001, 2003*a*,*b*). These alterations may alter the force–frequency relation.

We hypothesized that in control and failing hearts the force–frequency relation is determined not only by changes in the intracellular Ca^{2+} transient but also by frequency-dependent changes in Ca^{2+} sensitivity of the myofilaments through different phosphorylation levels of contractile proteins. To discriminate between the contribution of Ca^{2+} handling and myofilament Ca^{2+} sensitivity, the frequency dependency of force, pressure, intracellular $[Ca^{2+}]_i$, Ca^{2+} sensitivity and phosphorylation levels of contractile proteins were determined in isolated cardiomyocytes, in trabeculae and in hearts from control and monocrotaline (MCT)-treated failing rats. A single injection of MCT, which induces pulmonary hypertension, causes right ventricular (RV) heart failure (Leineweber *et al.* 2000; Seyfarth *et al.* 2000; Korstjens *et al.* 2002; Kogler *et al.* 2003; Buermans *et al.* 2005). This model was chosen because it allows measurements in cardiac trabeculae with dimensions suitable for accurate force and intracellular Ca^{2+} measurements (Janssen *et al.* 2002). Our results indicated that not only changes in Ca^{2+} handling but also changes in Ca^{2+} sensitivity of the myofilaments, through altered phosphorylation of contractile proteins, contribute to the negative force–frequency relation in failing hearts.

Methods

Design of the study

Male Wistar rats ($n = 64$) were randomly assigned to two experimental groups. At a body weight of 175 g, animals received a single subcutaneous injection of saline (control) or 80 mg kg^{-1} monocrotaline (failing). During its first passage through the pulmonary circulation MCT damages the pulmonary endothelium, thereby inducing pulmonary hypertension, causing right ventricular (RV) hypertrophy resulting eventually in RV heart failure (Leineweber *et al.* 2000; Seyfarth *et al.* 2000; Korstjens *et al.* 2002; Kogler *et al.* 2003; Buermans *et al.* 2005). The experiments on isolated cardiac trabeculae, isolated cardiomyocytes or isolated hearts were all performed 4 weeks after injection. All protocols were in accordance with the guidelines of the Animal Experimental Welfare Committee of the VU University Medical Center (VUMC).

Force and intracellular Ca^{2+} measurements

The effects of changes in stimulation frequency on force development and intracellular $[Ca^{2+}]_i$ transients were determined in isolated cardiac trabeculae by means of fura 2-AM, as previously described (Lamberts *et al.* 2002).

Under intraperitonial pentobarbital anaesthesia, the hearts of 22 male Wistar rats (control $n = 11$, failing $n = 11$) were quickly removed and perfused via the aorta with a modified Krebs–Henseleit solution (see below) to which 20 mmol l^{-1} 2,3-butanedione monoxime was added. A suitable trabecula from the right ventricle was dissected, transferred to the experimental bath and attached between a force transducer and a micromanipulator. The muscles were constantly superfused with a modified Krebs–Henseleit solution, kept at 27°C and continuously stimulated at 0.5 Hz. The modified Krebs–Henseleit solution consisted of (mmol l^{-1}): 118 NaCl, 4.5 KCl, 1 $CaCl_2$, 0.33 NaH_2PO_4, 1 $MgCl_2$, 25 $NaHCO_3$ and 10 glucose. The solution was gassed with 95%O_2–5%CO_2 (pH 7.45). After mounting, the preparations were stimulated for 60 min to allow equilibration. To impose similar stretch levels the muscles in both groups were stretched to the length (L_{max}) at which isometric developed force was maximal. The muscles were loaded for 30 min with the cell-permeant acetoxymethyl ester form of the fluorescent intracellular Ca^{2+} indicator fura 2-AM (Molecular Probes, Eugene, OR, USA; F1221, final concentration 10 μmol l^{-1}) to measure $[Ca^{2+}]_i$ transients. During fura 2-AM loading electrical stimulation was turned off and temperature was increased to 37°C. After 30 min of dye washout, during which electrical stimulation was resumed and temperature returned to 27°C, the experimental protocol was started.

At 27°C, a force–frequency relation (0.5, 1, 2 and 3 Hz) at 1 mmol l^{-1} external Ca^{2+}, a force–Ca^{2+} dose–response curve (0.25, 0.5, 1, 3 mmol l^{-1} Ca^{2+}) at 0.5 Hz and maximal force (F_{max}, at 1 mmol l^{-1} external Ca^{2+}) attained during post-extrasystolic potentiation (ter Keurs *et al.* 1987) were determined. Hereafter, the temperature was increased to 37°C and a force–frequency relation (0.5, 1, 2, 3, 4 and 6 Hz) at 1 mmol l^{-1} Ca^{2+} and F_{max} were determined. During all interventions, force and $[Ca^{2+}]_i$

transients were recorded. Force was normalized to cross sectional area calculated from the diameter measured in two perpendicular directions in the middle part of the preparation at L_{max}, assuming an ellipsoidal cross-section. The time from stimulus to half-relaxation (tHR) was used as relaxation parameter.

The signal of fura 2 fluorescence at 520 nm following excitation at wavelengths 340 nm and 380 nm was collected with a photomultiplier. In all experiments, the fura 2 signal was at least 5 times above background level (autofluorescence), which was subtracted. In some preparations after switching from 27°C to 37°C, fura 2 leakage precluded accurate measurements of the $[Ca^{2+}]_i$ transients at 37°C, so these data were discarded.

Right ventricular pressure measurements

In isolated hearts the RV pressure–frequency relation was determined, as previously described (Lamberts *et al.* 2007). The hearts of 38 animals (control $n = 19$, failing $n = 19$) were rapidly dissected, placed in ice-cold modified Krebs–Henseleit solution and the aorta was cannulated for retrograde Langendorff perfusion at constant coronary perfusion pressure of 100 mmHg at 37°C. The modified Krebs–Henseleit solution had the following composition (mmol l^{-1}): 118.5 NaCl, 4.7 KCl, 1.4 $CaCl_2$, 25 $NaHCO_3$, 1.2 $MgCl_2$, 1.2 KH_2PO_4 and 11 glucose. The solution was continuously gassed with 95% O_2–5% CO_2 (pH 7.4).

A drainage cannula was inserted into the apex. The hearts were paced at 5 Hz using two electrodes, one attached to the drain and the other to the right atrium. A custom-made plastic balloon, filled with degassed distilled water, was inserted in the RV. RV pressure was measured using a catheter tip manometer. A pressure–volume relation was recorded to determine the volume (V_{max}) at which maximal isovolumic pressure developed. The RV balloon volume was then adjusted to 80% of V_{max}.

After mounting, the hearts were left to stabilize for 20 min and a pressure–frequency relation (3, 6, 9 Hz) was determined. During all interventions diastolic and systolic pressures and the maximal rate of force development ($+dP/dt$) were recorded. The time from stimulus to half-relaxation (tHR) and the ratio between the minimal rate of pressure development ($-dP/dt$) and developed pressure (P_{dev}) were taken as relaxation parameters.

Thereafter, control and failing hearts were divided in four subgroups. Three groups (all $n = 5$) were paced for 60 min at 3, 6 or 9 Hz. In addition in another subgroup ($n = 4$) hearts were kept quiescent for 60 min by adding 0.37 mmol l^{-1} lidocaine hydrochloride to the perfusate. After this 60 min period, the hearts of all four groups were immediately placed in ice-cold modified Krebs–Henseleit solution. The free wall of the RV was rapidly dissected, frozen in liquid nitrogen, freeze-dried and stored at −70°C.

In a few experiments, the β-adrenergic receptor blocker atenolol (0.02 mmol l^{-1}) was added to rule out a possible sympathetic effect of electrical stimulation of residual nerve endings in isolated hearts on the force–frequency response, an effect that is distinct from the well-known long-term effect of β-blockers on adrenergic receptor density and sensitivity. We found no effect of atenolol on the force–frequency relation (data not shown).

Ca^{2+} sensitivity measurements

Frequency-dependent alterations in Ca^{2+} sensitivity of the contractile apparatus were studied in isolated skinned cardiomyocytes obtained from RV tissue of the above-mentioned isolated control and failing hearts frozen at a pacing frequency of 0 and 9 Hz.

Cardiomyocytes from control and failing hearts were mechanically isolated as previously described (van der Velden *et al.* 1998). In short, tissue was homogenized and cells were permeabilized with 0.5% Triton and washed. Single cardiomyocytes ($n = 10$ in each group, from $n = 4$ hearts in 0 Hz group and $n = 5$ hearts in 9 Hz group) with uniform striation pattern were selected and glued to thin stainless steel needles with silicon adhesive. Sarcomere length measured in relaxing solution was adjusted to 2.2 μm. Measurements were performed at 15°C. The Ca^{2+} sensitivity of force was determined by measuring isometric force at maximal $[Ca^{2+}]$ (pCa4.5; $pCa = -\log[Ca^{2+}]$) and submaximal $[Ca^{2+}]$ before and after treatment with the catalytic subunit of protein kinase A (PKA) (100 U ml^{-1} in relaxing solution with 6 mmol l^{-1} dithiothreitol (DTT) for 40 min at 20°C). Mean ΔpCa_{50} values were calculated from the shift in pCa_{50} upon PKA treatment from individual cardiomyocytes in each group.

Protein analysis

Freeze-dried RV tissue obtained from the control ($n = 19$) and failing ($n = 19$) hearts frozen at different pacing frequencies (0, 3, 6 and 9 Hz) was used to determine the frequency-dependent phosphorylation of several contractile proteins.

The phosphorylation levels of myosin light chain 1 (MLC-1), myosin light chain 2 (MLC-2) and troponin T (TnT) were assessed by two-dimensional polyacrylamide gel electrophoresis (2-D-PAGE) (van der Velden *et al.* 2003*b*). Samples were treated with trichloroacetic acid to preserve the phosphorylation status of the proteins (Morano *et al.* 1988) and loaded (200 μg dry weight) on immobiline strips with a pH gradient of 4.0 to 7.0 (Amersham Pharmacia Biotech). In the second dimension, proteins were separated by SDS-PAGE. Gels were stained with Coomassie blue, scanned and analysed using AIDA (Raytest, Germany). The phosphorylation levels were expressed as a percentage of the total protein content.

Table 1. Body weight and wet lung and ventricle weights on the day of the experiment

	Control ($n = 11$)	Failing ($n = 10$)
BW (g)	333 ± 10	231 ± 8*
LW (g)	1.63 ± 0.05	2.35 ± 0.16*
RV (mg)	220 ± 15	412 ± 20*
RV/BW (mg g^{-1})	0.66 ± 0.04	1.81 ± 0.11*
LV (mg)	968 ± 69	839 ± 55
LV/BW (mg g^{-1})	2.90 ± 0.18	3.65 ± 0.20
RV/LV	0.23 ± 0.01	0.50 ± 0.03*

BW, body weight; LW, lung weight; RV, right ventricle weight; LV, weight of left ventricle + septum. Values are expressed as means ± S.E.M. *$P < 0.05$ *versus* control.

Phosphorylation of troponin I (TnI) was determined by non-equilibrium isoelectric focusing gel electrophoresis (NEIEF) as described earlier (O'Farrell *et al.* 1977; Madden, 1995; Kobayashi *et al.* 2005). The NEIEF gels contained 8 M urea, 5% acrylamide (acrylamide/bis-acrylamide 29 : 1; optional 2% Triton X-100), 0.8% ampholyte (3.5–10.0) and 1.2% ampholyte (7.0–9.0) (Amersham, NJ). Sample loading buffer contained 8 mol l^{-1} urea, 2.5 mol l^{-1} thiourea, 2 mmol l^{-1} EDTA, 0.5%, ampholytes (3–10), 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 10 mmol l^{-1} DTT, 2 mmol l^{-1} TBP and a protease inhibitor cocktail. Unlike typical IEF, the lower and upper reservoirs were filled with 20 mol l^{-1} NaOH and 10 mmol l^{-1} H_3PO_4, respectively, and the positive and negative ports were reversed. Electrophoresis was carried out at 100 V for 20 min, 200 V for 50 min followed by 500 V for 10 min without pre-run. For the Western blot, after separation, proteins were transferred to nitrocellulose membrane overnight at 30 V at 4°C. Membranes were blocked for 1 h in 5% non-fat milk in Tris-Buffered Saline Tween-20 (TBST), washed and incubated with monoclonal anti-TnI (1 : 5000 in TBST; clone C5 from Research Diagnostics Inc.) for 3 h at room temperature. After washing, they were further incubated with anti-mouse antibody conjugated to horseradish peroxidase Horseradish Peroxidase, correct (HRP). Proteins were visualized with Electrochemiluminescence (ECL) (Amersham) and quantified by densitometric analysis using NIH Image.

In addition, the phosphorylation levels of myosin binding protein C (MyBP-C) MLC1, MLC2, TnT and TnI were assessed by phosphospecific staining. Proteins were separated on a one-dimensional 12% SDS-PAGE and the gels were stained with Pro-Q Diamond phosphoprotein stain (Molecular Probes) (Jweied *et al.* 2005). After visualization of the phosphoproteins, the same gel was stained with colloidal Coomassie blue to verify equal protein loading in each lane. Images were quantified by using ImageQuant analysis software (Bio-Rad). Pro-Q Diamond signals were normalized to total actin protein content.

The amount of PKCα protein and phosphorylation levels were assessed by gel electrophoresis and stained with antibodies for PKCα (dilution 1 : 500, Research & Diagnostics Antibodies (R & D) Systems) and phosphorylated PKCα at Ser^{657} (dilution 1 : 200, Santa Cruz Biotechnology). The immunoreactive bands were visualized by chemiluminescence and quantified with AIDA software. Protein content was determined by Ponceau staining. The amount of phosphorylation of the blots was normalized to the actin levels on the Ponceau-stained blot. To circumvent difficulties in the analysis of experimental groups between different gels we applied on a gel with 12 lanes/slots 2 times five samples ($n = 10$) of failing hearts at 0 Hz or 9 Hz stimulation frequencies, respectively. On the same gel two additional samples were applied, which each consisting of a pool of five control hearts samples paced at 0 Hz or 9 Hz, respectively. This procedure was repeated on a second gel for control and failing hearts, but on this second gel the individual control heart samples were applied and the failing heart samples were pooled.

Analysis and statistics

Statistical differences within groups were tested with one- or two-way ANOVA followed by a Bonferroni *post hoc* test; $P < 0.05$ was considered significant. All data are expressed as mean ± S.E.M., with n indicating the number of animals per group, unless indicated otherwise. Fura 2 signals of five consecutive contractions were averaged to obtain accurate $[Ca^{2+}]_i$ transients. The force–pCa relation was fitted to the following Hill equation:

$$F(Ca^{2+})/F_o = [Ca^{2+}]^{n_H}/\left(Ca_{50}^{n_H} + [Ca^{2+}]^{n_H}\right)$$

where F is steady state force, F_o denotes the steady state force at saturating Ca^{2+} concentration determined at pCa = 4.5, n_H represents the steepness of the relationship, and Ca_{50} or pCa_{50} represent the Ca^{2+} concentration at which force is half of F_o.

Results

MCT-induced right ventricular heart failure

On the day of the experiment, MCT-treated animals had an almost 2-fold increased RV weight, an almost 3-fold increased RV weight/body weight ratio and a 2-fold increased RV/LV weight ratio in comparison to the controls, indicating marked RV hypertrophy (Table 1). Furthermore, MCT-treated animals displayed a progressive loss of body weight during several days before killing and showed an increased lung weight (Table 1) with pleural effusion (fluid in the lungs), indicative of congestive heart failure (Leineweber *et al.*

2000; Seyfarth *et al.* 2000; Buermans *et al.* 2005). RV hypertrophy was also reflected in the cross-sectional area of the trabeculae studied: $0.031 \pm 0.004\ mm^2$ in the control and $0.046 \pm 0.009\ mm^2$ in the failing group. Although, in the present study, an increase in LV/body weight ratio was observed (Table 1), this increase does not reflect LV hypertrophy, but is mainly due to the fact of increased body weight of the MCT-treated rats with unaltered LV weight (Leineweber *et al.* 2000; Seyfarth *et al.* 2000).

Frequency dependency of force and intracellular Ca^{2+}

Force and $[Ca^{2+}]_i$ transients obtained at different stimulation frequencies for control and failing trabeculae are shown in Fig. 1. In Fig. 2 the averaged diastolic and systolic force and $[Ca^{2+}]_i$ levels are shown.

A positive force–frequency relation was observed in control trabeculae, which was accompanied by a frequency-dependent increase in systolic $[Ca^{2+}]_i$, while diastolic force and diastolic $[Ca^{2+}]_i$ remained constant. Failing trabeculae clearly displayed a negative systolic force–frequency relation, while diastolic force remained constant. In the failing group systolic $[Ca^{2+}]_i$ was depressed compared with control, but peak systolic $[Ca^{2+}]_i$ increased with frequency. Diastolic $[Ca^{2+}]_i$ tended to be elevated ($P = 0.07$) in the failing trabeculae compared with controls, but was not affected by frequency. The disparity of the force–frequency and $[Ca^{2+}]_i$–frequency relations in the failing group suggests the presence of frequency-dependent changes in myofilament Ca^{2+} sensitivity. This is further illustrated in Fig. 3 (upper panel) where the impact of an increase in frequency on developed force and the amplitude of the $[Ca^{2+}]_i$ transient is shown: the decrease in developed force with increasing pacing frequency in the failing muscle is accompanied by a small increase in the amplitude of $[Ca^{2+}]_i$ rather than a decrease. Figure 3 shows that the magnitudes of the frequency-dependent effects in Ca^{2+} sensitivity at 27°C (upper panel) and 37°C (lower panel) were very similar.

The relaxation of force was slowed in failing muscle (time to half-relaxation (tHR) at 0.5 Hz at 27°C: control 282 ± 9 ms; failing 314 ± 10 ms, $P < 0.05$; and at 37°C: control 130 ± 4 ms; failing 152 ± 5 ms, $P < 0.05$, all groups $n = 10$) and was accompanied in the failing muscles with prolonged relaxation of the $[Ca^{2+}]_i$ transient (Fig. 1). Although relaxation was slower, the frequency-dependent acceleration of relaxation remained and was even more pronounced in the failing trabeculae than in control muscles (change in time to half-relaxation from 0.5 to 3 Hz at 27°C: control 78 ± 5 ms; failing 92 ± 6 ms, $P < 0.05$; and from 0.5 to 6 Hz at 37°C: control 21 ± 3 ms; failing 39 ± 11 ms, $P = 0.07$).

Maximal force (F_{max}) attained during post-extrasystolic potentiation was not affected in the failing muscles (control $75.2 \pm 7.4\ mN\ mm^{-2}$, failing $62.7 \pm 9.6\ mN\ mm^{-2}$, $P > 0.05$, $n = 10$).

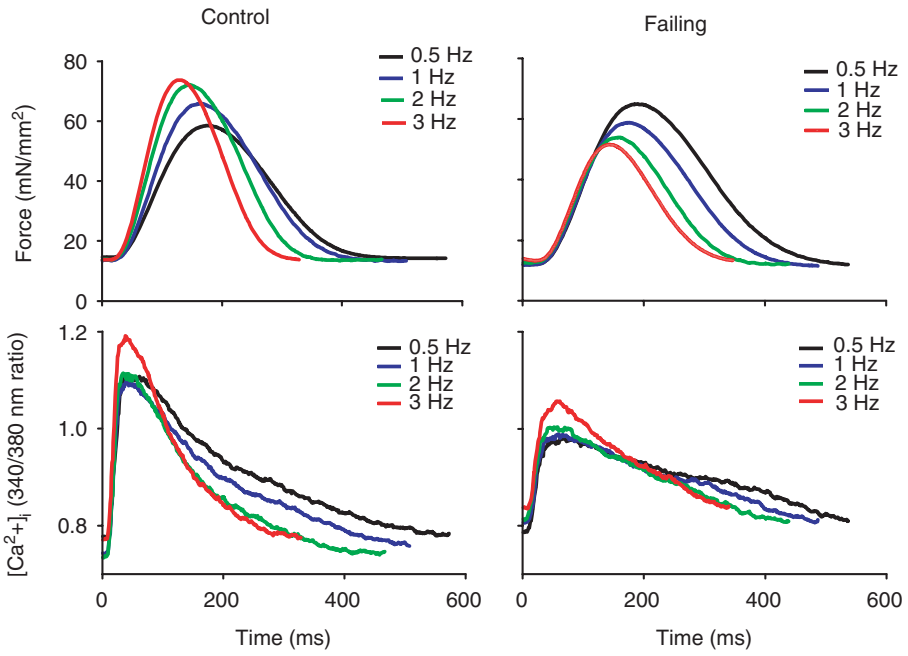

Figure 1. Averaged force twitches (upper panels, $n = 10$) and $[Ca^{2+}]_i$ transients (lower panels) at different stimulation frequencies from control (left panels) and failing (right panels) right ventricular trabeculae

In the control group a positive force–frequency relation with increased frequency-dependent acceleration of relaxation (of force and Ca^{2+} transient) exists, while in the failing group a negative force–frequency relation with a preserved frequency-dependent acceleration of relaxation was observed.

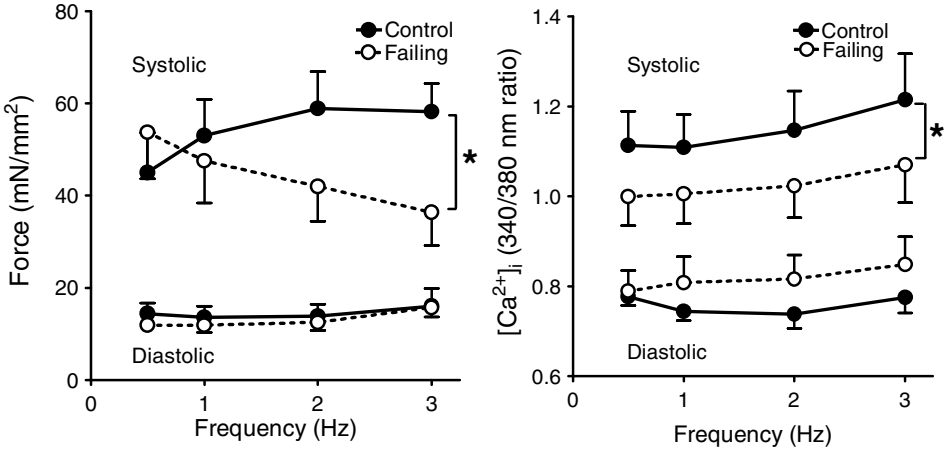

Figure 2. Averaged diastolic (lower values) and peak systolic (upper values) force (left panel) and $[Ca^{2+}]_i$ (right panel) at different stimulation frequencies at 1 mmol l^{-1} Ca^{2+} from control ($n = 10$) and failing ($n = 10$) right ventricular trabeculae
In controls, systolic force and $[Ca^{2+}]_i$ increased with frequency, whereas in failing muscles, systolic force decreased and $[Ca^{2+}]_i$ increased with frequency. In both groups, diastolic force and diastolic $[Ca^{2+}]_i$ remained constant. Values are expressed as means ± S.E.M. $^*P < 0.05$ *versus* control group.

Figure 4 shows the normalized force–external $[Ca^{2+}]_o$ relation (left panel) and the amplitude of intracellular $[Ca^{2+}]_i$ (right panel) for both groups. Developed force as well as the amplitude of $[Ca^{2+}]_i$ increased with an increase in external $[Ca^{2+}]_o$ in both groups. The leftward shift in the force–$[Ca^{2+}]_o$ relation in failing compared with control muscles, indicating increased sensitivity of the failing cardiomyocytes to external calcium, was

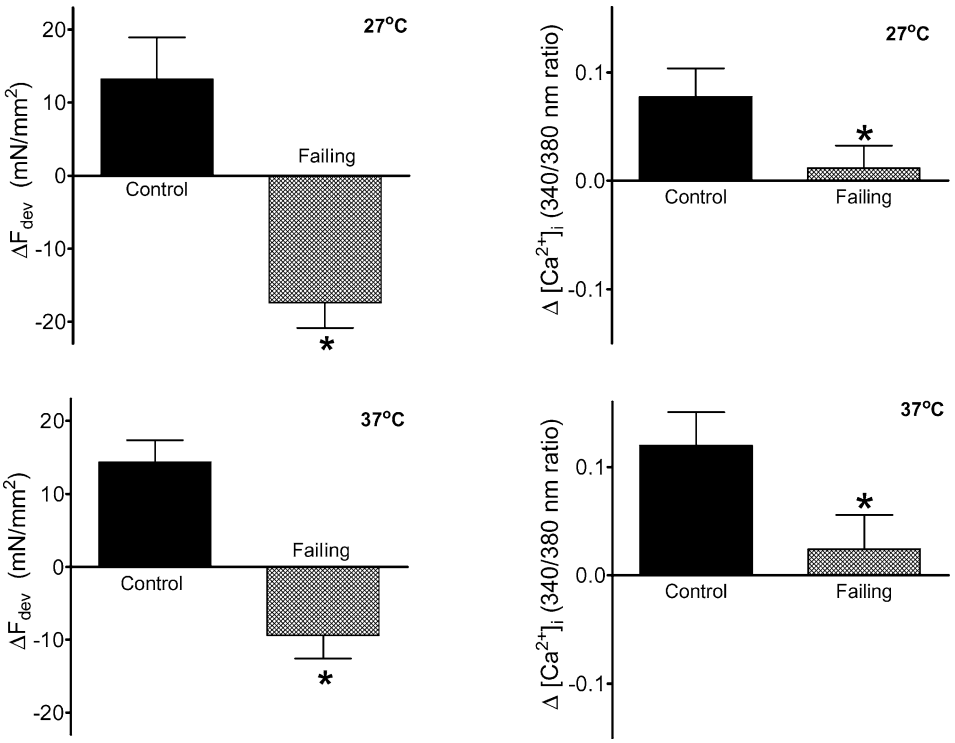

Figure 3. Changes in developed force (left panels) and changes in the amplitude of the $[Ca^{2+}]_i$ transient (right panels) with an increase in stimulation frequency from 0.5 to 3 Hz at 27°C (upper panels) and from 0.5 to 6 Hz at 37°C (lower panels) from control ($n = 10$) and failing ($n = 10$) right ventricular trabeculae
In the control group as well as in the failing muscles, the frequency-dependent increase in developed force was accompanied by an increase in amplitude of $[Ca^{2+}]_i$, although the amplitude of $[Ca^{2+}]_i$ was decreased in the failing muscles compared with controls. Values are expressed as means ± S.E.M. $^*P < 0.05$ *versus* control group.

accompanied by a decreased amplitude of the $[Ca^{2+}]_i$ transient.

Frequency dependency of RV pressure

The upper panels of Fig. 5 show that in the control hearts a flat RV pressure–frequency relation existed and that $+dP/dt_{max}$, an often-used index of contractility in isolated hearts, increased with stimulation frequency. In failing hearts the pressure–frequency and $+dP/dt_{max}$–frequency relation were both negative, similar to the force–frequency relations in the isolated trabeculae (Figs 1 and 2). The lower panels of Fig. 5 show that in the failing hearts relaxation (parameters $(-dP/dt)/P_{dev}$ and tHR) was slower compared with the control hearts, while the frequency-dependent acceleration of relaxation remained.

Frequency dependency of Ca^{2+} sensitivity

To directly test whether the Ca^{2+} sensitivity of the myofilaments is frequency dependent, isometric force–pCa relations were determined in isolated skinned cardiomyocytes from quiescent control and failing hearts and after pacing at 9 Hz.

Figure 6*A* shows that in control cardiomyocytes the force–pCa curve was not affected by an increase in frequency from 0 Hz to 9 Hz. The pCa_{50} values (Table 2) were not significantly different, indicating that Ca^{2+} sensitivity was not altered. In contrast, in failing cardiomyocytes an increase in frequency from 0 Hz to 9 Hz reduced Ca^{2+} sensitivity as reflected in the rightward shift of the force–pCa relation and the decreased pCa_{50} values (Fig. 6*B*, Table 2).

Table 2. pCa_{50}, ΔpCa_{50} and n_H values before and after PKA treatment

			Control	Failing
Before PKA	pCa_{50}	0 Hz	5.55 ± 0.02	5.62 ± 0.01*
		9 Hz	5.58 ± 0.01	5.57 ± 0.01†
	n_H	0 Hz	2.87 ± 0.10	2.70 ± 0.13
		9 Hz	2.68 ± 0.12	2.79 ± 0.07
After PKA	pCa_{50}	0 Hz	5.48 ± 0.01	5.47 ± 0.01
		9 Hz	5.48 ± 0.02	5.49 ± 0.01
	ΔpCa_{50}	0 Hz	0.08 ± 0.01	0.15 ± 0.01*
		9 Hz	0.10 ± 0.01	0.08 ± 0.01†
	n_H	0 Hz	3.12 ± 0.12	3.19 ± 0.14
		9 Hz	2.90 ± 0.12	3.24 ± 0.15

Values are expressed as means ± S.E.M. from $n = 10$ cardiomyocytes in each group. *$P < 0.05$ *versus* control, †$P < 0.05$ *versus* 0 Hz. Mean ΔpCa_{50} values were calculated from the shift in pCa_{50} upon PKA treatment from individual cardiomyocytes in each group.

The force–pCa curve from quiescent failing cardiomyocytes was significantly shifted to the left compared with the curve from quiescent control cardiomyocytes (Fig. 6*C*, Table 2), indicating an increase in basal Ca^{2+} sensitivity in failing myocardium. In all groups the force–pCa curve was shifted rightwards to a similar end-value after PKA treatment (Fig. 6*D*, Table 2), suggesting that the frequency-dependent changes in Ca^{2+} sensitivity observed predominantly result from differences in phosphorylation levels of TnI and/or MyBP-C. The steepness of the curves, as indicated by the Hill coefficients, did not differ between groups (Table 2). The maximum force generated by the control and failing cardiomyocytes was very similar (Control: 22.0 ± 1.5 kN mm^{-2} *versus* Failing: 25.4 ± 1.7 kN mm^{-2}, $P = 0.15$).

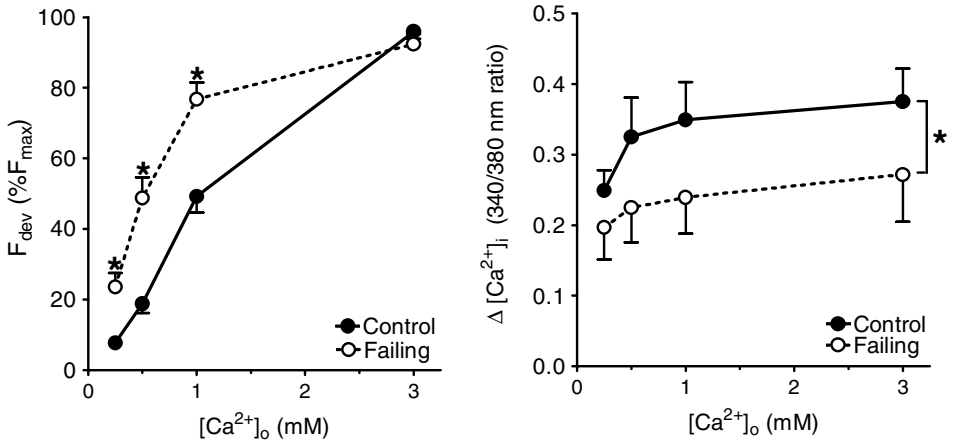

Figure 4. Effect of external $[Ca^{2+}]_o$ on developed force (left panel, normalized to F_{max}) and the amplitude of intracellular $[Ca^{2+}]_i$ (right panel) at 0.5 Hz and 27°C from control ($n = 10$) and failing ($n = 10$) right ventricular trabeculae

A leftward shift in the force–$[Ca^{2+}]_o$ relation in the failing group indicates an increased sensitivity of the cardiomyocytes to external calcium, which is accompanied by a decreased amplitude of $[Ca^{2+}]_i$ as compared with control preparations. Force and amplitude of $[Ca^{2+}]_i$ increased with $[Ca^{2+}]_o$ in both groups. Values are expressed as means ± S.E.M. *$P < 0.05$ *versus* control group.

Frequency dependency of protein phosphorylation

In Fig. 7*A* an example of a Coomassie stained 2-D SDS-PAGE gel (left panel) is shown of a control heart paced at 3 Hz. The specific MLC2 sections of control and failing hearts at 0 and 9 Hz are shown in the right panel of Fig. 7*A*. Figure 7*B* and the included linear regression lines show that MLC2 phosphorylation increases with frequency in failing hearts, but not in control hearts ($n = 5$ samples per frequency in control and failing groups, with 2 runs per sample, which yielded an overall reproducibility of $4.5 \pm 0.4\%$). The mean phosphorylation levels of MLC2 in the range of frequencies studied were less in failing hearts ($37.4 \pm 1.6\%$, $P < 0.05$) than in control hearts ($43.6 \pm 1.3\%$). Figure 8*A* shows an example of a NEIEF gel from samples of the control (left panel) and failing group (right panel). Figure 8*B* and the included linear regression lines show that TnI phosphorylation increases with frequency in failing hearts, but not in control hearts (Control: 0 Hz ($n = 4$); 3, 6 and 9 Hz ($n = 5$); Failing: 3 Hz ($n = 5$); 0, 6 and 9 Hz ($n = 6$), with a total average of 3.6 ± 0.2 repeated measurements per sample, which yielded an overall reproducibility of $6.5 \pm 0.7\%$). The mean levels of TnI phosphorylation in the range of frequencies studied were not different between both groups ($36.7 \pm 2.0\%$ *versus* $37.5 \pm 1.4\%$ phosphorylation, control and failing, respectively). The differences in phosphorylation levels of MLC2 and TnI found using the Pro-Q-Diamond stain were consistent with the differences found on the 2-D and NEIEF gel, respectively. Analysis of the MLC1 and TnT spots on the 2-D gels and of MLC1, TnT and MyBP-C on the Pro-Q Diamond gels revealed no frequency dependency of their phosphorylation levels and also no differences between control and failing hearts

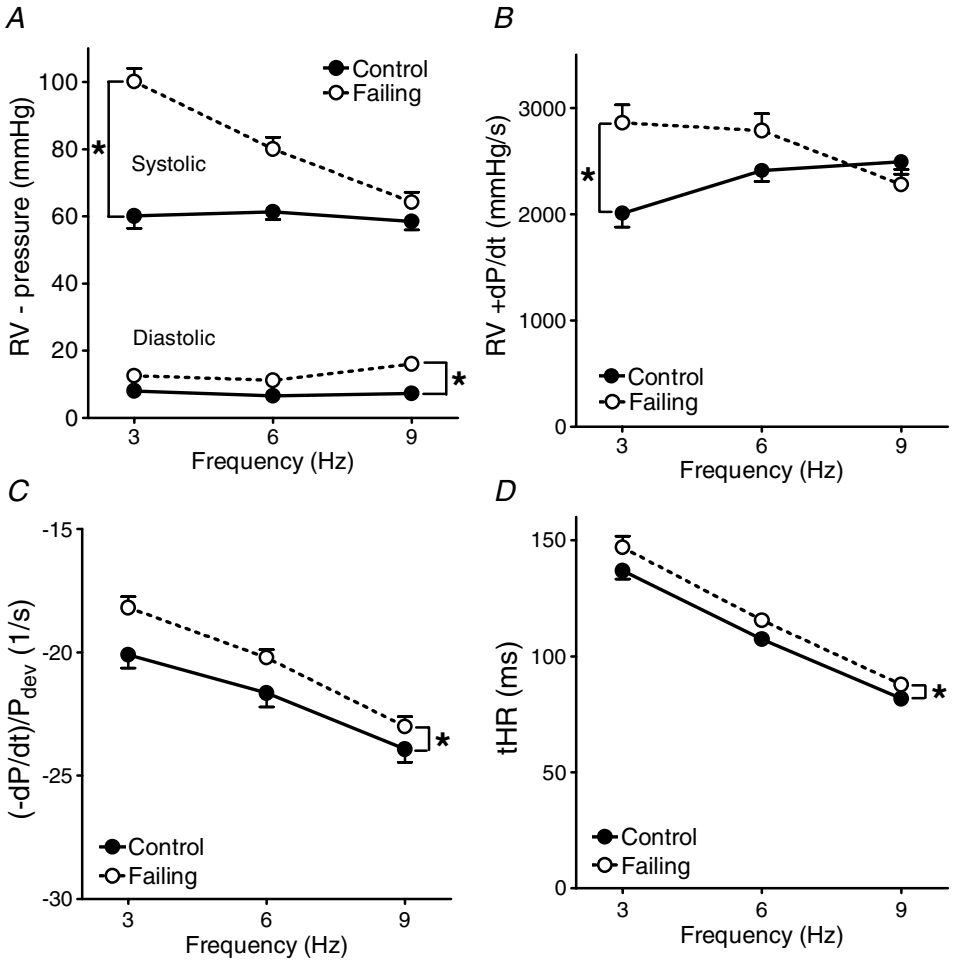

Figure 5. Averaged diastolic and systolic RV pressures (*A*), +d*P*/d*t* (*B*), (−d*P*/d*t*)/P_{dev} (C) and tHR (*D*) at different stimulation frequencies in control and failing Langendorff perfused hearts
In the control group a flat pressure–frequency relation and positive +dP/dt_{max}–frequency relation was found, whereas in the failing group a negative pressure–frequency and +dP/dt_{max}–frequency relation was found. In the failing group, relaxation (*C* and *D*) was slower, but the frequency-dependent acceleration of relaxation remained. Values are expressed as means ± S.E.M. $^*P < 0.05$ *versus* control.

(data not shown). In Fig. 9*A* is shown the total amount of protein expression of PKCα of control (pooled, $n = 10$) and failing hearts (individual, $n = 10$). In the failing hearts, the amount of PKCα protein expression is significantly increased compared with controls. On a second gel, with control (individual, $n = 10$) and failing hearts (pooled, $n = 10$), a similar increase in the amount of PKCα protein expression was found (data not shown). In Fig. 9*B* is shown the phosphorylation levels of PKCα, normalized to the amount of PKCα protein, of control and failing hearts at 0 or 9 Hz stimulation frequencies. The phosphorylation level of PKCα was increased by frequency in the failing hearts ($P < 0.05$), but not in controls.

Discussion

In the control group a positive force–frequency relation was found with a considerable increase in amplitude of the $[Ca^{2+}]_i$ transient with frequency, while Ca^{2+} sensitivity of force, measured in permeabilized cardiomyocytes, remained constant. In the failing group, the negative force–frequency relation was accompanied by a small increase in amplitude of the $[Ca^{2+}]_i$ transients with frequency and a frequency-dependent *decrease* in Ca^{2+} sensitivity. Thus, frequency-dependent Ca^{2+} desensitization of the myofilaments contributes to the negative force–frequency relation in failing rat myocardium This frequency-dependent Ca^{2+} desensitization was associated with a frequency-dependent increase in phosphorylation of TnI, MLC2 and PKCα.

Frequency dependency of force and phosphorylation in the control group

Under physiological conditions, a positive force–frequency relation is found in a number of mammalian species

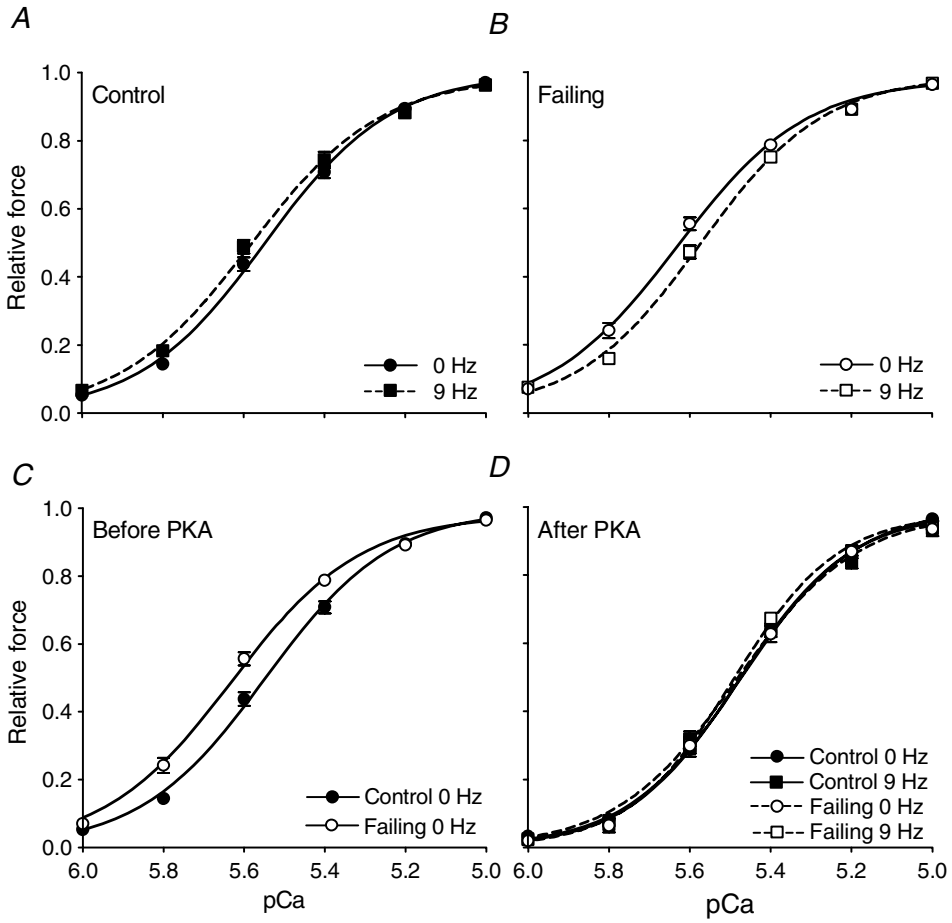

Figure 6. Isometric force–pCa relations in isolated skinned cardiomyocytes from control (*A*) and failing (*B*) quiescent (0 Hz) or stimulated (9 Hz) right ventricles before (*C*) and after (*D*) PKA treatment
In the failing cardiomyocytes the force–pCa curve shifted to the right with an increase in frequency. At 0 Hz, the force–pCa relation was shifted leftward in failing compared with control cardiomyocytes. After PKA treatment the force–pCa curves were similar for all groups. Values are expressed as means ± S.E.M. from $n = 10$ cardiomyocytes in each group.

including man. The increase in sarcolemmal Ca^{2+} influx per unit of time, resulting in increased SR Ca^{2+} loading, is well established as an underlying mechanism for the increase in myocardial force (Gao *et al.* 1998; Layland & Kentish, 1999; Pieske *et al.* 1999; Stuyvers *et al.* 2002). However, because of the highly non-linear force–Ca^{2+} relation, a possible change in myofilament Ca^{2+} sensitivity, through phosphorylation of contractile proteins, cannot be ruled out as a contributing factor. For instance, experiments on rabbit septal preparations revealed a significant frequency-dependent increase in MLC2 phosphorylation (Silver *et al.* 1986; Sweeney *et al.* 1993). Also, a positive correlation was found between heart rate and MLC2 phosphorylation in rats studied at different levels of treadmill exercise or β-adrenergic stimulation and inhibition (Fitzsimons *et al.* 1989). More recently, a decreased Ca^{2+} sensitivity was observed in rabbit trabeculae at higher frequencies, which was accompanied by changes in MLC2 and TnI phosphorylation (Varian & Janssen, 2007).

Our experiments on permeabilized isolated isometric contracting cardiomyocytes (Fig. 6*A*, Table 2) revealed that Ca^{2+} sensitivity was not affected by an increase in frequency in the control group. Moreover, our data revealed that phosphorylation levels of MLC1, MLC2, MyBP-C, TnT and TnI of isovolumic contracting control hearts were not frequency dependent. This is in line with the results of Takimoto *et al.* (2004) who showed in transgenic mice with aspartic acid substitutions for the serine sites targeted by PKA on TnI, mimicking constitutive phosphorylation at the PKA sites, that the frequency-dependent increase in isometrically contracting trabeculae was not affected. In our study, in isometrically contracting control cardiomyocytes, PKA treatment, which does not affect MLC2 phosphorylation (Strang *et al.* 1994), induced a shift in Ca^{2+} sensitivity which was similar in quiescent and in paced (9 Hz) hearts (Fig. 6*C* and *D*). This supports the concept that TnI phosphorylation may enhance the frequency-dependent regulation of cardiac contraction in the *in vivo* working heart, but not in

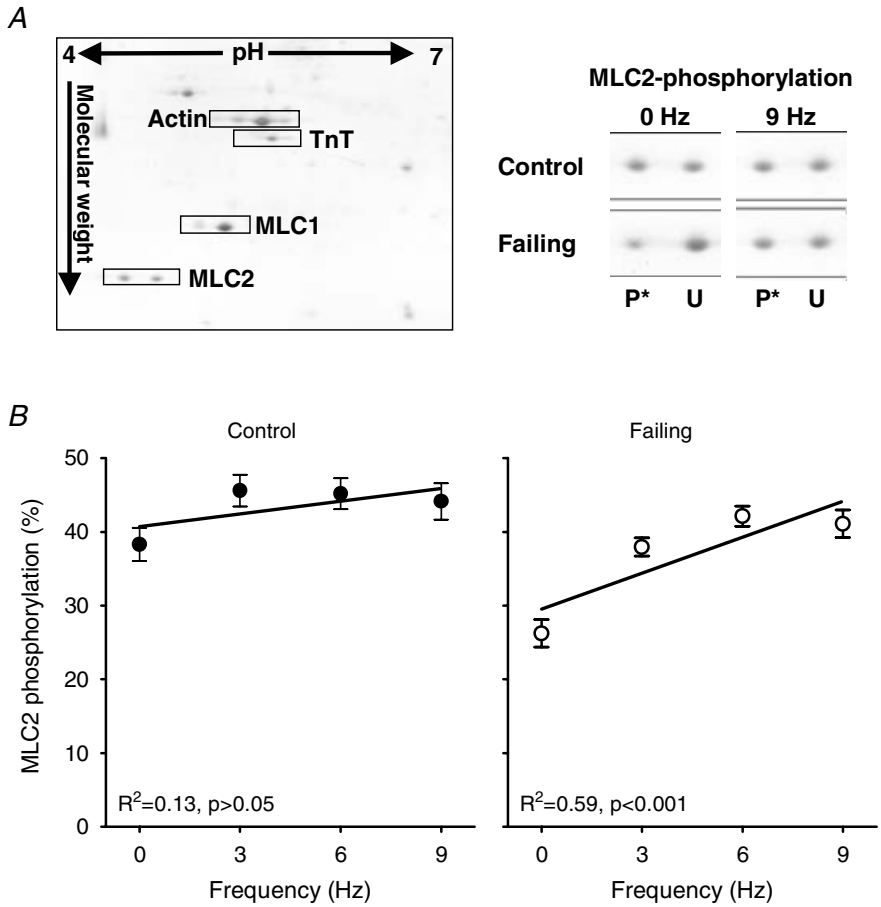

Figure 7. MLC2 phosphorylation at different stimulation frequencies in control and failing hearts
A, total coomassie stained 2-D gel of RV tissue of a control heart at 3 Hz (left panel) and specific MLC2 sections (right panel) of control and failing hearts at 0 and 9 Hz. *B*, frequency dependency of MLC2 in failing hearts but not in control. Abbreviations used: MLC2, myosin light chain 2; U, unphosphorylated; and P*, phosphorylated. Values are expressed as means ± S.E.M., $n = 5$ per frequency.

isometric contractions (Layland & Kentish, 2002; Takimoto *et al.* 2004). Recently, it was also demonstrated in transgenic mice, which expressed a non-phosphorylatable MLC2 protein, that phosphorylation of MLC2 was not an essential determinant of the force–frequency relation (Dias *et al.* 2006).

Thus, our experiments in control hearts are consistent with the notion that the positive force–frequency relation is mainly due to an increase in sarcolemmal Ca^{2+} influx per unit of time and that alterations in Ca^{2+} sensitivity, through phosphorylation of contractile proteins, do not contribute to the positive force–frequency relation in healthy myocardium.

Frequency dependency of force and phosphorylation in the failing group

The negative, blunted or flat force–frequency relation is a hallmark of the failing heart (Gwathmey *et al.* 1990; Mulieri *et al.* 1992; Schwinger *et al.* 1993; Eising *et al.* 1994; Pieske *et al.* 1995, 1999; Janssen *et al.* 2000; Brixius *et al.* 2002; Kogler *et al.* 2003), and has mainly been attributed to alterations in Ca^{2+} handling (Pieske *et al.* 1999). Altered Ca^{2+} homeostasis in failing hearts was evident from a reduced amplitude of the $[Ca^{2+}]_i$ transient (Fig. 2), an increased sensitivity of developed force to extracellular calcium (Fig. 4) and an elevated systolic function at low pacing frequencies (Figs 4 and 5). The half-times of relaxation of force of the trabeculae and isolated hearts were prolonged, consistent with the deceleration of Ca^{2+} re-uptake and Ca^{2+} extrusion (Pieske *et al.* 1999; Bers *et al.* 2003), which was supported by the prolonged relaxation of the $[Ca^{2+}]_i$ transients in the failing muscles (Fig. 1). The maximal forces (F_{max}) attained in trabeculae during post-extrasystolic potentiation and in skinned cardiomyocytes were the same in control and failing hearts. Together with the increased force at low stimulation frequencies in failing trabeculae this indicates that the contractile capacity of failing cardiomyocytes is not impaired but that developed force is closer to saturation, which reduces contractile reserve (Korstjens *et al.* 2002; Kogler *et al.* 2003), probably due to enhanced SR load (Pieske *et al.* 1999; Bers *et al.* 2003).

In our animals with heart failure, the Ca^{2+} sensitivity was increased under basal conditions, which is consistent with findings in the MCT model (Kogler *et al.* 2003) and in

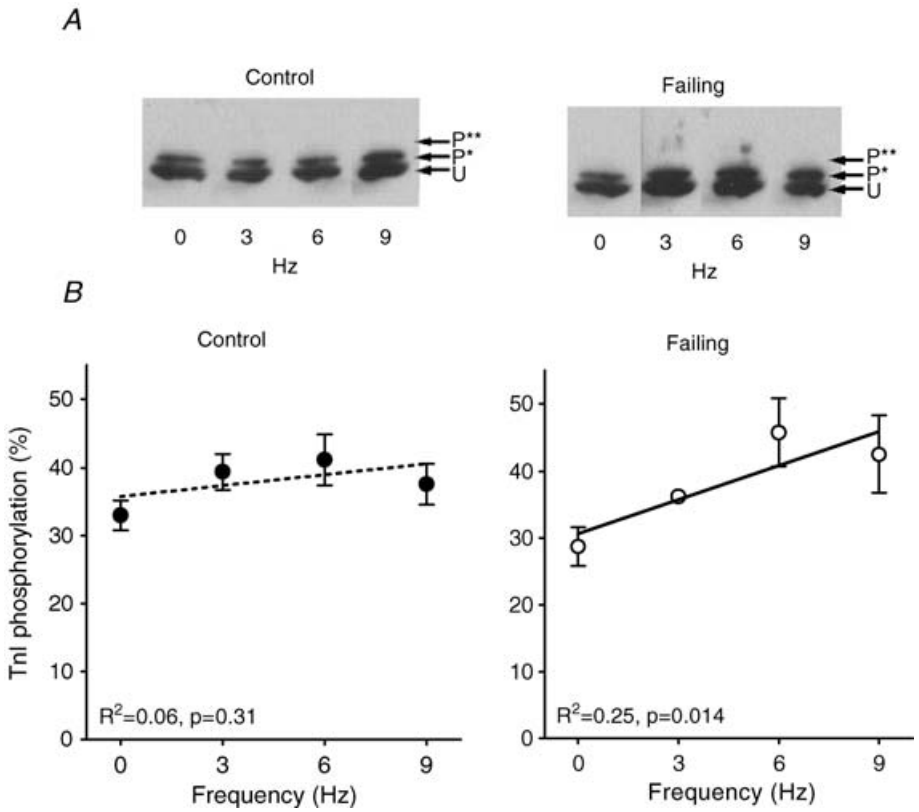

Figure 8. TnI phosphorylation at different stimulation frequencies in control and failing hearts
A, Western immunoblot of a NEIEF gel, stained with TnI antibody of failing hearts. *B*, frequency dependency of TnI in failing hearts but not in control. Abbreviations used: TnI, troponin I; U, unphosphorylated; P*, monophosphorylated; and P**, biphosphorylated. Values are expressed as means ± S.E.M. (Control: 0 Hz ($n = 4$); 3, 6 and 9 Hz ($n = 5$); Failing: 3 Hz ($n = 5$), 0, 6 and 9 Hz ($n = 6$).

human failing myocardium (van der Velden *et al.* 2003*a*,*b*). Interestingly, in failing trabeculae the net influx of Ca^{2+} per unit of time increased with an increase in frequency (Fig. 3) and the relaxation of the $[Ca^{2+}]_i$ transient was prolonged (Fig. 1). Thus, with Ca^{2+} sensitivity increased, and force not yet saturated (Fig. 4) in failing hearts this would be expected to accentuate the frequency-dependent alterations in Ca^{2+} handling resulting in increased myocardial force at higher frequencies. Nevertheless the force–frequency relation is negative and this reflects a frequency-dependent desensitization of the myofilaments.

The increased basal Ca^{2+} sensitivity in failing myocardium cannot be explained by alterations in phosphorylation of MLC2 or TnI. Recently, the importance of site-specific alterations in phosphorylation of TnI on the force–frequency relation was demonstrated in transgenic mice (Bilchick *et al.* 2007). Hence, the most likely explanation is that the intricate alterations in phosphatases and kinases involved result in a specific pattern of phosphorylation, and that also the location of phosphorylation site(s) may be important to explain the functional effects.

Frequency-induced changes in contraction and Ca^{2+} handling can be different at temperatures lower than body temperature (Layland & Kentish, 1999; Janssen *et al.* 2002); however, in our trabeculae at 27 and 37°C we found qualitatively similar results in the frequency-induced changes in force and $[Ca^{2+}]_i$. Thus, the frequency-dependent Ca^{2+} desensitization of the contractile apparatus also exists in failing hearts at body temperature. This is confirmed by the direct Ca^{2+} sensitivity measurements in the isolated cardiomyocytes in this study (Fig. 6*B*, Table 2).

As mentioned above, a frequency-dependent increase in MLC2 phosphorylation has been found in healthy myocardium (Silver *et al.* 1986; Fitzsimons *et al.* 1989; Sweeney *et al.* 1993). From our study it is clear that the frequency-dependent increase in MLC2 phosphorylation is also present in failing rat myocardium (Fig. 7*B*). This frequency dependence of MLC2 phosphorylation may originate from a CaMK-II-dependent increase in MLCK activity. MLC2 phosphorylation results in a stereospecific increase in the probability of force-generating cross-bridge formation (Sweeney *et al.* 1993; Olsson *et al.* 2004). However, since MLC2 phosphorylation leads to increased Ca^{2+} sensitivity (Sweeney *et al.* 1993; Olsson *et al.* 2004), it cannot explain the frequency-dependent Ca^{2+} desensitization observed in failing cardiomyocytes. Thus, the frequency-dependent phosphorylation of MLC2 counteracts a more potent mechanism responsible for the negative force–frequency relation in heart failure.

The frequency dependence of Ca^{2+} sensitivity of the contractile apparatus in failing cardiomyocytes was abolished by PKA treatment (Fig. 6*D*). TnI and myosin

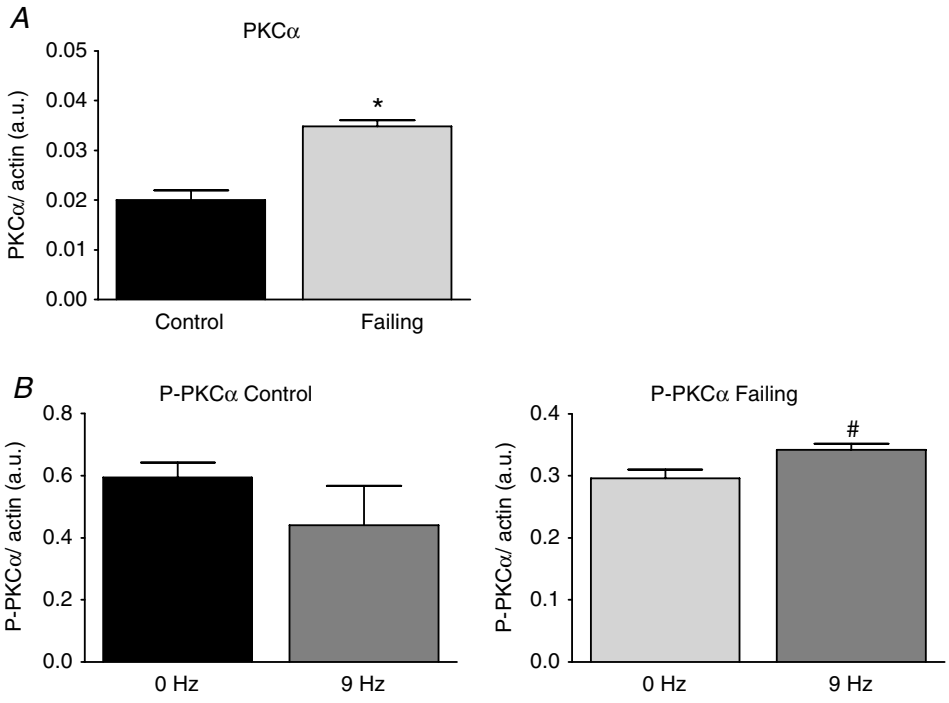

Figure 9. Expression and phosphorylation levels of PKCα in quiescent (0 Hz) and stimulated (9 Hz) control and failing hearts

A, increased expression of PKCα in failing hearts compared with control. *B*, frequency dependency of PKC phosphorylation in failing hearts but not in control. Abbreviations used: PKCα, protein kinase C α; P-PKCα, phosphorylated PKCα. Values are expressed as means ± S.E.M. $^*P < 0.05$ *versus* control, $\#P < 0.05$ *versus* 0 Hz.

binding protein C (MyBP-C) represent the main targets for PKA (Strang *et al.* 1994), and phosphorylation of both proteins is accompanied by a decreased Ca^{2+} sensitivity (Garvey *et al.* 1988; Harris *et al.* 2002). In our failing hearts, MyBP-C phosphorylation remained constant, while TnI phosphorylation increased with frequency (Fig. 8*B*). This indicates that phosphorylation of TnI and not MyBP-C is responsible for the frequency-dependent Ca^{2+} desensitization in failing myocardium. Furthermore, in quiescent cardiomyocytes the shift upon PKA treatment was larger in the failing group than in controls. However, at 9 Hz the PKA-induced shift was not different between failing and controls. This is in agreement with the observation that β-adrenergic stimulation blunts the force–frequency relation in failing myocardium (Schwinger *et al.* 1993; Eising *et al.* 1994). The absence of a significant shift in Ca^{2+} sensitivity by PKA in failing hearts is not in conflict with the modest frequency-dependent increase in MLC2 phosphorylation ($26.2 \pm 1.9\%$ at 0 Hz to $41.1 \pm 1.9\%$ at 9 Hz), as was found by others in rat (Olsson *et al.* 2004) and mice (Stelzer *et al.* 2006). Together these results indicate that the functional implications of the increase in MLC2 phosphorylation observed is only modest, as can be deduced also from the results observed in transgenic mice with a non-phosphorylatable MLC2 protein (Dias *et al.* 2006).

What could be the reason for the frequency-dependent increase in TnI phosphorylation and why is it evident only in failing hearts? Since the time-averaged intracellular Ca^{2+} concentration increases with pacing frequency, the answer to these questions should reside in an altered balance of Ca^{2+}-dependent kinase or phosphatase activity. Hence the main candidates would be the classical Ca^{2+}-dependent isoforms of PKC and the Ca^{2+}-dependent phosphatase calcineurin (PP2B). Both enzymes are up-regulated in heart failure (Bowling *et al.* 1999; Lim & Molkentin, 1999; Braz *et al.* 2004) and thus their contribution may be more conspicuous in failing than in control hearts. In agreement with these findings, our study clearly shows an increase in protein expression of the Ca^{2+}-dependent PKCα isoform in failing hearts (Fig. 9*A*).

Evidence suggests that PKCs phosphorylate not only the PKC-specific sites on TnI (Ser-42, Ser-44 and Thr-143), but also the PKA sites (Ser-23 and Ser-24) (Swiderek *et al.* 1990; Noland *et al.* 1995; Kobayashi *et al.* 2005). Recently, in transgenic mice with mutated PKA-TnI sites, to mimic dephosphorylation, and PKC sites mutated, to mimic constitutive phosphorylation, a blunted force–frequency response was found (Bilchick *et al.* 2007). Our study revealed a modest but significant increase in the phosphorylation level of PKCα with frequency in the failing hearts (Fig. 9*B*). This suggests a novel role for PKC-mediated TnI phosphorylation in modulating the force–frequency relation. Altered expression of PP2B might be involved as well, but only if this phosphatase would relieve the inhibition of a kinase involved in the phosphorylation of TnI, a route which might involve PKCs as well.

Conclusion

In control hearts, the positive force–frequency relation is primarily due to an increase in Ca^{2+} influx per unit of time. In failing rat hearts, frequency-dependent Ca^{2+} desensitization of the myofilaments together with alterations in Ca^{2+} homeostasis contributes to the negative force–frequency relation in failing rat myocardium. This frequency-dependent Ca^{2+} desensitization was associated with a frequency-dependent increase in phosphorylation of TnI and was counteracted by a frequency-dependent increase in MLC2 phosphorylation. This suggests that the negative force–frequency relation can be opposed by Ca^{2+} sensitizers and may explain why levosimendan improves the negative force–frequency relation in human failing myocardium (Janssen *et al.* 2000; Brixius *et al.* 2002) and acts more pronounced at higher heart rates (Janssen *et al.* 2000).

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