Functional effects of protein kinase C-mediated myofilament phosphorylation in human myocardium

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Abstract

Objective: In human heart failure β-adrenergic-mediated protein kinase A (PKA) activity is down-regulated, while protein kinase C (PKC) activity is up-regulated. PKC-mediated myofilament protein phosphorylation might be detrimental for contractile function in cardiomyopathy. This study was designed to reveal the effects of PKC on myofilament function in human myocardium under basal conditions and upon modulation of protein phosphorylation by PKA and phosphatases.

Methods: Isometric force was measured at different [Ca2+] in single permeabilized cardiomyocytes from non-failing and failing human left ventricular tissue. Basal phosphorylation of myofilament proteins and the influence of PKC, PKA, and phosphatase treatments were analyzed by one- and two-dimensional gel electrophoresis, Western immunoblotting, and ELISA.

Results: Troponin I (TnI) phosphorylation at the PKA sites was decreased in failing compared to non-failing hearts and correlated well with myofilament Ca2+ sensitivity (pCa50). Incubation with the catalytic domain of PKC slightly decreased maximal force under basal conditions, but not following PKA and phosphatase pretreatments. PKC reduced Ca2+ sensitivity to a larger extent in failing (ΔpCa50 = 0.19 ± 0.03) than in non-failing (ΔpCa50 = 0.08 ± 0.01) cardiomyocytes. This shift was reduced, though still significant, when PKC was preceded by PKA, while PKA following PKC did not further decrease pCa50. Protein analysis indicated that PKC phosphorylated PKA sites in human TnI and increased phosphorylation of troponin T, while myosin light chain phosphorylation remained unaltered.

Conclusion: In human myocardium PKC-mediated myofilament protein phosphorylation only has a minor effect on maximal force development. The PKC-mediated decrease in Ca2+ sensitivity may serve to improve diastolic function in failing human myocardium in which PKA-mediated TnI phosphorylation is decreased.

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Keywords: Protein kinase C; Heart failure; Myofilament function; Protein phosphorylation

1. Introduction

Cardiac pump function is controlled by β-adrenergic receptor-mediated activation of PKA [1] and by endothelin, angiotensin II or α-adrenergic receptor-mediated activation of PKC [2]. During the development of cardiac disease the contributions of endothelin, angiotensin II and adrenergic stimuli to cardiac pump function change in an intricate way...
via complex patterns of receptor up- or down-regulation and/or enhanced stimulation [1,3,4]. Due to β-adrenergic desensitization and receptor down-regulation, PKA activity is decreased in human heart failure, while an increase in PKC has been reported [5–8]. Consequently, the regulatory role of PKC in cardiac contractility will be enhanced in diseased human myocardium.

Myocardial contraction is partly regulated via phosphorylation of myofilament proteins. PKA-mediated TnI phosphorylation at serines 23/24 decreases myofilament Ca\(^{2+}\)-sensitivity [9,10] and contributes to an acceleration of cardiac relaxation [11,12]. In vitro studies [13,14] have shown that Tnl, troponin T (TnT), myosin light chain 2 (MLC-2) and myosin binding protein C (MyBP-C) are the myofilament substrates for PKC. In addition to phosphorylation of Tnl at PKC-sites (serines 43/45, threonine 144), PKC could phosphorylate PKA-sites (serines 23/24) [15–18]. Functional studies in rodent myocardium indicated a central role for PKC-mediated phosphorylation of Tnl (serines 43/45) [19] and TnT [20] in decreasing maximal actomyosin Mg-ATPase activity [20–22] and maximal force [19,20,23]. In a recent in vitro study on human myocardial thin filaments using in vitro motility assays an increased PKC isoform expression was related to a decrease in an index of thin filament related maximal force, suggesting that this also may be the case in human tissue [8]. The direction of the effect of PKC on myofilament Ca\(^{2+}\)-sensitivity in studies in rodent myocardium is still a matter of debate [20–23]. Despite recent advances in rodents, little is known about the effect of PKC on myofilament protein phosphorylation and function in human myocardium.

The effect of PKC on myofilament function depends on the combined activities of kinases/phosphatases present in the heart, which set different basal phosphorylation levels of myofilament proteins in healthy and diseased myocardium. In this respect, we have shown that dephosphorylation of MLC-2 by protein phosphatase-1 (PP-1), which is up-regulated in human heart failure [5,24], decreased myofilament Ca\(^{2+}\)-sensitivity to a larger extent in failing than in non-failing human myocardium, although basal MLC-2 phosphorylation was significantly lower in failing hearts [25]. Therefore, to reveal the full dynamic range of PKC on myofilament function it is prerequisite to define and modulate the basal phosphorylation status of myofilament proteins present within the heart.

The present study was designed to investigate whether or not PKC exerts a different effect on myofilament function in non-failing and failing human myocardium, which exhibit inherent differences in basal myofilament phosphorylation status [10]. Moreover, the dynamic range of PKC was explored in detail by modulation of basal protein phosphorylation status by PKA and phosphatases. Since several PKC isoforms are expressed in the myocardium, we studied the effect of the highly conserved catalytic domain of PKC on force in single permeabilized cardiomyocytes from donor and end-stage failing human myocardium. Protein analysis was performed to characterize basal phosphorylation status of myofilament target proteins for PKC and to assess the effects of the kinase/phosphatase treatments.

2. Methods

2.1. Biopsies

Left ventricular (LV) transmural tissue samples were obtained during heart transplantation surgery from 7 patients with end-stage dilated cardiomyopathy (NYHA IV) and from 5 non-failing donors (Table 1). The tissue was collected in cardioplegic solution and 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 [Cardiovasc Res 1997;35:2–4].

2.2. Force measurements

Cardiomyocytes were mechanically isolated, Triton-permeabilized and mounted in the experimental set-up at a sarcomere length of 2.2 μm as described previously [10,25]. During mechanical isolation of cardiomyocytes no phosphatase inhibitors were present. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) did not reveal differences in the phosphorylation status of contractile proteins between cardiomyocyte-samples taken before and after mechanical isolation [25]. The pCa (−log;\(_{10}([Ca^{2+}])\) of the solutions ranged from 9 (relaxing) to 4.5 (maximal activating). After maximal activation 4 to 5 measurements were carried out at sub-maximal [Ca\(^{2+}\)] (pCa > 4.5) by a maximal activation. Force values obtained in solutions with sub-maximal [Ca\(^{2+}\)] were normalized to the interpolated maximal force values (pCa 4.5). The decrease in maximal force during a force–pCa series did not significantly differ between cardiomyocytes from donor (14 ± 1%) and failing (12 ± 1%) hearts. Rate of force redevelopment

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Characteristics of donors and patients</th>
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<tr>
<td>Sex</td>
<td>Age</td>
</tr>
<tr>
<td>Donor 1</td>
<td>Female</td>
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<tr>
<td>Donor 2</td>
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<td>Patient 5</td>
<td>Male</td>
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<tr>
<td>Patient 6</td>
<td>Female</td>
</tr>
<tr>
<td>Patient 7</td>
<td>Female</td>
</tr>
</tbody>
</table>

Abbreviations: EF, ejection fraction; LVEDVI, LV end-diastolic volume index; ACEI, angiotensin-converting-enzyme inhibitors; Amio, amiodarone; Diu, diuretics; \(\beta\), beta-blocker.
was determined at pCa values ranging from 4.5 to 5.6 using the slack-test [25]. At low [Ca\(^{2+}\)] (pCa>5.6) force redevelopment could not be fitted accurately due to the low signal-to-noise ratio.

After the initial force–pCa series, the myocyte was incubated for 40 min at 20 °C in relaxing solution containing 6 mmol/l dithiothreitol (DTT) without kinase (time-control), with the catalytic domain of PKC (Sigma batch 93K0330; 0.25 U/ml) or the catalytic subunit of PKA (Sigma batch 35H9522; 100 U/ml). After incubation the force–pCa series was repeated (Fig. 1). In several cardiomyocytes a third force–pCa series was performed to study sequential effects, i.e. PKC after PKA or visa versa. All force measurements were performed at 15 °C.

The effect of PKC on Ca\(^{2+}\)-sensitivity was also studied upon pretreatment with alkaline phosphatase (AP; calf intestinal; New England BioLabs; 1000 U/ml) for 60 min in relaxing solution containing 6 mmol/l dithiothreitol (DTT) without protease inhibitor cocktail (PIC; 1000 U/ml; Sigma) with AP (1000 U/ml) or protein phosphatase-2A\(_{1}\) (PP2A\(_{1}\); bovine kidney; Calbiochem; 1 U/ml) [8] which was followed by a PKC incubation and subsequent \(F_{\text{max}}\) measurement.

### 2.3. Analysis of endogenous protein phosphorylation

Enzyme-linked-immunosorbent-assay (ELISA) was used to determine basal phosphorylation of TnI using specific monoclonal antibodies against total TnI (phosphorylated and dephosphorylated; clone 16A11; Research Diagnostics) and against dephosphorylated TnI (antibody against dephosphorylated PKA-sites; serines 23/24; clone 22B11; Research Diagnostics). This latter antibody does not cross-react with mono- or bisphosphorylated TnI [26]. Tissue samples were treated with trichloroacetic acid (TCA) to preserve the phosphorylation status of myofilament proteins. Homogenates of 0.5 μg/μl total protein concentration were diluted in phosphate-buffered-saline (PBS-HCl) and applied in triplicate to ELISA plates. Plates were incubated overnight at 4 °C. Specific binding sites were blocked with PBS-HCl containing 5% milk and 0.3% (v/v) Tween-20 for 1 h at room temperature. Thereafter, plates were incubated with specific TnI antibodies for 1 h at room temperature. TnI antibody binding was detected using polyclonal goat-antimouse immunoglobulins/HRP (Dako Cytomation). To visualize the residual immunocomplexes, plates were incubated with 0.1% (w/v) tetra-methyl-benzidine and 0.02% hydrogen peroxide in 0.11 M sodium acetate (pH 5.5). The color reaction was stopped by adding 1 N \(\text{H}_2\text{SO}_4\). The plates were read at 450 nm (Dynatech, MR 7000) relative to a blank obtained by adding PBS to the wells instead of tissue homogenate in the first step. Variability of the triplicate measurements obtained for each biopsy sample was less than 5%.

TnI phosphorylation was quantified using purified non-phosphorylated human cardiac TnI protein as a standard. TnI calibration curves for both antibodies were linear up to 5 ng TnI. All TnI values were well within the linear range. The level of basal TnI dephosphorylation present in the tissue samples was obtained by normalizing the signal of dephosphorylated TnI (22B11) to that of total TnI (16A11). The same antibodies were employed in Western immunoblotting for analysis of the TnI phosphorylation status in myocardial tissue treated with kinase or phosphatase (see below).

2D-PAGE was performed to determine the endogenous phosphorylation status of myosin light chain 1 (MLC-1), MLC-2 and TnT [10,25]. Samples, containing DeStreak Reagent (Amersham Biosciences; 15 mg/ml rehydration solution) to prevent protein oxidation were loaded on immobiline strips with a pH gradient of 4.5 to 5.5 (Amersham Pharmacia Biotech). In the second dimension proteins were separated by SDS-PAGE using an acrylamide to bis-acrylamide ratio of 25:1 in the stacking gel (3.5% acrylamide, pH 9.3) and of 20:1 in the separating gel (3.5% acrylamide, pH 6.8). Gels were stained with Coomassie blue, scanned and analyzed using Image Quant (Molecular Dynamics).

### 2.4. Alterations in myofilament protein phosphorylation

To investigate the effect of PKC, PKA and AP on myofilament protein phosphorylation 400 μg (dry weight) of tissue was incubated for 60 min at 20 °C in 60 μl of (1) relaxing solution containing 0.4 μl tributylphosphine (TTP) and 0.2 μl PIC (control), (2) relaxing solution with 0.4 μl TTP, 0.2 μl PIC, 10 μl phosphatase inhibitor cocktail (P2850; Sigma) and PKC (17 U/ml) or PKA (2500 U/ml) or (3) relaxing solution with 0.4 μl TTP, 0.2 μl PIC and AP (2500 U/ml). Subsequently, tissue was treated with TCA and analyzed by 2D-PAGE (350 μg dry weight) to determine phosphorylation of MLCs and TnT and by 1D-PAGE (1 μg dry weight) containing 15% total acrylamide (acrylamide to bis-acrylamide ratio 37.5:1) followed by...
3. Results

3.1. Basal myofilament function and phosphorylation status

Force measurements were performed in 34 cardiomyocytes from 5 donor hearts and in 29 myocytes from 7 failing hearts. Mean dimensions of the preparations did not differ between both groups. Average maximally developed isometric tension ($F_{\text{max}}$) was slightly, though not significantly, higher in donor than in failing hearts (31.9 ± 2.6 versus 27.7 ± 2.1 kN/m²). Passive force ($F_{\text{pas}}$) did not significantly differ between the two groups (3.8 ± 0.3 and 5.1 ± 0.8 kN/m² in donor and failing, respectively). Ca$^{2+}$-sensitivity of force ($\text{pCa}_{50}$) was significantly increased in failing (5.74 ± 0.03) compared to donor (5.56 ± 0.01) myocardium, while the steepness of the normalized force–Ca²⁺ curves, nh, was significantly lower in failing (2.34 ± 0.13) than in donor (2.96 ± 0.16) hearts (Fig. 2A). A significant positive correlation ($r^2=0.58$; $P<0.05$) was present between $F_{\text{pas}}$ and LV end-diastolic volume index (LVEDVI; Table 1). No other significant correlations were found between cardiomyocyte force characteristics and clinical patient data.

Basal phosphorylation of the PKA-sites of TnI determined by ELISA was significantly higher in donor compared to failing myocardium. This was also evident from Western immunoblotting (inset Fig. 2B). The average level of dephosphorylated TnI (i.e. dephosphorylated both at serine 23 and 24) to total TnI amounted to 3.1% ± 1.7% in donor and 24.1% ± 4.2% in failing myocardium ($P<0.05$). In Fig. 4B a 2D-gel is shown from failing myocardium, which illustrates basal phosphorylation of TnT, MLC-1 and MLC-2. Noteworthy, our previous 2D-PAGE [25] revealed 4 protein spots at the level of MLC-1, which were all recognized by a specific antibody against MLC-1. However, these 4 spots do not represent different isoforms or phosphorylated forms of MLC-1, but are oxidative modifications of thiol groups, since addition of DeStreak Reagent (Methods section), which transforms thiol groups into a stable disulphide, reduced the number of MLC-1 spots to two. Hence our present 2D-gels (Fig. 4B) allow quantification of the unphosphorylated and the phosphorylated forms of MLC-1. Phosphorylation of both MLC-1 and MLC-2 was significantly lower in failing compared to donor myocardium. The amount of phosphorylated TnT did not differ between donor and failing hearts. An overview of basal protein phosphorylation is given in Table 2.

<table>
<thead>
<tr>
<th>Myofilament protein</th>
<th>Donor</th>
<th>Donor</th>
<th>Donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>TnIP (ELISA)</td>
<td>69.9 ± 1.7</td>
<td>75.9 ± 4.2*</td>
<td></td>
</tr>
<tr>
<td>TnTP</td>
<td>63.2 ± 4.6</td>
<td>63.3 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>MLC-1P</td>
<td>12.3 ± 2.2</td>
<td>5.6 ± 0.4*</td>
<td></td>
</tr>
<tr>
<td>MLC-2</td>
<td>36.2 ± 3.5</td>
<td>53.5 ± 3.0*</td>
<td></td>
</tr>
<tr>
<td>MLC-2*</td>
<td>20.4 ± 1.2</td>
<td>18.9 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>MLC-2P</td>
<td>32.4 ± 2.7</td>
<td>21.8 ± 2.5*</td>
<td></td>
</tr>
<tr>
<td>MLC-2*P</td>
<td>11.0 ± 1.7</td>
<td>5.8 ± 0.7*</td>
<td></td>
</tr>
</tbody>
</table>

Protein values, obtained in ELISA (TnI) or 2D-gel analysis, are given as percentage of total TnI. TnT, MLC-1 or 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 failing.
A significant correlation was also found between percentage of dephosphorylated TnI and pCa50 ($r^2 = 0.65; P < 0.05$) and nH ($r^2 = 0.37; P < 0.05$). Moreover, significant inverse correlations were observed between pCa50 and percentage of phosphorylated MLC-1 ($r^2 = 0.40; P < 0.05$) and of phosphorylated MLC-2 ($r^2 = 0.50; P < 0.05$).

### 3.2. Effect of PKC on myofilament function

Exogenous treatment of cardiomyocytes with PKC slightly decreased $F_{\text{max}}$ (~12% in both donor and failing cardiomyocytes), though this effect was not significant within the two groups (Table 3). Similarly, PKC treatment decreased $F_{\text{pas}}$ and the correlation between $F_{\text{pas}}$ and LVEDVI found under basal conditions was absent after PKC treatment ($r^2 = 0.05; \text{NS}$).

At sub-maximal [Ca$^{2+}$] force was markedly reduced after PKC (Fig. 1B), which reflects a decreased Ca$^{2+}$-sensitivity. This PKC induced decrease in Ca$^{2+}$-sensitivity was smaller in donor than in failing cardiomyocytes (Fig. 3A,B; Table 3). The PKC effect was saturated under our experimental conditions, because doubling of the PKC

### Table 3

<table>
<thead>
<tr>
<th></th>
<th>Donor</th>
<th>Failing</th>
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<tbody>
<tr>
<td></td>
<td>$F_{\text{max}}$</td>
<td>$F_{\text{pas}}$</td>
</tr>
<tr>
<td>Control incubation</td>
<td>4 hearts; 5 myocytes</td>
<td>5 hearts; 7 myocytes</td>
</tr>
<tr>
<td>Before</td>
<td>22.8 (4.2)</td>
<td>2.3 (0.5)</td>
</tr>
<tr>
<td>After</td>
<td>21.2 (4.3)</td>
<td>2.4 (0.6)</td>
</tr>
<tr>
<td>PKC incubation</td>
<td>5 hearts; 9 myocytes</td>
<td>7 hearts; 11 myocytes</td>
</tr>
<tr>
<td>Before</td>
<td>28.8 (2.5)</td>
<td>4.7 (0.4)</td>
</tr>
<tr>
<td>After</td>
<td>25.4 ($^*$.2.3)</td>
<td>4.0 ($^*$.0.4)</td>
</tr>
<tr>
<td>PKA incubation</td>
<td>4 hearts; 6 myocytes</td>
<td>4 hearts; 5 myocytes</td>
</tr>
<tr>
<td>Before</td>
<td>41.1 (7.6)</td>
<td>6.0 (1.4)</td>
</tr>
<tr>
<td>After</td>
<td>38.8 (7.5)</td>
<td>5.3 (0.7)</td>
</tr>
</tbody>
</table>

$F_{\text{max}}$ and $F_{\text{pas}}$ are given in kN/m$^2$. $^*P<0.05$, before versus after incubation in two-way ANOVA. $^1P<0.05$ in Bonferroni post hoc test. $^2P<0.05$, donor versus failing in two-way ANOVA. SEM is given between brackets.

$F_{\text{max}}$ and $F_{\text{pas}}$ are given in kN/m$^2$. A significant correlation was also found between percentage of dephosphorylated TnI and pCa50 (Fig. 2B; $r^2 = 0.65; P < 0.05$) and nH ($r^2 = 0.37; P < 0.05$). Moreover, significant inverse correlations were observed between pCa50 and percentage of phosphorylated MLC-1 ($r^2 = 0.40; P < 0.05$) and of phosphorylated MLC-2 ($r^2 = 0.50; P < 0.05$).
concentration (0.5 U/ml) nor prolongation of the incubation time (60 min) did not further decrease Ca\textsuperscript{2+}-sensitivity. Despite differences in basal Ca\textsuperscript{2+}-sensitivity values, similar pCa\textsubscript{50} values were obtained for failing and donor hearts after PKC treatment.

To compare the functional effects of PKA- and PKC-mediated myofilament phosphorylation donor and failing cardiomyocytes were also treated with PKA. The dose of PKA was based on a previous study [28], in which an activity of 100 U/ml proved to be saturating. In addition, experiments with different incubation times (ranging from 10 to 70 min) indicated that the maximal functional effect in human cardiomyocytes was reached after 30 min. In donor cardiomyocytes PKA caused a smaller shift in pCa\textsubscript{50} (\(\Delta p\text{Ca}_{50} = 0.04 \pm 0.01\)) compared to PKC. Similarly, in failing myocytes the shift upon PKA (\(\Delta p\text{Ca}_{50} = 0.13 \pm 0.03\); \(P < 0.05\)) was smaller compared to the shift upon PKC. In agreement with previous findings [10], \(F_{\text{max}}\) and \(F_{\text{pas}}\) were not affected by PKA in both groups. Time-controls did not significantly alter \(F_{\text{max}}\), \(F_{\text{pas}}\) or Ca\textsuperscript{2+}-sensitivity in both donor and failing tissue. The steepness of the force–pCa relations was significantly decreased during all incubations (with and without kinase) and seems to be an intrinsic property of the preparations. Force characteristics are summarized in Table 3.

Both in donor and failing cardiomyocytes a curvilinear relationship was found when \(K_r\) was plotted as a function of relative force (Fig. 3C,D), which is consistent with previous observations in skeletal and cardiac tissue [29,30]. After incubation without kinase (time-controls) or upon treatment with PKC (Fig. 3C,D) or PKA the relation between \(K_r\) and relative force did not differ from before incubation in both groups. This indicates that force-dependency of \(K_r\) remained unaltered upon kinase treatment.

Fig. 4. (A) Incubation of pure human TnI protein (50 ng/lane; left panels) and failing cardiac tissue (1 \(\mu\)g/lane; right panels) in relaxing solution with PKC or PKA increased phosphorylation status of TnI compared to the control incubation without kinase (C) as shown by Western immunoblotting using a specific antibody against dephosphorylated PKA-sites on TnI (serines 23/24). Values given indicate percentage of dephosphorylated PKA-sites compared to the value found for (C), which was set to 100% of dephosphorylated TnI. Within cardiac tissue samples the value for TnI was normalized to the signal obtained for TnT within the same sample. (B) Coomassie-stained 2D-gel (IEF, isoelectric focusing) to illustrate TnT, MLC-1 and MLC-2 composition in failing hearts. Phosphorylated forms are indicated with a P; MLC-2 is composed of two isoforms (2 and 2*), which are both partly phosphorylated. (C) Coomassie-stained gels to illustrate the effect of tissue incubations with PKC, PKA and alkaline phosphatase (AP) on phosphorylation status of TnT, MLC-1 and MLC-2. Control incubation (C) abolished phosphorylation of MLC-2, while TnT and MLC-1 phosphorylation were preserved compared to the basal phosphorylation status before incubation (BI). PKC increased TnT phosphorylation, but did not alter phosphorylation of the MLCs. PKA did not alter phosphorylation of TnT and both MLCs. AP abolished TnT phosphorylation resulting in a single TnT protein spot. 0P, 1P and 2P: no, mono- and bisphosphorylation, respectively.
3.3. Effect PKC on myofilament protein phosphorylation

It has been described that PKC is able to phosphorylate TnI at PKA-sites [15,18]. Indeed, Western blot analysis (Fig. 4A) of pure human TnI protein and failing myocardial tissue incubated without (Control) or with PKC or PKA, using the antibody against dephosphorylated PKA-sites on TnI (serines 23/24), showed that PKC was able to phosphorylate TnI in human cardiac tissue, although it was less potent than PKA. This can be seen from the decrease in the amount of dephosphorylated TnI upon incubation with PKC and PKA (Fig. 4A).

To further identify the myofilament targets of PKC, PKC treated tissue from a failing heart sample was analyzed by 2D-PAGE (Fig. 4C). During a control incubation of tissue in relaxing solution without added kinases (sample C), phosphorylation of TnT (left panel) was essentially identical (65.6%) to the basal TnT phosphorylation status of the tissue sample prior to the control incubation (sample BI, 66.5%). Phosphorylation of MLC-1 (middle panel) was only

Fig. 5. Upon the first force–pCa series the myocyte was incubated for 40 min with PKC or PKA, after which the force–pCa series was repeated. Thereafter, the cell was again incubated for 40 min with PKC or PKA and a third force–pCa series was performed. PKC further reduced Ca²⁺-sensitivity after PKA in donor (4 hearts, 6 cardiomyocytes) and failing (4 hearts, 5 cardiomyocytes) myocardium (A–C; P < 0.05 in repeated measures-ANOVA), while PKC treatment abolished the effect of PKA on Ca²⁺-sensitivity in both groups (5 cardiomyocytes from 4 donor (D) and 4 failing (E) hearts (D–F). No significant effect was found on Fmax. *Significant in post hoc Bonferroni’s Multiple Comparison Test.
slightly changed compared to basal MLC-1 phosphorylation, while both isoforms of MLC-2 (right panel) were completely dephosphorylated during the control incubation. PKC did not phosphorylate MLC-2 nor altered MLC-1 phosphorylation, while TnT phosphorylation was markedly increased (91.7%). Upon PKC treatment a shift occurred from the unphosphorylated TnT protein spot towards monophosphorylated (56.2%) and bisphosphorylated (35.5%) TnT. PKA did not phosphorylate MLC-2, TnT (68.4%) or MLC-1. Tissue incubated with alkaline phosphatase (AP) served as a “negative” control in order to establish the position of dephosphorylated protein forms. AP dephosphorylated TnT resulting in a single TnT protein spot, while MLC-1 phosphorylation remained unaltered.

![Image of 2D-gel showing PKC and PKA phosphorylation](image)

Fig. 6. (A) Silver-stained 2D-gel of the TnT region showing that PKC phosphorylated TnT upon AP treatment. (B) AP decreased, whereas PKC and PKA increased phosphorylation of TnI at serines 23/24 in failing cardiac tissue, expressed relative to the control incubation (C), which was set to 100% of dephosphorylated TnI. Upon dephosphorylation of myofilament proteins with AP Ca²⁺-sensitivity was decreased by PKC in both donor (C) and failing (D) cardiomyocytes. (E) Effect of PKC on $F_{\text{max}}$ in donor cardiomyocytes upon prior phosphatase incubation (AP or PP2A₁).
3.4. Effect of PKC after PKA pretreatment

The functional effects of PKC were also studied upon modulation of basal protein phosphorylation by pretreatment of cardiomyocytes with PKA. After PKA Ca\(^{2+}\)-sensitivity was further decreased upon PKC treatment both in donor and failing cardiomyocytes (Fig. 5A,B). Relative to the myofilament Ca\(^{2+}\)-sensitivity after PKA treatment, PKC significantly reduced pCa\(_{50}\) by 0.07±0.01 units in donor and by 0.06±0.01 units in failing cardiomyocytes (Fig. 5C). In contrast, after PKC pretreatment PKA was not able to further reduce Ca\(^{2+}\)-responsiveness (Fig. 5D–F). PKC did not alter F\(_{\text{max}}\) upon PKA pretreatment, nor did PKA alter F\(_{\text{max}}\) after PKC pretreatment.

3.5. Effect of PKC after phosphatase pretreatment

The functional effects of PKC were also studied upon dephosphorylation of myofilament proteins with phosphatase. 2D-gels of AP/PKC-treated tissue (Fig. 6A) showed that TnT phosphorylation was markedly reduced after AP and following PKC treatment increased to approximately 52%. AP also decreased TnI phosphorylation at serines 23/24 (Fig. 6B). After AP treatment, which did not significantly alter pCa\(_{50}\) or nH, PKC significantly decreased Ca\(^{2+}\)-sensitivity by 0.08±0.02 U in donor and by 0.18±0.03 units in failing cardiomyocytes (Fig. 6C,D). The lack of an effect of AP on Ca\(^{2+}\)-sensitivity is surprising. However, it could be due to the opposing actions of MLC-2 and TnT/TnI dephosphorylation on Ca\(^{2+}\)-sensitivity.

Recent studies in rodent myocardium indicated central roles for TnT and TnI phosphorylation in reducing F\(_{\text{max}}\) upon PKC activation [19,20,23]. To investigate if PKC could decrease the maximal force generating capacity in human myocardium under conditions where both TnT and TnI were dephosphorylated, F\(_{\text{max}}\) measurements were performed in donor cardiomyocytes pretreated with phosphatase (AP or PP2A). Both AP and PP2A treatments did not significantly alter F\(_{\text{max}}\). Moreover, PKC did not alter F\(_{\text{max}}\) both after treatment with AP (7 cardiomyocytes, 3 hearts) or PP2A (7 cardiomyocytes, 2 hearts)(Fig. 6E). Therefore, under conditions where a maximum effect of PKC would be expected, no effect on F\(_{\text{max}}\) was observed.

4. Discussion

4.1. Effect of PKC on myofilament Ca\(^{2+}\)-sensitivity

Our data extend previous observations [15–18] indicating that PKC is able to phosphorylate PKA-sites on TnI (Fig. 4A). Although PKC was less potent than PKA in phosphorylating serines 23/24, no additional effect of PKA on Ca\(^{2+}\)-sensitivity was observed (Fig. 5D–F), indicating that the maximal functional effect may be achieved without saturation of TnI phosphorylation at serines 23/24. However, the effect of PKC on Ca\(^{2+}\)-sensitivity did not originate solely from phosphorylation of the PKA-sites, since PKC further reduced myofilament Ca\(^{2+}\)-sensitivity after PKA treatment. Our results indicate that in human myocardium PKC decreases Ca\(^{2+}\)-sensitivity via phosphorylation of PKA-sites in TnI and via phosphorylation of PKC-sites within thin (TnI, TnT) and/or thick myofilament related proteins (MyBP-C, titin).

Until now, the effects of PKC have been mainly studied in rodent myocardium. These studies reported no change [16], a decrease [19,22] and an increase [21] in myofilament Ca\(^{2+}\)-sensitivity. These differences may be caused by the complexity of the PKC cascade involving different isoforms and site-specific phosphorylation. More detailed insight in the effects of PKC on Ca\(^{2+}\)-sensitivity has been obtained using site-specific phosphorylation of TnI mutant proteins [16,17,19]. From these studies it became apparent that several phosphorylation sites within TnI are crucial for regulation of Ca\(^{2+}\)-sensitivity [16]. PKC-mediated phosphorylation of PKA- or PKC-sites (serines 43/45, threonine 144) both decreased Ca\(^{2+}\)-sensitivity of in vitro MgATPase activity [16]. Force measurements in mouse muscle fibres [19] containing mutant TnI forms with specific phosphorylation at PKC-sites pointed towards a role for threonine 144 (143 in human) in the reduction of myofilament Ca\(^{2+}\)-sensitivity, which in view of our results may also be the case in human myocardium.

4.2. Effect of PKC on maximal force

Studies in rodent myocardium indicated a pivotal role for TnI and TnT in PKC-mediated depression of F\(_{\text{max}}\) [19,20,23]. In a recent study using an in vitro motility assay, an increased PKC expression in failing human hearts was associated with a reduction in thin filament maximal force, based on the minimal amount of α-actinin needed to arrest thin-filament motility, which was reversed upon treatment with PP2A [8]. Our experiments addressed the functional effects of PKC-mediated phosphorylation of both thin and thick myofilament proteins. It should be mentioned that the catalytic domain of PKC, used in our experiments, may exert different effects than the various PKC isoforms present in the heart due to target selectivity. However, our protein analysis provided evidence that the PKC catalytic domain was able to phosphorylate the two main myofilament proteins, TnI and TnT, implicated in the reduction of F\(_{\text{max}}\) in rodent studies [20,23]. Treatment of human cardiomyocytes with the catalytic domain of PKC decreased F\(_{\text{max}}\) by ~12% under basal conditions, though this effect was not significant within the donor and failing group. Moreover, PKC did not change F\(_{\text{max}}\) upon PKA or phosphatase pretreatment. Hence, F\(_{\text{max}}\) remained unaltered by either kinase or phosphatase treatment both in donor and failing myocardium. Overall, our experiments do not provide strong evidence in favor of PKC-mediated depres-
sion of the maximal force generating capacity in human myocardium.

4.3. PKC in heart failure

Increased expression of PKC isoforms has been reported in human heart failure [5-8], which based on previous studies [8,19,20,23] could reduce the maximal force generating capacity of the heart via troponin phosphorylation. In the present and previous studies [10,25] we did not find a difference in \( F_{\text{max}} \) between failing and healthy human myocardium. Although our study indicates that PKC treatment increases phosphorylation of both TnI and TnT in human myocardium, basal TnT phosphorylation was not increased in failing compared to donor hearts. Moreover, PKC induced a similar shift in myofilament \( \text{Ca}^{2+} \)-sensitivity after PKA pretreatment in donor and failing cardiomyocytes (0.07 versus 0.06 pCa units), suggesting that basal phosphorylation of PKC-sites within the myofilaments of donor and failing hearts are similar. The apparent discrepancy between increased PKC isoform expression reported in several studies [5-8] and the increased \( \text{Ca}^{2+} \)-responsiveness in end-stage failing human myocardium might be related to compartmentalization involving translocation and anchoring of PKC isoforms to specific target proteins within the cardiomyocyte, which depends on the manner of stimulation and may even be transient [31]. Overall, our data indicate that increased PKC isoform expression does not alter basal protein phosphorylation and function, but may have a transient, more dynamic role in myocardial contraction upon stimulation of the heart.

Although we did not find evidence for increased PKC-mediated myofilament protein phosphorylation in failing compared to donor myocardium, the reduction in basal phosphorylation of both MLCs (Table 2) might result from an indirect action of PKC. Only recently it has been reported that PKC may alter the phosphorylation status of proteins involved in \( \text{Ca}^{2+} \)-handling, specifically phospholamban, indirectly via phosphorylation of protein phosphatase inhibitor-1 (I-1) resulting in enhanced protein phosphatase-1 (PP-1) activity [5]. Opposite to PKC, PKA reduces PP-1 activity via phosphorylation of I-1. Thus, in failing human myocardium increased PKC and reduced PKA activity together could, via phosphorylation of I-1 [32], result in increased PP-1 activity [5,32]. Such an increase in PP-1 activity has been reported in failing human myocardium [33] and might explain the reduced basal phosphorylation of MLC-1 and MLC-2. The significant negative correlations observed between percentage of dephosphorylation of MLC-1 and basal levels of MLC phosphorylation are indicative for a possible interaction between kinase and phosphatase activity in human myocardium.

In healthy human hearts the \( \beta \)-adrenergic pathway represents the main pathway to enhance pump function during exercise via an increase in pressure generation (positive inotropy), relaxation rate (lusitropy) and frequency of contraction. Part of the enhanced relaxation originates from the decreased myofilament \( \text{Ca}^{2+} \)-sensitivity resulting from PKA-mediated TnI phosphorylation [12,13]. In diseased human myocardium the response to \( \beta \)-adrenergic receptor stimulation is blunted [1]. It has been shown that TnI phosphorylation status is decreased in end-stage failing myocardium [34], which could explain the increased \( \text{Ca}^{2+} \)-responsiveness observed in end-stage human heart failure [9,10]. In the present study, the reduction in \( \text{Ca}^{2+} \)-sensitivity upon PKA or PKC treatment was significantly larger in failing than in donor cardiomyocytes (Fig. 3). Moreover, an excellent correlation was found between basal phosphorylation at the PKA-sites and myofilament \( \text{Ca}^{2+} \)-sensitivity. These observations can be explained by the reduced level of TnI phosphorylation at PKA-sites in failing compared to donor myocardium. Since the present study showed that PKC is able to decrease \( \text{Ca}^{2+} \)-responsiveness both via phosphorylation of PKA- and PKC-sites, activation of PKC in the failing human heart may represent an alternative route in order to preserve positive lusitropy during exercise.

Noteworthy, although no significant difference was found in \( F_{\text{paw}} \) between donor and failing hearts, a significant correlation was present between \( F_{\text{paw}} \) and LVEDVI. Moreover, this correlation was absent after PKC treatment. A previous study [26] revealed that patients with diastolic heart failure (DHF) had elevated \( F_{\text{paw}} \), which correlated with LV end-diastolic pressure and was reduced to control values by PKA treatment. Within the latter study no difference was observed in TnI phosphorylation between DHF and control samples and it was suggested that the phosphorylation deficit responsible for increased \( F_{\text{paw}} \) might involve other sarcomeric proteins like MyBP-C or titin. The present data indicate that such a deficit might be reversed by both PKA and PKC.

4.4. Study limitations

The present study indicates that within the intact human sarcomere the catalytic domain of PKC is able to phosphorylate both PKA- and PKC-sites of TnI and TnT. Caution should be exerted in extrapolation of the current results to the situation in the working heart.

The low level of TnI dephosphorylation in donor myocardium merely reflects simultaneous dephosphorylation of both PKA-sites. Possibly one of the PKA-sites is constitutively phosphorylated and additional functional effects may occur via phosphorylation of the remaining PKA-site [35]. The relatively high level of basal TnI phosphorylation status in donor myocardium may be a reflection of high catecholamine release during tissue procurement, whereas in failing myocardium, this effect is blunted as a result of desensitization of the \( \beta \)-adrenergic pathway. Therefore, we cannot exclude that the magnitude of the observed difference in \( \text{Ca}^{2+} \)-sensitivity might differ from the in vivo situation. However, myofilament \( \text{Ca}^{2+} \)-sensitivity in cardiomyocytes from patients with less severe
forms of cardiomyopathy (NYHA class II/III) was similar to that in to in healthy rat cardiomyocytes upon PKA treatment [38]. Moreover, we found reduced TnI phosphorylation and an increase in myofilament Ca\textsuperscript{2+}-sensitivity in post-infarct remodeled pig myocardium compared to sham-operated animals, which was abolished upon PKA treatment [27]. In these animals cardiac samples were obtained when noradrenaline levels were similar in both infarct and sham group (~50 pg/mL).

The question remains which PKC isoforms are specifically targeted to the myofilaments in healthy and failing human myocardium. Differences may exist between PKC isoforms in their preference to phosphorylate certain myofilament proteins or specific sites within a myofilament protein. Such a site-specific phosphorylation was demonstrated in a study on TnI phosphorylation, in which PKC\textsubscript{α} preferred to phosphorylate serines 43/45, while PKC\textsubscript{δ} favored phosphorylation of PKA-specific sites [17]. Moreover, accessibility of phosphorylation sites may depend on basal phosphorylation status of the cardiac tissue. Therefore, further studies are required to address the site-specific preferences of the different PKC isoforms at rest and during activation in human myocardium.

4.5. Conclusion

In the present study no indications were found that increased PKC-mediated myofilament phosphorylation contributes to decreased pump function via a reduction in maximal force generating capacity in human heart failure. Activation of PKC and the subsequent decrease in myofilament Ca\textsuperscript{2+}-sensitivity due to troponin phosphorylation may rather counteract the detrimental effect of reduced PKA-mediated TnI phosphorylation on diastolic pump function in failing human myocardium.

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