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Effect of Troponin I Ser23/24 Phosphorylation on Ca²⁺-Sensitivity in Human Myocardium Depends on the Phosphorylation

Background

Viola Kooij¹, Martina Saes², Kornelia Jaquet², Ruud Zaremba¹, D. Brian Foster³, Anne M. Murphy⁴, Cris dos Remedios⁵, Jolanda van der Velden¹, and Ger J.M. Stienen¹

¹ Laboratory for Physiology, Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, The Netherlands ² St Josef-Hospital/Bergmannsheil, Clinic of the Ruhr-University of Bochum, Bochum, Germany ³ Institute of Molecular Cardiobiology, Division of Cardiology, Johns Hopkins University School of Medical, Baltimore, USA ⁴ Department of Pediatrics, Johns Hopkins University School of Medical, Baltimore, USA ⁵ Muscle Research Unit, Institute for Biomedical Research, The University of Sydney, Sydney, Australia

Abstract

Protein kinase A (PKA)-mediated phosphorylation of Ser23/24 of cardiac troponin I (cTnI) causes a reduction in Ca²⁺-sensitivity of force development. This study aimed to determine whether the PKA-induced modulation of the Ca²⁺-sensitivity is solely due to cTnI phosphorylation or depends on the phosphorylation status of other sarcomeric proteins. Endogenous troponin (cTn) complex in donor cardiomyocytes was partially exchanged (up to 66±1%) with recombinant unphosphorylated human cTn and in failing cells similar exchange was achieved using PKA-(bis)phosphorylated cTn complex. Cardiomyocytes immersed in exchange solution without complex added served as controls. Partial exchange of unphosphorylated cTn complex in donor tissue significantly increased Ca²⁺-sensitivity (pCa₅₀) to 5.50±0.02 relative to the donor control value (pCa₅₀=5.43±0.04). Exchange in failing tissue with PKA-phosphorylated cTn complex did not change Ca²⁺-sensitivity relative to the failing control (pCa₅₀=5.60±0.02). Subsequent treatment of the cardiomyocytes with the catalytic subunit of PKA significantly decreased Ca²⁺-sensitivity in donor and failing tissue. Analysis of phosphorylated cTnI species revealed the same distribution of un-, mono- and bis-phosphorylated cTnI in donor control and in failing tissue exchanged with PKA-phosphorylated cTn complex. Phosphorylation of myosin-binding protein-C in failing tissue was significantly lower compared to donor tissue.

These differences in Ca²⁺-sensitivity in donor and failing cells, despite similar distribution of cTnI species, could be abolished by subsequent PKA-treatment and indicate that other targets of PKA are involved the reduction of Ca²⁺-sensitivity. Our findings suggest that the sarcomeric phosphorylation background, which is altered in cardiac disease, influences the impact of cTnI Ser23/24 phosphorylation by PKA on Ca²⁺-sensitivity.

Corresponding author: Viola Kooij, Laboratory for Physiology, VU University Medical Center (VUmc), Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands, Phone: +31 20 4448124 Fax: 0+31 20 4448255, v.kooij@vumc.nl.

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Keywords

Myocardium; single cardiomyocyte; troponin I; phosphorylation; protein kinase A; Ca^{2+} -sensitivity; sarcomere

Introduction

During exercise and stress, β -adrenergic receptors stimulation causes an increase in cardiac output to meet the demands of the body. Stimulation of β -adrenergic receptors leads to an increased level of intracellular cAMP, which in turn activates protein kinase A (PKA) [1;2]. Cardiomyocyte contractility is increased by PKA-mediated phosphorylation of proteins involved in calcium handling and myofilament proteins. The increase in heart rate requires faster myocardial relaxation, which is in part caused by increased re-uptake of cytosolic calcium into the sarcoplasmic reticulum. Myofilament proteins such as cardiac troponin I (cTnI), cardiac myosin-binding protein-C (cMyBP-C) and titin are substrates for PKA [3;4]. PKA phosphorylation of cTnI at Ser23/24 reduces Ca^{2+} -sensitivity of the myofilaments, which is also considered to be beneficial for relaxation of the heart [5–7].

In cardiac disease, the β -adrenergic receptor pathway is chronically over-stimulated to maintain cardiac output. However, chronic over-stimulation results in desensitization of the β -adrenergic receptor pathway and this could even lead to a reduced phosphorylation of the PKA target proteins. Indeed, previous studies showed reduced phosphorylation of cTnI and cMyBP-C in end-stage failing human hearts compared to non-failing donor myocardium [8–12]. In addition, reduced phosphorylation of myosin light chain 2 (MLC-2) has been reported in diseased myocardium [13] which has been associated with increased protein phosphatase 1 expression [13–15]. As a consequence of the disturbed balance between kinases and phosphatases, baseline phosphorylation of myofilament proteins differs between failing and donor hearts. To assess if a difference in baseline phosphorylation background alters the myofilament response to PKA-mediated cTnI phosphorylation, we investigated myofilament response to alterations in Ser23/24 cTnI bis-phosphorylation in non-failing donor and end-stage failing myocardium.

The elegant cardiac troponin (cTn) exchange method was applied to explore the direct effects of cTnI phosphorylation by PKA. The advantage of this technique is that it allows determination of the direct effects of cTnI phosphorylation by PKA on contractility in human cardiac preparations without alteration of phosphorylation of other contractile proteins. Endogenous cTn in non-failing donor tissue, which has a high baseline cTnI phosphorylation level, was replaced by unphosphorylated recombinant human cTn complex. PKA-pretreated, bis-phosphorylated cTn complex was exchanged in failing tissue, in which cTnI is largely unphosphorylated. Our data show that baseline phosphorylation of myofilament proteins influences the impact of PKA phosphorylation of cTnI on Ca^{2+} -sensitivity. These results suggest that the other myofibrillar targets of PKA, cMyBP-C and/or titin, play an important role in modulating the effect of cTnI phosphorylation on Ca^{2+} -sensitivity in human myocardium.

Materials and methods

A detailed description of Materials and methods is given in the online supplement.

Exchange of human troponin complex

Human recombinant cTn complex was prepared as described before [16]. The complex was maximally bis-phosphorylated at Ser23/24 by incubation with the catalytic subunit of protein kinase A. Single cardiomyocytes were mechanically isolated with a glass tissue homogenizer

[17], and permeabilized by means of Triton X-100. In cardiomyocytes from explanted non-failing donor hearts (n=5) the endogenous complex, predominantly containing phosphorylated cTnI, was replaced by unphosphorylated cTn, while in cells from idiopathic dilated end-stage failing myocardium (IDCM, NYHA Class IV; n=7), endogenous cTn was replaced by bis-phosphorylated cTn complex. In a limited set of experiments (n=2 in both donor and failing tissue), phosphorylated cTn (0.5 mg/ml) was exchanged in donor cells and unphosphorylated cTn was exchanged in failing cells. Exchange was performed by overnight incubation of the cardiomyocytes with 0.5 or 1.0 mg/ml recombinant cTn complex. Donor and failing cardiomyocytes kept in exchange solution without cTn complex added served as controls. Human cardiac tissue collection was approved by the Human Research Ethics Committee of The University of Sydney (#7326).

Isometric force measurements in single human cardiomyocytes

Force measurements on permeabilized cardiomyocytes were performed as described previously [16;17]. After an initial series of measurements at different Ca^{2+} -concentrations, myocytes were incubated with PKA and subsequently force measurements were repeated. The concentration dependence of the effect of PKA on force development was studied in single cardiomyocytes isolated from end-stage failing tissue.

Protein analyses

The phosphorylation levels of sarcomeric proteins was determined using ProQ Diamond stained 1D-gels, Western blotting and ELISA as described previously [18]. The distribution of phosphorylated forms of cTnI was analysed using Phos-tagTM acrylamide gels [19]. This combination of techniques provides a detailed picture of the overall and site-specific phosphorylation of the contractile proteins.

Data analysis

Data in non-failing donor and failing samples were compared using an unpaired Student *t*-test. Effects of PKA incubations were tested with paired Student *t*-test and the effects of the cTn exchange in combination with PKA effects were tested using two-way ANOVA followed by a Bonferroni post-hoc test. Values are given as means $\pm$ S.E.M. of *n* myocytes.

Results

Phosphorylation of sarcomeric proteins and recombinant cTn complex

Baseline phosphorylation was determined in donor and failing heart samples separated by 1D gel electrophoresis, stained with ProQ Diamond in conjunction with Sypro Ruby as shown in Figure 1A. As described previously [7;20], the major differences in phosphorylation between tissue from donor and end-stage failing patients observed reside in the level of cMyBP-C and cTnI phosphorylation, which are significantly lower in failing samples (Figure 1B). Phos-tag analysis yields the distribution between un-, mono- and bis-phosphorylated species in the donor and failing tissue. Donor tissue shows high mono- and bis-phosphorylation whereas failing tissue shows most dominantly the un-phosphorylated cTnI form (Figure 1C).

The catalytic subunit of PKA was used to selectively phosphorylate recombinant human cardiac cTn at the PKA-mediated phosphorylation sites Ser23/24 of cTnI. Samples were taken at different time points to assess the rate and extent of phosphorylation. The time course, shown in Figure 1D, indicates that cTnI phosphorylation saturated after 90 minutes of incubation. Hence, this incubation time was used to obtain phosphorylated cTn complex used during the exchange experiments.

Exchange of recombinant cTn complex in donor and failing tissue

Exchange experiments were performed in different donor (n=5) and failing (n=5) patients. One-way ANOVA analysis showed no significant difference in cardiomyocyte force measurements between patients (Table 1). Western blot analysis indicated that the level of bis-phosphorylated cTnI decreased in donor upon exchange with unphosphorylated cTn complex, while cTnI bis-phosphorylation increased in failing samples upon exchange with phosphorylated cTn complex (Figure 2A). Overnight incubation of donor tissue in exchange solution without added cTn complex (control) resulted in dephosphorylation of cTnI by ~19%, relative to the phosphorylation level in untreated donor tissue (Figure 2A). Exchange with 0.5 and 1.0 mg/ml unphosphorylated cTn complex decreased bis-phosphorylation respectively to $44.1 \pm 7.0\%$ and $28.8 \pm 3.5\%$ of the value in untreated donor tissue. Bis-phosphorylation of cTnI in failing tissue kept overnight in exchange solution without added cTn complex (failing control) amounted to $5.8 \pm 1.8\%$. Exchange of PKA phosphorylated cTn (cTn+P) in failing cardiomyocytes increased bis-phosphorylated cTnI levels to $37.9 \pm 6.6\%$ (0.5 mg/ml cTn+P) and $74.7 \pm 8.0\%$ (1.0 mg/ml cTn+P) of the values observed in untreated donor tissue.

Quantification of the degree of exchange in donor (n=4) and failing (n=6) tissue was performed using ELISA with the same cTnI Ser23/24 bis-phosphorylation antibody as used for Western blotting. To determine the percentages of exchange, the phosphorylation level of the donor control (no cTn complex during overnight incubation in exchange solution) was set at 100%, to correct for the loss of phosphorylation during the overnight incubation. In donor tissue addition of 0.5 mg/ml cTn resulted in $52.6 \pm 6.1\%$ and 1.0 mg/ml cTn in $65.9 \pm 0.9\%$ exchange. Addition of 0.5 mg/ml cTn+P resulted in $36.6 \pm 2.6\%$ and 1.0 mg/ml cTn+P in $69.0 \pm 1.1\%$ exchange in failing tissue. These latter values did not differ significantly from the corresponding values in donor tissue. The distribution between un-, mono- and bis-phosphorylated species in the donor and failing exchange samples was determined using Phos-tag gels. Exchange using 1.0 mg/ml unphosphorylated cTn in donor cells decreased mono- and bis-phosphorylated cTnI and resulted in a similar pattern as observed in control failing cells (no complex during overnight incubation). Exchange using 1.0 mg/ml phosphorylated cTn complex in failing cardiomyocytes increased mono- and bis-phosphorylated cTnI and resulted in a similar distribution as found in control donor cells (Figures 2B and C).

Phosphorylation level of cMyBP-C is significantly lower in failing than in donor tissue

Some dephosphorylation of sarcomeric proteins occurred during overnight incubation in exchange solution at 4 °C, despite the presence of high concentrations of phosphatase inhibitor cocktails in the solution. Phosphorylation levels of myofilament proteins were analyzed using ProQ Diamond in conjunction with Sypro stained gels from donor (n=6) and failing (n=12) samples. In addition to the differences in cTnI phosphorylation described above, cMyBP-C showed a significantly lower phosphorylation level in failing compared to donor tissue. The phosphorylation levels of cMyBP-C and cTnT after overnight exchange were the same as the basal levels shown in Figure 1B. However, Desmin and MLC-2 were significantly dephosphorylated after overnight incubation, but the reductions in phosphorylation in donor and failing tissue were similar (data not shown). Hence, the baseline differences in phosphorylation status between donor and IDCM tissue were largely preserved.

Ca²⁺-sensitivity after exchange is altered in donor cells but not in failing cells

In Figure 3A and Table 1 the results are presented of the force measurements in permeabilized cardiomyocytes at different Ca²⁺-concentrations. It can be seen that in cardiomyocytes from donor tissue, Ca²⁺-sensitivity (pCa₅₀) significantly increased after exchange with 0.5 mg/ml and 1.0 mg/ml unphosphorylated cTn complex. The pCa₅₀ values were increased to 5.55 ± 0.02 (0.5 mg/ml cTn) and 5.50 ± 0.02 (1.0 mg/ml cTn) relative to the values obtained after control incubation: 5.43 ± 0.03 . However, pCa₅₀ was not altered in failing cardiomyocytes upon

exchange with 0.5 mg/ml and 1.0 mg/ml phosphorylated cTn (cTn+P). In failing tissue pCa₅₀ values were 5.57±0.02 (0.5 mg/ml cTn+P), 5.60±0.02 (1.0 mg/ml cTn+P) and 5.60±0.01 in control (Figure 3B).

These results indicate that even though exchange of 1.0 mg/ml cTn+P in failing tissue resulted in a similar phosphorylation pattern as found in donor control (no cTn complex during overnight incubation), pCa₅₀ did not decrease to the level of the donor control.

After these measurements the cardiomyocytes were treated with PKA to achieve maximal PKA-mediated phosphorylation of the contractile proteins. Figure 3C illustrates the effects of subsequent PKA incubation on Ca²⁺-sensitivity. The control donor cells showed only a small decrease in pCa₅₀ by 0.03±0.01. The effect of PKA was significantly larger in donor cells exchanged with 0.5 and 1.0 mg/ml cTn compared to the donor control (in both cases ΔpCa₅₀ amounted to 0.10±0.01). All failing cells (control and exchanged with cTn+P) showed a strong response to PKA as illustrated by the shift in pCa₅₀ (control 0.10±0.01; 0.5 mg/ml cTn+P 0.13±0.01; 1.0 mg/ml cTn+P 0.12±0.02). PKA incubation decreased pCa₅₀ values in failing cells to the level of the donor control, suggesting the involvement of PKA target proteins other than cTnI in the regulation of Ca²⁺-sensitivity.

Maximal force in donor and failing cells exchanged with 0.5 mg/ml cTn was slightly but significantly decreased upon incubation with PKA, but not in the cells exchanged with 1.0 mg/ml cTn. PKA incubation of the donor control cells resulted in a significant decrease of the steepness of the force – pCa relation (nH) and resulted in a significant increase in the rate of tension redevelopment (Ktr). However, Ktr did not differ significantly in the other donor and failing groups (Table 1A and B).

In a limited set of experiments (n=2 in both donor and failing tissue; 10 cardiomyocytes each), we tested the effects of the exchange of phosphorylated cTn (0.5 mg/ml) in donor cells and of unphosphorylated cTn in failing cells. In both cases Ca²⁺-sensitivity after exchange did not differ from the values obtained in the control groups, before as well as after subsequent PKA incubation.

Impact of PKA incubation on Ca²⁺-sensitivity in end-stage failing tissue

The lack of an effect of phosphorylated cTn exchange on Ca²⁺-sensitivity in failing tissue could at least partly be due to the fact that higher levels of cTnI phosphorylation would be required than achieved after exchange. Therefore, the concentration dependence of the effect of PKA on Ca²⁺-sensitivity was studied in failing cardiomyocytes after incubation with PKA for 40 minutes. In parallel experiments the effect of the incubation on cTnI and cMyBP-C phosphorylation was assessed by ProQ Diamond staining, Western blotting and Phos-tag.

ProQ Diamond staining showed that cTnI and cMyBP-C phosphorylation increased with an increase in PKA (0–320 U/ml), whereas cTnT phosphorylation remained constant. Saturation of cTnI phosphorylation was reached after incubation with 80 U/ml PKA, whereas cMyBP-C phosphorylation reached saturation already after incubation with 10 U/ml (Figure 4A and B). The distribution between un-, mono- and bis-phosphorylated cTnI after the incubations was determined using Phos-tag gels. These results showed a decrease of unphosphorylated cTnI, while bis-phosphorylated cTnI gradually increased upon incubation with PKA (Figure 4C). Mono-phosphorylated cTnI showed a biphasic time course. It initially increased, but at larger concentrations it eventually decreased, most likely because it became bis-phosphorylated.

ΔpCa₅₀ (differences of midpoints of force-pCa curves) plotted against cTnI and cMyBP-C phosphorylation indeed confirmed that the effect of PKA on the Ca²⁺-sensitivity was largest at 40 U/ml of PKA and above (Figure 5A), when cTnI and cMyBP-C were highly

phosphorylated (Figure 5B and C). However, these experiments do not account for the difference in Ca^{2+} -sensitivity between control donor cells and failing cells after exchange with 1 mg/ml phosphorylated cTnI, where the distributions of un-, mono- and bis-phosphorylated cTnI are very similar.

Discussion

Our data show that the phosphorylation background of myofilament proteins influences the impact of PKA phosphorylation of cTnI on Ca^{2+} -sensitivity. These results suggest that the other myofilament targets of PKA, notably cMyBP-C and/or titin might play an important role by modulating the effect of cTnI phosphorylation on Ca^{2+} -sensitivity in human myocardium.

Differences in endogenous phosphorylation between donor and failing tissue

In agreement with previous studies [8–11], our study clearly showed differences in the endogenous levels of protein phosphorylation between explanted donor and idiopathic dilated end-stage failing tissue (IDCM). ProQ Diamond staining revealed a more than twofold decrease in the overall cMyBP-C phosphorylation and a six-fold decrease in cTnI phosphorylation, whereas the phosphorylation of Desmin, cTnT and MLC-2 were rather similar. Desensitisation of the β -adrenergic receptors observed in failing hearts [21] could result in a reduction of the PKA-mediated phosphorylation of target proteins such as cMyBP-C and cTnI. In addition, a reduction of PKA activity could increase PP-1 activity [14], and thereby indirectly reduce MLC-2 phosphorylation [13], which however was not evident in this patient group. *Moreover, it has been reported that the activities of Ca^{2+} -dependent calmodulin kinase II [22] and protein kinase C [23;24] are increased in failing human hearts.* The functional significance of this latter observation is still a matter of debate [25–27]. Our data shows similar TnT phosphorylation in donor and failing while cTnI phosphorylation in failing was much smaller than in donor. However, it should be noted that overall phosphorylation levels do not completely rule out the possibility of differences in phosphorylation between sites. Moreover, we would like to emphasize that the phosphorylation status observed in donor tissue might be influenced by the inotropic support used and therefore does not necessarily represent the baseline values in healthy tissue. Nevertheless, it is clear that marked and potentially site specific differences are present in the phosphorylation background of donor and IDCM samples. This allowed us to investigate the functional effects of cTnI Ser23/24 phosphorylation as the difference in phosphorylation background between both groups was largely preserved after exchange.

Differences in baseline Ca^{2+} -sensitivity in donor and failing tissue

Our study showed a marked difference in baseline Ca^{2+} -sensitivity between control donor ($\text{pCa}_{50} 5.43 \pm 0.03$) and failing tissue ($\text{pCa}_{50} 5.60 \pm 0.01$) (Table 1). This difference is rather similar to the difference in untreated donor and failing tissue [20;28] indicating that protein dephosphorylation that occurred during overnight incubation in exchange solution did not affect the baseline Ca^{2+} -sensitivity to a significant extent. These previous studies also showed, in agreement with our current findings, that the difference in Ca^{2+} -sensitivity in these human preparations could be eliminated or largely reduced by application of the catalytic subunit of PKA.

In our control donor cells, with high endogenous phosphorylation levels, PKA treatment resulted in a small reduction in Ca^{2+} -sensitivity ($\Delta \text{pCa}_{50} = 0.04$), suggesting that compensation of the ~19% loss in cTnI phosphorylation had little effect on Ca^{2+} -sensitivity.

The effect of cTn exchange in donor and failing tissue

Our results showed that a decrease in bis-phosphorylation of cTnI at Ser23/24 in donor tissue did result in an increase in Ca^{2+} -sensitivity, whereas unexpectedly, an increase in bis-phosphorylation of cTnI at Ser23/24 in failing tissue did not result in a decrease in Ca^{2+} -sensitivity.

Subsequent incubation with exogenous PKA after cTn exchange decreased Ca^{2+} -sensitivity both in donor and in failing cardiomyocytes. It should be noted that the maximum levels of cTn exchange reached using 1 mg/ml cTn complex amounted to 65% and 69% in donor and failing tissue, respectively. Since the level of cTnI bis-phosphorylation in failing tissue reached 75% of the level observed in untreated donor myocardium (Figure 2A), there is a sufficient reserve (~25%) for exogenous PKA-mediated cTnI phosphorylation to explain the decrease in Ca^{2+} -sensitivity in failing tissue exchanged with bis-phosphorylated cTn (Figure 3C).

However, careful analysis of cTnI phosphorylation profile in failing tissue after exchange indicates that phosphorylation of cTnI is not the only determinant of Ca^{2+} -sensitivity in human myocardium. After exchange using 1.0 mg/ml cTn complex in failing tissue the level of bis-phosphorylated cTnI was comparable to the level found in donor controls (Figure 2A). To assess the distribution of un-, mono- and bis-phosphorylated forms of cTnI Phos-tag analysis was performed. Exchange using 1 mg/ml phosphorylated cTn complex in failing cardiomyocytes resulted in a similar distribution as found in control donor cells. Despite these correspondences, Ca^{2+} -sensitivity of force development differed markedly under these conditions. Since subsequent PKA treatment after the exchange largely reduced these differences in Ca^{2+} -sensitivity, we conclude that PKA-mediated phosphorylation of myofilament proteins other than cTnI is involved.

The involvement PKA substrates in the alteration of the Ca^{2+} -sensitivity

The human cTn complex used for the exchange experiments was completely unphosphorylated to start with and within this trimeric complex cTnI is the only target of PKA. This implies that upon exchange, the overall phosphorylation of cTnT is reduced in the cardiomyocytes. However, the endogenous cTnT phosphorylation levels in donor and failing tissue were very similar and since the degrees of exchange were also very similar, we consider it highly unlikely that differences in cTnT phosphorylation would contribute to the observed differences in Ca^{2+} -sensitivity between donor and failing tissue. From the correspondence in MLC-2 phosphorylation levels in our donor and failing samples we conclude that this is also not the case for MLC-2. Since exchange may also result in a loss of other post translational modifications within the cTn complex, we also tested the effects of the exchange of phosphorylated cTn in donor cells and of unphosphorylated cTn in failing cells. In both cases Ca^{2+} -sensitivity after exchange did not differ from the values obtained in the control groups, before as well as after subsequent PKA incubation. This strongly suggests that post translational modifications other than PKA-mediated phosphorylation that may occur as a result of the use of recombinant cTn during the exchange have little impact on Ca^{2+} -sensitivity.

Ryanodine receptors, L-type calcium channels, phospholamban, cMyBP-C and titin are known sarcomeric PKA target proteins and might therefore be candidates to cooperate with cTnI to decrease Ca^{2+} -sensitivity. Cardiomyocytes were permeabilized in a solution containing 0.5% Triton X-100. This solubilized the sarcolemmal and sarcoplasmatic membranes. Hence the involvement of ryanodine receptors, L-type calcium channels and phospholamban in our experiments was ruled out.

The endogenous cMyBP-C phosphorylation level was significantly lower in failing than in donor samples. PKA treatment in failing tissue caused an almost two-fold increase in cMyBP-

C phosphorylation (Figure 4A), indicating that cMyBP-C phosphorylation could play a role in the functional effects of PKA on Ca^{2+} -sensitivity. Previous studies using transgenic approaches indicated that cMyBP-C and its phosphorylation status are critical to regulation of cardiac function [5;29–32] and that PKA-mediated phosphorylation of cMyBP-C caused an acceleration of crossbridge kinetics involved in stretch activation [33]. Kentish et al. [5] showed in transgenic mice, overexpressing the unphosphorylatable slow skeletal Tn in the myocardium that the effect of PKA on Ca^{2+} -sensitivity was absent, but they mentioned that this did not rule out a potential permissive effect, in which phosphorylation of cMyBP-C is necessary for the effects of TnI phosphorylation to be seen. A pioneering study of Hofmann et al. [34] showed that extraction of cMyBP-C increased Ca^{2+} -sensitivity. Together, these data suggest that cMyBP-C phosphorylation may influence Ca^{2+} -sensitivity by increasing the likelihood of crossbridge attachment.

Titin is the predominant determinant of cardiomyocyte passive stiffness over the physiological sarcomere length range [35]. Phosphorylation of titin by PKA and protein kinase G reduced stiffness [4;36;37], whereas PKC α -mediated phosphorylation increased passive stiffness [38]. In failing human myocardium an elevated cardiomyocyte stiffness was observed [39; 40], which could be corrected by exogenous PKA and was associated with reduced phosphorylation of the stiff N2B isoform [41]. These data indicate that PKA-mediated phosphorylation of titin may be altered in end-stage failing human myocardium. Indeed, Kruger et al. [36] reported a clear trend towards decreased basal titin phosphorylation in end-stage failing tissue compared to donor tissue. It has been proposed that titin-based passive tension impacts Ca^{2+} -sensitivity in rat myocardium by a reduction of lattice spacing, which increases the likelihood of actomyosin interaction [42]. Thus a difference in the titin phosphorylation might underlie the diverse PKA-mediated effects on Ca^{2+} -sensitivity observed in the present study as well.

Titration of the effect of Ser23/24 phosphorylation on Ca^{2+} -sensitivity

The maximal reduction in Ca^{2+} -sensitivity upon incubation of failing tissue with increasing PKA activity was found at relatively high phosphorylation levels of Ser23/24 (Figure 4A). Mono-phosphorylation decreased with increasing PKA activity, while bis-phosphorylated cTnI steadily increased. Previous studies showed that the mono-phosphorylated cTnI is phosphorylated at Ser24 [43;44]. Phosphorylation at Ser23 occurs only after phosphorylation of Ser24 and at a slower rate. Evidence suggests that phosphorylation of both serine residues is required for the reduction in Ca^{2+} -sensitivity [43;45;46]. This is supported by the rather abrupt reduction in Ca^{2+} -sensitivity observed between 20 U/ml and 40 U/ml PKA in failing myocytes (Figure 5A) where the balance between mono- and bis-phosphorylated cTnI was shifted to the latter form, and the mono-phosphorylated form started to decrease (Figure 4B).

PKA-mediated phosphorylation of cMyBP-C was saturated faster than cTnI phosphorylation. Despite saturated cMyBP-C phosphorylation, the maximal reduction in Ca^{2+} -sensitivity was not attained (Figure 5A and B). These data indicate that the PKA sites on cMyBP-C are largely phosphorylated when the maximal effect of PKA on Ca^{2+} -sensitivity can be observed. However, they do not exclude the possibility that the maximum effect can also be obtained through complete phosphorylation of both serine residues on cTnI.

Functional implications

This study shows that the PKA-mediated decrease of Ca^{2+} -sensitivity through cTnI depends on the phosphorylation background and that other targets of PKA besides cTnI are involved. This phosphorylation background is determined by the balance between site-specific kinase and phosphatase activity. The origin as well as stage of heart failure might influence this balance of kinases and phosphatases [47]. The coordinated nature of the effects of β -adrenergic

stimulation may eventually cumulate into an increased Ca^{2+} -sensitivity in end-stage failing myocardium, resulting in an enhanced contraction even if Ca^{2+} -handling is compromised.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Endogenous phosphorylation of donor and failing tissue and PKA-mediated phosphorylation of recombinant troponin complex. **A.** 1D gradient gel, stained with ProQ Diamond and Sypro Ruby, shows the expression and phosphorylation of myofilament proteins from healthy donor and end-stage failing tissue. **B.** Endogenous phosphorylation of myosin binding protein C (cMyBP-C) and troponin I (cTnI) was significantly lower in failing ($n=7$) compared to donor ($n=5$) samples. Phosphorylation of myofilament proteins was expressed relative to the Sypro Ruby-stained cMyBP-C bands to correct for differences in protein loading. $*P<0.05$, donor vs. failing in unpaired t-test. cTnT, troponin T; MLC-2, myosin light chain 2. **C.** The distribution of un- (0P), mono- (1P) and bis-phosphorylated (2P) cTnI in donor ($n=5$) and in failing ($n=7$)

tissue determined using Phos-tag[™] acrylamide 1D gels. **D.** Time course of PKA-mediated phosphorylation of recombinant human cardiac troponin complex. Samples were taken at different time points and run on a 1D gradient gel stained with ProQ Diamond in conjunction with Sypro Ruby staining. Time constant of the exponential fitted to the data points: 32 min.

Figure 2.

Changes in cTnI phosphorylation in donor and failing cells upon exchange of cTn-complex. **A.** Western blot analysis of donor (n=2) tissue, exchanged with unphosphorylated cTn, and failing (n=2) tissue, exchanged with bis-phosphorylated cTn, using a Ser23/24 phosphospecific cTnI antibody. Ser23/24 bis-phosphorylation was expressed relative to actin to correct for differences in protein loading. The Ser23/24 signal obtained in untreated donor (n=2) tissue was set at 100%. Control denotes results obtained in tissue kept in exchange buffer without recombinant complex added. Exchange using unphosphorylated cTn resulted in a decrease in Ser23/24 phosphorylation in donor tissue, while exchange using phosphorylated cTn in failing tissue resulted in an increase in Ser23/24 phosphorylation. **B.** The effect of cTn exchange on

the distribution of un- (0P), mono- (1P) and bis-phosphorylated (2P) cTnI in donor (n=4) and (C.) in failing (n=4) tissue. Note that the distribution of phospho species in failing tissue after exchange with 1 mg/ml bis-phosphorylated cTn was very similar to distribution in control donor tissue.

Figure 3.

Effect of cTn exchange on the Ca^{2+} -sensitivity of force production in donor ($n=33$) and failing ($n=35$) cells. **A.** Isometric force plotted as a function of the free Ca^{2+} concentration. **B.** Ca^{2+} -sensitivity derived from the midpoint of the force – pCa relationship (pCa_{50}) increased upon exchange with unphosphorylated cTn complex in donor tissue, while exchange with phosphorylated cTn complex in failing cells did not result in a decrease in pCa_{50} . * $P<0.05$, control vs. 0.5 mg/ml cTn and 1.0 mg/ml cTn in paired t -test. **C.** The effect of subsequent PKA incubation on Ca^{2+} -sensitivity after exchange. In donor tissue, PKA incubation resulted in a decrease in Ca^{2+} -sensitivity (ΔpCa_{50}), which was more pronounced after exchange with unphosphorylated cTn complex than after the control incubation. In failing tissue, the PKA-

induced shift (ΔpCa_{50}) was similar in all groups. $*P < 0.05$, control vs. 0.5 mg/ml cTn and 1.0 mg/ml cTn in paired *t*-test.

Figure 4.

PKA-mediated phosphorylation of cTnI and cMyBP-C in failing tissue. **A.** Failing tissue was incubated with increasing concentrations of PKA up to 320 U/ml. The phosphorylation of cTnI and cMyBP-C, determined from the ProQ- and Sypro-stained gels increased in a concentration dependent manner. The increase in cTnI bis-phosphorylation was also analysed using Western blotting by means of a Ser23/24 cTnI antibody. The level of cTnI bis-phosphorylation was expressed relative to actin to correct for differences in protein loading. **B.** The effect of PKA incubation on the distribution of cTnI phosphospecies using Phos-tag™ acrylamide gels. 0P, unphosphorylated cTnI; 1P, mono-phosphorylated cTnI; 2P, bis-phosphorylated cTnI.

Figure 5.

A. Effect of PKA incubation on Ca^{2+} -sensitivity of force development in failing cardiomyocytes. PKA incubation for 40 minutes resulted in a rather abrupt decrease in Ca^{2+} -sensitivity (ΔpCa_{50}) between 20 and 40 U/ml. **B.** ΔpCa_{50} plotted as a function of the phosphorylation levels of cTnI and cMyBP-C determined by Pro-Q Diamond and Sypro-staining. **C.** ΔpCa_{50} plotted as a function of the cTnI Ser23/24 bis-phosphorylation determined by Western blotting.

Table 1

Overview of cardiomyocytes force measurements

Donor					
Incubation	F _{max}	pCa ₅₀	ΔpCa ₅₀	nH	Ktr
<i>Control</i>					
<i>5 donor hearts; 13 myocytes</i>					
Basal	26.8±3.8	5.43±0.03		2.7±0.2	0.67±0.07
After PKA	24.8±3.7	5.40±0.03	0.03±0.01	2.6±0.2*	0.92±0.10*
<i>cTn [0.5mg/ml]</i>					
<i>5 donor hearts; 19 myocytes</i>					
Basal	26.7±3.2	5.55±0.02		2.9±0.2	0.66±0.05
After PKA	23.1±2.5*	5.46±0.02*	0.10±0.01	2.8±0.2	0.69±0.04
<i>cTn [1.0mg/ml]</i>					
<i>5 donor hearts; 14 myocytes</i>					
Basal	21.1±3.2	5.50±0.02		2.6±0.2	0.64±0.03
After PKA	20.0±3.0	5.41±0.02*	0.10±0.01	2.8±0.3	0.62±0.05
<i>Failing</i>					
Incubation	F _{max}	pCa ₅₀	ΔpCa ₅₀	nH	Ktr
<i>Control</i>					
<i>5 failing hearts; 15 myocytes</i>					
Basal	31.5±3.8	5.60±0.01		3.6±0.3	0.59±0.04
After PKA	30.1±4.0	5.50±0.01*	0.10±0.01	3.9±0.3	0.75±0.10
<i>cTn+P [0.5mg/ml]</i>					
<i>5 failing hearts; 18 myocytes</i>					
Basal	21.4±1.6	5.57±0.02		2.9±0.1	0.65±0.06
After PKA	18.4±1.5*	5.44±0.02*	0.13±0.01	2.9±0.1	0.66±0.06
<i>cTn+P [1.0mg/ml]</i>					
<i>5 failing hearts; 17 myocytes</i>					
Basal	20.9±2.3	5.60±0.02*		3.3±0.2	0.71±0.10
After PKA	20.1±2.0	5.48±0.02	0.12±0.02	3.3±0.3	0.64±0.04

Control denotes results obtained from cardiomyocytes kept in exchange buffer without cTn complex added. Abbreviations: F_{max}, maximal force at saturating calcium concentration in kN/m²; pCa₅₀ and nH, midpoint and steepness of the force-pCa curves, respectively; Ktr, rate of tension redevelopment (s⁻¹) at saturating calcium concentration.

* P<0.05, effect of PKA in paired *t*-test.