Length-dependent activation is modulated by cardiac troponin I bisphosphorylation at Ser23 and Ser24 but not by Thr143 phosphorylation

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1Laboratory for Physiology, Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, The Netherlands, 2Department of Pediatrics/Division of Cardiology, Johns Hopkins University School of Medicine, Baltimore, Maryland, 3Muscle Research Unit, Bosch Institute, University of Sydney, Sydney, Australia; 4Department of Physics and Astronomy, VU University, Amsterdam, The Netherlands; and 5Interuniversity Cardiology Institute of the Netherlands-Netherlands Heart Institute, Utrecht, The Netherlands

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Wijnker PJ, Sequeira V, Foster DB, Li Y, dos Remedios CG, Murphy AM, Stienen GJ, van der Velden J. Length-dependent activation is modulated by cardiac troponin I bisphosphorylation at Ser23 and Ser24 but not by Thr143 phosphorylation. Am J Physiol Heart Circ Physiol 306: H1171–H1181, 2014. First published February 28, 2014; doi:10.1152/ajpheart.00580.2013.—Frank-Starling’s law reflects the ability of the heart to adjust the force of its contraction to changes in ventricular filling, a property based on length-dependent myofilament activation (LDA). The threonine at amino acid 143 of cardiac troponin I (cTnI) is a known target of protein kinase C (PKC) whose activity is increased in cardiac disease. Thr143 phosphorylation may modulate length-dependent myofilament activation in failing hearts. Therefore, we investigated if pseudo-phosphorylation at Thr143 modulates length dependence of force using troponin exchange experiments in human cardiomyocytes. In addition, we studied effects of protein kinase A (PKA)-mediated cTnI phosphorylation at Ser23/24, which has been reported to modulate LDA. Isometric force was measured at various Ca2+ concentrations in membrane-permeabilized cardiomyocytes exchanged with recombinant wild-type (WT) troponin or troponin mutated at the PKC site Thr143 or Ser23/24 into aspartic acid (D) or alanine (A) to mimic phosphorylation and dephosphorylation, respectively. In troponin-exchanged donor cardiomyocytes experiments were repeated after incubation with exogenous PKA. Pseudo-phosphorylation of Thr143 increased myofilament Ca2+ sensitivity compared with WT without affecting LDA in failing and donor cardiomyocytes. Subsequent PKA treatment enhanced the length-dependent shift in Ca2+ sensitivity after WT and 143D exchange. Exchange with Ser23/24 variants demonstrated that pseudo-phosphorylation of both Ser23 and Ser24 is needed to enhance the length-dependent increase in Ca2+ sensitivity. cTnI pseudo-phosphorylation did not alter length-dependent changes in maximal force. Thus phosphorylation at Thr143 enhances myofilament Ca2+ sensitivity without affecting LDA, while Ser23/24 bisphosphorylation is needed to enhance the length-dependent increase in myofilament Ca2+ sensitivity.

myofilament function; protein phosphorylation; troponin I

FRANK-STARLING’S LAW OF THE HEART describes the ability of the heart to adjust the force of its contraction (stroke volume) to changes in ventricular filling (end-diastolic volume), a property based on length-dependent activation (LDA) of the myofilaments (7, 8). An increase in sarcomere length within the working range of the heart enhances the maximal force-generating capacity and the sensitivity of the myofilaments to calcium. Length-dependent myofilament activation thus represents an important cellular mechanism to adjust cardiac performance in response to increased preload of the heart.

Cardiac troponin I (cTnI) is an important regulator of LDA of the myofilaments. This has been elegantly demonstrated via replacement of endogenous cTnI by slow skeletal troponin I (ssTnI) in cardiac muscle, which significantly reduced the increase in myofilament Ca2+ sensitivity upon an increase in sarcomere length (2, 16, 33). More recently, it was demonstrated that the threonine residue 143 (Thr144 in rat and mice, Thr143 in human) of the inhibitory region of cTnI is essential for length-dependent alterations in myofilament Ca2+ sensitivity. Substitution of the threonine for a proline at 143 (cTnIThr143Pro), as present in ssTnI and fast skeletal TnI, largely ablated the length-dependent increase in myofilament Ca2+ sensitivity in cardiac muscle (33).

Interestingly, Thr143 is a well-known target of protein kinase C (PKC), and its phosphorylation has been implicated in heart failure (25, 32, 43) where an increase in PKC-mediated cTnI phosphorylation has been found together with a decreased phosphorylation of the protein kinase A (PKA) sites (43). PKC-mediated Thr143 phosphorylation may, either alone or in concert with downregulated PKA-mediated phosphorylation, underlie changes in LDA in the heart. In the present study we therefore first investigated the effect of Thr143 phosphorylation on LDA in human failing cardiomyocytes. Troponin exchange experiments were used to study the functional effects of pseudo-phosphorylated cTnI at Thr143 (mimicked by aspartic acid) compared with unphosphorylated (mimicked by alanine) and wild-type (WT) cTnI.

The effect of Thr143 phosphorylation may depend on the phosphorylation status of Ser23/24, since it has been demonstrated that PKA-mediated cTnI phosphorylation enhances the length-dependent increase in myofilament Ca2+ sensitivity (16). Therefore, secondary experiments were performed with and without exogenous PKA incubation after cTnI exchange in nonfailing donor cardiomyocytes. Also, as previous experiments showed that PKA-bisphosphorylation at Ser23 and Ser24 is required to decrease Ca2+ sensitivity (39, 44), we investigated effects of pseudo-mono- and pseudo-bisphosphorylation at cTnI-Ser23/24. To study the effect of Ser23/24 phosphorylation on LDA, experiments were performed in failing cardiomyocytes that have a low baseline level of cTnI-Ser23/24 phosphorylation.

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Our data show that pseudo-phosphorylation of Thr143 in human cardiomyocytes increases myofilament Ca\(^{2+}\) sensitivity without affecting LDA of the sarcomeres. Moreover, in human myocardium Ser23/24 bisphosphorylation is required to increase the length-dependent shift in myofilament Ca\(^{2+}\) sensitivity.

**MATERIALS AND METHODS**

**Human myocardial tissue.** Troponin exchange experiments were performed in cardiomyocytes from end-stage failing idiopathic dilated cardiomyopathy (IDCM) hearts (2 males/1 female; left ventricular ejection fraction 16.7 ± 4.4%; age: 54.3 ± 1.9 yr) or from nonfailing donor myocardium, obtained during heart transplantation surgery. The tissue was collected in cardioplegic solution and stored in liquid nitrogen. Samples were obtained after informed consent and with approval of the Human Research Ethics Committee of The University of Sydney (#7326). The investigation conforms with the principles outlined in the Declaration of Helsinki (1997). The human cardiac samples used were extensively characterized (cardiomyocyte force characteristics and cTn phosphorylation) in a previous study (10).

**Expression and purification of recombinant troponin subunits.** Recombinant human troponin complex was produced as described in detail previously (39). Shortly, six different cardiac troponin I (cTnI) mutants [troponin C (cTnC), myc-tag labeled cTnT (cTnT-myc), WT cTnI and 23A/24D, and 23A/24A. cDNA encoding human cardiac isoforms (A) to mimic dephosphorylation: 143D, 143A, 23D/24D, 23D/24A, 23A/24D, and 23A/24A, cDNA encoding human cardiac isoforms [troponin C (cTnC), myc-tag labeled cTnT (cTnT-myc), WT cTnI and cTnT mutants] were transformed in Rosetta2 (22) and cultured under carbenicillin/chloramphenicol selection in Overnight Express LB medium (EMD Biosciences). Cultures were harvested by pellets were stored at −80°C until use.

Troponin subunits were purified using fast protein liquid chromatography (AKTA-FPLC System; Amersham Biosciences) as described previously (22).

**Reconstitution of troponin complexes.** Fractions containing equal ratios of cTnI, cTnC, and cTnT subunits were pooled, subsequently purified by AKTA-FPLC chromatography using a Resource Q to remove residual uncomplexed troponin subunits and finally dialyzed against 10 mM imidazole, 200 mM KCl, 5 mM MgCl\(_2\), 2.5 mM EGTA, 1 mM DTT, and 0.1 mM PMSF (pH 6.9; 2 times, 1 liter each) before concentrating the complexes to a final concentration of >2 mg/ml by centrifugation using Centriprep YM-10 centrifugal filters (Millipore).

**Exchange of human troponin complex.** Exchange of recombinant cTn in human cardiomyocytes was done as described previously (39). Briefly, single cardiomyocytes were mechanically isolated with a glass needle homogenizer and permeabilized by Triton X-100 (0.5%; v/v) for 5 min. They were subsequently incubated overnight at 4°C in exchange solution containing 1 mg/ml of recombinant human cTn complex with the addition of 4 mM CaCl\(_2\), 4 mM DTT, 5 μM/ml protease inhibitor cocktail (P8340; Sigma), and 10 μM/ml phosphatase inhibitor cocktail 2 and 3 (P5726 and P0044; Sigma) (pH 6.9). The next day, the cardiomyocytes were washed twice in rigor solution and finally in relaxing solution (5.95 mM Na\(_2\)ATP, 6.04 mM MgCl\(_2\), 2 mM EGTA, 139.6 mM KCl, and 10 mM imidazole pH 7.0). This method results in a homogenous distribution of recombinant cTn complex within the exchanged cardiomyocytes (22).

**Determination of the degree of troponin exchange.** The degree of troponin exchange was determined as described previously (39). Briefly, to determine the degree of cTn exchange and to assess protein phosphorylation status, part of the suspension of cells was treated with 2D-clean-up kit (GE Healthcare) as described by the manufacturer protocol after overnight cTn exchange. Subsequently, tissue pellets were homogenized in sample buffer containing 15% glycerol, 62.5 mM Tris (pH 6.8), 1% (wt/vol) SDS, and 2% (wt/vol) DTT. Protein concentration measured with RCDC Protein Assay Kit II (Bio-Rad) ranged between 2 to 4 mg/ml.

**Immunoblotting.** Immunoblotting was used to determine the degree of exchange of endogenous cTnT by recombinant cTnT complex. Therefore, recombinant cTnT was labeled with a Myc-tag to allow discrimination between endogenous and recombinant cTnT complex. In a previous study we demonstrated that Myc-tag labeling does not interfere with myofilament function (39). Proteins were separated on a one-dimensional 13% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane (Hybond) using the protocol supplied by the manufacturer in 1 h at 75 V. A specific monoclonal antibody against cTnT (Clone JLT-12; Sigma; dilution 1:1,250) was used to detect endogenous and recombinant cTnT by chemiluminescence (ECL; Amersham Biosciences). We have previously demonstrated that the affinity of the cTnT antibody was the same for cTnT compared with cTnT-Myc and that cTnT loading was within the linear range (39).

**Myofilament protein phosphorylation.** The phosphorylation levels of sarcomeric proteins was determined before and after cTn exchange using Pro-Q-Diamond-stained 1D-gels, as described previously (39). The phosphorylation signals were normalized to the intensities of the SYPRO Ruby stained myosin light chain 2 bands to correct for small differences in protein loading. The PeppermintStick Phosphoprotein marker (Molecular Probes) was used to correct for differences in staining between gels (41). The ratio of the intensities of Pro-Q-Diamond and SYPRO Ruby stained ovalbumin band was used to correct for inter-gel differences.

**The distribution of endogenous phosphorylated species of cTnT was analyzed using Phos-tag acrylamide gels (FMS Laboratory; Hiroshima University, Japan) as described before (10, 18).

**Analysis of titin isoform composition.** Tissue samples were weighed and pulverized in liquid nitrogen using a mortar and a pestle. Tissue powder was solubilized in 8 M urea buffer with DTT and 50% glycerol solution with protease inhibitors (0.16 mmol/l leupeptin, 0.04 mmol/l E-64, and 0.2 mmol/l PMSF). Samples were loaded in triplicate on 1% agarose gels stained with SYPRO Ruby to determine titin isoform composition as described previously (27).

**Isometric force measurements.** Force measurements in cardiomyocytes exchanged with recombinant cTnT were performed as described previously (17, 37). Isometric force was measured at 15°C and LDA was determined by measuring force at different Ca\(^{2+}\) concentrations, first at a sarcomere length of 1.8 μm and subsequently at 2.2 μm. The following parameters were determined: passive force at pCa 9.0 (F\(_{\text{pass}}\)), maximal force at pCa 4.5 (F\(_{\text{max}}\); total force minus F\(_{\text{pass}}\)), Ca\(^{2+}\) sensitivity of force development (pC\(_{\text{CaSO}}\)), and steepness of the sigmoidal force-pCa relation [Hill coefficient (n\(_{\text{Hill}}\))]. Only a minor decrease in maximal force development was observed during force measurements at a sarcomere length of 1.8 (2.1 ± 2.5%) and 2.2 (7.8 ± 1.9%) μm. Force decline during the entire experiment did not differ between cells exchanged with the different troponin complexes. Troponin-exchanged donor cardiomyocytes were incubated with exogenous PKA for 40 min at 20°C in relaxing solution containing the catalytic subunit of PKA (100 U/incubation; Sigma) after which isometric force measurements were performed at 15°C.

**Data analysis.** Data analysis was performed as previously described using the Hill equation to fit force-Ca\(^{2+}\) relations:

\[
F(Ca^{2+})/F_0 = [Ca^{2+}]^{nHill}/(Ca_{SO}^{Hill} + [Ca^{2+}]^{nHill})
\]

where F is steady-state force, F\(_0\) the steady-state force at the saturating Ca\(^{2+}\) concentration, n\(_{\text{Hill}}\) the steepness of the relationship, and Ca\(_{SO}\) (or pcCaSO) represents the midpoint of the relation. One-way ANOVA followed by a Bonferroni post hoc test was used to compare the amount of exchange of the different cTn-complexes. Two-way ANOVA repeated-measures followed by a Bonferroni post hoc test was used to compare groups exchanged with the different cTn complexes (#P < 0.05, significant difference compared with control; for WT, see Table 2–4; for 23A/24A, see Table 5). When two-way
amounted to 68.4 exchange percentage of the three cTn complexes in IDCM recombinant cTnT and the total amount of cTnT. The average cTn exchange was calculated from the ratio of myc-tagged and therefore two cTnT bands are found. The percentage of more slowly through the gel compared with endogenous cTnT Thr143 (143D), and WT cTnI. Myc-tagged cTnT migrates represent a typical immunoblot loaded with IDCM cardiomyocytes depicts a detection of the myc-tag labeled cTnT. Figure 1 depicts a distribution of cardiac troponin I (cTnI) phosphospecies of the 3 idiopathic dilated cardiomyopathy (IDCM) samples and the donor sample used in the exchange experiments determined via a Phos-tag-blot. B: immunoblot stained with an antibody against troponin T (cTnT) that recognizes both endogenous cTnT (lower band) and recombinant Myc-tag-labeled cTnT (cTnT-myc). An example is shown of a suspension of IDCM cardiomyocytes exchanged with recombinant cTn (143A, 143D, or WT). The bar graphs indicate average percentages of cTn exchange in cardiomyocytes after overnight incubation in exchange solution containing the different cTn species (average values from 3 IDCM hearts). No significant differences were found in exchange percentage between the 3 complexes. Error bars are visible when larger than symbol size. 143A, pseudo-dephosphorylated cTnT at Thr143; 143D, pseudo-phosphorylated cTnT at Thr143; WT, wild-type cTnT.

ANOVA revealed a significant effect for sarcomere length (P < 0.05), paired t-tests were performed to compare cell measurements at two different sarcomere lengths in each cTn-exchange group (*P < 0.05, 1.8 vs. 2.2 μm). Data values represent an average of all cells of the different IDCM hearts. No significant differences were found between the three IDCM hearts after cTn exchange with the same complex for all force parameters measured. Values are given as means ± SE of n myocytes.

RESULTS

Pseudo-phosphorylation of Thr143 increases Ca\(^{2+}\) sensitivity without affecting length-dependent activation in IDCM cardiomyocytes. As the effect of pseudo-phosphorylated Thr143 may be modulated by cTnI phosphorylation at Ser23/24, we first analyzed baseline cTnI phosphorylation in the IDCM samples used in the exchange experiments. Phos-tag analysis showed a relatively low cTnI phosphorylation in IDCM hearts (n = 3): 7.5 ± 0.9% bisphosphorylated, 27.2 ± 4.7% monophosphorylated and 65.3 ± 5.1% unphosphorylated cTnI (Fig. 1A).

Troponin exchange was determined using immunoblot detection of the myc-tag labeled cTnT. Figure 1B depicts a representative immunoblot loaded with IDCM cardiomyocytes incubated overnight with recombinant cTn containing pseudo-dephosphorylated Thr143 (143A), pseudo-phosphorylated Thr143 (143D), and WT cTnT. Myc-tagged cTnT migrates more slowly through the gel compared with endogenous cTnT and therefore two cTnT bands are found. The percentage of cTn exchange was calculated from the ratio of myc-tagged recombinant cTnT and the total amount of cTnT. The average exchange percentage of the three cTn complexes in IDCM amounted to 68.4 ± 3% and did not significantly differ (P = 0.58) between the three cTn complexes (Fig. 1B), indicating similar incorporation in the myofilaments.

Previous studies indicated that sarcomeric proteins, other than cTnT, may modify length-dependent activation (9, 19). To assess if cTn exchange affected phosphorylation of myofilament proteins other than troponin, ProQ-Diamond staining was performed before and after cTn exchange. In agreement with our previous study (39), no significant differences in protein phosphorylation were induced in the other contractile proteins studied (cardiac myosin-binding protein-C, myosin light chain 2, and desmin) upon cTn exchange (Table 1). Since endogenous cTnT phosphorylation level was low, no significant reduction in cTnT phosphorylation could be detected upon exchange with unphosphorylated whole troponin complex via ProQ-Diamond staining. The level of cTnT phosphorylation did not differ between cells exchanged with the different cTn complexes (Table 1).

To determine the effects of Thr143 phosphorylation on myofilament force development, force measurements were performed in failing cardiomyocytes exchanged with 143D. As controls, 143A was used, where alanine was used to mimic dephosphorylation at Thr143, and unphosphorylated WT cTnT. Ca\(^{2+}\) sensitivity of force development was measured at 2.2 μm sarcomere length in the cTn-exchanged IDCM cardiomyocytes (1 mg/ml recombinant cTn; 3 IDCM hearts, 11–13 cells in total). Myofilament Ca\(^{2+}\) sensitivity was significantly increased in 143D, evident from the leftward shift of the force-pCa curve for 143D compared with WT (Fig. 2A), while 143A did not change Ca\(^{2+}\) sensitivity compared with WT. The midpoint of the force-pCa relation (pCa\(_{50}\)) was significantly higher in 143D-exchanged cells compared with both controls (WT and 143A; Fig. 2B).

To study the effect of Thr143 phosphorylation on length-dependent myofilament activation, force measurements were performed in failing cardiomyocytes exchanged with 143D. As controls, 143A was used, where alanine was used to mimic dephosphorylation at Thr143, and unphosphorylated WT cTnT. Ca\(^{2+}\) sensitivity of force development was measured at 2.2 μm sarcomere length in the cTn-exchanged IDCM cardiomyocytes (1 mg/ml recombinant cTn; 3 IDCM hearts, 11–13 cells in total). Myofilament Ca\(^{2+}\) sensitivity was significantly increased in 143D, evident from the leftward shift of the force-pCa curve for 143D compared with WT (Fig. 2A), while 143A did not change Ca\(^{2+}\) sensitivity compared with WT. The midpoint of the force-pCa relation (pCa\(_{50}\)) was significantly higher in 143D-exchanged cells compared with both controls (WT and 143A; Fig. 2B).

Table 1. Myofilament protein phosphorylation before and after troponin exchange

<table>
<thead>
<tr>
<th></th>
<th>cMyBPc</th>
<th>Desmin</th>
<th>MLC2</th>
<th>cTnT</th>
</tr>
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<tbody>
<tr>
<td>Before exchange</td>
<td>0.39 ± 0.02</td>
<td>0.47 ± 0.13</td>
<td>0.35 ± 0.10</td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td>143A</td>
<td>0.37 ± 0.02</td>
<td>0.48 ± 0.14</td>
<td>0.36 ± 0.10</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>143D</td>
<td>0.37 ± 0.03</td>
<td>0.46 ± 0.17</td>
<td>0.33 ± 0.09</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>WT</td>
<td>0.35 ± 0.03</td>
<td>0.45 ± 0.08</td>
<td>0.29 ± 0.03</td>
<td>0.17 ± 0.02</td>
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Values are means ± SE of the ProQ/SYPRO intensity ratio. Shown is myofilament protein phosphorylation determined with ProQ-Diamond staining before and after exchange in 3 idiopathic dilated cardiomyopathy samples. Troponin exchange did not affect myofilament protein phosphorylation (by one-way ANOVA comparing before exchange to the exchanged groups). cMyBP-C, cardiac myosin-binding protein-C; MLC2, myosin light chain 2; cTnT, cardiac troponin T; A, alanine substitution; D, aspartic acid substitution; WT, wild type.
Also performed at 1.8 μm (Fig. 2C). The effect of sarcomere lengthening on myofilament Ca\(^{2+}\) sensitivity is displayed as the ΔpCa\(_{50}\), which is the difference in pCa\(_{50}\) at a sarcomere length of 1.8 μm (Fig. 2C) and 2.2 μm (Fig. 2A). For all cTn complexes, Ca\(^{2+}\) sensitivity significantly increased upon cardiomyocyte lengthening. The increase in pCa\(_{50}\) did not differ among groups (ΔpCa\(_{50}\): WT: 0.04 ± 0.01; 143D: 0.05 ± 0.01; and 143A: 0.03 ± 0.01; Fig. 2D). Sarcomere lengthening from 1.8 to 2.2 μm significantly increased maximal (F\(_{\text{max}}\)) and passive (F\(_{\text{pas}}\)) force in all three cTn-exchanged groups (Table 2), which is in accordance with the well-known length-force relationship (1). The n\(_{\text{Hill}}\) slightly decreased upon sarcomere lengthening, only reaching a significant difference for 143A (Table 2). No significant differences in F\(_{\text{max}}\), F\(_{\text{pas}}\), and n\(_{\text{Hill}}\) were found between cells exchanged with the three cTn complexes at both sarcomere lengths. Exchange in IDC myocardium, as a reduced length-dependent increase in myofilament Ca\(^{2+}\) sensitivity has been reported in failing compared with donor cardiac tissue (30). In our recent study we found a ΔpCa\(_{50}\) of 0.10 ± 0.01 in six donor samples (31), which is significantly larger than the value found in the IDC myocardium used in this study without cTn exchange (ΔpCa\(_{50}\) = 0.04 ± 0.01). Such a blunted length-dependent increase in Ca\(^{2+}\) sensitivity in failing cardiomyocytes may be due to the low baseline cTn phosphorylation but could also be related to changes in myofilament proteins other than tropinin. Therefore, we studied the effects of Thr143 pseudo-phosphorylation in combination with PKA-mediated effects on LDA in nonfailing donor cardiomyocytes. The effects of pseudo-phosphorylation at Thr143 (143D) and at Ser23/24 (23D/24D) were studied without and with treatment with exogenous PKA. The average cTn exchange percentage in donor cardiomyocytes amounted to 82.6 ± 3.1% and did not differ among the three complexes (WT, 143D, and 23D/24D). Phosphorylation of other myofilament proteins than cTnI in exchanged donor samples corresponded to cMyBP-C: 0.57 ± 0.04; desmin: 0.47 ± 0.06; MLC2: 0.28 ± 0.01; and cTnT: 0.24 ± 0.02.

Similar to IDC, 143D significantly increased Ca\(^{2+}\) sensitivity compared with WT in one donor sample at both sarcomere lengths, while Ca\(^{2+}\) sensitivity was significantly lower compared with WT in cells exchanged with 23D/24D (Fig. 3, A–C). No differences were found in ΔpCa\(_{50}\) between WT and 143D, while the length-dependent change in pCa\(_{50}\) was higher in cells exchanged with 23D/24D (Fig. 3 D and Table 3).
The relatively low $\Delta pC_{50}$ in WT and 143D compared with donor cardiomyocytes without cTn exchange (4 cells; $\Delta pC_{50} = 0.10 \pm 0.03$) and 23D/24D-exchanged cells (Fig. 3D) may be explained by removal of phosphorylated endogenous cTnI upon exchange with recombinant unphosphorylated cTn complex. It is known that donor cardiomyocytes are relatively highly phosphorylated at Ser23/24 at baseline in the donor tissue used in this study (Fig. 1A). Treatment of cTn-exchanged donor cardiomyocytes with exogenous PKA reduced Ca$^{2+}$ sensitivity in WT and 143D (Fig. 3E) compared with cTn-exchanged cells without PKA treatment (Fig. 3C). Moreover, PKA treatment increased $\Delta pC_{50}$ in WT and 143D (Fig. 3, F vs. D). ProQ-Diamond staining illustrated that cTnI and cMyBP-C phosphorylation was $5.5 \pm 0.4$ and $1.8 \pm 0.1$ times higher, respectively, in cTn-exchanged donor cells treated with PKA compared with untreated cTn-exchanged donor cells (Fig. 3G). This is in line with our previous study (31), where selective PKA-mediated phosphorylation of cMyBP-C and cTnI was found. After PKA treatment, WT and 143D displayed a similar length-dependent increase in myofilament Ca$^{2+}$ sensitivity as 23D/24D-exchanged cells (Fig. 3F). No effect of PKA-mediated phosphorylation on the length-dependent increase in Ca$^{2+}$ sensitivity was found in donor cardiomyocytes after exchange with 23D/24D compared with 23D/24D without PKA treatment (Fig. 3, D and F). This most likely demonstrates that PKA-mediated cTnI phosphorylation at Ser23/24 was responsible for the observed enhancement in the length-dependent increase in Ca$^{2+}$ sensitivity in human donor cardiomyocytes after exchange with WT and 143D. However, this does not exclude that phosphorylation of other PKA-mediated phosphorylation targets like titin and cMyBP-C may affect the length-dependent increase in Ca$^{2+}$ sensitivity, since it is possible that phosphorylation of cMyBP-C and titin exerted opposite effects on the length-dependent change in Ca$^{2+}$ sensitivity (6, 9).

In line with our experiments in IDCM cardiomyocytes, a sarcomere length increase from 1.8 to 2.2 $\mu$m in donor cardiomyocytes without (Table 3) or with (Table 4) PKA treatment increased $F_{\text{max}}$ and $F_{\text{pas}}$ for all cTn complexes, without changing $n_{\text{Hill}}$. Between groups (WT, 143D, and 23D/24D), no significant differences were found for $F_{\text{max}}$ and $F_{\text{pas}}$ (Tables 3 and 4). The $n_{\text{Hill}}$ was significantly higher in 143D compared with WT at a sarcomere length of 1.8 $\mu$m without PKA treatment (Table 3).

Overall, our data from failing and nonfailing human cardiomyocytes show that cTnI pseudo-phosphorylation at the PKC site Thr143 does not modulate length-dependent myofilament activation.

Both Ser23 and Ser24 need to be phosphorylated to increase length-dependent activation. Our data in donor cardiomyocytes confirm a previous study (16) that indirectly demonstrated that phosphorylation of cTnI-Ser23/24 enhances the increase in Ca$^{2+}$ sensitivity upon an increase in sarcomere length. However, while monophosphorylation of Ser23 or Ser24 has been reported in the human heart (42, 43), no functional effect of monophosphorylation has been found (39, 44). Therefore, we investigated if monophosphorylation of Ser23 or Ser24 would affect LDA of the myofilaments. For these experiments IDCM cardiomyocytes were used, which showed $< 7\%$ endogenous bisphosphorylation at Ser23/24 (Fig. 1A). Due to the low baseline cTnI phosphorylation, the remaining endogenous Ser23/24 bisphosphorylation after cTn exchange does not interfere with functional effects of mono- and bisphosphorylated cTn complexes. IDCM cardiomyocytes (6–9 cells per cTn complex) were exchanged with four different pseudo-phosphorylation variants of Ser23/24. The average cTn exchange percentage was 76.6 $\pm$ 1.9% and did not differ between the complexes. In accordance with our previous observations in nonfailing donor cardiomyocytes (39), only pseudo-bisphosphorylation decreased Ca$^{2+}$ sensitivity compared with mono- and dephosphorylated Ser23/24 in IDCM cardiomyocytes (Fig. 4A). Compared with pseudo-dephosphorylated Ser23/24 (23A/24A), pseudo-monophosphorylation at Ser23 (23D/24A) or Ser24 (23A/24D) did not affect LDA (Fig. 4B and Table 5). Upon exchange with pseudo-bisphosphorylated Ser23/24 (23D/24D), the length-dependent increase in Ca$^{2+}$ sensitivity was significantly increased compared with 23A/24A (Fig. 4B). The enhanced length-dependent increase in Ca$^{2+}$ sensitivity in 23D/24D was significantly higher compared with control IDCM cardiomyocytes without exchange. The sarcomere length increase from 1.8 to 2.2 $\mu$m increased $F_{\text{max}}$ and $F_{\text{pas}}$ for all cTn complexes, without changing $n_{\text{Hill}}$ (Table 5).

Length-dependent myofilament activation and titin-based passive stiffness. Titin-based passive stiffness has been shown to exert a profound effect on length-dependent activation. A high expression of the stiff N2B isoform in cardiac tissue has been associated with a higher passive force and larger length-dependent increase in myofilament Ca$^{2+}$ sensitivity compared with donor cardiomyocytes.
Fig. 3. Phosphorylation of Ser23 and Ser24 regulates the length-dependent increase in Ca^{2+} sensitivity in donor cardiomyocytes. Myofilament force development was measured at a sarcomere length of 1.8 (A) and 2.2 μm (B) at various Ca^{2+} concentrations in donor cardiomyocytes in which endogenous troponin complex was partially exchanged (82.6 ± 3.1%) with recombinant myc-tag-labeled troponin complexes (4–5 cardiomyocytes per group). C: compared with unphosphorylated wild-type cTnI (WT), Ca^{2+} sensitivity derived from the midpoint of the force-pCa relationship (pCa_{50}) was significantly increased after exchange with pseudophosphorylated cTnl at Thr143 (143D) and significantly decreased upon pseudo-bisphosphorylation of Ser23/Ser24 (23D/24D) at 2.2 μm. D: length-dependent increase in Ca^{2+} sensitivity (ΔpCa_{50}) did not differ between 143D and WT. However, exchange with 23D/24D significantly increased the length-dependent Ca^{2+} sensitivity increase compared with WT cTnI-exchanged donor cardiomyocytes were incubated with exogenous PKA. E: PKA treatment decreased Ca^{2+} sensitivity in cells exchanged with WT and 143D compared with untreated cardiomyocytes after exchange but had no effect in 23D/24D cells (Fig. 3, E vs. C). F: PKA treatment significantly increased the length-dependent increase in Ca^{2+} sensitivity for WT and 143D compared with untreated cells after exchange (Fig. 3, D vs. F). No significant differences in ΔpCa_{50} were found after PKA treatment of cells exchanged with the 3 different cTn complexes. *P < 0.05, WT vs. 143D and 23D/24D complexes in posttest Bonferroni analyses of one-way ANOVA. PKA treated cardiomyocytes were compared with untreated cardiomyocytes after exchange with the same complex via a Student’s t-test. G: myofilament proteins of cardiomyocytes after cTn exchanged without (lane 1) and with (lane 2) PKA treatment were separated by 1D-gel electrophoresis. Staining differences between gels were corrected with phosphorylated ovalbumin of the peppermint marker (PM). Phosphorylation signal on ProQ Diamond-stained gels was divided by the SYPRO signal of the same protein to correct for minor differences in loading. Phosphorylation levels of cTnI and cMyBP-C increased after PKA treatment.
with tissue with a high expression of compliant N2BA isoform (9). Thus changes in titin isoform composition, as have been reported in human heart failure (21, 23), may alter length-dependent activation. In our exchange experiments, passive tension did not differ between the different experimental groups (Tables 2–5). Moreover, no differences were observed in Fmax and titin isoform composition (Fig. 5) between the failing and donor samples used in the present study.

Discussion

This study demonstrates that cTnl pseudo-phosphorylation at the PKC-site Thr143 increases myofilament Ca2+ sensitivity without affecting the length-dependent increase in maximal force or Ca2+ sensitivity in human failing and nonfailing donor cardiomyocytes. The length-dependent increase in Ca2+ sensitivity is enhanced by pseudo-phosphorylation of Ser23/24 only upon phosphorylation of both sites.

Phosphorylation of Thr143 increases Ca2+ sensitivity without affecting length-dependent activation. Tachampa et al. (33) showed that substitution of a single amino acid at position 143 of cTnl (from Thr to Pro) greatly diminished the length-dependent increase in myofilament Ca2+ sensitivity. Since Thr143 is a well-known PKC-mediated phosphorylation site that has been implicated in heart failure (25, 32, 43), we investigated if phosphorylation of Thr143 may modify LDA of the myofilaments in human cardiomyocytes. We show that pseudo-phosphorylation of Thr143 does not affect the length-dependent increase in maximal force and Ca2+ sensitivity irrespective of the level of cTnl phosphorylation at PKA sites.

Table 3. Force measurements in donor cardiomyocytes after exchange with recombinant troponin without PKA treatment

<table>
<thead>
<tr>
<th>Without PKA</th>
<th>WT</th>
<th>143D</th>
<th>23D/24D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fmax, kN/m²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.8 µm</td>
<td>12.4 ± 0.5</td>
<td>13.3 ± 1.4</td>
<td>11.7 ± 1.1</td>
</tr>
<tr>
<td>2.2 µm</td>
<td>16.2 ± 0.5*</td>
<td>16.7 ± 1.2*</td>
<td>16.3 ± 0.9*</td>
</tr>
<tr>
<td>Fmax, kN/m²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.8 µm</td>
<td>2.2 ± 0.3</td>
<td>2.5 ± 0.6</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>2.2 µm</td>
<td>3.9 ± 0.6*</td>
<td>4.1 ± 0.8*</td>
<td>4.0 ± 0.3*</td>
</tr>
<tr>
<td>pCa50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.8 µm</td>
<td>5.55 ± 0.05</td>
<td>5.69 ± 0.02†</td>
<td>5.37 ± 0.01†</td>
</tr>
<tr>
<td>2.2 µm</td>
<td>5.57 ± 0.05</td>
<td>5.71 ± 0.02†</td>
<td>5.46 ± 0.01†</td>
</tr>
<tr>
<td>nHill</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.8 µm</td>
<td>1.9 ± 0.1</td>
<td>2.7 ± 0.4*</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>2.2 µm</td>
<td>1.9 ± 0.1</td>
<td>2.4 ± 0.3</td>
<td>1.7 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. One donor sample exchanged with 143D, 23D/24D, and WT troponin complex (4–5 myocytes per complex). Myofilament force was measured at sarcomere lengths of 1.8 and 2.2 µm at different Ca2+ concentrations. Ca2+ sensitivity derived from the midpoint of the force–pCa relationship (pCa50) increased at 2.2 µm compared with 1.8 µm for all complexes; however, the difference was only significant in 23D/24D. Stretching of cardiomyocytes from 1.8 to 2.2 µm increased maximal (Fmax) and passive force (Fpass) in all groups. There was no significant effect of sarcomere length on nHill (steepness of the force–pCa curves). Compared with WT, 143D significantly increased Ca2+ sensitivity and 23D/24D decreased Ca2+ sensitivity at both sarcomere lengths. No significant differences among the 3 cTn complexes for Fmax and Fpass were found. nHill was significantly higher in 143D compared with WT only at sarcomere length 1.8 µm. A two-way ANOVA repeated measures followed by Bonferroni post hoc test was used to compare groups exchanged with the different cTn complexes (†P < 0.05, significant difference compared with WT). When the two-way ANOVA revealed a significant effect for sarcomere length (P < 0.05, paired t-tests were performed to compare cell measurements at 2 different sarcomere lengths in each cTn-exchange group (§P < 0.05, 1.8 vs. 2.2 µm)).

The finding that Thr143 phosphorylation does not affect LDA is not in conflict with the previous finding that a substitution of Thr143 by a proline, as present in ssTnI and fast skeletal TnI, severely blunts LDA in cardiac muscle. A proline is a neutral amino acid with very specific properties [reviewed by Williamson (40)]. Replacement of a Thr by a Pro alters the amino acid charge differently compared with threonine phosphorylation. Likewise, mutations in the inhibitory region of cTnl, which are associated with inherited forms of cardiomyopathies, may disrupt LDA. We recently reported blunted LDA in myocardium from patients with hypertrophic cardiomyopathy harboring a mutation (R145W) in cTnl (31). Overall, these results indicate that mutation-induced changes in amino acids in the inhibitory region of cTnl and phosphorylation-mediated changes of Thr143 exert different effects on sarcomere function. While the threonine at position 143 is key for LDA in cardiac muscle, phosphorylation at this site does not modulate LDA.

Pseudo-phosphorylation of Thr143 using aspartic acid at amino acid 143 increased Ca2+ sensitivity both in IDCM and donor cardiomyocytes. In contrast, exchange in mouse fibers with pseudo-phosphorylated Thr143 mimicked by a glutamate did not change (§) or nonsignificantly decreased Ca2+ sensitivity (32) compared with WT exchange. Our data are in line with a study of Wang et al. (38) where incorporation of phosphate instead of pseudo-phosphorylation was used. By using genetically modified mice harboring alanine mutations at the different phosphorylation sites of cTnl, they demonstrated that PKC-βII preferentially phosphorylates Thr143 and thereby increases Ca2+ sensitivity both in IDCM and donor cardiomyocytes after exchange with recombinant troponin and subsequent PKA treatment

<table>
<thead>
<tr>
<th>With PKA</th>
<th>WT</th>
<th>143D</th>
<th>23D/24D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fmax, kN/m²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.8 µm</td>
<td>12.8 ± 1.1</td>
<td>11.7 ± 1.1</td>
<td>11.1 ± 1.9</td>
</tr>
<tr>
<td>2.2 µm</td>
<td>14.9 ± 1.6*</td>
<td>14.5 ± 1.6</td>
<td>16.2 ± 2.2*</td>
</tr>
<tr>
<td>Fmax, kN/m²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.8 µm</td>
<td>2.0 ± 0.2</td>
<td>2.0 ± 0.2</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>2.2 µm</td>
<td>2.9 ± 0.2*</td>
<td>3.4 ± 0.5*</td>
<td>3.6 ± 0.3*</td>
</tr>
<tr>
<td>pCa50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.8 µm</td>
<td>5.34 ± 0.06</td>
<td>5.47 ± 0.01†</td>
<td>5.37 ± 0.01†</td>
</tr>
<tr>
<td>2.2 µm</td>
<td>5.44 ± 0.08*</td>
<td>5.56 ± 0.02*</td>
<td>5.48 ± 0.01*</td>
</tr>
<tr>
<td>nHill</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.8 µm</td>
<td>2.1 ± 0.1</td>
<td>2.3 ± 0.2</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>2.2 µm</td>
<td>2.1 ± 0.1</td>
<td>2.4 ± 0.2</td>
<td>1.7 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. One donor sample exchanged with 143D, 23D/24D and WT troponin complex with PKA treatment (4–5 myocytes per complex). Myofilament force was measured at sarcomere lengths of 1.8 and 2.2 µm at different Ca2+ concentrations. Ca2+ sensitivity derived from the midpoint of the force–pCa relationship (pCa50) significantly increased at 2.2 µm compared with 1.8 µm for all complexes. Stretching of cardiomyocytes from 1.8 to 2.2 µm increased maximal (Fmax) and passive force (Fpass) in all groups, without affecting nHill (steepness of the force–pCa curves). Compared with WT, 143D increased Ca2+ sensitivity, reaching significance at sarcomere length 1.8 µm (†). pCa50 of WT did not significantly differ from 23D/24D after PKA treatment. No significant differences among the 3 cTn complexes for Fmax, Fpass, and nHill were found. A two-way ANOVA repeated measures followed by a Bonferroni post hoc test was used to compare groups exchanged in the different cTn complexes (†P < 0.05, significant difference compared with WT). When the two-way ANOVA revealed a significant effect for sarcomere length (P < 0.05, paired t-tests were performed to compare cell measurements at 2 different sarcomere lengths in each cTn-exchange group (§P < 0.05, 1.8 vs. 2.2 µm)).

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increased Ca\(^{2+}\) sensitivity. This suggests that an aspartic acid pseudo-phosphorylation at Thr143 more closely resembles PKC-mediated phosphorylation of Thr143 compared with a glutamate pseudo-phosphorylation.

The absence of an effect of Thr143 pseudo-phosphorylation on LDA is in line with the preserved LDA at the myofilament level in a mouse model of heart failure induced by overexpression of PKCε (20), which has been demonstrated to phosphorylate Thr143 (15). Thr143 phosphorylation did not increase the relatively small length-dependent increase in Ca\(^{2+}\) sensitivity when Ser23/24 are largely unphosphorylated in failing cells. One possibility to explain this may be the elevated Ca\(^{2+}\) sensitivity, limiting further Ca\(^{2+}\) sensitivity enhancement upon lengthening. However, pseudo-phosphorylation of Thr143 in combination with phosphorylation of Ser23/24 also did not change the enhanced length-dependent increase in Ca\(^{2+}\) sensitivity.

In donor cardiomyocytes, WT without PKA showed a pC\(_{50}\) of 5.57 ± 0.05 and a relatively low length-dependent...
increase in Ca$$^{2+}$$ sensitivity (Fig. 3, C and D), while 143D after PKA displayed the same pCa$_{50}$ (5.56 ± 0.02) with an enhanced length-dependent Ca$$^{2+}$$ sensitivity increase (Fig. 3, E and F). These observations support the conclusion that an enhanced length-dependent increase in Ca$$^{2+}$$ sensitivity is not directly related to a lower myofilament Ca$$^{2+}$$ sensitivity induced by phosphorylation of Ser23/24. This is in line with our previous study where a low Ca$$^{2+}$$ sensitivity at a sarcomere length of 2.2 μm was found in a patient with a cTnl mutation (R145W) after treatment with exogenous PKA together with a blunted length-dependent increase in Ca$$^{2+}$$ sensitivity (31). Titin isoform composition did not differ between the samples used for exchange experiments and therefore most likely did not contribute to the changes observed in length-dependent myofilament activation in our exchange experiments. In summary, our data show that PKC-mediated phosphorylation at Thr143 sensitizes the myofilament to Ca$$^{2+}$$ without affecting length dependence of the sarcomeres independent of the initial value of Ca$$^{2+}$$ sensitivity, which is set by the phosphorylation status of other troponin sites and sarcomeric proteins.

**Phosphorylation of both Ser23 and Ser24 increases length-dependent activation.** Exchange with WT troponin complex did not alter the length-dependent increase in Ca$$^{2+}$$ sensitivity in IDCM cardiomyocytes compared with (unexchanged) control cardiomyocytes, whereas it significantly reduced the length-dependent increase in Ca$$^{2+}$$ sensitivity in donor cardiomyocytes compared with control cardiomyocytes. An important difference in cTn between donor and IDCM cardiomyocytes is that donor cardiomyocytes are highly bisphosphorylated at Ser23/24 (>50%), in contrast to end-stage IDCM (<10%) (10, 39). LDA has been reported to be regulated by PKA-mediated phosphorylation of cTnl at these sites, although a reduction (14) and enhancement (16) of the length-dependent change in Ca$$^{2+}$$ sensitivity has been reported upon PKA incubation. In addition, contrasting results have been reported when pseudo-phosphorylated cTnl at Ser23/24 was exchanged, showing a blunted (28) or enhanced (24) change in length-dependent Ca$$^{2+}$$ sensitivity compared with WT exchange. The reason for these differences is unclear; however, one possible explanation for the blunted length-dependent increase in Ca$$^{2+}$$ sensitivity that was found in previous studies may be that a different protocol was used (14, 28). In the present study we first performed all force measurements at a low sarcomere length of 1.8 μm and thereafter performed measurements at 2.2 μm. We did not stretch cardiomyocytes to a sarcomere length of 2.3 μm to prevent damage to the sarcomeres. However, Rao et al. (28) and Kajiwara et al. (14) first measured at a sarcomere length of 2.3 μm and thereafter at a shorter sarcomere length of 2.0 μm.

In this study, we demonstrate using PKA-mediated phosphorylation and the exchange of pseudo-phosphorylated Ser23/24 that phosphorylation of Ser23/24 enhances the length-dependent increase in Ca$$^{2+}$$ sensitivity in human cardiomyocytes because 1) exchange with WT complex removed endogenous phosphorylation at Ser23/24 in donor cardiomyocytes and blunted the length-dependent increase in Ca$$^{2+}$$ sensitivity compared with baseline measurements in donor cells. 2) PKA treatment after WT exchange restored the length-dependent increase in Ca$$^{2+}$$ sensitivity in donor cardiomyocytes. 3) Exchange with pseudo-phosphorylated Ser23/24 preserved the length-dependent increase in Ca$$^{2+}$$ sensitivity in donor cardiomyocytes and enhanced the length-dependent increase in Ca$$^{2+}$$ sensitivity in IDCM cardiomyocytes to levels observed in donor. In support, it has been demonstrated that PKA-mediated phosphorylation shifts a shallow length-tension relationship to a steeper length-tension relationship in rat cardiomyocytes (12). In accordance, exchange with pseudo-phosphorylated Ser23/24 in rat cardiomyocytes was associated with a steepened length-tension relationship providing evidence that phosphorylation of cTnl-Ser23/24 is essential to enhance length-dependent activation (11). In addition, we demonstrated using mono- and bisphosphorylated cTnl complexes that phosphorylation at both Ser23 and Ser24 is required to enhance the length-dependent increase in myofilament Ca$$^{2+}$$ sensitivity.

**Implication of cTnl phosphorylation at Thr143 and Ser23/24 and in health and disease.** In the present study, we provide evidence that phosphorylation of the PKC site Thr143 does not affect LDA, while bisphosphorylation of the PKA-sites Ser23/24 enhances the length-dependent increase in Ca$$^{2+}$$ sensitivity in human cardiomyocytes (Fig. 6). In the healthy heart, sympathetic activation during stress and exercise increases heart rate and stroke volume to meet the demands of the body. This is accomplished via activation of β1-adrenergic receptors, which leads to activation of PKA. PKA phosphorylation of Ca$$^{2+}$$ handling proteins and myofilament proteins enhances cardiomyocyte contraction and relaxation (for reviews: see Refs. 3, 35). The enhanced length-dependent increase in Ca$$^{2+}$$ sensitivity induced upon PKA phosphorylation of cTnl-Ser23/24 will increase the range of myofilament Ca$$^{2+}$$ sensitivities at which the heart can operate within a heart cycle (between short and long sarcomere lengths) as is illustrated in Fig. 6 (WT vs. WT + PKA, 23D/24D, and 23D/24D + PKA, which resembles activation of the heart upon β-adrenergic receptor stimulation).

In end-stage heart failure, the Frank-Starling relationship has been reported to be depressed (13, 29). LDA is believed to be the cellular basis of the Frank-Starling mechanism (26, 34).
Schwinger et al. (30) previously reported a reduced length-dependent increase in Ca$^{2+}$ sensitivity in skinned fibers from terminally failing human myocardium. In our study, replacement of endogenous cTnI with pseudo-bisphosphorylated cTnI at Ser23/24 enhanced the length-dependent increase in myofilament Ca$^{2+}$ sensitivity in failing cardiomyocytes (Fig. 4B). This suggests that the blunted length-dependent increase in myofilament Ca$^{2+}$ sensitivity, which has been reported previously (30) in end-stage heart failure, may be at least partly caused by reduced bisphosphorylation at Ser23/24 of cTnI relative to explanted donor tissue, which has been reported in several studies (4, 18, 36, 42).

Apart from reduced PKA-mediated cTnI phosphorylation, increased PKC-mediated phosphorylation of Thr143 may modify LDA of cardiac muscle in heart failure since this amino acid has been demonstrated to play a key role in the length-dependent increase in Ca$^{2+}$ sensitivity (33). Pseudo-phosphorylation of the PKC site Thr143 did not affect LDA properties of human heart muscle but did increase Ca$^{2+}$ sensitivity as illustrated in Fig. 6 (WT vs. 143D; WT + PKA vs. 143D + PKA). Based on our results, PKC-mediated phosphorylation of Thr143, which has been implicated in cardiac disease (25, 32, 33), does not restore the blunted length-dependent Ca$^{2+}$ sensitivity increase in the failing heart, although it does increase Ca$^{2+}$ sensitivity. The high Ca$^{2+}$ sensitivity may aid to maintain cardiac output of the failing heart; however, it may also contribute to diastolic dysfunction.

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20. Montgomery DE, Rundell VL, Goldspink PH, Urboniene D, Geenen S, Carlucci DB, Murphy AM, van Eyk JE, Tesi C, Poggesi C, van der Velden J, Hoekstra AJ. Length dependence of calcium sensitivity in failing cardiomyocytes (Fig. 4B).


