Protein kinase C \( \alpha \) and \( \varepsilon \) phosphorylation of troponin and myosin binding protein C reduce Ca\(^{2+}\) sensitivity in human myocardium

Viola Kooij · Nicky Boontje · Ruud Zaremba · Kornelia Jaquet · Cris dos Remedios · Ger J. M. Stienen · Jolanda van der Velden

Received: 27 April 2009 / Revised: 20 July 2009 / Accepted: 22 July 2009 / Published online: 5 August 2009
© The Author(s) 2009. This article is published with open access at Springerlink.com

Abstract Previous studies indicated that the increase in protein kinase C (PKC)-mediated myofilament protein phosphorylation observed in failing myocardium might be detrimental for contractile function. This study was designed to reveal and compare the effects of PKC\( \alpha \)- and PKC\( \varepsilon \)-mediated phosphorylation on myofilament function in human myocardium. Isometric force was measured at different [Ca\(^{2+}\)] in single permeabilized cardiomyocytes from failing human left ventricular tissue. Activated PKC\( \alpha \) and PKC\( \varepsilon \) equally reduced Ca\(^{2+}\) sensitivity in failing cardiomyocytes (\( \Delta p_{Ca_{50}} = 0.08 \pm 0.01 \)). Both PKC isoforms increased phosphorylation of troponin I- (cTnI) and myosin binding protein C (cMyBP-C) in failing cardiomyocytes. Subsequent incubation of failing cardiomyocytes with the catalytic subunit of protein kinase A (PKA) resulted in a further reduction in Ca\(^{2+}\) sensitivity, indicating that the effects of both PKC isoforms were not caused by cross-phosphorylation of PKA sites. Both isoforms showed no effects on maximal force and only PKC\( \alpha \) resulted in a modest significant reduction in passive force. Effects of PKC\( \alpha \) were only minor in donor cardiomyocytes, presumably because of already saturated cTnI and cMyBP-C phosphorylation levels. Donor tissue could therefore be used as a tool to reveal the functional effects of troponin T (cTnT) phosphorylation by PKC\( \alpha \). Massive dephosphorylation of cTnT with alkaline phosphatase increased Ca\(^{2+}\) sensitivity. Subsequently, PKC\( \alpha \) treatment of donor cardiomyocytes reduced Ca\(^{2+}\) sensitivity (\( \Delta p_{Ca_{50}} = 0.08 \pm 0.02 \)) and solely increased phosphorylation of cTnT, but did not affect maximal and passive force. PKC\( \alpha \)- and PKC\( \varepsilon \)-mediated phosphorylation of cMyBP-C and cTnI as well as cTnT decrease myofilament Ca\(^{2+}\) sensitivity and may thereby reduce contractility and enhance relaxation of human myocardium.

Keywords Protein kinase C · Cardiac · Heart failure · Myofilament function · Contractile proteins · Phosphorylation

Introduction

The protein kinase C (PKC) family consists of a number of different isoforms with different substrate specificities [16]. In the failing human heart PKC activity and the expression of the Ca\(^{2+}\) dependent isoform PKC\( \alpha \) are increased [6]. PKC\( \alpha \) has been shown to play an important role in the development of cardiac hypertrophy and heart failure [7, 12, 13]. PKC\( \alpha \) directly phosphorylates regulatory myofilament proteins such as cardiac troponin I (cTnI) and cardiac troponin T (cTnT) [17]. PKC\( \alpha \) also phosphorylates protein phosphatase inhibitor 1, which alters the activity of protein phosphatase 1 [7, 31]. PKC\( \varepsilon \) is another (Ca\(^{2+}\) independent) PKC isoform, which translocates upon activation to the...
myofilaments (and the sarcolemma) [10, 15, 16]. Several myofilament regulatory proteins [myosin binding protein C (cMyBP-C), cTnT, cTnI] serve as targets for PKCε [24, 40]. Moreover, transgenic mice overexpressing constitutively active PKCε slowly develop dilated cardiomyopathy with failure [11].

Studies in rodent myocardium indicated that cTnT and cTnI phosphorylation influence Ca²⁺ sensitivity and were involved in the PKC-mediated depression of the maximal force generating capacity [8, 18, 32, 33]. Using an in vitro motility assay, an association was observed between the increased PKC expression in failing human hearts and a reduction of the force exerted on thin filaments [29]. Moreover, a recent study using two different animal models of congestive heart failure, in which PKCε expression and activation was considerably increased, provided evidence that augmented PKCε-induced myofilament protein phosphorylation contributes to myofilament dysfunction by a reduction of maximum force and of Ca²⁺ sensitivity [2].

The expression of the different PKC isoforms, with different substrate specificity, varies between rodents and humans and with different disease states [1, 19]. This may affect the basal phosphorylation status of the myocardium and thus influence the effects of the kinases on Ca²⁺ sensitivity and force development per se. Specific information on the impact of the different PKC isoforms on contractile function in human myocardium is lacking. Therefore, we investigated the impact of PKCα and PKCε in human tissue from donor and end-stage failing hearts obtained during transplant surgery.

Materials and methods

Human ventricular tissue

Left ventricular (LV) transmural tissue samples were obtained during heart transplantation surgery from ten patients with end-stage dilated cardiomyopathy (NYHA IV) and four non-failing donor hearts (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 Committee (St Vincents’ Hospital Human Research Ethics Committee; File number: H03/118; Title: Molecular Analysis of Human heart Failure). The investigation conforms with the principles outlined in the Declaration of Helsinki (Cardiovascular Research 1997;35:2–4).

Force measurements

Cardiomyocytes were mechanically isolated, Triton X-100 permeabilized and mounted in the experimental set-up at a sarcomere length of 2.2 μm. Force measurements and maximal rate of force redevelopment measurements were performed as described previously [28, 35–37].

Briefly, a single cardiomyocyte was mounted between a force transducer and piezoelectric motor. After transferring the cardiomyocyte from relaxing solution (5.89 mM Na₂ATP, 6.48 mM MgCl₂, 40.76 mM propionic acid (Kprop), 100 mM Bis-2-aminoethanesulfonic acid (BES), 7.0 mM ethylene glycol tetra acetate acid (EGTA), 14.5 mM phosphocreatine disodium salt (CrP), pH 7.10 at 15°C, pCa 9) to activating solution (5.97 mM Na₂ATP, 6.28 mM MgCl₂, 40.64 mM Kprop, 100 mM BES, 7.0 mM CaEGTA, 14.5 mM CrP, pH 7.10 at 15°C, pCa 4.5), isometric force was measured. After reaching steady force, the cardiomyocyte was 20% reduced in length within 2 ms and restretched after 30 ms (slack test). During this slack test, force first dropped to zero and after the restretch quickly redeveloped to the original steady state level. A single exponential was fitted to estimate the rate constant of force redevelopment at maximal activation (Kₜ-max). Calcium sensitivity (pCa₅₀) was determined by measuring force in activating solutions with submaximal [Ca²⁺] (pCa values ranged from 5.0 to 6.0) obtained by appropriate mixing of the activating and relaxing solutions. The initial control force at maximal [Ca²⁺] (i.e., pCa 4.5) was used to calculate the maximal force per cross-sectional area. The force values at submaximal [Ca²⁺] were normalized to the interpolated control values [38].

After the initial force-pCa series, the myocyte was incubated for 60 min at 20°C in a Ca²⁺-containing solution (pCa 5.9) containing 6 mM dithiothreitol (DTT), 1 μM PMA (phorbol 12-myristate 13-acetate; Sigma), 50 nM calyculin A (calA, Sigma) without kinase (time control),

| Table 1 Characteristics from donors and patients |
|-----------------------------------------------|
| Sex | Age | LVEF (%) | Medication |
|-----------------------------------------------|
| Donor 1 Female 24 – – |
| Donor 2 Male 13 – – |
| Donor 3 Male 21 – – |
| Donor 4 Female 38 – – |
| Patient 1 Female 41 – ACEI, Diu |
| Patient 2 Female 45 8 ACEI, Diu, β |
| Patient 3 Male 65 – ACEI,Diu |
| Patient 4 Male 61 12 ACEI, Amio, Diu, β |
| Patient 5 Male 55 15 ACEI, Diu |
| Patient 6 Male 56 15 ACEI, Diu, β |
| Patient 7 Male 56 20 ACEI,Diu,β |
| Patient 8 Male 27 – ATII, Diu |
| Patient 9 Female 23 15 ACEI, Diu, β |
| Patient 10 Female 65 14 ACEI, Diu |

LVEF left ventricular ejection fraction, ACEI angiotensin-converting-enzyme inhibitors, ATII angiotensin II receptor antagonist, Amio amiodarone, Diu diuretics, β beta-blocker
with 10 µg/mL human recombinant PKCζ (Sigma P1782; batch 93K0330) or 10 µg/mL human recombinant PKCε (Sigma P1164; batch 060K1890). CalA was added to the incubation buffer to prevent protein dephosphorylation during incubation. The concentrations of the active PKC isoforms were saturating as no further effect on cardiomyocyte function were seen when the concentration was doubled. A separate group of failing myocytes was incubated for 40 min at 20°C in relaxing solution (see above) containing 6 mM DTT and 100 U/mL of the catalytic subunit of protein kinase A (PKA, Sigma P2645; batch 35H9522). After the incubation the force-pCa series was repeated. A third force-pCa series was performed to study sequential effects, i.e., PKC isoforms after PKA or visa versa.

The effect of PKCζ was also investigated upon pre-treatment of failing and donor cardiomyocytes with alkaline phosphatase (AP, calf intestinal, New England Biolabs; 2,000 U/mL) in relaxing solution (pCa 9) containing 6 mM DTT and 50 µM protease inhibitor cocktail (PIC, Sigma, P8340) for 60 min at 20°C. All force measurements were performed at 15°C.

Protein analysis

Endogenous protein phosphorylation

To preserve the endogenous phosphorylation status, frozen tissue samples (~1 mg dry weight) were homogenized in liquid nitrogen and resuspended in 1 mL cold (−20°C) 10% trichloroacetic acid solution (TCA; dissolved in acetone containing 0.1% (w/v) DTT) [27]. Tissue homogenates were kept for 1 h at −80°C and slowly heated to room temperature by stepwise increments in temperature as follows: 20 min at −20°C, 20 min at 4°C and 20 min at room temperature (20 ± 2°C). The homogenates were thoroughly mixed on a vortex between all steps. Thereafter, the tissue homogenates were centrifuged at 12,000g for 15 min and tissue pellets were washed with 1 mL of 0.2% (w/v) DTT-acetone solution and shaken for 5 min at room temperature. Centrifugation, washing and shaking was repeated three times. Thereafter tissue pellets were freeze-dried and homogenized in 1D-sample buffer containing 15% glycerol, 62.5 mM Tris (pH 6.8), 1% (w/v) SDS and 2% (w/v) DTT (final concentration 2.5 µg dry weight/µL).

To determine basal phosphorylation status of myofilament proteins, myocardial samples (TCA treated 20 µg/lane) were separated on gradient gels (Criterion Tris–HCl 4–15% gel, BioRad) and stained with Pro-Q diamond phosphoprotein gel stain (Molecular Probes) in conjunction with SYPRO Ruby staining (Molecular Probes) of the gels [43]. The phosphorylation signals for myofilament proteins were normalized to the intensities of the SYPRO Ruby-stained myosin binding protein C (cMyBP-C) bands. All signals were quantified using the luminescent image analyzer LAS-3000 (Fuji Science Imaging Systems) and Aida image analyzer software (Isotopenmeßgeräte GmbH, Staubenhardt, Germany).

Alterations in myofilament protein phosphorylation

To investigate the effect of the PKC isoforms as well as of PKA and AP on myofilament protein phosphorylation 600 µg (dry weight) of tissue was treated in with Triton X-100 (0.5%) in relaxing solution (pCa 9) containing PIC (5 µL/mL), phosphatase inhibitor cocktail (PhIC2, Sigma, P5726, 5 µL/mL) and calA (50 nM) for 5 min at room temperature, washed twice with relaxing solution without Triton X-100 and subsequently incubated for 60 min or 40 min for (PKA) at 20°C in 100 µL of (1) relaxing solution containing PIC (50 µL/mL), PhIC2 (50 µL/mL), calA (50 nM) and DTT (6 mM) (control incubation); (2) activating solution (pCa 5.9) containing PIC (50 µL/mL), PhIC2 (50 µL/mL), calA (50 nM), DTT (6 mM), PMA (1 µM) and PKCε (10 µg/mL) or (3) PKCε (10 µg/mL); (4) relaxing solution containing PIC (50 µL/mL), PhIC2 (50 µL/mL), calA (50 nM), DTT (6 mM) and PKA (100 U/mL); (5) relaxing solution containing PIC (50 µL/mL), DTT (6 mM) and AP (2,000 U/mL). Part of the tissue treated with AP was washed and subsequently incubated with PKCζ (see incubation no. 2). Subsequently, tissue was treated with TCA and analyzed using ProQ-stained gradient gels as described above.

To investigate the effect of PKCζ phosphorylation of titin, tissue samples with a final concentration of ~10 µg/µL dry weight were homogenized in 100-150 µL sample buffer consisting of 0.55 M Tris HCL, 0.55 M EDTA, 10% glycerol, 1% β-mercaptoethanol, 20% (w/v) SDS, 8 µL/mL leupeptin (Peptin-institute, Japan), 10 µL/mL PhIC1 (Sigma, P2850) and PhIC2, pH6.8. Myocardial samples (~100 µg/lane) were separated on agarose strengthened 2% sodium dodecyl sulfate-polyacrylamide gels and stained with Pro-Q diamond phosphoprotein gel stain in conjunction with Sypro Ruby staining [5]. Staining and analysis as described above.

Data analysis

Force-pCa relations were fit to the Hill equation as described previously [37]. Values are given as mean ± SEM of n experiments. Cardiomyocyte force values for donor and failing samples were compared using an unpaired Student t test. Effects of incubations without or with PKCζ, PKCε, PKA or AP were tested with paired Student t test. Repeated measures ANOVA followed by a Bonferroni post hoc test was used when studying the
effects of sequential incubations with the PKC isoforms, PKA or AP. P < 0.05 was considered significant.

**Results**

PKC\(\varepsilon\) and PKC\(\varepsilon\) phosphorylation of cTnI and cMyBP-C decrease Ca\(^{2+}\) sensitivity in failing myocardium

Direct incubation of the cardiomyocytes from failing hearts with saturating amounts of PKC\(\varepsilon\) (n = 14 cardiomyocytes) and PKC\(\varepsilon\) (n = 7 cardiomyocytes) resulted in a decrease in Ca\(^{2+}\) sensitivity (pCa\(_{50}\)) (Fig. 1a, b). Both PKC isoforms had no effect on maximal force (\(F_{\text{max}}\)). Passive force (\(F_{\text{pas}}\)) was reduced after PKC\(\varepsilon\) treatment, whereas PKC\(\varepsilon\) had no significant effect on \(F_{\text{pas}}\) (Table 2a). \(F_{\text{max}}\), \(F_{\text{pas}}\) and pCa\(_{50}\) did not change in the absence of kinases (time control; \(n = 6 \) failing cardiomyocytes), while the steepness of the force-pCa relationship (nH) slightly decreased (Table 2a).

Figure 1c shows a phosphoprotein (ProQ) stained 1D gel of failing tissue samples treated with PKC\(\varepsilon\) and PKC\(\varepsilon\), at concentrations of 10 and 50 \(\mu\)g/mL. The phosphorylated active forms of PKC\(\varepsilon\) and PKC\(\varepsilon\) became apparent in the incubations with 50 \(\mu\)g/mL kinase. Gels were subsequently stained with SYPRO Ruby to correct for differences in protein loading. The phosphorylation levels with 50 \(\mu\)g/mL kinase were similar to the incubations with 10 \(\mu\)g/mL kinase, indicating that the standard concentrations of 10 \(\mu\)g/mL kinase yielded the maximum obtainable phosphorylation level. To further investigate if PKC\(\varepsilon\) and PKC\(\varepsilon\) are able to phosphorylate cTnI, recombinant human tropinin complex [43] was incubated with both isoforms. The results on the recombinant, non-phosphorylated tropinin complex clearly indicate that PKC\(\varepsilon\) and PKC\(\varepsilon\) both phosphorylate cTnT and cTnI and that the efficacy of PKC\(\varepsilon\) was less than that of PKC\(\varepsilon\) (Fig. 1d).

Figure 1e shows that PKC\(\varepsilon\) (n = 5) and PKC\(\varepsilon\) (n = 2) caused pronounced (2- to 3-fold) increases in cTnI and cMyBP-C phosphorylation in failing myocardium (relative to the control incubation without kinase which was set to 1). The relative increase in cMyBP-C phosphorylation by PKC\(\varepsilon\) (3.4 ± 0.5) was somewhat higher to that observed with PKC\(\varepsilon\) (2.1 ± 0.4), while phosphorylation of cTnI with PKC\(\varepsilon\) (1.6 ± 0.1) was lower compared with PKC\(\varepsilon\) (2.9 ± 0.6). A minor increase in desmin phosphorylation was observed with both isoforms, while only PKC\(\varepsilon\) slightly increased cTnT phosphorylation. No effects were observed on phosphorylation of myosin light chain 2 (MLC-2). As PKC\(\varepsilon\) lowered passive force, the effect of PKC\(\varepsilon\) incubation on the phosphorylation of cardiac titin was analyzed in failing (n = 5) and donor (n = 6) tissue. Incubation with PKC\(\varepsilon\) did not result in a significant change in titin phosphorylation in both donor and failing tissue (Fig. 2a, b).

To address the origin of the shift in Ca\(^{2+}\) sensitivity in failing hearts, cells were treated with PKA after incubation with PKC\(\varepsilon\) and \(\varepsilon\). This resulted in a further reduction in Ca\(^{2+}\) sensitivity (Fig. 3a, b), without any significant effect on \(F_{\text{max}}\) and \(F_{\text{pas}}\) (Table 2a). Reversal of the PKA and PKC\(\varepsilon\)/PKC\(\varepsilon\) sequences yielded similar reductions in Ca\(^{2+}\) sensitivity for PKA and PKC\(\varepsilon\) and \(\varepsilon\), indicating that the effects of PKC\(\varepsilon\) and \(\varepsilon\) on Ca\(^{2+}\) sensitivity were independent of that of PKA (Fig. 3c, d; Table 2a).

On average, \(K_{\text{tr-max}}\) [tension redevelopment rate (s\(^{-1}\)] at maximal Ca\(^{2+}\) concentration] was significantly increased after PKC\(\varepsilon\), PKC\(\varepsilon\) and PKA incubation in failing cardiomyocytes (Table 2a). However, it must be noted that the change in \(K_{\text{tr-max}}\) in failing cells upon kinase treatments did not significantly differ from the increase observed in the (time) control incubations (Table 2a). These results suggest that the kinases and phosphatase used have little, if any, effect on the kinetics of force redevelopment at saturating calcium concentration.

PKC\(\varepsilon\) decreases Ca\(^{2+}\) sensitivity via cTnT phosphorylation

As observed in a previous study [14, 36], analysis of baseline endogenous myofilament protein phosphorylation revealed significantly higher cMyBP-C and cTnI phosphorylation in donor samples in comparison to failing samples, while no significant differences in phosphorylation were observed for desmin, cTnT and MLC-2 (Fig. 4a). \(F_{\text{max}}\) and \(F_{\text{pas}}\) did not significantly differ between failing (\(n = 56\); \(F_{\text{max}}\) 31.7 ± 1.5 kN/m\(^2\) and \(F_{\text{pas}}\) 1.6 ± 0.1 kN/m\(^2\)) and donor (\(n = 20\); \(F_{\text{max}}\) 33.8 ± 3.0 kN/m\(^2\) and \(F_{\text{pas}}\) 1.8 ± 0.2 kN/m\(^2\)) cardiomyocytes, while Ca\(^{2+}\) sensitivity of the myofilaments was significantly higher in failing (5.66 ± 0.01; n = 54) compared to donor (5.50 ± 0.01; n = 20) myocardium. In addition the steepness of the pCa-force relationship (nH) was slightly lower in failing (3.48 ± 0.09; n = 54) compared to donor (3.85 ± 0.19; n = 20) cells (\(P < 0.05\)). \(K_{\text{tr-max}}\) was slightly, though not significantly, higher in failing (0.84 ± 0.02 s\(^{-1}\); n = 56) than in donor (0.77 ± 0.03 s\(^{-1}\); n = 20) cells. Previous analysis using two-dimensional gel electrophoresis revealed that endogenous cTnT phosphorylation is high (~63%), both in donor and failing myocardium [36]. To uncover functional effects of PKC\(\varepsilon\)-mediated cTnT phosphorylation in human tissue, PKC\(\varepsilon\) incubations were repeated after pretreatment of failing and donor cardiomyocytes with AP. AP significantly reduced cTnT phosphorylation to 15% of its original level in donor and to 17% in failing myocardium without dephosphorylation of cTnI (Fig. 5a, b). AP treatment also significantly reduced phosphorylation of cMyBP-C (~30%) in donor myocardium, and of desmin (~30%) in failing tissue. Subsequent
phosphorylation by PKCζ of samples dephosphorylated by AP resulted in significant increases in cTnT phosphorylation in failing (170%) and in donor tissue (95%). In addition, PKCζ increased phosphorylation of cMyBP-C (*23%), desmin (*113%), cTnI (*160%) and MLC-2 (*80%) in failing tissue, while in AP pre-treated donor tissue, cTnT was the only protein phosphorylated by PKCζ (Fig. 5c).

After the massive dephosphorylation of cTnT, the effects of PKCζ on cardiomyocyte function were studied. AP treatment resulted in an increase in Ca^{2+} sensitivity, which was similar in failing and donor tissue (Fig. 6a, b; ΔpCa_{50} = 0.09). Subsequent treatment with PKCζ resulted in a decline in Ca^{2+} sensitivity to a level (pCa_{50} after AP-PKCζ: 5.60 ± 0.01 and 5.51 ± 0.02 in failing and donor, respectively) similar to that observed after PKCζ treatment, without AP pretreatment (Table 2b). The steepness of the force-pCa relation decreased upon AP in failing and donor cells and remained low after PKCζ (Table 2b). A similar decline in steepness of the Ca^{2+} sensitivity curve was observed in the time control experiments in the absence of kinase or phosphatase. AP treatment resulted in a small but significant increase in F_{max} in failing cells (Fig. 6c). In donor cells (Fig. 6d) no effect on F_{max} was observed. PKCζ applied after AP treatment did not affect F_{max} in both groups. AP also caused a significant increase in F_{pas} in both
failing and donor cells (Fig. 6e, f), but subsequent PKCα treatment failed to restore the original levels.

Discussion

This study shows that PKC-mediated phosphorylation of cMyBP-C and cTnI as well as of cTnT cause a reduction of Ca^{2+} sensitivity of force development in human myocardium. No effects on maximal force generating capacity of the myofilaments were observed, neither upon direct PKC treatment nor following phosphatase pretreatment. On the basis of these data we propose that increased PKC activity in failing myocardium may depress systolic function of the heart as for the same intracellular Ca^{2+} concentration, the myofilament force generation and thereby developed pressure will be reduced. On the other hand, the reduced myofilament Ca^{2+} sensitivity may help myocardial relaxation as Ca^{2+} concentrations are increased in failing hearts [3, 9].

PKCα and PKCε phosphorylation of cTnI and cMyBP-C decrease Ca^{2+} sensitivity in failing myocardium

Direct incubation of the cardiomyocytes from failing hearts with saturating amounts of active PKCα and PKCε resulted in a decrease in Ca^{2+} sensitivity (Fig. 1). PKCα and PKCε incubations of failing tissue resulted in increased phosphorylation of cMyBP-C and cTnI. PKCα incubation resulted in higher phosphorylation of cTnI and PKCε in

Table 2  Overview of the force measurements with PKCα, PKCε and PKA incubations (a) and AP and PKCα incubations (b)

| Incubation | Cardiomyocyte measurements | F_{max} | F_{pas} | pCa_{50} | nH | K_{ir-max} (s^{-1}) |
|------------|-----------------------------|---------|---------|----------|----|---------------------|
| (a) PKCα   | 7 failing hearts; 14 myocytes |         |         |          |    |                     |
| Before     | 28.2 ± 2.4                  | 1.5 ± 0.2 | 5.67 ± 0.03 | 3.43 ± 0.18 | 0.69 ± 0.03 |
| After      | 28.6 ± 2.4                  | 1.4 ± 0.2*| 5.59 ± 0.03*| 3.28 ± 0.24 | 0.81 ± 0.04* |
| PKCε       | 5 failing hearts; 7 myocytes |         |         |          |    |                     |
| Before     | 30.3 ± 3.9                  | 1.6 ± 0.3 | 5.68 ± 0.03 | 3.86 ± 0.20 | 0.60 ± 0.05 |
| After      | 31.2 ± 3.1                  | 1.4 ± 0.2*| 5.60 ± 0.02*| 3.42 ± 0.13 | 0.74 ± 0.05* |
| PKA        | 7 failing hearts; 14 myocytes |         |         |          |    |                     |
| Before     | 22.8 ± 2.1                  | 1.4 ± 0.2 | 5.65 ± 0.02 | 3.34 ± 0.07 | 0.68 ± 0.02 |
| After      | 22.9 ± 2.0                  | 1.4 ± 0.1*| 5.53 ± 0.01*| 3.57 ± 0.13*| 0.84 ± 0.04* |
| Control#   | 2 failing hearts; 6 myocytes |         |         |          |    |                     |
| Before     | 22.7 ± 2.5                  | 1.5 ± 0.1 | 5.66 ± 0.03 | 3.16 ± 0.12 | 0.64 ± 0.06 |
| After      | 22.1 ± 2.5                  | 1.3 ± 0.1 | 5.67 ± 0.03 | 2.65 ± 0.11*| 0.74 ± 0.04 |
| (b) AP     | 5 failing hearts; 11 myocytes |         |         |          |    |                     |
| Before     | 35.7 ± 3.6                  | 1.8 ± 0.3 | 5.64 ± 0.02 | 3.68 ± 0.11 | 0.76 ± 0.07 |
| After      | 38.6 ± 4.5*                 | 2.5 ± 0.3*| 5.73 ± 0.03*| 2.85 ± 0.13*| 0.68 ± 0.04 |
| AP + PKCα  | 5 failing hearts; 10 myocytes |         |         |          |    |                     |
| Before     | 35.5 ± 3.8                  | 2.6 ± 0.3 | 5.73 ± 0.03 | 2.84 ± 0.14 | 0.56 ± 0.06 |
| After      | 35.1 ± 3.9                  | 2.4 ± 0.4 | 5.60 ± 0.01*| 2.99 ± 0.15 | 0.66 ± 0.09 |
| AP         | 4 donor hearts; 8 myocytes   |         |         |          |    |                     |
| Before     | 34.1 ± 4.5                  | 1.5 ± 0.2 | 5.48 ± 0.02 | 4.04 ± 0.19 | 0.62 ± 0.09 |
| After      | 34.3 ± 4.9                  | 2.0 ± 0.4*| 5.57 ± 0.02*| 3.33 ± 0.18*| 0.67 ± 0.07 |
| AP + PKCα  | 4 donor hearts; 7 myocytes   |         |         |          |    |                     |
| Before     | 31.6 ± 4.4                  | 2.2 ± 0.4 | 5.59 ± 0.02 | 3.25 ± 0.18 | 0.57 ± 0.04 |
| After      | 31.3 ± 4.8                  | 2.0 ± 0.4 | 5.51 ± 0.02*| 3.30 ± 0.15 | 0.76 ± 0.07* |

F_{max}, maximal force at maximal calcium concentration in kN/m²; F_{pass}, passive force measured in free calcium solution in kN/m²; nH, steepness of the force-pCa curves

* P < 0.05, before versus after incubation in paired t test

# Time control incubations
higher phosphorylation levels of cMyBP-C, indicating iso- 
zyme specific substrate affinity. Although phosphoryla-
tion effects were diverse, the functional consequences of both 
PKC isoforms were quite similar.

It has previously been determined that cMyBP-C has 
PKC-phosphorylatable residues [24, 40]. However, so far 
no data has been published (to our knowledge) that directly 
link cMyBP-C phosphorylation with alterations in Ca\textsuperscript{2+} 
sensitivity. Our data suggest involvement of PKC-mediated 
cMyBP-C phosphorylation in the reduced Ca\textsuperscript{2+} sensitivity 
observed in failing tissue.

Kobayashi et al. [20] indicated that PKC\textsubscript{e} was able to 
phosphorylate PKA sites on mouse cTnI. Our previous 
study also showed that the active catalytic domain of PKC 
caus ed cross-phosphorylation of the PKA sites (Ser23/24) 
[35]. We addressed this issue for PKC\textsubscript{α}- and PKC\textsubscript{e}-medi-
ated phosphorylation by performing experiments where 
icubations of PKC\textsubscript{α} or PKC\textsubscript{e} were followed by PKA as 
well as by incubations in the reverse order. The results 
showed that the shifts in Ca\textsuperscript{2+} sensitivity in failing myocar-
dium did not depend on the sequence of application of 
PKA and PKC\textsubscript{α/e}. This indicates that the effects of the 
PKC isoforms and PKA were independent and that the 
PKC\textsubscript{α/e}-mediated effects were not caused by cross-phos-
phorylation of the PKA sites under our conditions. 
Kobayashi et al. [20] used the recombinant mouse cTnI and 
an excess of PKC\textsubscript{α}, therefore it is possible that the cross-
phosphorylation they observed does not occur under more 
physiological conditions or that it is more pronounced in 
mice than in human cTn.

PKC\textsubscript{α} decreases Ca\textsuperscript{2+} sensitivity via cTnT 
phosphorylation.

Direct incubation of donor cells with PKC\textsubscript{α} did not alter 
Ca\textsuperscript{2+} sensitivity (Fig. 4b), which is in line with minor effects 
of PKC\textsubscript{α} on protein phosphorylation of cMyBP-C, desmin, 
cTnI, cTnT and MLC2 (Fig. 4c). The absence of functional 
effects of PKC\textsubscript{α} is most likely explained by the relatively 
high baseline phosphorylation status of cMyBP-C, cTnI and 
cTnT in donor myocardium. Because endogenous cMyBP-C 
and cTnI phosphorylation levels in donor tissue were high 
(Fig. 4a) and no major effects of PKC\textsubscript{α} on Ca\textsuperscript{2+} sensitivity 
and protein phosphorylation were observed (Fig. 4b, c), the 
donor tissue was used as a tool to reveal functional effects of 
PKC\textsubscript{α}-mediated phosphorylation of cTnT.

AP treatment resulted in a massive decrease in cTnT 
phosphorylation in failing and donor myocardium. Sub-
sequent treatment with PKC\textsubscript{α} solely increased phosphory-
lation of cTnT in donor myocardium, which made it 
possible to identify the role of cTnT upon phosphorylation 
by PKC\textsubscript{α}. Treatment with PKC\textsubscript{α} following AP treatment 
showed a significant reduction in Ca\textsuperscript{2+} sensitivity in both 
failing and donor tissue. These results indicate that AP is 
able to dephosphorylate sites on cTnT, which are subse-
quently targeted by PKC\textsubscript{α} and when phosphorylated reduce 
Ca\textsuperscript{2+} sensitivity.

Overall, our results are in agreement with previous 
animal studies [17, 24, 40] indicating that functionally 
relevant targets of PKC\textsubscript{α}- and PKC\textsubscript{e}-mediated Ca\textsuperscript{2+} 
desensitization of the myofilaments are located on cardiac
troponin. Both cTnI and cTnT contain a number of different PKC-phosphorylatable sites. On the basis of biochemical in vitro studies Ser-42 and Ser-44 and Thr-143 on cTnI [30] and Thr-194, Ser-198, Thr-203 and Thr-284 on cTnT [17, 34] are considered the main sites (phosphorylated sites based on human sequence). It has been proposed that the relative balance of phosphorylation of the three PKC sites on cTnI is important for the regulation of its function [32]. Pseudophosphorylation of Ser-42/Ser-44 significantly reduced the myofilament response to calcium and phosphorylation of Thr-143 induced an increase in sensitivity to calcium [7, 39]. Among the four phosphorylation sites on cTnT, Thr-203 appeared to be the most important for modulation of cTn function in that it inhibited force as well as its Ca$^{2+}$ sensitivity [33].

PKC$\alpha$ reduces passive force, but not maximal force

Rodent studies indicate that PKC-mediated phosphorylation results in a reduction of the maximum force generating capacity of the cardiomyocytes [8, 26, 32, 33]. A study using an in vitro motility assay suggested that this might also be the case in human tissue [2]. However, the present results did not show depression of force upon PKC treatment and indicate that caution should be exerted when extrapolating data obtained in rodent studies to force development in human cardiomyocytes. Cardiac cTnI and cTnT phosphorylation by PKC may occur at different sites in different species and may explain the absence of an effect of PKC$\alpha$ on $F_{\text{max}}$ in human cardiomyocytes. Only recently, using mass spectrometry Zabrouskov et al. [42] revealed a novel phosphorylation site (Ser-76 or Thr-77) in human cTnI, while no phosphorylation was observed at the sites mentioned above. A study in mice revealed that the PKC$\alpha$-induced reduction in $F_{\text{max}}$ critically depended on cTnT phosphorylation at Thr-203 [33]. Future studies should be performed to reveal which sites are phosphorylated by PKC in human myocardium. Recently Molnár et al. [25] showed that PKC$\alpha$ contributes to the maintenance of contractile force in human cardiomyocytes, which is in line with our
results that PKCα treatment does not negatively affect maximal force. AP treatment resulted in a small (5%) but significant increase in $F_{\text{max}}$. However, subsequent treatment with PKCα did not result in a reduction of force development. This suggests that AP is able to dephosphorylate at donor tissue.

Fig. 5 Effect of alkaline phosphatase (AP) treatment and the subsequent effect of PKCα on myofilament protein phosphorylation in donor and failing myocardium. a 1D gradient gel stained with ProQ diamond from donor and failing myocardium incubated without kinase or phosphatase (C), with PKCα, with AP and with AP followed by PKCα (AP + PKCα). MM molecular weight marker, PM Pepper-mint Stick Phosphoprotein marker (Molecular Probes). b Changes in phosphorylation of cTnT by AP relative to time control without phosphatase in failing (n = 5) and donor (n = 6) myocardium. c Changes in phosphorylation by PKCα upon AP pretreatment relative to the values obtained after AP pretreatment (n = 4 in both groups). *$P < 0.05$, PKCα relative to control in paired t test.
least to some extent one of the contractile proteins determining $F_{\text{max}}$. However, in human tissue the impact is rather small and the link with PKC\(\alpha\) is not evident.

PKC\(\alpha\) treatment resulted in a small reduction of $F_{\text{pas}}$ both in donor and in failing cardiomyocytes, whereas PKC\(\varepsilon\) had no significant effect but did show a similar trend (Table 2). AP treatment caused a significant increase in $F_{\text{pas}}$ in failing and donor tissue and remained elevated after PKC\(\alpha\) treatment. *$P < 0.05$, before versus after phosphatase/kinase treatment in Bonferroni post hoc test.

As high $F_{\text{pas}}$ has been associated with high left ventricular end-diastolic pressure in patients with diastolic dysfunction [4], the reduction in passive stiffness by PKC\(\alpha\)-mediated phosphorylation might improve diastolic filling of the heart.
Limitations and conclusions

In this study the effects of the kinases and phosphatases on the phosphorylation levels are represented relative to basal phosphorylation levels for each protein. Although the changes in protein phosphorylation levels were relatively small, the functional changes were significant and may be physiologically relevant.

Since the transition to end-stage heart failure is accompanied by an increase in PKCα activity [6] it is surprising that the effects of PKCα are observed only in end-stage failing hearts and become visible in donor hearts only after dephosphorylation by alkaline phosphatase. It should be noted, however, that the endogenous phosphorylation levels in the explanted myocardium studied not necessarily reflect the in vivo situation. The lower levels of cMyBP-C and cTnI phosphorylation observed in end-stage failing compared to donor myocardium may reflect desensitisation of the β-adrenergic pathway. Moreover, it has been suggested that high catecholamine levels at the time of tissue procurement underlies the relative high levels of cMyBP-C and cTnI phosphorylation levels in donor myocardium [23]. Future studies in biopsy samples taken under well-controlled hemodynamic conditions with parallel measurements of blood catecholamines should be performed to address this issue. Nevertheless, the data presented in this study clearly indicate that changes in expression and activation of PKCα and PKCe in response to pathophysiological stressors or increased hemodynamic demands during exercise translate into alterations in contractile function in human myocardium.

Open Access This article is distributed under the terms of the Creative Commons Attribution Noncommercial License which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

References

1. Bayer AL, Heidkamp MC, Patel N, Porter M, Engman S, Samarel AM (2003) Alterations in protein kinase C isoenzyme expression and autophosphorylation during the progression of pressure overload-induced left ventricular hypertrophy. Mol Cell Biochem 242:145–152
2. Belin RJ, Sumandea MP, Allen EJ, Schoenfelt K, Wang H, Solaro JR, de Tombe PP (2007) Augmented Protein Kinase C-α-induced myofilament protein phosphorylation contributes to myofilament dysfunction in experimental congestive heart failure. Circ Res 101:195–204
3. Bekenkes I, Aronsen JM, Birkeland JA, Henriksen UL, Louch WE, Sjaastad I, Sejersted OM (2008) Slow contractions characterise failing rat hearts. Basic Res Cardiol 103:328–344
4. Borbély A, van der Velden J, Bronzwaer JGF, Papp Z, Édes I, Stienen GJM, Paulus WJ (2005) Cardiomyocyte stiffness in diastolic heart failure. Circulation 111:774–781
5. Borbély A, Falcao-Pires I, van Heerebeek L, Hamdani N, Édes I, Gavina C, Leite-Moreira AF, Bronzwaer JGF, Papp Z, van der Velden J, Stienen GJM, Paulus WJ (2009) Hypophosphorylation of the stiff N2B titin isoform raises cardiomyocyte resting tension in failing human myocardium. Circ Res 104:780–786
6. Bowling N, Walsh RA, Song G, Estridge T, Sandusky GE, Fouts RL, Mintze K, Pickard T, Roden R, Bristow MR, Sabbah HN, Mizrahi JL, Gromo G, King GL, Vlahos CJ (1999) Increased protein kinase C activity and expression of Ca2+-sensitive isofoms in the failing human heart. Circulation 99:384–391
7. Braz JC, Gregory K, Pathak A, Zhao W, Sahin B, Klevisky R, Kimball TF, Lorenz NJ, Nairn AC, Liggett SB, Bodi I, Wang S, Schwartz A, Lakatta EG, DePaoli-Roach AA, Robbins J, Hewett TE, Bibb JA, Westfall MV, Kranias EG, Molkenstj JD (2004) PKC-β regulates cardiac contractility and propensity toward heart failure. Nature 10:248–254
8. Burkart EM, Sumandea MP, Kobayashi T, Nili M, Martin AF, Homsher E, Soloro RJ (2003) Hypophosphorylation or glutamic acid substitution at protein kinase C sites on cardiac troponin I differentially depress myofilament tension and shortening velocity. J Biol Chem 278:11265–11272
9. Del Monte F, Johnson CM, Stepaneck AC, Doye AA, Gwathmey JK (2002) Defects in calcium control. J Card Fail 8:421–431
10. Dorn GWII, Souroujan MC, Liron T, Chen CH, Gray MO, Zhou HZ, Cukai M, Wu G, Lorenz NJ, Moehly-Rosen D (1999) Sustained in vivo cardiac protection by a rationally designed peptide that causes α protein kinase C translocation. Proc Natl Acad Sci USA 96:12798–12803
11. Goldsprink PH, Montgomery DE, Walker LA, Ubroniene D, McKinney RD, Geenen DL, Solaro RJ, Buttrick PM (2004) Protein kinase C epsilon overexpression alters myofilament properties and composition during the progression of heart failure. Circ Res 95:424–432
12. Hahn HS, Marreez Y, Odley A, Sterbling A, Yussman MG, Hilty KC, Bodi I, Liggett SB, Schwartz A, Dorn GW 2nd (2003) Protein kinase C alpha negatively regulates systolic and diastolic function in pathological hypertrophy. Circ Res 93:1111–1119
13. Hambleton M, Hahn H, Pleger ST, Kuhn MC, Klevisky R, Carr AN, Kimball TF, Hewett TE, Dorn GW 2nd, Koch WJ, Molkenstj JD (2006) Pharmacological- and gene therapy-based inhibition of protein kinase C alpha/beta enhances cardiac contractility and attenuates heart failure. Circulation 114:574–582
14. Hammerstand N, Kooij V, van Dijk S, Merkus D, Paulus WJ, dos Remedios C, Duncker DJ, Stienen GJM, van der Velden J (2008) Sarcomeric dysfunction in heart failure. Cardiovasc Res 77:649–658
15. Huang XP, Pi Y, Lokuta AJ, Greaser ML, Walker JW (1997) Arachidonic acid stimulated protein kinase C-ε redistribution in heart cells. J Cell Science 110:1625–1634
16. Huang XP, Walker JW (2003) Myofilament anchoring of protein kinase C-ε-signalisation in cardiac myocytes. J Cell Science 117:1971–1978
17. Jideama NM, Noland TA Jr, Raynor RL, Gloe BC, Fabbro D, Kazantzeff MG, Blumberg PM, Hannun YA, Kuo JF (1996) Phosphorylation specificities of protein kinase C isozymes for bovine cardiac troponin I and troponin T and sites within these proteins and regulation of myofilament properties. J Biol Chem 271:23277–23283
18. Kobayashi T, Solaro RJ (2005) Calcium, thin filaments, and the integrative biology of cardiac contractility. Annu Rev Physiol 67:39–67
19. Johnsen DD, Kacimi R, Anderson BE, Thomas TA, Said S, Gerdes AM (2005) Protein kinase C isoforms in hypertension and hypertrophy: insight from SHHF rat hearts. Mol Cell Biochem 270:63–69
20. Kobayashi T, Yang X, Walker LA, Van Breemen RB, Solaro RJ (2005) A non-equilibrium isoelectric focusing method to determine states of phosphorylation of cardiac troponin I: identification of Ser-23 and Ser-24 as significant sites of phosphorylation by protein kinase C. J Mol Cell Cardiol 38:213–218

21. Krüger M, Kötter S, Grützner A, Lang P, Andresen C, Redfield MM, Butt E, dos Remedios C, Linke WA (2009) Protein kinase G modulates human myocardial passive stiffness by phosphorylation of the titin springs. Circ Res 104:87–94

22. Linke WA (2008) Sense and stretchiability: the role of titin and titin-associated proteins in myocardial stress-sensing and mechanical dysfunction. Cardiovasc Res 77:637–648

23. Marston SB, de Tombe PP (2008) Troponin phosphorylation and myofilament Ca$^{2+}$-sensitivity in heart failure: Increased or decreased? J Mol Cell Cardiol 45:603–607

24. Mohamed AS, Dignam JD, Schlender KK (1998) Cardiac myosin-binding protein C (MyBP-C): identification of protein kinase A and protein kinase C phosphorylation sites. Arch Biochem Biophys 358:313–319

25. Molnár A, Borbély A, Czuriga D, Ivetta SM, Szilágyi S, Her telendő Z, Pásztor ET, Galajda Z, Szerafin T, Jaquet K, Papp Z, Edes I, Tóth A (2008) Protein kinase C contributes to the maintenance of contractile force in human ventricular cardiomyocytes. J Biol Chem 284:1031–1039

26. Montgomery DE, Chandra M, Huang Q, Jin J, Solaro RJ (2001) Transgenic incorporation of skeletal TnT into cardiac myofilaments blunts PKC-mediated depression of force. Am J Physiol Heart Circ Physiol 280:H1011–H1018

27. Morano I, Arndt H, Gartner C, Reung JC (1988) Skinned fibers of human atrium and ventricle: myosin isozymes and contractility. Circ Res 62:632–639

28. Narolska NA, Piroddi N, Belus A, Boontje NM, Scellini B, Morano I, Arndt H, Gartner C, Reung JC (1988) A role for protein kinase C phosphorylation by protein kinases. Eur J Biochem 190:575–582

29. Noguchi T, Hunlich M, Camp PC, Begin KJ, El-Zarou M, Patten R, Leavitt BJ, Iittleman FP, Alpert NR, LeWinter MM, VanBuren P (2004) Thin filament-based modulation of contractile performance in human atrial heart failure. Circulation 110:982–987

30. Noland TA, Raynor RL, Kuo JP (1989) Identification of sites phosphorylated in bovine cardiac troponin I and troponin T by protein kinase C and comparative substrate activity of synthetic peptides containing the phosphorylation sites. J Biol Chem 264:20778–20785

31. Pathak A, del Monte F, Zhao W, Schultz JE, Lorenz JN, Bodi I, Weiser D, Hahn H, Carr AN, Syed F, Mavila N, Jha L, Qian J, Marrezz Y, Chen G, McGraw DW, Heist EK, Guerrero JL, DePaoli-Roach AA, Hajjar RJ, Kranias EG (2005) Enhancement of cardiac function and suppression of heart failure progression by inhibition of protein phosphatase1. Circ Res 96:756–766

32. Solaro RJ, Rosevear P, Kobayashi T (2008) The unique functions of cardiac troponin I in the control of cardiac muscle contraction and relaxation. Biochem Biophys Res Commun 369:82–87

33. Sumandea MP, Pyle WG, Kobayashi T, de Tombe PP, Solaro RJ (2003) Identification of a functionally critical protein kinase C phosphorylation residue of cardiac troponin T. J Biol Chem 278:35135–35144

34. Swidersk K, Jaquet K, Meyer HE, Schächtele C, Hofmann F, Heilmeyer LM Jr (1990) Sites phosphorylated in bovine cardiac troponin T and I. Characterization by 31P-NMR spectroscopy and phosphorylation by protein kinases. Eur J Biochem 190:575–582

35. Van de Velden J, Narolska NA, Lamberts RR, Boontje NM, Borbély A, Zaremba R, Bronzwaer JG, Papp Z, Jaquet K, Paulus WJ, Stienen GJM (2006) Functional effects of protein kinase C-mediated myofilament phosphorylation in human myocardium. Cardiovasc Res 69:876–887

36. Van de Velden J, Papp Z, Boontje NM, Zaremba R, de Jong JW, Janssen PML, Hasenfuss G, Stienen GJM (2003) The effect of myosin light chain 2 dephosphorylation on Ca$^{2+}$-sensitivity of force is enhanced in failing human hearts. Cardiovasc Res 57:505–514

37. Van de Velden J, Papp Z, Zaremba R, Boontje NM, de Jong JW, Owen VJ, Burton PB, Goldmann P, Jaquet K, Stienen GJM (2003) Increased Ca$^{2+}$-sensitivity of the contractile apparatus in end-stage human heart failure results from altered phosphorylation of contractile proteins. Cardiovasc Res 57:37–47

38. Verduyn SC, Zaremba R, van der Velden J, Stienen GJM (2007) Effects of contractile protein phosphorylation on force development in permeabilized rat cardiac myocytes. Basic Res Cardiol 102:476–487

39. Wang H, Grant JE, Doede CM, Sadayappan S, Robbins J, Walker JW (2006) PKC-betaII sensitizes cardiac myofilaments to Ca$^{2+}$ by phosphorylating troponin I on threonine-144. J Mol Cell Cardiol 41:823–833

40. Xiao L, Zhao Q, Du Y, Yuan C, Solaro RJ, Buttrick PM (2007) PKC's increases phosphorylation of the cardiac myosin binding protein C at serine 302 both in vitro and in vivo. Biochemistry 46:7054–7061

41. Yamasaki R, Wu Y, McNabb M, Greaser M, Labeit S, Granzier H (2002) Protein kinase A phosphorylates titin's cardiac-specific N2B domain and reduces passive tension in rat cardiac myocytes. Circ Res 90:1181–1188

42. Zabrouskov V, Ge Y, Schwartz J, Walker JW (2008) Unraveling molecular complexity of phosphorylated human cardiac troponin I by top-down electron capture dissociation/electron transfer dissociation mass spectrometry. Moll Cell Proteomics 7:1838–1849

43. Zaremba R, Merkus D, Hamdani N, Lamers JMJ, Paulus WJ, dos Remedios C, Duncker DJ, Stienen GJM, van der Velden J (2007) Quantitative analysis of myofilament protein phosphorylation in small cardiac biopsies. Proteomics Clin Appl 1:1285–1290